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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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African Journal of Biotechnology

Table of Contents: Volume 13 Number 14, 2 April, 2014

ARTICLES

Table of Contents: Volume 13 Number 14, 2 April, 2014

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Full Length Research Paper

Assessment of the genetic diversity and pattern of relationship of West African sorghum accessions using microsatellite markers

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An understanding of the extent, distribution and patterns of genetic variation is useful for estimation of any possible loss of genetic diversity and assessment of genetic variability and its potential use in breeding programs, including establishment of heterotic groups. This study assessed patterns of genetic diversity and relationships among 30 West African sorghum accessions using 22 microsatellite markers. Population structure and within population genetic diversity was also assessed using the same markers. Genotypic data was generated using the ABI Prism 3730 and alleles called and sized using genemapper software version 3.7. Molecular data was analyzed using DARwin 4.0, powermarker 3.0 and Arlequin version 3.11. The average marker quality index was 0.27 while a mean PIC of 0.54 was observed across the 22 SSR markers. Among the 30 accessions, the markers detect a total of 146 alleles with an average of 6.6 alleles per marker. Results from the various statistical analyses performed revealed a wide range of polymorphism from 22.7 to 86.4%. The mean heterozygosity was relatively low at 0.28 while the average Nei's genetic diversity among the 30 populations was 0.57. The within population Nei's genetic diversity assessed from 49 individuals in 10 populations was lower at 0.54 and the average heterozygosity was also lower at 0.21. Cluster and principal coordinate analysis of the 30 populations revealed two distinct groups independent of their geographic origins The examination of the hierarchical partitioning of genetic variation by AMOVA demonstrated that genetic differentiation was significant at P < 0.00. Of the total diversity, 8.9% was attributed to country differences, 54.11% was attributed to population differences within the countries while 36.99% was attributed to differences within populations. The F_{ST} value (0.63) indicated a very high genetic differentiation as expected for selfing species. This study demonstrates the utility of SSR markers in detecting polymorphism, estimating genetic diversity and establishment of genetic clusters for heterotic studies.

Key words: Genetic diversity, heterozygosity, SSR microsatellites, heterotic grouping, alleles, polymorphism.

INTRODUCTION

Sorghum (*Sorghum bicolor* (L.) Moench) is the fifth most important cereal crop worldwide after wheat (*Triticum species*), rice (*Oryza species),* maize (*Zea mays*) and barley (*Hordeum vulgare*) with an annual average production of 61 million tones over the past decade (FAO, 1995; FAOSTAT, 2004; http://appsfaoorg/defaulthtm). Sorghum, together with pearl millet (*Pennisetum americanum* (L.) and finger millet (*Eleusine coracana* (L.), represent Africa's main contribution to the world food supply (de Vries and Toenniessen, 2001). The crop can be grown under a wide range of soil and climatic conditions and thus plays a major role in semi-arid regions of the world where drought, heat and poor soil conditions make production of other cereals difficult (Dogget, 1988; House, 1995). The crop occupies 25% or more of arable land in Mauritania, Gambia, Mali, Burkina Faso, Ghana, Niger, Somalia and Yemen, and > 10% of this area in Nigeria, Chad, Sudan, Tanzania and Mozambique.

Approximately 120 million people living in semi-arid West and Central Africa (WCA) depend on pearl millet and sorghum as the major food crops and sources of income. Because of its global socio-economic importance, there has been substantial interest in characterizing the levels of genetic diversity present within sorghum using both phenotypic and molecular markers (Dean et al., 1999; Dje et al., 2000; Ghebru et al., 2002; Grenier et al., 2000a, b; Menz et al., 2004; Casa et al., 2005). These analyses have provided a foundation of genetic data for making informed decisions regarding the management and utilization of genetic resources (Casa et al., 2005). Understanding the structure of genetic diversity provides the foundation for effective and sustained population breeding and hybrid development in sorghum. The genetic diversity in the germplasm of a breeding program affects the potential genetic gain through selection. Information about genetic diversity also permitted the classification of germplasm into heterotic groups, which are particularly important to hybrid breeding. Even though the genetic mechanisms that explain heterosis are not fully understood, it is well documented that crosses between unrelated and consequently genetically distant parents, show greater hybrid vigor due to an enhanced degree of heterozygosity than crosses between closely related parents (Stuber, 1994; Hallauer, 1999; Menz et al., 2004). Estimates of molecular-marker based genetic distance have been proven to be a useful way to describe existing heterotic groups to identify new heterotic groups and to assign inbreeds of unknown genetic origin to established heterotic groups (Dubreuil et al., 1996; Saghai et al., 1997; Hongtrakul et al., 1997; Pejic et al., 1998; Casa et al., 2002). The research presented here was to provide an initial assessment of the pattern of sorghum diversity of West Africa in order to provide insights for tapping this diversity in current and future breeding efforts. We used SSR loci to determine diversity and evaluate genetic relationships and population structure within and among 30 sorghum accessions.

MATERIALS AND METHODS

Plant materials

Seed samples of 30 sorghum accessions (ICRISAT B lines and parents from heterosis study) were collected from different countries and diverse geographic origins in West Africa and their passport data is provide in Table 1.

DNA extraction, quantification and normalization

Individual and bulking sampling methods were used. Approximately 6 cm of 4 seedlings leaf-tissue of each accession was pooled for all the 30 accessions. 10 accessions were selected randomly and 5 seedlings per accession sampled individually. Isolation of DNA was done using a modified CTAB protocol (Mace et al., 2004). Determination of the quality, quantity and purity of the isolated DNA was essential for PCR optimization and were done using agarose (0.7%) gel electrophoresis stained with ethidium bromide (10 mg/ml) and Nanodrop spectrophotometer. All DNA samples were diluted to the required concentration (5 ng/μl). This was necessary to ensure uniformity in results.

PCR optimization and amplification

All the 22 primers were run with the BTX623 standard DNA and optimized by varying the primer (2 pmol/ul) and $MgCl_2$ (10 nM/ul) between 0.6 - 2.0 ul. The dNTPs (2 mM/ul and enzyme (5 U/ul) were varied between 0.4 - 0.75 and 0.04 -0.08 respectively. The annealing temperatures of the primers were optimized using the touchdown PCR amplification procedure as described by Folksterman et al. (2005). PCR reactions conditions were set in 10 μl volumes in 384-well PCR plates (ABGene, Rochester, N.Y.). Temperature cycling was carried out using the GeneAmp PCR systems 9600 (PE-Applied Biosystems). A final extension of 20 min at 72°C was included to minimize the +A overhang (Smith et al., 1997). The PCR products was run on 2.5% agarose gel (Figure 1) and amplification checked with the DNA analyzer.

Capillary electrophoresis

Genotyping was carried out by capillary electrophoresis using the ABI PRISM 3730 (Applied Biosystems), a fluorescent based

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Table 1. Sorghum accessions selected for the study.

Entry	Identification	Origin	Race	Breeding origin
1	IS 3534B	Sudan	Guinea	Identified maintainer line
2	FambéB	Mali	Guinea	Maintainer, product of mutation breeding
3	IPS001B	Mali	Guinea	Maintainer, product of mutation breeding
4	CSM219B	Mali	Guinea	Maintainer, product of mutation breeding
16	(GPN99 271-20-2)	West African	Guinea	Maintainer, derived from broad-based Guinea population
17	ICSB 38	ICRISAT-Nigeria	Caudatum	Pedigree breeding
19	IS 6731bf	Burkina Faso	Guinea	Landrace
21	IS 27564bf	BurkinaFaso	Guinea	Landrace
22	Seguetana CZ	Mali	Guinea	Landrace
34	GPN01 267-9-1	ICRISAT-Mali	Guinea	Derivative of Guinea Population
40	IS 15629ca	Cameroon	Guinea	Landrace
43	Fara Fara-17	Nigeria	Guinea/Caudatum	Landrace
48	Kaura-Katsina	Nigeria	Durra/Caudatum	Landrace
49	Guinea-Katsima	Nigeria	Guinea	Landrace
50	CSM388	Mali	Guinea	Landrace
105	90SN3	Niger	Caudatum	Pedigree breeding
107	90SN5	Niger	Caudatum	Pedigree breeding
110	A223	USA	Caudatum	Pedigree breeding
111	AT623	USA	Caudatum	Pedigree breeding
139	98-BE-F5P-82	IER-Mali	Guinea/Caudatum	Pedigree breeding derivative
140	97-SB-F5DT-150 B	IER-Mali	Guinea/Caudatum	Pedigree breeding derivative
141	02-SB-F5DT-12 B	IER-Mali	Guinea/Caudatum	Pedigree breeding derivative
142	02PR-3009K B	IER-Mali	Guinea/Caudatum	Pedigree breeding derivative
144	00-KO-F5DT-19	IER-Mali	Guinea/Caudatum	Pedigree breeding derivative
145	03-F4T-38	IER-Mali	Guinea/Caudatum	Pedigree breeding derivative
146	02-SB-F4FT-298	IER-Mali	Guinea/Caudatum	Pedigree breeding derivative
147	02-SB-F4FT-189	IER-Mali	Guinea/Caudatum	Pedigree breeding derivative
153	SK5912	Nigeria	Durra/Caudatum	Derived from landrace
154	Buhu Banza	Nigeria	Durra/Caudatum	Landrace
155	Fara Fara	Nigeria	Guinea/Caudatum	Landrace

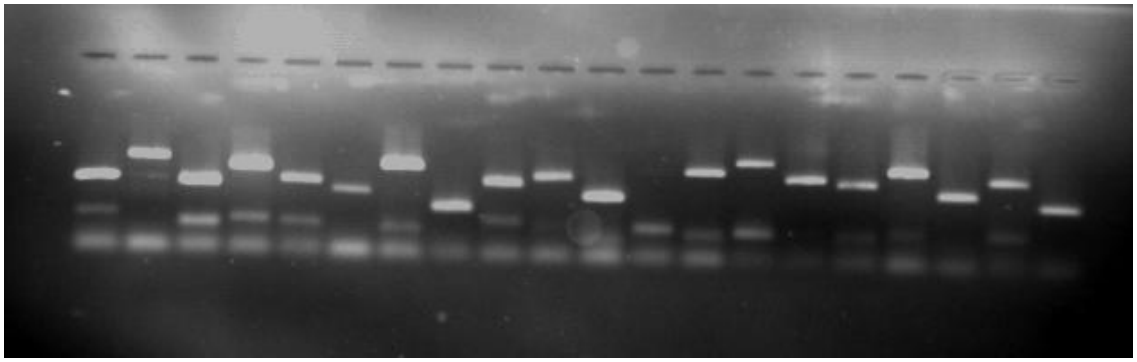

Figure 1. Primer amplification products obtained with the optimized PCR protocol.

capillary detection system that uses polymer as the separation matrix. This facilitated the accurate sizing of the microsatellite alleles to within ± 0.3 base pairs (Buhariwalla and Crouch, 2004). PCR products were co-loaded post-PCR based on dye label, fragment size and fluorescence to reduce the unit cost of high throughput genotyping (Table 2). 0.5 - 1 µl of labelled PCR

Table 2. Summary of the 22 SSR markers, primer multiplex and co-loading sets used in the study.

Marker name	Dye label	Multiple x set	Chromosome	Repeat motif	Reference
gpsb123	VIC		8	(CA)7+(GA)5	Unpublished*
mSbCIR223	HEX	Set 2	2	(AC)6	Unpublished
mSbCIR240	HEX	Set 7	8	(TG)9	Unpublished
mSbCIR276	NED	Set 7	3	(AC)9	Unpublished
mSbCIR283	NED	Set 5	10	(CT)8 (GT)8.5	Unpublished
mSbCIR286	6-FAM	Set 6	1	(AC)9	Unpublished
SbAGB02	VIC	Set 5	7	$(AG)_{35}$	Taramino et al. (1997)
Xcup11	NED	Set 2	3	$(GCTA)_4$	Schloss et al. (2002)
Xgap072 = Sb4-72	VIC	Set 3	6	(AG)16	Brown et al. (1996)
Xgap084=Sb6-84	VIC	Set 2	2	(AG)14	Brown et al. (1996)
Xisep0310	HEX	Set 1	2	(CCAAT)4	unpublished, ICRISAT
Xtxp012	PET	Set 6	4	$(CT)_{22}$	Kong et al. (2000)
Xtxp021	6-FAM	Set 4	4	$(AG)_{18}$	Kong et al. (2000)
Xtxp114	6-FAM	Set 3	3	$(AGG)_8$	Bhattramakki et al. (2000)
Xtxp136 (Kaf3)	VIC	Set 6	5	$(GCA)_5$	Bhattramakki et al. (2000)
Xtxp141	NED	Set 3	10	$(GA)_{23}$	Bhattramakki et al. (2000)
Xtxp265	VIC	Set 4	6	$(GAA)_{19}$	Bhattramakki et al. (2000)
Xtxp273 (Pbbf)	6-FAM	Set 5	8	$(TTG)_{20}$	Bhattramakki et al. (2000)
Xtxp278	NED	Set 1	7	$(TTG)_{12}$	Bhattramakki et al. (2000)
Xtxp286	6-FAM	Set 7	2.00	$(GCA)_4ACA(GCA)_5A$ $(CAA)_5 + (AAC)_9$	Kong et al. (2000)
Xtxp320 (PhyB)	NED	Set 4	1	$(AAG)_{20}$	Bhattramakki et al. (2000)
Xtxp321	PET	Set 1	8	$(GT)_4 + (AT)_6 + (CT)_{21}$	Bhattramakki et al. (2000)

* Unpublished, Agropolis-CIRAD-Genoplante.

products were loaded and mixed with formamide (PE-Applied Biosystems) and ROX-labelled GS500LIZ-3730 size standard (PE-Applied Biosystems). DNA fragments were denatured and size-fractioned using capillary electrophoresis on an ABI 3730 automatic DNA sequencer (PE-Applied Biosystems). The peaks were sized and the alleles called using genemapper software and the internal ROX GS500LIZ-3730 size standard (Figure 2). This system has the advantages of automated filling of capillaries, automated sample loading and rapid electrophoresis (Buhariwalla and Crouch, 2004). To verify the repeatability of each PCR and each capillary eletrophoresis run, a control sample (accession BTx623) was included during the PCR of each SSR marker and during each capillary electrophoresis run. Only alleles with a relative fluorescent unit of > 500 were scored.

Data analysis

All SSR markers showed high reproducibility with high consistency in the amplified product between the PCR and ABI runs of the control BTx623. Allele sizing and calling was done using genemapper ver 3.7 software. Adjustment for allele size inconsistencies and checking quality of markers was done by Allelobin. Genetic diversity parameters that is polymorphic information content (PIC), heterozygosity and number of alleles for each marker, % of polymorphic loci estimates, genetic diversity within and among the accessions and countries, genetic distances within and among accessions and genetic distances within and among the countries were done using powermarker version 3.25 and tools for population genetics analysis (TFPGA) softwares. TFPGA provided 3 estimates of heterozygosity, direct count, expected heterozygosities under Hardy-Weinburg equilibrium (H-W) and Nei`s (1978) unbiased heterozygosity. PIC values give the information that each marker impacts into the study, which is the measure of the usefulness of each marker in distinguishing one individual from another. Darwin version 5.0 software was used to calculate the principle component analysis (PCA) and clustering within and among the accessions and countries. To determine the genetic relationships and differentiation, the accessions were clustered based on the matrix of genetic similarities using the un-weighted pair group method using arithmetic averages (UPGMA) clustering algorithm. Dissimilarity was calculated from alleleic data, where dissimilarity index was calculated by simple matching coefficient. Genetic distances were computed for microsatellite data and trees constructed using the neighbour-joining method in Darwin version 5.0 software.

The principle component scores were not standardized and thus had variance equal to the corresponding eigen values. The UPGMA results were used to generate dendrograms. The robustness of the phylogenies was evaluated by bootstrapping (1000 permutations) replicates over loci. An exact test was used to determine possible deviations from Hardy-Weinberg equilibrium and the existence of non-random associations of genotypes across polymorphic co-dominant loci (Guo and Thompson, 1992; Weir, 1990). Exact tests were performed using TFPGA software. Analysis of molecular

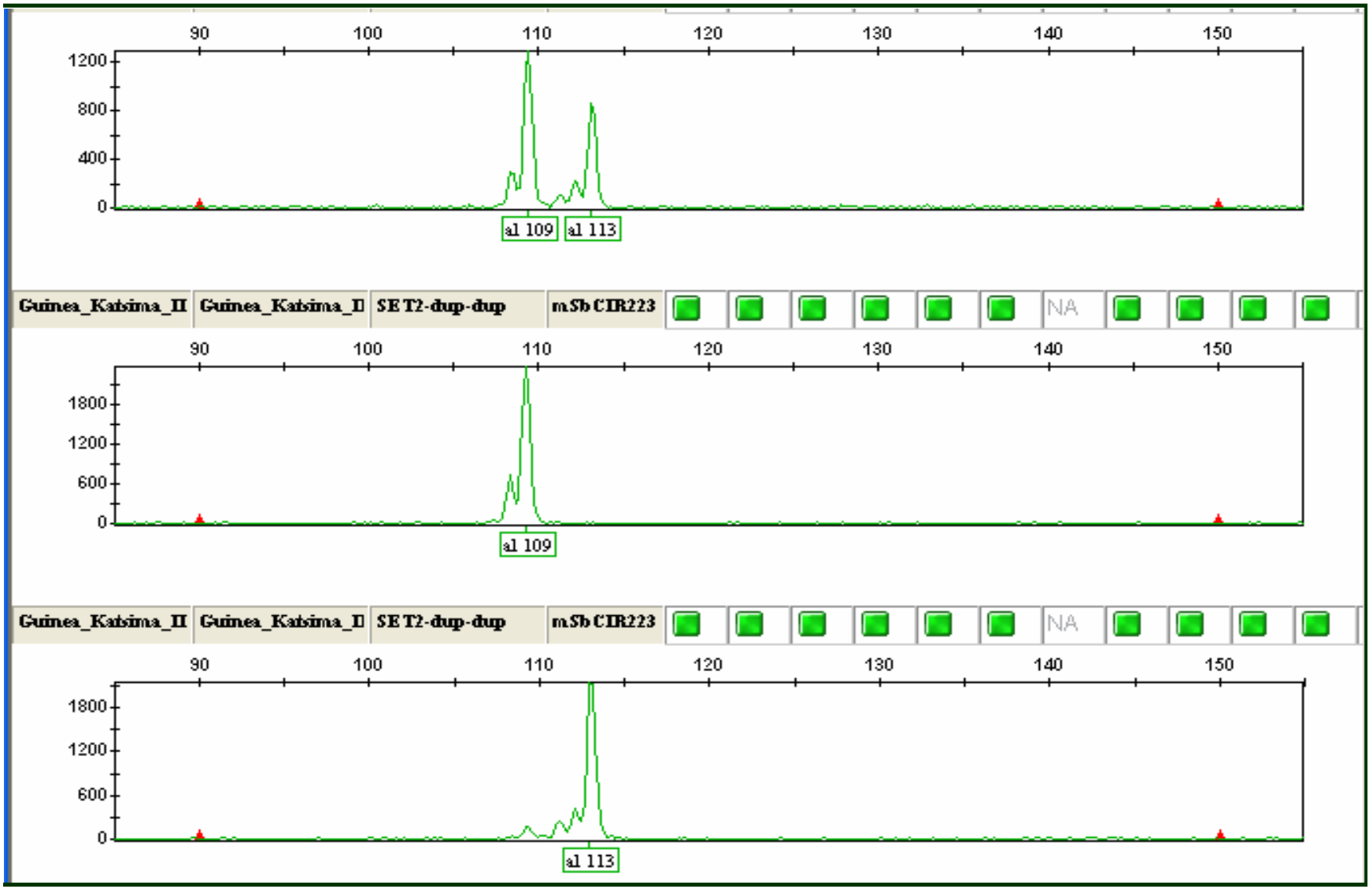

Figure 2. Electrophenograms from ABI 3730 capillary sequencer showing heterozygous individual (top) and homozygote individuals (middle and bottom). The numbers at the bottom of each peak indicate allele size in base pairs (bp).

Table 3. Performance of all markers in the bulks (Allelobin software).

Marker name	Repeat length	Quality index	Total alleles	Minimum allele	Maximum allele	Allele (%)	Rare alleles (< = 5%)
ISEP310	5	0.2009	2	159	204	204 (93.55)	
Xtxp321	2	0.1776	14	190	222	202 (22.58)	190, 192, 200, 206, 208, 210, 212, 222
Xtxp278	3	0.2191	4	249	285	249 (90.62)	252, 270, 285
Xcup11	4	0.26	3	154	170	170 (59.52)	154
CIR223	2	0.2139	7	108	126	116 (56.82)	120,126
sb6-84	2	0.3142	8	168	196	182 (42.86)	168,196
Sb4-72	2	0.1157	5	186	196	186 (57.89)	
Xtxp114	3	0.0719	2	211	214	214 (70.59)	
Xtxp141	2	0.2042	9	147	191	151 (51.35)	153,161,165,183
Xtxp21	2	0.4299	6	176	188	176 (61.29)	188
Xtxp265	3	0.4844	15	171	228	189 (16.67)	171, 198, 201, 204, 216, 219, 222, 228
Xtxp320	3	0.2985	8	266	290	278 (29.73)	
Xtxp273	3	0.202	11	163	211	193 (33.33)	163,184,199, 202, 211
CIR283	2	0.4556	10	99	147	117 (22.86)	131,141, 143,147
sbAG02	2	0.1923	5	96	120	96 (50.00)	116, 118, 120
Xtxp12	2	0.4695	9	171	195	175 (27.27)	179, 187, 191
Xtxp136	3	0.1702	2	241	244	241 (88.24)	
CIR286	2	0.3483	5	111	149	113 (64.71)	149
Xtxp286	3	0.4271	6	110	209	113 (37.93)	110, 194
CIR240	2	0.2117	7	112	158	112 (59.52)	136, 152, 154
CIR276	2	0.46	4	229	251	229 (39.39)	251
gpSb123	2	0.2236	4	288	296	290 (58.97)	294
Total			146				

variance (AMOVA, Excoffier et al. 1992) was used to partition SSR variation among-groups and among-populations within-groups. Within-population components were computed by a non-parametric permutation procedure, using 1000 permutations. AMOVA and F_{st} indices were calculated using the ARLEQUIN version 3.11 (http://cmpg.unibe.ch/software/arlequin3).

RESULTS

SSR marker performance

A total of 22 SSR markers were optimized and used to genotype 30 sorghum bulks and 49 individuals from 10 accessions with BTx623 being used as an internal control accession. Marker quality indices (Table 3) for both the bulk accessions and individual plants samples varied from 0.07 in Xtxp114 to 0.48 in Xtxp265 and 0.11 in SbAGB02 to 0.48 in Xtxp286, respectively, and the overall average quality index for the 22 markers was 0.27. The 22 SSR markers revealed a total of 146 different alleles among the 30 accessions, an average of 6.6 alleles per marker. The number of alleles detected in each of the 22 SSR markers was highly variable and ranged from 2 in Xisep0310, Xtxp114 and Xtxp136 to 15 by markers Xtxp 321 and Xtxp 265 while markers Sb4-72, Xtxp114, Xtxp 320 and Xtxp136 did not detect any rare alleles.

For the 49 individuals from 10 populations, the 22 SSR markers revealed a total of 108 alleles, with an average of 4.9 alleles per marker. The total number of alleles detected in the individual plants ranged from 2 in 4 markers (Xcup11, Xisep0310, Xtxp136 and Xtxp278) to 9 in Xtxp265 and Xtxp321.

In the bulk populations, polymorphism information content (PIC) ranged from 0.09 in markers ISEP0310 and Xtxp278 to 0.86 in marker Xtxp 265 while the average across all the markers was 0.54. The polymorphic information content (PIC) in the individuals from the 10 populations was much lower. The values ranged from 0.02 in Xtxp136 to 0.81 in marker mSbCIR283, with an average 0.49 across all the markers.

Pattern of genetic diversity and relationships

The total number of alleles in each of the 30 bulked accessions varied from 23 to 37 with an average of 30.17. The highest number of alleles (37) was observed in accessions CSM388 and 02SBF4189 whereas the lowest (23) was observed in accession FaraFara17, an accession originating from Nigeria. The number of alleles in the accessions was varying even among accessions of the same origin. The inter-racial pedigree breeding derivatives with 8 accessions had the most varying number of alleles between 25 - 37 while the least variability of alleles was found among the Guinea population derivatives (West African Guineas) with two accessions (between 28 to 29). The inter-racial pedigree breeding derivative that had the highest number of alleles was Nigeria with 26.85% and while the lowest was Cameroon with 2.98%.

The Nei's unbiased estimate of genetic diversity (H) among the 30 accessions across the 22 loci was relatively low at a mean value of 0.58. The mean heterozygosity for all the 30 accessions across the 22 loci was 0.29 which was lower than the mean Nei's unbiased estimate of gene diversity (H). There were relatively minimal differences between the observed heterozygosity and the Nei's unbiased estimate of gene diversity for all the microsatellite markers, which is characteristic of inbreeding species. The mean Nei's unbiased estimate of gene diversity (H) within countries was variable, ranging from the highest (H = 0.4518) in Mali to the lowest (H = 0.09) in Sudan. The average within country diversity was (H = 0.2931). The average heterozygosity was quite low (below 0.5) in all the countries. The highest level of heterozygosity was 0.3409 observed in Burkina Faso accessions while the lowest was 0.1818 in Cameroon and Sudan accessions. The difference between the average gene diversity and average heterozygosity was relatively small.

There was a high genetic differentiation within the accessions, which was significant at $p < 0.05$. Polymorphism in the ten accessions ranged from 22.73 to 86.36% across the 22 loci with a mean of 45.54%. The average Nei's gene diversity (H) within the 10 accessions was 0.54 across the 22 polymorphic loci while the average heterozygosity was 0.17. The population Nei's genetic diversity (H) was higher than within population genetic diversity, which is expected of selfing species as a result of low levels of inter-population hybridization.

The Roger's modified genetic distance was calculated to determine the relationships among the 30 populations. Pairwise genetic similarities between accessions were assessed based on Dice's genetic similarity co-efficients. The most distant accessions were ICSB38 (21), which is an introduced Caudatum, and IS3534B (25) (GD = 0.82 from and Sudan. The closest accessions were 02SB51 (5) which is a Guinea derivative and 97SB51 (8) from Burkina Faso. The genetic similarities between accessions calculated using pairwise dice's (1945) was the lowest (0.160) between IS1562 and FaraFara17 accessions from Cameroon and Nigeria respectively and the highest (0.833) between FambeB from Mali and AT623 of USA. Cluster analysis performed using the similarity matrix revealed five major groups for all acce-ssions except GPN99271 from Guinea, which appeared to be distant from all other groups (Figure 3). The number of acces-sions for each of the 5 groups varied from 2 to 8 and the patterns of grouping seemed to be irrespective of their geographical origins.

A multidimensional scatter plot (MDS) was used to assess the genetic relationships further and the genetic patterns were confirmed by principal co-ordinate analysis (PCoA) (Figure 4). The first 5 principal components from PCoA explained 57.2% of the total variation. A scatter plot of PC1 (24.8%) and PC2 (12.2%) revealed the same patterns of grouping as the cluster analysis. The first eigen vector explained 27.94 % variation. A scatter plot of the first and second axes of non-metric multi-dimensional scaling (MDS) revealed five clusters of inter-relationships among accessions. The analysis showed that the accessions generally clustered on the basis of the geographical origins. Accessions from Nigeria were distributed in two clusters B and E which also had accession IS 2756 from Burkina Faso while Niger accessions were in their own cluster C. Accession GPN 99 271 from Guinea was placed in its own cluster A. All accessions from Mali were in cluster D and grouped with accessions from Burkina Faso. Accessions in cluster B were widely distributed. The allele frequency based pair-wise genetic distances between countries calculated using powermarker V3.25 (Liu and Muse, 2004) revealed the relatedness of accessions on a country by country basis (Table 4). The countries pair-wise comparisons revealed that accessions from Niger and Sudan were the most distant, whereas accessions from Nigeria and Cameroon were the closest genetically. The accessions from Nigeria and Mali are also quite close. Materials from Nigeria and Guinea, Nigeria and Niger, Sudan and Guinea were equal.

The matrix of pair-wise genetic distances between countries was subjected to sequential agglomerative hierarchical nested (SAHN) using un-weighted pair-group analysis (UPGMA) and resulted in 4 distinct clusters (Figure 5). Niger, Cameroon and Nigeria were closely related and grouped together in cluster.

Population structure

The examination of the hierarchical partitioning of genetic variation by AMOVA in Arlequin version 4.0 demonstrated that genetic differentiation was significant at $P < 0.00$ using the exact value for population differentiation

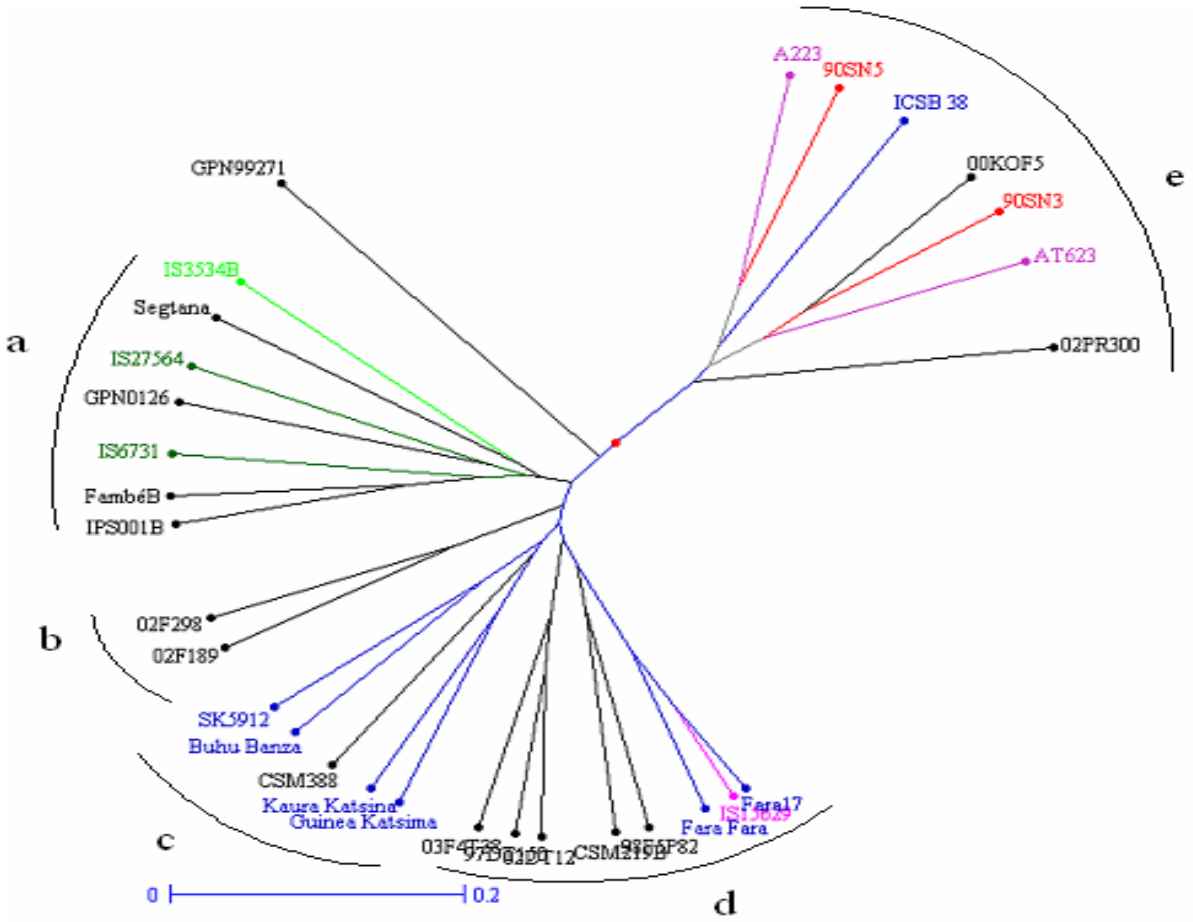

Figure 3. Neighbour joining tree showing relationships between 30 accessions from different origins using the UPGMA method of Darwin Ver. 4.0 (same colour code for all accessions that belong to the same country).

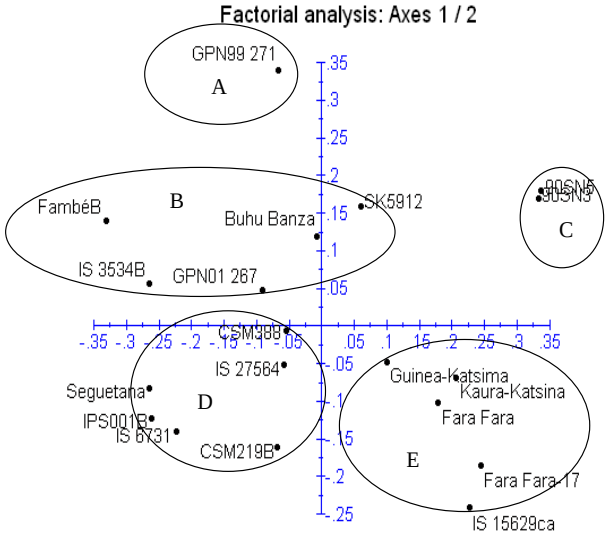

Figure 4. A Multidimensional scatter plot (MDS) showing the relationship of the accessions from West Africa.

(Raymond and Rousset, 1995) (Table 5). There was a clear genetic differentiation both among countries, among populations within countries and within the populations using significance tests based on 1000 permutations calculated according to Weir (1990; 1996). The genetic variation was higher among populations within countries with a variance component of 3.50 than within populations (with a variance component of 2.39). The genetic variation was lowest among countries with variance components of 0.576. Of the total diversity, 8.90% was attributed to country differences, 54.11% was attributed to population differences within the countries while 36.99% was attributed to differences within populations. The Fst index was 0.630, indicating a high level of genetic differentiation among the populations. The value of F, which is equivalent to F_{IT} and estimates the overall (total population) level of inbreeding, was 0.70 indicating a relatively high level of inbreeding while the value of theta (P) was 0.2342 showing an excess of homozygotes.

Table 4. Genetic distance matrices between countries calculated according to Nei and Li (1979) using powermarker ver. 3.25.

	Burkina Faso	Cameroon	Guinea	Mali	Niger	Nigeria	Sudan
Burkina Faso	****	****	****	****	****	****	****
Cameroon	0.52	****	****	****	****	****	****
Guinea	0.51	0.67	****	****	****	****	****
Mali	0.41	0.57	0.45	****	****	****	****
Niger	0.68	0.53	0.58	0.68	****	****	****
Nigeria	0.48	0.39	0.47	0.42	0.47	****	****
Sudan	0.55	0.66	0.47	0.51	0.75	0.61	****

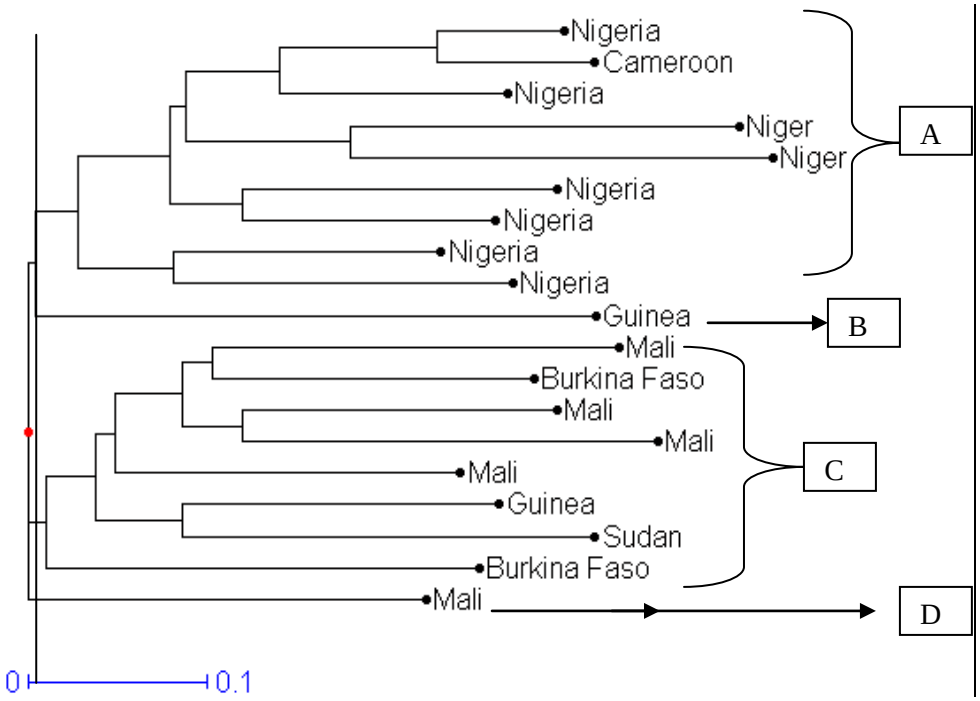

Figure 5. Dendrogram of distribution of diversity between Western Africa countries using binary data (Darwin 4.0).

The Hardy-Weinberg equilibrium assumes a random mating system, which can be assessed by computing the deviation of the observed heterozygosity from Nei`s unbiased estimate of gene diversity (H). The mean chi-square exact p-value test for deviation of the observed heterozygosities from H (Nei's unbiased estimate of gene diversity) using Markov chain method showed that the deviation from Hardy-Weinberg equilibrium was significant (p = 0.05) when the entire dataset was analyzed. The deviation from Hardy-Weinberg equilibrium was highly significant in all the 22 loci (P = 0.0000) except in loci Xtxp278 and Xtxp136. The deviation from the Hardy-Weinberg equilibrium is expected inbreeding species as the mating system systems is not expected to be random.

DISCUSSION

Most of the SSR markers that were used in this study showed high polymorphic information contents (PIC) with very good marker quality indices (Table 3). Brown et al. (1996) as well as Anas and Yoshinda (2004) while working on sorghum observed similarly high levels of polymorphisms. The mean diversity index per each SSR locus was 0.53 in the individuals and 0.58 in the bulks, which allowed us to discriminate each of the sorghum accessions. The SSRs used in this study covered all the sorghum genome (Dean et al., 1999). Thus, these markers should represent the genetic diversity among these sorghum lines. All the 22 SSR loci were poly-

Table 5. AMOVA calculated according to weir, (Weir, 1996).

Source of variation	d.f	Sum of squares	Variance component	% of variation	P-value	Fst
Among groups (country)	3	144.96	0.58	8.9	0.094	
Among accessions within group	6	218.38	3.50	54.11	< 0.001	0.63
Within accessions	88	210.58	2.39	36.99	< 0.001	

morphic in the individuals (except locus mSbCIR 286) and bulks, as seen in previous studies of sorghum germplasm with diverse geographic origins (Grenier et al., 2000b; Casa et al., 2005).

The average number of alleles per locus identified in this study was lower in individuals (4.91) and higher in bulks (6.27) than the average of 5.9 previously reported in elite sorghum lines (Smith et al., 2000), though both were lower than obtained in other studies (Menz et al., 2004; Uptmoor et al., 2003; Dje et al., 2000; Grenier et al., 2000). The SSR loci were able to uniquely identify each of our accessions. Fragment sizes obtained in this study, were usually across a wider range than previously reported in studies of the same loci in other sorghum varieties (Brown et al., 1996; Dean et al., 1999; Dje et al., 2000; Ghebru et al., 2002). The number of observed alleles for most of the loci was higher than those observed by Schlötterer (1998). This suggests that the sorghum accessions studied may be exceptionally polymorphic, providing more size variation within 30 accessions compared to other studies (Brown et al., 1996; Dean et al., 1999; Dje et al., 2000; Grenier et al., 2000; Ghebru et al., 2002). This could also be due to the]inclusion of interracial varieties in the study.

Within accessions, we observed substantial inbreeding (F_{IS} = 0.70), which would be expected as a consequence of self-fertilization at a rate of S = 2 (F_{IS}/ (1+FIS) = 0.71. A similar level of selfing has been reported for sorghum by Ellstrand and Foster (1983). Similar values of inbreeding coefficient (F_{IS} = 0.70) were obtained using both alloenzyme and microsatellite markers in cultivated sorghum sampled *in situ* in North-Western Morroco (Dje et al., 1999). The coefficients obtained were higher than those of Dje et al. (2000). The large F_{IS} values show the large degree of relatedness among the individuals within the sorghum accessions studied. This may be due to high levels of selection for uniformity exercised by farmers in the collection areas (Ghebru et al., 2002; Ghebru, personal interview of local farmers; Araya et al. 1997; Moa, 1999; Dje et al., 2000).

Wright (1978) cited by Kiambi et al. (2005); Semagn et al. (2001) and Hartl (1987), suggested that an Fst range of 0 - 0.05 indicates little differentiation, 0.05 - 0.15 moderate and 0.15 - 0.25 large differentiation and above 0.25 indicates very large differentiation. In this study, the level of population differentiation was Fst = 0.63, which is very large using the suggested parameters. This F_{ST} value was slightly lower than in other sorghum population genetics studies. Dje et al. (2000) reported Fst = 0.68 for landraces on the basis of only three different SSR loci. The western African sorghums show a high level of allelic fixation similar to previous reports by Ghebru et al. (2002) in sorghum landrace populations. Apparently, this indicates an increased level of inbreeding and hence high levels of homozygosity. The Fst values observed in this study were relatively high, indicating an increased degree of allele fixation. New alleles may be generated because of outcrossing and subsequent intralocus recombination including gene conversion. Because local farmers practice a lot of selection, effective population sizes are reduced, therefore increasing the opportunity for fixation of alleles.

In this study, a high inter-population differentiation was found (54.11%). Isoenzyme studies have shown that selfing species have nearly 60% of their genetic diversity distributed among populations while less than 25% of the genetic diversity of mixed mating and outcrossing species is found among populations (Semagn et al. 2001; Hamrick and Godt, 1997).

Sorghum accessions from western Africa tends to have a wide genetic background, the accessions divided into 4 groups A - E. In this study, with a few exceptions, a clear tendency of clustering was observed based on the accessions race and breeding origin as well as their country of origin, similar clustering was also observed by Geleta et al. (2006). Most of the accessions given the same name or similar identification characters by farmers were grouped together, similar results were also obtained by Ghebru et al. (2002) based on the accession names (given by farmers) and again on the basis of their country of origin. Similar results were also obtained by Anas and Yoshinda (2004). In many instances, species exhibit a spatial structure of genetic variation across their ecogeographic range. Different levels of genetic diversity among countries may be due to several factors including mating systems, rate of mutation, migration and dispersal mechanisms, biotic and abiotic selection intensities which are determined by geographic location, climate and soil (Kiambi et al., 2005).

Information about the relationship among breeding materials and the genetic diversity in the available germplasm is important in making choices of parents in

breeding programs. This applies particularly to hybrid breeding where recognition and exploitation of heterotic patterns between different sources of germplasm are important for success. The principal coordinate analysis PCoA based on genetic distance (GD) estimates determined by SSR data for the 49 individuals, provided a distinct separation of lines from different germplasm groups in West African sorghum. The ICRISAT B lines, which are also maintainer lines, were close together though they did not appear in the same cluster while the other hybrid parents from heterosis study also classified as landraces clustered in the same axis but in two groups. Similar results were also observed in maize by (Melchinger et al., 1991; Messmer et al., 1992; Dubreuil et al., 1996) and barley (Melchinger et al., 1994). Likewise, cluster analysis (CA) of 148 US maize inbreeds (Dubreuil et al., 1996) as well as B- and R- lines in sorghum (Ahnert et al., 1996) and sunflower (Hongtrakul et al., 1997) partitioned the lines in accordance with their origin from different breeding groups and pedigree information.

The cluster analysis based on genetic similarity among the 30 bulked sorghum accessions examined showed a clear demarcation of the germplasm mainly according to their races and breeding origin and not to their B- or parental classification. The sorghum races represented in this study included guinea, durra/caudatum, guinea/ caudatum and caudatum while the breeding origins included pedigree breeding lines and their derivatives, landraces and landrace derivatives, maintainer lines, maintainer products of mutation breeding, and maintainer derivative from guinea populations. Each of the clusters E, D and B were clearly differentiated from other groups and all represented the guinea race, landraces (D and E) and a maintainer line from guinea derivative (cluster B). Clusters A and C had the guinea/caudatum and durra/ caudatum races respectively while cluster F contained predominantly the guinea race. Cluster G had representatives from caudatum and guinea/caudatum while cluster H had only guinea/caudatum. All materials in both clusters were of pedigree breeding and their derivatives breeding origin. Cluster I was a mixture of guinea/ caudatum, durra/caudatun and guinea races but the materials were all landraces. Similar results were obtained in sorghum by Menz et al. 2004 where the sorghum lines were grouped according to their working groups.

This study provides a detailed analysis and quantification of genetic diversity in sorghum accessions of West African origin. The data also reaffirm the power of SSR markers to distinctly group closely related accessions. Several authors have indicated that SSR technology is highly cost effective (Smith et al., 2000) and that this technology could easily be employed in resource-poor countries. It could provide efficient and fast screening for both germplasm conservation and crop improvement. The data demonstrates that the West African sorghum accessions contain a great deal of genetic diversity as indicated by the observed number of alleles. The high genetic diversity value among the sorghum accessions (landraces, breeding entries and maintainer lines) indicates that the level of genetic diversity was not influenced by breeding activities. The consistent clustering of most breeding entries/races from Western Africa close to each other in the present study apparently substantiates that the marker system used has a high potential in quantifying the level of similarity and relationships among sorghum germplasm.

The results of this study suggest that elite sorghum germplasm should be grouped by genetic background and not by existing B- or R-line classification. Moreover, the traditional inter-group crossing approach utilized by public breeders may actually dilute potential heterotic patterns. The results reported by Ahnert et al. (1996) also support this conclusion.

REFERENCES

Ahnert D, Lee M, Austin DF, Livini C, Woodman WL (1996). Genetic Diversity among Elite Sorghum Inbred Lines Assessed with DNA Markers and Pedigree Information. Crop Sci. 36(5):1385-1392.

Anas Yoshida T (2004). Genetic diversity among Japanese cultivated sorghum assessed with simple sequence repeats markers. Plant Product. Sci. 7(2):217-223.

Araya D, Tesfamariam S, Sium T (1997). Survey of traditional breeding practices and maintenance of the landraces in Zoba Debub University of Asmara, Eritrea.

Bhattramakki D, Dong JM, Chhabra AK, Hart GE (2000). An intergrated SSR and RFLP limkage map of *Sorghum bicolor* (L.) Moench Genome 43:988-1002.

Brown SM, Hopkins MS, Mitchell SE, Senior ML, Wang TY, Duncan RR, Gonzalez-Candelas F, Kresovich S (1996). Multiple methods for the identification of polymorphic simple sequence repeats (SSRs) in sorghum *sorghum bicolor* (L.) Moench. Theor. Appl. Genet. 93:190-198.

Buhariwalla HK, Crouch JH (2004). Optimization of marker screening protocol to assess the degree and distribution of genetic diversity in landrecas of pigeonpea. In: Bramel, PJ (2004). Assesing the risk of losses of Biodiversity in Traditinal Cropping Systems: A case study of pigeonpea in Andhara Pradesh. International Crops Institute for the semi-Arid tropics Pantacheru, 502, 324, Andhra Pradesh, India. p. 168

Casa AM, Mitchell SE, Hamblin MT, Sun H, Bowers JE, Paterson AH, Aquadro CF, Kresovich S (2005). Diversity and selection in sorghum: simultaneous analyses using simple sequence repeats. Theor. Appl. Genet. 111(1):23-30.

Casa AM, Mitchell SE, Smith OS, Register III JC, Wessler SR, Kresovich S (2002). Evaluation of *Hbr* (MITE) markers for assessment of genetic relationships among maize (*Zea mays* L) Inbred lines. Theor. Appl. Genet. 104:104-110. darwinnmsuedu/~molbio/plants/sorghumhtml

Dean RE, Dahlberg JA, Hopkins MS, Mitchell SE, Kresovich S (1999). Genetic redundancy and diversity among 'Orange' accessions in the US national sorghum collection as assessed with simple sequence repeat (SSR) markers. Crop Sci. 39: 1215-1221

Dje` Y, Forcioli D, Ater M, Lefe`bvre C, Vekemans X (1999). Assessing population genetic structure of sorghum landraces from Northwestern Morocco using allozyme and microsatellite markers. Theor. Appl. Genet. 99: 157-163

Dje` Y, Heuertz M, Lefe`bvre C, Vekemans X (2000). Assessment of genetic diversity within and among germplasm accession in cultivated sorghum using microsatellite markers.

De Vries J, Toenniessen G (2001) Securing the harvest: biotechnology, breeding and seed systems for African crops: CABI Publ Wallingford 59-60.

Dogget H (1988) Sorghum, 2nd edition Longman, UK , Burnt Mill, Harlow, Essex, England.

Dubreuil P, Dufour P, Krejci E, Causse M, De Vienne D, Gallais A, Charcosset A (1996). Organization of RFLP diversity among inbred lines of maize representing the most significant heterotic groups. Crop Sci. 36:790-799.

Ellstrand NC, Foster KW (1983). Impact of population structure on the apparent outcrossing rate of grain sorghum (*Sorghum bicolor*). Theor. Appl. Genet. 66: 323-327.

FAO (1995) Production Yearbook Vol. 49, FAO. Roe, Italy

FAOSTAT (2004) http://appsfaoorg/defaulthtm.

Folksterman RT, Fredrick H, Rattunde W, Chandra S, Raju SG, Hash TC (2005). The pattern of genetic diversity of Guinea-race *Sorghum bicolor* (L) Moench landraces as revealed with SSR markers.

Hallauer AR (1999). Temperate maize and heterosis pp. 353-361 *In:* Coors j, Pandey S (ed). Genetics and Exploitation of Heterosis in Crops CIMMYT, Mexico City, 17-22 Aug 1997 ASA, Madison, WI.

Hamrick JL, Godt MJW (1997). Allozyme diversity in cultivated crops. Crop Sci. 37: 26-30

Hartl DL (1987). A primer of population genetics 2nd Edition Sinauer, Associates Inc Publishers Sunderland, Massachusetts http://cmpgunibech/software/arlequin3.

Hongtrakul V, Huestis GM, Knapp SJ (1997). Amplified fragment length polymorphisms as a tool for DNA fingerprinting sunflower germplasm: Genetic diversity among oilseed inbred lines. Theor. Appl. Genet. 95:400-407.

House LR (1995). Sorghum: One of the world's great cereals. Afr. Crop Sci. J. 3: 135-142

Geleta N, Maryke T, Labuschagne Viljoen CD (2006). Genetic diversity analysis in sorghum germplasm as estimated by AFLP, SSR and morpho-agronomical markers. Biodivers. Conserv. 15:3251-3265.

Ghebru B, Schmidt RJ, Bennetzen JL (2002). Genetic diversity of Eritrean Sorghum landraces assessed with simple sequence repeat (SSR) markers. Theor. Appl. Genet. 105:229-236.

Grenier C, Bramel-Cox PJ, Noirot M, Prasada Rao KA, Hamon P (2000a). Assessment of genetic diversity in three subsets constituted from ICRISAT sorghum collection using random vs non-random sampling procedures A using morpho-agronomical and passport data. Theor. Appl. Genet. 101:190-196.

Grenier C, Deu M, Kresovich S, Bramel-Cox PJ, Hamon P (2000b). Assessment of genetic diversity in three subsets constituted from ICRISAT sorghum collection using random vs non-random sampling procedures B Using molecular markers. Theor. Appl. Genet. 101:197-202

Guo SW, Thompson EA (1992). Performing the exact test of Hardy-Weinberg proportion for multiple alleles. 48: 361-372.

Kiambi DK, Newbury HJ, Ford-Lloyd BV, Dawson I (2005). Contrasting genetic diversity among *Oryza longistaminata* (A Chev et Roehr) from different geographic origins using AFLP. Afr. J. Biotechnol. Vol. 4(4):308-317.

Kong L, Dong J, Hart G (2000). Charecteristics, linkage-map positions, and allelic differentiation of sorghum bicolor (L) Moench DNA simple sequence repeats (SSRs). Theor. Appl. Genet. 101:438-448

Liu K, Muse S (2004). POWERMARKER: New genetic data analysis software Version 30 Free program distributed by the author over the internet from http://www Powermarkernet.

Mace ES, Hutokshi k, Buhariwalla Crouch JH (2004). A high-throughput DNA extraction protocol for tropical molecular breeding programs. Plant Mol. Biol. Rep. 21:459a-459h.

Melchinger AE, Messmer MM, Lee M, Woodman WL, Lamkey KR (1991). Diversity and relationships among US maize inbreds revealed by restriction fragment length polymorphisms. Crop Sci. 31: 669-678.

Melchinger AE, Andreas G, Mahendra S, Messmer MM (1994). Relationships among European Barley Germplasm: I Genetic Diversity among Winter and Spring Cultivars Revealed by RFLPs. 34(5):1199-1205.

Menz MA, Klein RR, Unhruh NC, Rooney WI, Klein PE, Mullet JE (2004). Genetic diversity of public inbreds of sorghum determined by mapped AFLP and SSR markers. Crop Sci. 44:1236-1244.

Messmer MM, Melchinger AE, Boppenmaier J, Herrmann RG (1992). Relationships among early European maize (Zea maysL) inbred lines: II Comparison of pedigree and RFLP data. Crop Sci. 33:944-950.

Ministry of Agriculture (MOA) (1999). Lowland sorghum in Eritrea: Survey of local landraces of lowland sorghums as described by farmers, MOA, Asmara, Eritrea `1.

Nei M (1978). Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics 89: 583-590.

Nei M, Li W (1979) Mathematical model for studying genetic variation in terms of Restriction endonucleases. Proc. Natl. Acad. Sci. 76(10):5269-5273.

Pejic IP, Ajamone-Marsan M, Morgante M, Kozumplick V, Castiglioni P, Taramino G, Motto M (1998). Comparative analysis of genetic similarity among maize inbred lines detected by RFLPs, RAPDs, SSRs and AFLPs. Thoer. Appl. Genet. 97: 1248-1255.

Raymond M, Rousset F (1995). GENEPOP (ver 12): A population genetics software for exact test and ecumenicism. J. Hered. 86:248-249.

Saghai MA, Yang GP, Zhang Q, Gravois KA (1997). Correlation between molecular marker distance and hybrid performance in US Southern long grain rice. Crop Sci. 37:145-150.

Schloss SJ, Mitchell SE, White GM, Kukatla R, Bowers JE, Paterson AH, Kresovich S (2002). Charecterization of RFLP probe sequences for gene discovery and SSR development in *Sorghum bicolor* (L) moench. Theor. Appl. Genet. 105:912-920.

Schlötterer C, Ritter R, Harr B, Brem G (1998). High mutation rate of a long microsatellite allele in *Drosophila melanogaster* provides evidence for allele-specific mutation rates. Mol. Biol. Evol. 15:1269-1274.

Semagn K, Stedje B, Bjornstad A (2001). Analysis of genetic diversity and structure in Ethiopian populations of *Phytolacca dodecandra* using RAPD. 135:51-60.

Senior ML, Murphy JP, Goodman MM, Stuber CW (1998). Utility of SSRs for determining genetic similarities and relationships in maize using agarose gel system. Crop Sci. 38:1088-1098.

Smith JSC, Chin ECL, Shu H, Smith OS, Wall SJ, Senior ML, Mitchell SE, Kresovich S, Ziegle J (1997). An evaluation of the utility of SSR loci as molecular markers in maize (*Zea mays* L.): Comparisons with data from RFLPs and pedigree. Theor. Appl. Genet. 95:163-173.

Smith JSC, Kresovich S, Hopkins MS, Mitchell SE, Dean RE, Woodman WL, Lee M, Porter K (2000). Genetic diversity among elite sorghum inbred lines assessed with simple sequence repeats. Crop Sci. 40:226-232.

Stuber CW (1994). Hereosis in plant breeding. Plant Breed Rev. 12:227-251.

Taramino G, Tarchini R, Ferrario S, Lee M, Pe` ME (1997). Characterization and mapping of simple sequence repeats (SSRs) in *Sorghum bicolor* . Theor. Appl. Genet. 95: 66-72.

Uptmoor R, Wenzel W, Friedt W, Donaldson G, Ayisi K, Ordon F (2003). Comparative analysis on the genetic relatedness of Sorghum bicolor accessions from Sourthern Africa by RAPDs, AFLPs and SSRs. Thoer. Appl. Genet. 106: 1316-1325.

Weir BS (1990). Genetic data analysis Sinauer, Sunderland, MA.

Weir BS (1996). Genetic data analysis II. Sinauer, Sunderland.

Wright S (1978). Evolution and the genetics of populations Vol. 4 variability within and among natural populationsuniversity of Chicago Press Chicago.

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Influence of culture medium and age of zygotic embryos on *in vitro* germination of *Elaeis guineensis* Jacq.

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Oil palm cultivation has acquired economic importance due to rising demand for vegetable oils used in food, pharmaceutical, cosmetic and most recently, in biofuel industries. However, the commercial production increase of oil palm is limited by plantlet production, as it primarily uses seeds that require a long germination period and have low germination rates. In this study we evaluated the effect of embryo age (time after pollination), culture media and presence of carbohydrates on *in vitro* germination of zygotic embryos of oil palm (*Elaeis guineensis* Jacq.) Manicoré hybrid. Embryos at 80, 90, 100 and 110 days post-anthesis were inoculated in MS medium or modified Y3 medium, with or without 3% sucrose. Media were renewed after 30 days of inoculation, and plantlets were kept for 45 days until collection for histological analyses. The embryos did not germinate in medium without sucrose. Embryos of 100 days post-anthesis in MS medium showed the best result for germination rate (88%), which did not differ statistically from Y3 medium. As for the parameters, 90-day embryos showed better results for number and length of roots in Y3 medium. It was concluded that 90-day embryos cultivated in Y3 medium generate plantlets in better conditions to be transferred to acclimatization.

Key words: Oil palm (*Elaeis guineensis* Jacq.), germination, sucrose, Y3 medium, MS, anatomy, embryo culture.

INTRODUCTION

Oil palm (*Elaeis guineensis* Jacq.) is a monocot perennial and monoecious plant from the Arecaceae family. It is also an oilseed plant with a long life cycle (Morcillo et al., 2007). This crop has been economically important due to

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high oil yield of improved genotypes, approximately 6,000 kg ha^{-1} (Boari, 2008). It is even much higher than soy, the most widely cultivated oilseed crop, which produces approximately 500 kg ha^{-1}. Another relevant factor is the variety of oils produced, which may be intended for food, pharmaceutical, cosmetic, and biofuel production (Boari, 2008; Mya et al., 2010). Biofuel has enhanced interest in oil palm due to the need for fuel production through alternative and renewable energy sources, which contributes to environmental preservation, and also because fossil fuels are finite resources (Ghassan et al., 2003).

Scientific research on oil palm has enabled the generation of highly adapted and productive plants, such as the interspecific Manicoré hybrid from crossing between *E. guineensis* and *Elaeis oleifera.* This hybrid produces a large amount of oil, yielding approximately 30 tons per cluster / ha / year and is resistant to lethal yellowing, an anomaly of yet unknown cause that affects oil palm crops causing major damage (Campos, 2011). Brazil currently has 95,000 hectares intended for oil palm cultivation and approximately 75 million hectares of land suitable for palm growing (Agrianual, 2012). In this context, there is government action to stimulate culture expansion for approximately seven years, and production is estimated at 12 million seeds per year (Collares, 2011). This increasing demand must be accompanied by commercial-scale availability of plantlets. As it is a monocot plant, this species cannot be propagated by microcuttings. Propagation is usually by seeds that require a long time to germinate (1 to 3 years) and show low germination rates (approximately 30%) (Martine et al., 2009; Luis et al., 2010). Thus, there is need for developing techniques that improve germination and reduce time for establishing new plantings. Tissue culture can be used as a tool for optimizing embryo germination of *E. guineensis* Jacq through isolation and cultivation under appropriate conditions (Torres et al., 1998). Composition of culture medium is a major factor influencing resumption of *in vitro* embryo development, and it is necessary to establish an osmotic and nutrient balance suitable for embryo regeneration (Suranthran et al., 2011). Among the components in culture medium, sucrose stands out as the most commonly used carbohydrate source, which also regulates medium osmolarity. Immature embryos usually require carbohydrate sources for their development since they have no reserves, and the younger the embryo, the higher the osmolality required (Hu and Ferreira, 1998). Besides germination, proper development of anatomical structures of *in vitro* plantlets is of utmost importance for successful acclimatization. Development of shoot and root systems reflect the acclimatization ability of plantlets and can be assessed by histological analysis.

This study evaluated the effect of embryo age (time after pollination), culture medium, and presence of carbohydrates on embryo germination of *E. guineensis* Jacq. In addition, we analyzed plantlet development using anatomical parameters.

MATERIALS AND METHODS

Plant material

The trials were conducted at the Central Laboratory of Molecular Biology and Laboratory of Plant Anatomy, Federal University of Lavras, Brazil. Immature fruits of *E. guineensis* Jacq. Manicoré hybrid, at different days after pollination (80, 90, 100 and 110), were provided by Denpasa Company, state of Pará, for germination experiments.

Disinfestation and *in vitro* germination

Fruits were washed with sodium hypochlorite (1.25%) and then broken using a vice grip to remove epicarp, mesocarp and endocarp, and expose almonds. Almonds were washed with water and immersed in 70% alcohol for 30 s under laminar flow. Then, they were transferred to sodium hypochlorite solution (1.25%) containing three drops of Tween per 100 ml of solution and washed three times in autoclaved distilled water under continuous stirring. After disinfection, embryos were isolated from almonds, inoculated in MS medium (Murashige and Skoog, 1962) and modified Y3 medium (Eeuwens, 1978) without the addition of amino acids, supplemented or not with 3% sucrose, pH adjusted to 5.7 ± 0.1, and solidified with agar 0.6% (w/v). After inoculation, embryos were kept for 30 days, in 16 h photoperiods at 26 ± 2°C. Later they were transferred to Magenta jars containing the same culture medium, where they remained for 45 days under the same conditions mentioned above. Germination rate and embryo size were evaluated at 30 days after inoculation in embryos showing at least some radicles. After 75 days of embryo inoculation, plantlets were assessed for the following parameters: number of leaves, shoot size, presence of roots, and size of root system.

Anatomy of plantlets

After 75 days of germination, *in vitro* culture plantlets were collected and fixed in FAA70 (Johansen, 1940) for 48 h, then transferred to 70% ethanol and kept until analysis. Transverse sections of leaves and roots were carried out in table microtome according to the method described by Melo et al. (2007). Sections were cleared in 50% sodium hypochlorite for 10 min, washed twice in distilled water for 10 min, and stained with safranin solution and Astra blue (safrablau 7:3) for 30 s. Then, sections were rinsed in distilled water and mounted on slides with 50% glycerol (Kraus and Arduin, 1997). Slides were observed and photographed in photonic microscope coupled to a digital camera. These photomicrographs were used for measuring anatomical parameters in an image analysis program (ImageTool - UTHSCSA) calibrated with the same microscopic ruler and photomicrographed at the same magnifications. Four measurements were taken at each anatomical feature per replicate. Leaves were evaluated for the following characteristics: epidermal thickness of abaxial and adaxial surfaces, mesophyll thickness, cuticle thickness, hypodermal thickness, sclerenchyma thickness, phloem thickness, and diameter of metaxylem cells in the midrib. The roots were evaluated for epidermal thickness, cortex thickness, exodermis thickness, endoderm thickness, total root diameter, vascular cylinder diameter, metaxylem cell diameter, phloem thickness, total root area, and vascular cylinder area using the ImageJ® program. We used a completely randomized design with two types of culture medium (MS and Y3), two concentrations of

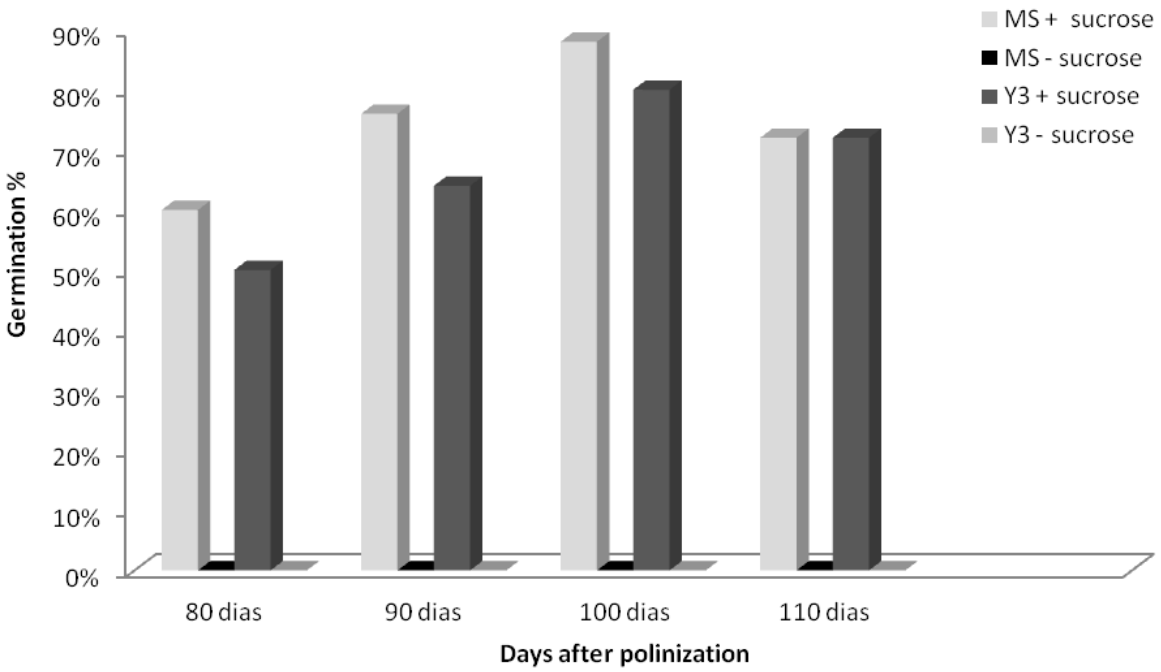

Figure 1. *In vitro* germination percentage of *E. guineensis* Jacq. hybrid Manicoré embryo with 80, 90, 100 and 110 days after anthesis, inoculated in different culture medium.

sucrose (presence or absence in the medium) and four embryo ages (80, 90, 100, 110 days) after pollination in five replicates, each containing five embryos. The data were subjected to analysis of variance, and means were compared by Tukey test at 5% probability using SISVAR ® program (Ferreira, 2008).

RESULTS AND DISCUSSION

Germination of embryos

Embryo germination of *E. guineensis* Jacq. Manicoré hybrid was influenced by culture medium composition, sucrose in the medium and embryo age, with statistical differences occurring between treatments. It was found that 80, 90, 100 and 110-day embryos cultivated on MS and Y3 media with addition of sucrose germinated, whereas those grown in the same culture media without addition of sucrose showed no germination (Figure 1). The highest germination rate (88%) was obtained in 100-day embryos inoculated with MS medium and sucrose. However, this rate did not differ significantly from treatments containing 90 and 110-day embryos in the same culture medium (72 and 76% germination respectively). Similar behavior was found in embryos in modified Y3 medium, with no statistical differences between treatments containing 100 and 110-day embryos. Carbohydrate sources were essential for embryo development of palm oil BRS Manicoré hybrid, regardless of growth stages in MS and Y3 culture media. Carbohydrate sources in medium influenced various metabolic processes, affecting growth and maintaining proper osmolarity for embryo development (Hu and Ferreira, 1998). Cardoso et al. (2010) evaluated germination of CN 470 oil palm hybrid in MS and full Y3 medium and found the highest germination rate (85.18%) in the treatment in MS medium.

Torres et al. (2005) found that addition of sucrose to culture medium was essential for embryo growth and development of *Heliconia rostrata*. Pereira et al. (2006) evaluated embryo germination of *Uncaria guianensis* (Wild) DC. and found 100% germination with 15 g L^{-1} sucrose, regardless of concentration in MS medium. Nunes et al. (2008) assessed different concentrations of sucrose and physiological stages of fruit ripening in germination of Jatropha and found that a higher concentration of sucrose (60 gL^{-1}) provided a higher number of germinated immature embryos (83.68%). Ribeiro et al. (2011) obtained no roots or leaf sheaths in plantlets from embryos of mature fruits grown in medium without sucrose, and associated with result of lack of carbohydrate reserves sufficient for full plant development.

In addition to *in vitro* germination, plantlet acclimatization has been limiting micropropagation of many species, especially palm trees. Transferring plants from a protected, sterile environment with carbohydrates and saturated humidity to non-sterile, low humidity environments without carbohydrates has been leading to plant loss, low growth rate and extended period in obtaining completely acclimatized plants (Souza Júnior et al.,

Table 1. Seedlings length - cm (SL) after 30 days and shoot length - cm (ShL), leaf number (LN), roots number (RN) and root length - cm (RL) of *Elaeis guineensis* Jacq. hybrid Manicoré seedlings after 75 days on MS medium are supplemented with sucrose and Y3.

Age (days)	Culture medium	SL (cm)	ShL (cm)	LN	RN	RL (cm)
80	MS	0.91^{B}	1.37^{B}	1.37^{B}	0.12^{A}	0.37^{A}
90		0.96^{B}	2.05^{A}	2.12^{A}	0.00	0.00
100		1.24^{A}	1.56^{B}	0.62^{B}	0.25^{A}	0.33^{A}
110		0.80^{B}	1.36^{B}	1.87^{A}	0.00	0.00
80	Y3	1.13^{a}	2.00^{a}	2.00^{a}	0.12^{b}	0.15^{b}
90		0.67^{b}	1.27^{b}	2.62^{a}	0.50^{a}	0.53 ^{b}
100		0.79^{b}	1.12^{b}	0.50^{b}	0.62^{a}	2.91^{a}
110		0.79^{b}	2.22^{a}	2.50^{a}	0.12^{b}	0.07^{b}

Means followed by the same capital letters do not differ, in the column, at 5% of probability and means followed by the same letters do not differ, in the column, at 5% of probability by Tukey.

2001). Thus, as structural changes that may occur in plantlets are detected, it is possible to optimize and even control *in vitro* conditions to maximize plant survival during acclimatization (Apóstolo et al., 2005).

In this study, we analyzed some growth parameters such as size of germinated embryos, size of shoots, number of leaves, presence of roots, and root size (Table 1). Y3 medium provided better conditions for parameters related to root formation. It should probably be related to the higher amount of salts in MS medium and the different nitrogen source, compared with Y3 medium. According to Assis and Teixeira (1998), the influence of culture medium on rooting is related to carbon-nitrogen relationship. Moderate nitrogen deficiency is usually more beneficial to rooting than excessive or even adequate levels. According to Ribeiro et al. (2011), the higher concentration of salts in MS medium (100%) inhibited root growth in oil palm plantlets thus confirming Ferreira et al. (2002), who assessed embryonic axes of cupuassu (*Theobroma grandiflorum* Schum.) in different salt concentrations and found that high nutrient availability may prevent plantlet rooting.

Anatomy of plantlets

Anatomical differences in leaves were also found in treatments in MS and Y3 media associated with different ages after pollination (Figure 2). With regard to epidermal thickness of abaxial and adaxial surfaces of plantlets grown in MS medium, all ages showed developed epidermis. Only plantlets from 100 day post-anthesis embryos showed lower results for both parameters, and the same behavior was found in Y3 medium. As for cuticle deposition, plantlets obtained from embryos at 110 days post-anthesis in MS medium and from 90-day embryos in Y3 medium showed better results. Regarding hypodermis, no statistical differences were found between treatments (Table 2). Leaf cuticle thickness can have different functions relating to leaf protection and resistance, such as reducing herbivore digestibility, limiting entry of pathogens and water and nutrient exudation, and also facilitates acclimatization of *in vitro* plants (Akin and Robinson, 1982; Rathi, 1998). Cuticle thickness varies with environmental conditions (Taiz and Zeiger, 2004), and lack of epicuticular wax on leaves of *in vitro* plants occurs as a result of high humidity in the culture flask. Rapid dehydration of *in vitro* plants transferred to a greenhouse is correlated, among other factors, with low deposition of cuticles and epicuticular wax, and high reduction of leaf mesophyll (Machado and Biasi, 2011).

With regard to leaf development, mesophyll, sclerenchyma and phloem thickness, and metaxylem diameter showed good results with plantlets from 110 day embryos in MS medium and 90 day embryos in Y3 medium, except phloem thickness (Table 3). Increase in leaf thickness, especially cell elongation or addiction is associated with decrease in mesophyll resistance to carbon dioxide (Nobel, 1977) and correlated with increase of potentially limiting factors to photosynthesis such as Rubisco, electron carriers and stomatal conductance (Bjorkman,1981). Chazdon and Kaufman (1993) found that photosynthetic capacity was correlated with mesophyll thickness in two species of *Piper* (Piperaceae). Development of plant support systems is important for acclimatization, as reduced carrying capacity (sclerenchyma and collenchyma) can limit this process (Campostrini and Otoni, 1996). Soares (2003) compared *in vitro* and *ex vitro* Ingá (*Inga vera*) leaves and reported that lack of support tissues in leaves of micropropagated plants makes plants less resistant and fragile looking. With regard to rooting, plantlets from 90 and 110 day embryos in MS medium showed no roots, whereas plantlets from 80 and 100 day embryos in MS medium and from 80, 90, 100 and 110 day embryos in Y3 medium showed rooting, thus it was possible to evaluate anatomical parameters (Figure 3). Treatments containing

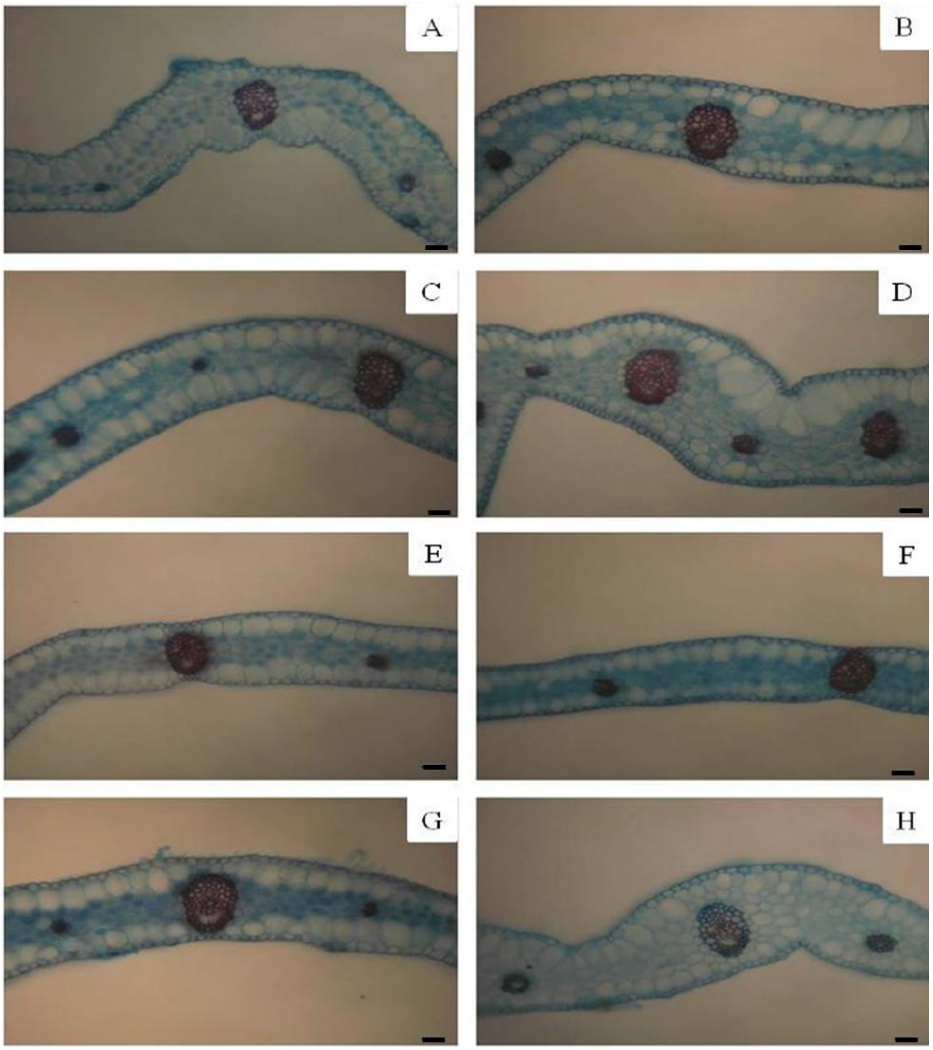

Figure 2. Photomicrographs of leaves from *E. guineensis* hybrid Manicoré seedlings germinated *in vitro* from embryos with different ages. Leaves of seedlings from embryos with 80, 90, 100 and 110 days after pollination were maintained on MS culture medium (A, C, E and G respectively) or Y3 culture medium (B, D, F and G respectively). Bars = 100 µm.

Table 2. Morophometric and anatomical characteristics of leaves from *E. guineensis* hybrid Manicoré seedlings germinated *in vitro* from embryos with different ages.

Age (days)	Culture medium	DET (µm)	BET (µm)	CT (µm)	DHT (µm)	BHT (µm)
80	MS	17.77^{A}	20.78^{AB}	8.33^{AB}	51. 66^{A}	65.92^{A}
90		17.09^{A}	18.93^{B}	9.03^{A}	51.64^{A}	73.11^{A}
100		13.98^{B}	15.02^{C}	7.24^{B}	47.86^{A}	67.85^{A}
110		18.57^{A}	22.36^{A}	9.11^{A}	53.32^{A}	68.04^{A}
80	Y3	16.74^{ab}	18.59^{a}	5.95^{c}	49.75^{a}	63.89^{b}
90		18.50^{a}	21.28^{a}	8.60^{a}	42.25^{a}	86.62^{a}
100		14.83^{b}	15.51^{b}	7.12^{bc}	45.54^{a}	68.79^{b}
110		17.28^{a}	21.44^{a}	8.33^{a}	45.30^{a}	59.66^{b}

Adaxial epiderms thickness (DET) and abaxial epiderms thickness (BET) and cuticle thickness (CT), adaxial hypodermis thickness (DHT) and abaxial hypodermis thickness (BHT). Means followed by the same capital letters do not differ, in the column, at 5% of probability and means followed by the same letters do not differ, in the column, at 5% of probability by Tukey.

Table 3. Morophometric and anatomical characteristics of leaves from *E. guineensis* hybrid Manicoré seedlings germinated *in vitro* from embryos with different ages.

Age (days)	Culture medium	MT (µm)	SE (µm)	FT (µm)	MD (µm)
80	MS	88.08^{AB}	58.79^{B}	46.05^{AB}	49.28^{A}
90		74.69^{B}	50.52^{AB}	35.16^{C}	38.04^{B}
100		89.71^{AB}	58.79^{AB}	57.86^{A}	43.88^{AB}
110		99.94^{A}	70.46^{A}	57.05^{AB}	45.72^{A}
80	Y3	95.54^{ab}	78.34^{a}	52.83^{b}	52.54^{a}
90		153.90^{c}	100.84^{b}	40.28^{a}	49.06^{ab}
100		81.55^{a}	69.37^{a}	37.86^{a}	40.98^{c}
110		110.74^{c}	73.44^{a}	39.88^{a}	42.35^{bc}

Mesophyll thickness (MT), sclerenchyma thickness (SE), phloem thickness (FT) and metaxylem diameter (MD). Means followed by the same capital letters do not differ, in the column, at 5% of probability and means followed by the same letters do not differ, in the column, at 5% of probability by Tukey.

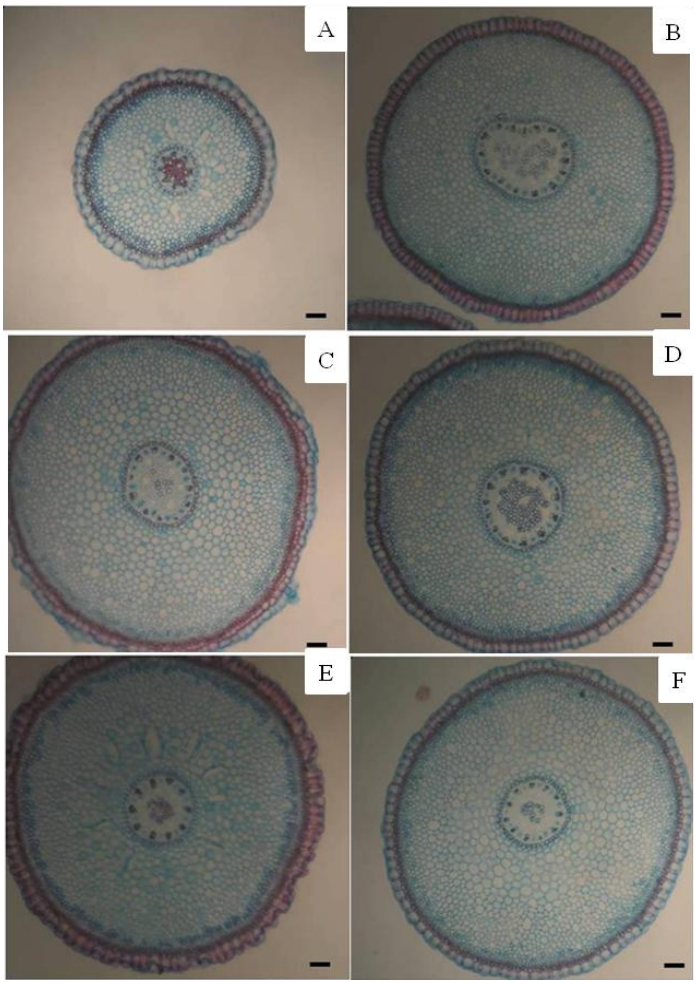

Figure 3. Photomicrographs of roots from *E. guineensis* hybrid Manicoré seedlings germinated *in vitro* from embryos with different ages. Roots of seedlings from embryos with 80 and 100 days after pollination were maintained on MS culture medium (A and B respectively) and from embryos with 80, 90, 100 and 110 days after pollination maintained on Y3 culture medium (C, D, E and F) respectively). Bars = 100 µm.

Table 4. Morophometric and anatomical characteristics of roots from *E. guineensis* hybrid Manicoré seedlings germinated *in vitro* from embryos with different ages.

Age (days)	Culture medium	RTA (mm^2)	RD (µm)	RET (µm)
80	MS	0.75^{B}	978.07^{B}	52.86^{A}
90		0.00	0.00	0.00
100		1.60^{A}	1430.10^{A}	48.54^{A}
110		0.00	0.00	0.00
80	Y3	1.84^{a}	1.53^{a}	45.49^{b}
90		1.68^{b}	1.46^{b}	50.65^{ab}
100		1.83^{a}	1.53^{a}	55.41^{a}
110		1.65^{b}	1.45^{b}	42.92^{c}

Root total area (RTA) and root diameter (RD), root epidermis thickness (RET). Means followed by the same capital letters do not differ, in the column, at 5% of probability and means followed by the same letters do not differ, in the column, at 5% of probability by Tukey.

Table 5. Morophometric and anatomical characteristics of roots from *E. guineensis* hybrid Manicoré seedlings germinated *in vitro* from embryos with different ages.

Age (days)	Culture medium	CT (mm)	VCA (mm^2)	VCD (mm)
80	MS	0.28^{B}	0.02^{B}	0.19^{B}
90		0.00	0.00	0.00
100		$O.41^{A}$	0.12^{A}	0.40^{A}
110		0.00	0.00	0.00
80	Y3	0.49^{a}	0.09^{b}	0.34^{a}
90		0.44^{b}	0.10^{a}	0.37^{a}
100		0.45^{b}	0.08^{b}	0.32^{b}
110		0.49^{a}	0.06^{b}	0.29^{c}

Cortex thickness (CT), vascular cylinder area (VCA) and vascular cylinder diameter (VCD). Means followed by the same capital letters do not differ, in the column, at 5% of probability and means followed by the same letters do not differ, in the column, at 5% of probability by Tukey.

MS medium associated with ages 100 and 80 days after pollination did not differ statistically for the parameters analyzed in roots: total root area, total root diameter, cortex thickness, vascular cylinder area, and vascular cylinder diameter (Tables 4 and 5). In Y3 medium, total root area, total root diameter and epidermal thickness excelled in 100 day embryos. Best results for cortex thickness were obtained with 80 and 110 day embryos. The highest results for vascular cylinder area and diameter were found with 90 day embryos, and vascular cylinder diameter did not differ statistically from 80 day embryos (Tables 4 and 5).

Results for exodermis and endoderm thickness, metaxylem diameter, and phloem thickness in plantlet roots in MS medium were not statistically different (Table 6), while plantlets from 80 day embryos in Y3 medium showed greater exodermis and endodermis thickness. Results did not differ statistically in plantlets from 90 day embryos (Table 6), which also showed greater metaxylem diameter. Vascular cylinder area, vascular cylinder diameter, and metaxylem diameter may affect acclimatization process to a greater extent, and showed better results in Y3 medium with 90 day embryos. They are also critical in the conduction of water and nutrients to the entire plant.

According to Jesus et al. (2010), root hydraulic conductivity is directly related to vascular cylinder diameter, thus its good development ensures adequate supply of water and minerals to shoots.

Conclusion

Sucrose is essential for germination of immature embryos of *E. guineensis* Jacq. Manicoré hybrid. MS medium with zygotic embryos at 90, 100 and 110 days post-pollination can provide high germination rates, likewise Y3 medium with embryos at 100 and 110 days post-anthesis. The treatment in Y3 medium with zygotic embryos at 90 days post-anthesis was outstanding in number and length of roots, as well as in anatomical parameters of leaves and roots associated with the best conditions for plantlet

Table 6. Morophometric and anatomical characteristics of roots from of *E. guineensis* hybrid Manicoré seedlings germinated *in vitro* from embryos with different ages.

Age (days)	Culture medium	EXT (µm)	ENT (µm)	MD (µm)	FT (µm)
80	MS	33.22^{b}	16.80^{b}	18.56^{b}	38.63^{b}
90		0.00	0.00	0.00	0.00
100		36.91^{b}	17.19^{b}	17.89^{b}	37.92^{b}
110		0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}
80	Y3	51.50^{c}	18.58^{b}	16.47^{a}	37.29^{b}
90		35.69^{a}	17.12^{ab}	19.33^{b}	38.56^{bc}
100		45.10^{b}	15.80^{a}	17.45^{a}	41.52^{c}
110		35.25^{a}	17.35^{ab}	16.48^{a}	34.16^{a}

Exodermis thickness (EXT), endoderm thickness (ENT), metaxylem diameter (MD) and phloem thickness (FT).
Means followed by the same capital letters do not differ, in the column, at 5% of probability and means followed by the same letters do not differ, in the column, at 5% of probability by Tukey.

acclimatization.

Conflict of Interests

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

REFERENCES

Agrianual (2012). Anuário da Agricultura Brasileira: Coco-da-baía. São Paulo: FNP, Consultoria e Agroinformativos. pp. 285-291.

Akin DE, Robinson EL (1982). Structure of leaves and stems of arrowleaf and crimson clovers as related to in vitro digestibility. Crop Sci. 22(1):24-29.

Apóstolo NM, Brutti CB, Llorente BE (2005). Leaf anatomy of *Cynara scolymus* L. in successive micropropagation stages. *In vitro* Cell. Dev. Biol. Plant 41(3):307-313.

Assis TF de, Teixeira SL (1998). Enraizamento de plantas lenhosas. In:Torres, AC; Caldas LS, Buso, JA. Cultura de tecidos e transformação genética de plantas. Brasília: Embrapa-SPI/Embrapa-CNPH. pp. 261-296.

Björkman O (1981). Responses to different quantum flux densities. In Encyclopedia of Plant Physiology, New Series, Lange OL, Nobel PS, Osmond CB, Zeigler H, eds., Springer, Berlin. 12A:57-107.

Boari A de J (2008). Estudos realizados sobre o amarelecimento fatal do dendezeiro (*Elaeis guineensis* Jacq. *EMBRAPA* - Documentos, pp. 348-359.

Campos JI (2011). Produtores podem encomendar sementes do híbrido de dendê BRS Manicoré. Embrapa. Available in <http://www.embrapa.br/imprensa/noticias/2011/julho/1a-semana/produtores-podem-encomendar-sementes-do-hibrido-de-dende-brs-manicore/> Acess: dezember, 19 of 2012.

Campostrini E, Otoni WC (1996). Aclimatização de plantas: abordagens recentes. ABCTP Notícias. 12p.

Cardoso J de NO, Miranda VS, Lemos OF de, Coelho IL (2010). Obtenção de plântulas de híbridos de dendezeiro por cultivo in vitro. Rev. Ciênc. Agr. 53(2):177-181.

Chazdon RL, Kaufman S (1993). Plasticity of leaf anatomy of two forest shrubs in relation to photosynthetic light asclimation. Functional Ecology, Oxford. 7:385-394.

Collares D (2011). Produção de cultivares e sementes de dendê na Embrapa. Agroenergia Rev. 2:30-31, maio.

Eeuwens CJ (1978). Mineral requirements for growth and callus initiation of tissue explants excised from mature coconut palms (*Cocos nucifera*) and cultured *in vitro* . Physiol. Plant 36(1):23-28.

Ferreira DF (2008). SISVAR: Um programa para análise e ensino de estatística. Rev. Symposium 6:36-4.

Ferreira MGR, Cárdenas FEN, Carvalho CHS, Carneiro AA, Damião Filho CF (2002). Resposta de eixos embrionários de cupuaçu (*Theobroma grandiflorum* Schum.) à concentração de sais, doses de sacarose e renovação do meio de cultivo. Revista Científica Symposium, Lavras. 24(1):246-248.

Ghassan TQ, Mohamad I, Al-Widan B, Ali O (2003). A combustion performance and missions of ethyl ester of a waste vegetable oil in a water-cooled furnace. Appl. Therm. Eng. 23:285-293.

Hu CY, Ferreira AG (1998). Cultura de embriões. In: TORRES AC, CALDAS LS, BUSO JA (Ed.). Cultura de tecidos e transformação genética de plantas. Brasília: Embrapa-SPI; Embrapa-CNPH. 1:371-393.

Jesus MAS, Jesus MAS, Carvalho SP de C, Castro EM de, Gomes CN (2010). Observações anatômicas em plantas de *Coffea arabica* L. obtidas por enraizamento de estacas. Rev. Ceres. 57(2):175-180.

Johansen DA (1940). Plant microtechnique. New York: McGraw Hill Book. 523p.

Kraus JE, Arduin M (1997). Manual básico de métodos em morfologia vegetal. Seropédica, UFRRJ. 198pp.

Luis ZG, Bezerra KMG, Scherwinski-Pereira JE (2010). Adaptability and leaf anatomical features in oil palm seedlings produced by embryo rescue and pre-germinated seeds. Braz. J. Plant Phys. 22(3):209-215.

Machado MP, Biasi LA (2011). Morfoanatomia das folhas do porta-enxerto de videira 'VR043-43' (*Vitis vinifera* L. × *Vitis rotundifolia* michx.) em diferentes condições de cultivo. Rev. Acad. Ciênc. Agr. Amb. 9(1):73-78.

Martine MB, Laurent KK, Pierre BJ, Eugené KK, Hilaire KT, Justin KY (2009). Effect of storage and heat treatments on the germination of oil palm (*Elaeis guineensis* Jacq.) seed. Afr. J. Agric. Res. 4(10):931-937.

Melo HC, Castro EM de, Soares AM, Melo LA de, Alves JD (2007). Alterações anatômicas e fisiológicas em *Setaria anceps* Stapf ex Massey e *Paspalum paniculatum* L. sob condições de déficit hídrico. Hoehnea 34:145-153.

Morcillo F, Gallard A, Pillot M, Jouannic S, Aberlenc-Bertossi F, Collin M, Verdeil JL, Tregear JW (2007). EgAP2-1, an AINTEGUMENTA-like (AIL) gene expressed in meristematic and proliferating tissues of embryos in oil palm. Planta 226(6):1353-1362.

Murashige T, Skoog F (1962). A revised médium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15(3):473-497.

Mya T, Apichart V, Somvong T, Chatchawan J (2010). Efficient and rapid plant regeneration of oil palm zygotic embryos cv. 'Tenera' through somatic embryogenesis. Acta Physiol. Plant 33(1):123-128.

Nobel PS (1977). Internal leaf area and cellular CO2 resistance: photosynthetic implications of variations with growth conditions and plant species. Physiol. Plant 40(2):137-144.

Nunes CF, Pasqual M, Santos DN dos, Custódio TN, Araujo AG (2008). Diferentes suplementos no cultivo *in vitro* de embriões de pinhão-

manso. Pesq. Agropec. Bras. 43(1):9-14.
Pereira R de CA, Pinto JEBP, Bertolucci SKV, Castro EM de, Silva FG (2006). Germinação, avaliação do ácido giberélico e posição do explante no alongamento *in vitro* de *Uncaria guianensis* (Aublet) Gmelin Rubiaceae (unha-de-gato). Ciênc. e Agrotec. 30(4):637-642.
Rathi AS (1998). Role of leaf cuticular thickness in resistance against powdery mildew disease in peas. Indian J. Pulse Res. 11(3):136-13.
Ribeiro LM, Neves S da C, Silva PO, Andrade IG (2011). Germinação de embriões zigóticos e desenvolvimento *in vitro* de coquinho-azedo. Rev. Ceres 58(2):133-139.
Soares GA (2003). Aspectos do cultivo *in vitro* do ingazeiro (Inga vera Willd, subsp. affinis (DC.) Penn TD). Dissertacao (Mestrado em Fisiologia Vegetal) - Universidade Federal de Lavras, Lavras. 107p.
Souza Júnior EE de, Barboza SBSC, Souza LAC (2001). Efeito de substratos e recipientes na aclimatação de plântulas de abacaxizeiro *Ananas comosus* (L.) Merril cv. pérola. Pesq. Agropec. Trop. 31(2):147-151.
Suranthran P, Sinniah UR, Subramaniam S, Aziz MA, Romzi N, Gantait S (2011). Effect of plant growth regulators and activated charcoal on in vitro growth and development of oil palm (*Elaeis guineensis* Jacq. var. Dura) zygotic embryo. Afr. J. Biotechnol. 10(52):10600-10606.
Taiz L, Zeiger E (2004). Fisiologia vegetal. Porto Alegre: Artmed. pp. 449-484.
Torres AC, Caldas LS, Buso JA (1998). Cultura de tecidos e transformação genética de plantas. Ed: CBAB Embrapa, Brasília, DF. 1:864.
Torres AC, Duval FG, Ribeiro DG, Barros AFF, Aragão FAD (2005). Efeito da sacarose, cinetina, isopentenil adenina e zeatina no desenvolvimento de embriões de *Heliconia rostrata in vitro*. Hortic. Bras. 23(3):789-792.

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Full Length Research Paper

Fruit, seed and embryo development of different cassava (*Manihot esculenta* Crantz) genotypes and embryo rescue

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Fruit, seed and embryo developments of different cassava (*Manihot esculenta* Crantz) genotypes, as well as embryo rescue, were investigated. The fruits of three genotypes after uncontrolled open pollination presented the same progressive development with similar sizes at different stages. There are large differences in the fruit set as well as the embryo development between different genotypes. Days after pollination (DAP) was found not to be an adequate predictor of embryo size as their size ranged from almost invisible to 8.7 mm in length at 32 DAP even within the different locules of the same fruit. The ideal stage for embryo rescue in cassava was from 32 to 36 DAP, because at that stage most embryos are visible (> 0.7 mm); and their excision without injury is feasible. Also, *in vitro* germination of the cotyledonary embryos at that stage had a high success rate. A half Murashige and Skoog (MS) medium supplemented with 1.0 mg/l GA_3, 2% sucrose and 0.2% gel rite proved to be adequate for embryo rescue.

Key words: *Manihot esculenta* Crantz, day after pollination (DAP), fruit set, seed size, embryo size, embryo rescue.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz), a member of the Euphorbiaceae family, is a perennial vegetative propagated shrub widely planted in tropical and subtropical regions of the world. Cassava, along with maize, sugarcane and rice, constitutes the most important sources of energy in the diet of most tropical countries of the world (Ceballos et al., 2004). It has played an important role in food security as a famine reserve crop historically, and also become potentially highly resilient to future climate changes (Jarvis et al., 2012). With the worldwide increasing importance of cassava for human consumption, animal feed and industrial applications, there will be an increasing need to develop cultivars with specific characteristics and for adaptation to different

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ecologies. Conventional breeding efforts have attempted to address many of the constraints facing cassava productivity, with outstanding success. Average productivity of cassava in countries like Thailand and Vietnam has doubled in the last two decades. Biotechnology provides new tools for overcoming some of the problems that currently hinder cassava improvement. Major efforts are currently undergoing to develop protocols for the production of doubled haploids with the ultimate objective of introducing inbreeding in the genetic enhancement of cassava. Embryo rescue is to nurture the immature or weak embryo, thus allowing it the chance to survive, and plays an important role in modern plant breeding (Sharma et al., 1996). Embryo rescue also demonstrates potential roles in cassava breeding: (1) rescue plantlets from the younger fruits when confronted with adverse climate (Uma et al., 2011); (2) rescue *in vitro* plantlets following interspecific hybridization (Clarke et al., 2011; Cisneros et al., 2013); (3) shorten the breeding cycle by recovering the plantlets from younger seeds, replace germinating the seeds harvested after breaking the dormancy (Kagithoju et al., 2013; Gbadamosi, 2013). But initial work required considerable amount of basic research because of the scarcity of information related to the biology or structural development of flower buds in cassava (Perera et al., 2013). So, this research was conducted to explore fruit, seed and embryo development of different cassava genotypes, and then recover the plantlets from younger seeds. This information is crucial for ongoing research to obtain doubled haploids through wide crosses with *Ricinus communis*.

MATERIALS AND METHODS

This study was carried out in the field and at the tissue culture laboratory of cassava genetics in the International Center for Tropical Agriculture (CIAT), Cali-Palmira, Colombia, from February to September, 2012. Three elite lines (HMC-1, SM1219-9 and MCOL1505) being used by the cassava program of CIAT were selected because of their profuse flowering and planted in the field. All three lines were grown in the field in clay loam soil, pH 7.2, at CIAT headquarters in Cali, Colombia (3°30′N, 76°19′W; 965 m above sea level). During 1993 to 2010, mean daily temperatures ranged from a maximum of 29.7ᴸ to a minimum of 19.2ᴸ, with a mean monthly temperature of 23.8ᴸ. The relative humidity averaged 78% and annual rainfall, 936 ± 34 mm. Each of the two experiment, at anthesis day, 100 female flowers on 15 to 20 inflorescences (in members of the Euphorbia family whose correct name is cyathium) from 10 healthy plants of three lines were marked with plastic labels (identifying them and stating date of anthesis) and left for natural, open pollinations carried out by insects. In the first experiment, the width of fruits at different development stages (7, 14, 21 and 28 days after pollination (DAP) was measured by using the electronic digital caliper (01407A, Neiko). Fruit set at different stages was calculated as the ratio of fruits that remained attached to the mother plant to the number of female flowers initially marked. When determining the width of fruits, those fruits with atypically smaller size were not considered, except at 7 DAP.

At 32 DAP, parts of the remaining fruits were harvested, and number of fruits scored. Fruits were then dissected and number of seeds and cotyledonary embryos counted. The width of fruits, the length of seeds, and cotyledonary embryos were then measured. The width of fruits was measured by using the electronic digital caliper. The length of both seeds and cotyledonary embryos were measured with a Wild M7A stereomicroscope connected to a digital micrometric ocular Wild MMS 235 (Heerbrugg, Switzerland). The fruit width, seed length and embryo length were presented as mean ± standard error. In a second experiment, fruits were harvested at different number of days after anthesis (26, 28, 30 and 32 DAP), and counted. Fruits were dissected and number of seeds and cotyledonary embryos was counted to determine the percentage of seeds with visible cotyledonary embryos that could be observed under Wild M7A stereomicroscope (10 × ocular with 6 × magnification). Parts of harvested cotyledonary embryos were used to recover plantlets via embryo rescue culture.

Embryo rescue was done at the tissue culture laboratory of cassava genetics in CIAT. The immature seeds dissected out of fruits were surfaced-sterilized by immersion in 70% alcohol for 1 min, followed by immersion in 0.5% sodium hypochlorite for 8 min, and then washed three times with sterile water. The seeds were split along the longitudinal axis utilizing sterile forceps and scalpel, using aseptic conditions, under the stereomicroscope. For each line, 50 excised cotyledonary embryos were used for embryo rescue. Excised cotyledonary embryos were placed radicle down on the preferred medium (M6 medium), that is, half Murashige and Skoog (1962) (MS) basal medium, supplemented with 1.0 mg/L GA_3, 2% sucrose and 0.2% gelrite (Sigma). The pH of media above was adjusted to 5.8 ± 0.1 before autoclaving at 121ºC for 20 min. The embryo cultures were incubated at 28 ± 1ºC under a 12/12 h (day/night) photoperiod with light supplied by white fluorescent tubes (25 µmol $m^{-2}s^{-1}$). After *in vitro* culture for two weeks, the germination of the cotyledonary embryos was scored.

RESULTS AND DISCUSSION

Fruit set and development data are summarized in Table 1. The fruits of three genotypes (HMC-1, SM1219-9 and MCOL1505) after uncontrolled open pollination presented the same progressive development with similar sizes at different stages. In contrast, there were marked differences for fruit set among the three cultivars employed (Table 1). At the initial stage (7 DAP), there were no apparent differences on fruit set. But at 14 DAP, fruit set of HMC-1 and MCOL1505 sharply declined to 62.1 and 39.7%, respectively, contrasting with that of SM1219-9 (95.7%). And at 28 DAP, the fruit set of SM1219-9 remained at 91.9%, much higher than that of the other two genotypes (48.5 and 38.2% respectively). The difference in fruit set is mainly attributed to the abscission of fruits during the second weeks probably due to the occurrence of abnormal gametes and/or (most likely) problems in the germination and growth of pollen tubes or in the fertilization to produce viable zygotes.

The genotype differences on fruit set had also been reported in other crops (Esen et al., 1978). There are also other factors affecting the fruit set, such as insects and diseases (Figure 1C). In one particular batch, the marked female flowers encountered moderate rain during the afternoon of anthesis day, and fruit set of all three genotypes dropped down at 14 DAP (data not shown). As illustrated in Table 2 and Figure 1A, there was considerable variation in seed and embryo developments among the

Table 1. Fruit development of different cassava genotypes at different days after pollination (DAP).

Genotype	7 DAP		14 DAP		21 DAP		28 DAP	
	Fruit width (mm)	Fruit set (%)	Fruit width (mm)	Fruit set (%)	Fruit width (mm)	Fruit set (%)	Fruit width (mm)	Fruit set (%)
HMC-1	5.4 ± 0.9	98.5	15.1 ± 3.1	62.1	19.9 ± 1.8	53.0	22.6 ± 1.3	48.5
SM1219-9	6.8 ± 1.0	100	13.9 ± 1.0	95.7	19.1 ± 0.7	95.7	20.5 ± 0.7	91.9
MCOL1505	5.3 ± 0.7	100	14.4 ± 0.8	39.7	17.9 ± 2.1	38.2	19.3 ± 1.9	38.2

Figure 1. A) Seeds and embryos of cassava (HMC-1) at 30 to 38 days after pollination (DAP). **B)** Seedlings germinated from young cotyledonary embryos. **C)** Seeds affected by insects and diseases in the field.

Table 2 Fruit, seed and embryo development of different cassava genotypes at 32 days after pollination (DAP).

Genotype	Number of fruits collected	Number of seeds	Number of embryos	Fruit width (mm)	Seed length (mm)	Embryo length (mm)		
						Average	Maximum	Minimum
HMC-1	35	71	51	22.8 ± 1.8	10.9 ± 0.6	5.6 ± 2.2	8.7	0.7
SM1219-9	31	79	52	20.7 ± 0.5	9.2 ± 0.3	4.2 ± 1.2	6.6	1.8
MCOL1505	31	23	18	19.3 ± 1.3	9.9 ± 0.4	3.6 ± 1.6	7.3	1.5

different genotypes considered. Cassava ovaries have three locules, each with one ovule (Alves, 2002). At 32 DAP, the average seed number of each fruit of HMC-1, SM1219-9 and MCOL1505 were 2.1, 2.6 and 0.7, respectively. Mostly, only two seeds were harvested in fruits from HMC-1, with the third locule containing a brown tiny ovule. Fruits from SM1219-9, often produced three seeds, as apposed to an average of less than one seed per fruit of MCOL1505.

Fruit and seed sizes of different genotypes were similar at the same number of days after anthesis; but the embryo size showed marked differences. On the other hand, there was wide variation of embryo length when harvested at 32 DAP. For example, in the case of HMC-1 embryo length ranged from 0.7 to 8.7 mm (Table 2). Moreover, large variation in embryo sizes was observed among the different locules of the same fruit, which would suggest that the embryo development of cassava is highly un-synchronized. It is worthy to emphasize the relative uniformity in embryo size as for SM1219-9, compared with

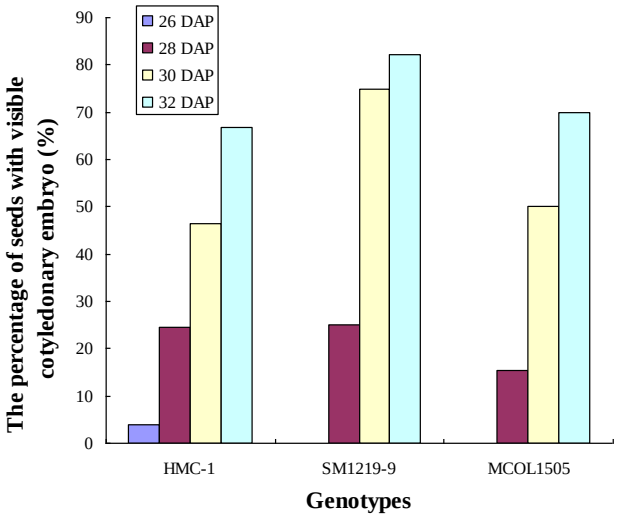

Figure 2. Embryo development of three cassava genotypes at different days after pollination (DAP).

that of HMC-1 and MCOL1505. This observation would further point SM1219-9 as an ideal elite parental material for the ongoing work to develop a protocol for the production of doubled haploids in cassava. In addition to genetic variation, other factors, such as temperature (Lang and Sutka, 1994), might affect the development of seed and embryo. However these factors would affect equally the three genotypes involved in this study (unless genotype x environment interactions are markedly significant in the reproductive biology of cassava) and therefore, would not properly explain the differences observed between the three genotypes.

An important and critical preliminary result of this work was the observation that the embryo and endosperm development remains very slow till the fourth week after pollination; and only at 22 DAP, it was possible to observe embryos at early developmental stage (globular phase) (L.N. Ramos, personal communication). Our results are showed in Figure 2, it was very difficult to visualize cotyledonary embryos inside the seed at 26 DAP, even under stereomicroscope. This was the case for the three genotypes used. Even at 28 DAP; the percentage of seeds with visible cotyledonary embryo was less than 30%. At 30 DAP, this figure rose up to over 70% in SM1219-9, and around 50% for both HMC-1 and MCOL1505. It is interesting to note that abortive embryos were found in the relatively smaller size of seeds of all three genotypes. During fruit development of each genotype, the color of seeds changed progressively from cream white to brown from 30 to 38 DAP; and also various sizes of embryo were observed (Figure 1A). At 38 DAP, most of the seeds are too hard to dissect out the embryo without injury. Seeds from fruits harvested at an earlier age are, in this regard, more suitable for embyo rescue purposes.

The cotyledonary embryos excised from the immature fruits were cultured on M6 medium, which was screened out by comparative experiments. Every cultured cotyledonary embryo could germinate and develop roots, internodes and shoots, independent of the genotype (Figure 1B). Our protocol improved the recovery efficiency of the seeds in cassava embryo rescue culture, compared to the 66% results reported in the literature (Fregene et al., 1999; Akinbo et al., 2010). The presence of GA_3 in the M6 medium probably promoted the development of immature embryos in agreement with the results reported by Anuradha and Rout (2005). It also found that the embryo without visible cotyledon was too small to germinate, and demonstrated that embryo size was positively correlated with success in embryo rescue and recovery of plantlets (Kapila and Sethi, 1993). Rescuing and culturing younger embryos when they are not readily visible may require the inclusion of other tissue such as embryo sac and even part of the ovary. Likewise, parental genetic, phenological and physiological differences can also contribute to variations in the *in vitro* responses of rescued embryos (Clarke et al., 2006).

Conclusion

In conclusion, this is an important contribution to our understanding of cassava fruit, seed and embryo development of different genotypes and an improvement on previous protocols for embryo rescue in cassava (Fregene et al., 1999). Large differences were found in fruit set and developmental rate as well as the embryo development between different genotypes. The ideal stage for embryo rescue in cassava was found to be from 32 to 36 DAP. A half MS medium supplemented with 1.0 mg/l GA_3, 2% sucrose and 0.2% gelrite proved to be adequate for embryo rescue. Embryo rescue is likely to play an important role in the production of doubled haploid plants from wide crosses or pollinations with irradiated pollen, and also shorten the breeding cycle.

Conflict of Interests

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

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REFERENCES

Akinbo O, Labuschagne M, Fregene M (2010). Embryo rescue as a method

to develop and multiply a backcross population cassava (*Manihot esculenta* Crantz) from an interspecific cross of *Manihot esculenta* ssp. *flabellifolia*. Afr. J. Biotechnol. 9(42):7058-7062.
Alves AAC (2002). Cassava botany and physiology. In: Hillocke RJ, Thresh JM, Bellotti AC (eds) Cassava: biology, production and utilization. CAB, International. pp 67-89.
Anuradha M, Rout GR (2005). Study of embryo rescue in floribunda rose. Plant Cell Tiss. Organ Cult. 81(1):113-117.
Ceballos H, Iglesias CA, Perez JC, Dixon AGO (2004). Cassava breeding: opportunities and challenges. Plant Mol. Biol. 56:503-516.
Cisneros A, Garcia RB, Tel-Zur N (2013). Creation of novel interspecific-interploid *Hylocereus* hybrids (Cactaceae) via embryo rescue. Euphytica 189:433-443.
Clarke HJ, Kumari M, Khan TN, Siddique KH (2011). Poorly formed chloroplasts are barriers to successful interspecific hybridization in chickpea following *in vitro* embryo culture. Plant Cell Tiss. Organ Cult. 106(3):465-473.
Clarke HJ, Wilson JG, Kuo I, Lulsdorf MM, Mallikarjuna N, Kuo J, Siddique KHM (2006). Embryo rescue and plant regeneration *in vitro* of selfed chickpea (*Cicer arietinum* L.) and its wild annual relatives. Plant Cell Tiss. Organ Cult. 85:197-204.
Esen A, Soost R, Geraci G (1978). Seed set, size, and development after 4x×2x and 4x×4x crosses in *Citrus*. Euphytica 27:283-294.
Fregene M, Ospina JA, Roca W (1999). Recovery of cassava (*Manihot esculenta* Crantz) plants from culture of immature zygotic embryos. Plant Cell Tiss. Organ Cult. 55:39-43.
Gbadamosi AE (2013). *In vitro* propagation of *Picralima nitida* (Stapf) through embryo culture. Afr. J. Biotechnol. 12(22): 3447-3454.
Jarvis A, Villegas JR, Campo BVH, Racines CN (2012). Is cassava the answer to African climate change adaptation? Tropical Plant Biol. 5(1):9-29.
Kagithoju S, Godishala V, Kairamkonda M, Nanna RS (2013). Embryo culture is an efficient way to conserve a medicinally important endangered forest tree species *Strychnos potatorum*. J. Forestry Res. 24(2):279-283.
Kapila RK, Sethi GS (1993). Genotype and age effect on in vitro embryo rescue of bread wheat × hexaploid triticale hybrids. Plant Cell Tiss. Organ Cult. 35:287-291.
Lang MM, Sutka J (1994). The effect of temperature on seed set and embryo development in reciprocal crosses of wheat and barley. Euphytica 78:53-58.
Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant 15:473-479.
Perera PIP, Quintero M, Dedicova B, Kularatne JDJS, Ceballos H (2013). Comparative morphology, biology and histology of reproductive development in three lines of *Manihot esculenta* Crantz (Euphorbiaceae: Crotonoideae). AoB Plants 5, pls046; doi: 10.1093/aobpla/pls046.
Sharma DR, Kaur R, Kumar K (1996). Embryo rescue in plants – a review. Euphytica 89(3):325-337.
Uma S, Lakshmi S, Saraswathi MS, Akbar A, Mustaffa M (2011). Embryo rescue and plant regeneration in banana (*Musa* spp.). Plant Cell Tiss. Organ Cult. 105(1):105-111.

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Full Length Research Paper

Assessment of an oil palm population from Nigerian Institute for Oil Palm Research (NIFOR) for simple sequence repeat (SSR) marker application

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Oil palm (*Elaeis guineensis* Jacq.), a monocotyledonous plant belonging to the Arecaceae family, is one of the most important oil crops in the world. In Nigeria, oil palm has benefited immensely from conventional breeding efforts resulting in high yields that have been achieved with this breeding material. However, oil palm breeding is slow and time-consuming due to a breeding cycle of about 10 years. In addition, the process of outcrossing leads to high variation in yield components and vegetative traits. Although DNA marker technologies offer great possibilities for plant breeding through marker-assisted selection, there are so far no reports of its application to oil palm breeding in Nigeria. In this study, 32 SSR markers were used for the assessment of marker application in an oil palm breeding population coming from the extensive breeding program at the Nigerian Institute for Oil Palm Research (NIFOR). Seven SSR markers out of the 32 tested (22%) segregated in the progeny 12 (*tenera* x Deli *dura*). SSR markers mEgCIR0059, mEgCIR1917, mEgCIR3260, mEgCIR3275, mEgCIR3533 and mEgCIR3557 proved to be fully informative markers following a segregation ratio of 1:1:1:1, while marker mEgCIR0074 segregated in a 1:1 ratio.

Key words: Oil palm, microsatellite marker, marker-assisted selection, NIFOR.

INTRODUCTION

Oil palm (*Elaeis guineensis* Jacq.; Arecaceae), a mono-cotyledonous plant, is one of the most important oil crops in the world (Low et al., 2008). There are currently two species assigned to the genus *Elaeis*: the African oil

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Abbreviations: AVROS, Algemene Verenigung van Rubberplanters ter Oostkust van Sumatra; **bp,** base pairs; **CNRA**, Centre National de Recherche Agronomique; **cM,** centiMorgan; **DF,** degree of freedom; **IRD,** infrared dye**; LG**, linkage group; **NCBI**, National Center for Biotechnology Information; **NIFOR**, Nigerian Institute for Oil Palm Research; **PCR,** polymerase chain reaction; **QTL,** quantitative trait loci; **RPO**, red palm oil; **SSR**, simple sequence repeat.

palm (*E. guineensis*) and the South American oil palm (*Elaeis oleifera*). The chromosome numbers for *E. guineensis* and *E. oleifera* were determined with 2n=2x=32 (Madon et al., 1998). The African oil palm (*E. guineensis*) has three fruit forms differing in their shell thickness: *dura* (thick-shell)*, pisifera* (shell-less) and *tenera* (thin-shell), which results from a cross between *dura* and *pisifera* (Hartley, 1988). Shell thickness is controlled by one major co-dominant gene called *Sh* (Beirnaert and Vanderweyen, 1941) and plays a major role in oil yield. In addition, different fruit types (*virescens, albescens, nigrescens* and *poissoni*) are distributed among the three fruit forms (Corley and Tinker, 2003). Oil palms allow the production of two distinct kinds of oils from two different tissues within the fruits: (red) palm oil from the fruit pulp (mesocarp) around the nut and palm kernel oil from the kernel (Akapanabiatu et al., 2001; Asemota et al., 2004). Red palm oil as a saturated fat contains 44% palmitic acid, 39% oleic acid and smaller percentage of stearic, linolenic, lauric, myristic and linoleic acid (Burri, 2012; Oguntibeju et al., 2009). In addition, RPO is rich in provitamin A carotenoids (alpha- and beta-carotene) as well as vitamin E (both tocopherols and tocotrienols) (Burri, 2012). Palm kern oil consists mostly of lauric acid, followed by myristic acid, oleic acid and palmitic acid. Comparing palm kernel oil from Nigeria (Akapanabiatu et al., 2001) with palm kernel oil from Malaysia (Kok et al., 2011), considerable differences in oleic acid and myristic acid content can be observed. Nigerian palm kern oil contained 15-19% oleic acid and only 45-48% lauric acid, whereas the palm kern oil from Malaysia had only 9-12% oleic acid and 51-53% lauric acid. About 90% of the palm oil produced is used for food products (Sambanthamurthi et al., 2000). Although the unrefined red palm oil has a high nutritional value (provitamin A and vitamin E), the refined commercial palm oil (color- and odorless) is used due to its low costs and its high oxidative stability (Burri, 2012; Ong and Goh, 2002; Matthäus, 2007).

In Nigeria, oil palm yield has benefited immensely from conventional breeding efforts. For example, average yields from the second cycle planting materials coming from the Nigerian Institute for Oil Palm Research range between 20 to 25 tones fresh fruit bunches per hectare per year in a well-managed plantation (NIFOR, 2008). However, oil palm breeding is slow and time consuming due to a breeding cycle of about 10 years. As an outcrossing crop, high variations in yield components or vegetative traits are observed in the offspring (NIFOR, 2008). Regarding the long generation time in oil palm breeding, the possibility of selection at the nursery stage has often been considered desirable. Exploitation of DNA marker technologies combining the knowledge from research in molecular genetics and genomics offers great possibilities in plant breeding (Collard and Mackill, 2008). However, there have been no reported cases of its application in breeding of oil palms in Nigeria, so far (NIFOR, 2008).

DNA markers can be used to detect the presence of allelic variation in the genes underlying traits of interest and have been applied to a range of crop species such as *Flammulina velutipes* (Physalacriaceae) (Zhang et al., 2010), *Brassica rapa* (Brassicaceae) (Kapoor et al., 2009), cereals (Gethi et al., 2002; Li et al., 2008; McCough et al., 2002; Shehata, et al., 2009; Zheng et al., 2008), *Cucumis sativus* (Cucurbitaceae) (Hu et al., 2010), *Spartina* spp. (Poaceae) (Baisakh et al., 2009), *Glycine max* (Fabaceae) (Xia et al., 2007), *Nelumbo nucifera* (Nelumbonaceae) (Kubo et al., 2009; Pan et al., 2007). In oil palm, a set of SSR markers have been developed and together with AFLP markers have been integrated into a general genetic map (Billotte et al., 2005). This genetic map consisted of 944 markers on 16 linkage groups, corresponding to the expected haploid chromosome number of oil palm, and covered 1,743 cM. Linkage group 4 carried the *Sh* gene responsible for the shell thickness. The progeny was obtained from a cross between two heterozygous *Elaeis guineensis* Jacq. parents: LM2T (*tenera* palm belonging to the CNRA La Mé oil palm breeding program, Ivory coast) and DA10D (*dura* palm selected from a Deli population introduced to Indonesia in the 19th century). QTL for phenotypic traits regarding fruit variety, yield components and vegetative growth were localized together with the *Sh* gene for shell thickness on this map (Billotte et al., 2010). Additional genetic maps were constructed by Seng et al. (2011), Singh et al. (2009) and Ting et al. (2013). In between, the *Sh* gene was isolated from *E. guineensis* (*dura* [Sh+/Sh+] and *pisifera* [Sh-/Sh-] form) and two independent mutations were identified in the *pisifera* forms, Congo AVROS and Nigerian T128, that lead to non-functional MADS box transcription factors that cannot support the formation of the shell (Singh et al., 2013a).

Simple sequence repeat markers (SSR), also known as microsatellites, are particularly interesting for plant breeding due to the properties of genetic co-dominance, high reproducibility, multi-allelic variation, easy amplify-cation by PCR, and production of results that are easy to interpret in self-pollinating species (Collard and Mackill, 2008). In addition, the technology is relatively easy to transfer from one laboratory to the other (Zheng et al., 2008). In view of the above mentioned advantages, SSR markers have been used for marker-assisted selection e.g. in rice (Shen et al., 2001), in sunflower (Tang et al., 2003) and peach (Sajer et al., 2012). However, in outbreeding crops like e.g. oil palm, eucalyptus, loblolly pine, cassava or potato, the identification of the segregation patterns of the SSRs in the progenies is challenging. Whereas, in inbred lines, the maximum number of alleles that can segregate in crosses is two (van Ooijen, 2011), the number of segregating alleles per locus in the offspring derived from a cross between two individuals of an outcrossing species (full-sib) family can range between one and four. This represents a major distinction between a progeny of inbred lines and a full-sib

family. An additional challenge has to be faced if the parents of the experimental breeding populations are not available. For mapping markers under these circumstances, the reconstruction of the parental genetic constitution from the observed segregation patterns in the full-sib family is required. Markers that allow this are called informative markers (van Ooijen, 2011). Markers with segregation type *ab x cd* provide information regarding the genotypes of the parents of the population. In this case, the four genotypes (*ac*, *ad*, *bc* and *bd*) can be considered the complete genotypes in an allogamous full-sib family. In addition, markers with a segregation type *ef x eg* also provide complete information regarding the genotypes of the parents of the population (van Ooijen, 2011). These fully informative markers follow segregation ratios of 1:1:1:1 as deduced from their banding patterns and the reports of Lespinasse et al. (2000) and Billotte et al. (2005).

The objective of this study was to carry out an assessment of an oil palm breeding population from NIFOR for its suitability for marker-assisted selection (MAS) using a new DNA isolation protocol and SSR markers. The parental material was not available, so that the genetic constitution had to be derived from the observed segregation patterns in the progeny. This is the first of series of experiments undertaken by NIFOR towards the incorporation of molecular markers into its breeding programs. SSR markers were chosen for this study because of the reliable ability to detect polymerphisms among closely related individuals (Shehata et al., 2009).

MATERIALS AND METHODS

Breeding population

The oil palm population (progeny 12) used for this study was derived from the Nigerian Institute for Oil Palm Research experimental oil palm breeding program (second cycle population) planted in 1987. Progeny 12 is a cross between 13.386 T (*tenera*) and 26.1074 D (Deli *dura)*. Ninety-two (92) individuals of the population were used for the molecular investigations.

DNA extraction

Genomic DNA was extracted from 92 individuals of progeny 12. Between 0.15 to 0.2 g of fresh leaf tissue was ground quickly in 800 µl of DNA extraction buffer (100 mM Tris-HCl, pH 8.0; 50 mM EDTA, pH 8.0; 500 mM NaCl; 5% SDS). The homogenate (buffer and ground tissue) was transferred into 1.5 ml Eppendorf tube. Extra 200 µl of DNA extraction buffer was added, mixed well and centrifuged for 2 min at 10,000 x g (4°C). The supernatant was collected into another Eppendorf tube and labeled. Then 200 µl of 5 M potassium acetate were added and mixed well. An equal volume of phenol-chloroform-isoamylalcohol (25:24:1) was added and mixed very well. The mixture was centrifuged for 5 min at 10,000 x *g* (4°C). The supernatant was carefully transferred into another Eppendorf tube and 800 µl of absolute ethanol was added. To precipitate the DNA, the mixture was kept on ice for 20 min and later centrifuged for 5 min at 10,000 x *g* (4°C). Finally, the DNA pellet was washed twice with 70% ethanol, air dried at ambient temperature and re-suspended in 200 µl of TE (10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0) and stored at -20°C.

DNA quantification and standardization

The DNA concentrations (ng/µl) and the optical density (OD) ratios 260/280 were calculated by measuring the OD at UV wavelength of 260 and 280 nm using a spectrophotometer (UltrospecTM 2 3100 pro, GE Healthcare Europe GmbH, Freiburg, Germany). The DNA was then diluted to 50 ng/µl and used for SSR analyses.

SSR analyses

The SSR loci that were used for this study were sequenced and mapped by Billotte et al. (2005). Sequences for a total of 32 SSR loci (Table 1) were downloaded from National Center for Biotechnology Information (NCBI) using the accession numbers given in Billotte et al. (2005). Due to the special breeding interest in linkage group 4 because of the presence of the *Sh* gene as well as QTLs for yield, bunch components and vegetative traits located on this linkage group (Billotte et al., 2010), most SSR markers came from this linkage group. Forward and reverse SSR primers were designed from the sequences (Table 1) using the online Primer3 version 4 (http://frodo.wi.mit.edu/).

Primer tailing

An M13 tailing procedure (Oetting et al., 1995) was applied for fluorescence labeling of the PCR amplification products. An M13 tail (5'-TTTCCCAGTCACGACGTT-3') was added to the 5'-end of all the designed forward primers. All unlabeled primers were ordered from Invitrogen and the M13-IRD800 (=DY-781) labeled primer was ordered from biomers.net (Ulm, Germany).

PCR amplification

The PCR amplification was performed in a GeneAmp® PCR System 2700 thermal cycler (Applied Biosystems, Darmstadt, Germany). For the PCR, 4 µl of DNA (50 ng/µl) was mixed with 11 µl master mix. The master mix contained 0.3 µl dNTPs (10 mM), 8.7 µl H_2O, 1.5 µl 10x PCR buffer (for FIREPol*Taq* polymerase), 0.15 µl M13-IRD800 primer (5 pmol/µl) for labeling, 0.05 µl FIREPol*Taq* polymerase (5 U/µl), 0.15 µl forward primer (5 pmol/µl) and 0.15 µl reverse primer (5 pmol/µl). The PCR conditions included an initial denaturation at 95°C for 5 min, followed by 36 cycles of 95°C for 20 s, 50°C for 20 s and 72°C for 30 s and a final elongation step at 72°C for 5 min.

Polyacrylamide gel analysis

The PCR products were mixed with equal volume (15 µl) of loading buffer (98 ml formamide, 2 ml of 0.5M EDTA pH 8.0 and 37.5 mg bromophenol blue). This mixture of PCR products and loading buffer was denatured for 2 min at 94°C and transferred onto ice before loading 0.5 µl into the well for separation. The PCR products were separated on 6% denaturing polyacrylamide gels using the DNA Analyzer Model 4300 (LI-COR Biosciences, NE, USA).

Segregation analyses

The number of alleles that segregated in our study was determined

Table 1. SSR primer combinations tested in progeny 12 from the NIFOR breeding program. SSR markers were named according the SSR loci sequenced by Billotte et al. (2005).

Marker name	NCBI accession number	LG	Primer sequences[1] (5'-3')	Product size (bp)[2]
mEgCIR3275	AJ578630	4	F-GGTGGAAGCTTTTTGTCTGC R-ATTGAAGAGGGCAGGGTTTT	192
mEgCIR3716	AJ578711	4	F-CAGACATGGCAGCAAAAAGA R-ATCTTGTCTGGGGGATGTTC	229
mEgCIR3194	AJ578625	4	F-TGGTGGTTGGGAGTGATT R-TGTTAGTTTGCTAACTTGAAACAGG	262
mEgCIR3413	AJ578665	4	F-GGAAGGAAAGAAGCGAGAGG R-ACATGTCCATGCTGTTGGAA	255
mEgCIR3286	AJ578633	4	F-ATTTTGGGGTCAGGGTTTGT R-CAGGTCCATGGGAAAAGAGA	184
mEgCIR3526	AJ578673	4	F-TGACAGAGAGAAAAGGGAGAGG R-TGGTGTTCATTCTGCTCGTC	216
mEgCIR1917	AJ578575	4	F-GCAATGGAAAGAGCTGGAAG R-GGTGGATCAGTCGAGCATTT	208
mEgCIR0786	AJ578541	4	F-AGTTCCGTGCACCCACTTAC R-CAGCAGACAGGGAGCTAACC	266
mEgCIR3439	AJ578670	4	F-TGACAAGCCAACTTGAAAGC R-GTTGACAACCTGACCAAGCA	248
mEgCIR3232	AJ578627	4	F-CAAGCCCCTTAGCTGCATAC R-TGGAGGAGCAGCTTTAGCAT	213
mEgCIR3310	AJ578644	4	F-TGGCCGATCTGTATTAACCA R-AAATTCTGAGCCCATGCCTA	169
mEgCIR3535	AJ578676	4	F-AAAAACAAAAGGTGGGGAAA R-CCAGCCATTGCCGTATCTAT	199
mEgCIR2423	AJ578598	4	F-ACCCCATGAGGAATTTGGAT R-TGTGCCCATATGTGTGTGTG	184
mEgCIR1716	AJ578569	4	F-TTTGTGGTAACATGTGGTTGC R-CCCCTTCCGCATGTAAATTAT	181
mEgCIR3693	AJ578706	4	F-TTGGCCACTTTGAAGAATCC R-TTTTCTGGTCAGGGTTAGCTG	170
mEgCIR3533	AJ578674	4	F-ACGGTCTATGGCTCTGTCGT R-ACATGAGGAAAGCGCTAGGA	199
mEgCIR1753	AJ578573	4	F-CGATTCATGAACATTACAAGCA R-TCCAAGGTGATGGTCTGTGA	189
mEgCIR3769	AJ578723	4	F-TACTTCCTACTGGCCCATGC R-CGGATAGCTGGTGACATCCT	225
mEgCIR0074	AJ578500	4	F-CGATGATGAGGCTTGTGCTA R-TGAATGGCTATGACCGTGAA	225
mEgCIR2595	AJ578611	4	F-CAATATCAAAGAGCCGCACA R-AATGCATCTCTGGTCCTTGC	265

Table 1. Contd.

mEgCIR3705	AJ578709	4	F-AACCATCCACCATGCAGAA R-TTCCACAATTCCATTCATTCAA	234
mEgCIR3040	AJ578622	4	F-GATCTCTTGTGGGTGCGTTA R-AGGTCCTCATCCGACTTGTG	228
mEgCIR3477	AJ578671	4	F-TAGCATGCAGACCACACACA R-ATGCTGGGAAAATCATGCTG	222
mEgCIR0059	AJ578499	4	F-TGCAGGGGATGCTTTTATTT R-GGCCCTTAATTCCTGCCTTA	189
mEgCIR3775	AJ578724	4	F-ATGTGGGAACTCCTGAAACG R-TCCTTAGCGGCTTCACTTGT	168
mEgCIR3557	AJ578682	4	F-CATTGCCATTCCCTTCAAGT R-TCCCCTCTGTTCACTCAAGC	226
mEgCIR0369	AJ578516	3	F-AACCAAGGGGTAGCAAACCT R-TTTTAATCCCTGCCTGATGC	212
mEgCIR3260	AJ578628	3	F-GGGCAAGTCATGTTTCCTACA R-TAAGGGCGAGGTATTTCTGC	236
mEgCIR0408	AJ578519	2	F-AGCGCAGTTGCTCGGTATAA R-CCCTGCAGTGTCCCTCTTTA	163
mEgCIR3683	AJ578703	2	F-CATCAGTAGCTTGAACCTGAAAAA R-CTGAGGTCTACAGGGCATGTT	190
mEgCIR0874	AJ578558	1	F-TGCTCCAGTTGTCGAGTTGT R-TTGCAGTTTATTTGGCTACCAG	185
mEgCIR3788	AJ578728	1	F-TGACCAAAGACAGCATGAGC R-CATGAGCGCAACATCAGACT	194

[1]Each forward primer contained in addition the M13 tail (TTTCCCAGTCACGACGTT) at the 5'-end. [2]The visible size on the gel will be 18 bp larger due to the M13 tail.

following the genotype configuration demonstrated by Ritter et al. (1990), Lespinasse et al. (2000) and Billotte et al. (2005). Alleles were labeled A_i with i ranging from 0 (null allele = no amplification product) to 4 in case of four segregation alleles. Patterns for the individual SSR markers were identified in the full-sib family (Tab. 2). Chi square tests $\chi^2 = \Sigma (o-e)^2/e$, in which o represents the observed values and e the expected values and P-value calculation were performed using the statistics functions in Excel to verify if the observed segregation pattern corresponds to the expected segregation pattern at $p > 0.05$. Degrees of freedom were calculated as the number of categories (patterns) in this population minus 1.

RESULTS

DNA isolation using the novel DNA protocol

High quality genomic DNA could be extracted from 92 individuals of progeny 12 (*tenera* x *Deli dura*) each showing one major band larger than 23 kilobase (kb) on an ethidium bromide agarose gel. No visible signs of degradation of the DNA were detectable (Figure 1). Estimation of the DNA concentrations showed OD 260/280 ratios ranging between 1.7 and 2.0, supporting the visual picture. This proved that the developed simplified DNA extraction protocol allows efficient isolation from oil palm leaves.

SSR analyses in progeny 12 (NIFOR)

From 32 tested primer combinations for SSR markers coming from four linkage groups (1, 2, 3 and 4), nine gave reproducible patterns in progeny 12 (13.386 T × 26.1074 D). Two of these SSR markers (mEgCIR3439 and mEgCIR3535) resulted in monomorphic banding patterns. However, seven SSR markers out of the 32 tested (22%) segregated in the progeny (Table 2). Their banding patterns (except for mEgCIR0074) are shown in Figures 2 and 3. One of the segregating SSR markers (mECIR3260) belongs to linkage group 3, all other SSR

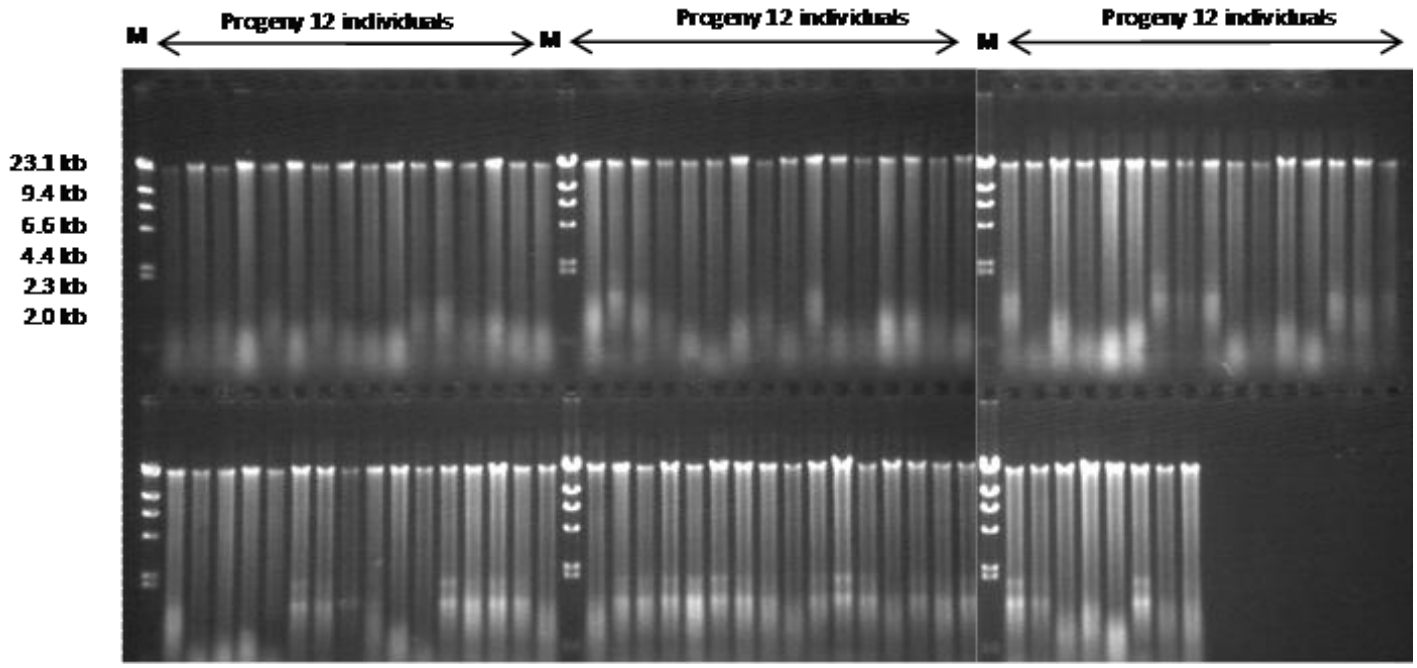


Figure 1. Separation of extracted total DNA from oil palm leaves of progeny 12 (92 individuals) for quality control. M: Lambda DNA HindIII digested (New England Biolabs) as marker. Five microliters of each sample (50 ng/µl) extracted with the new DNA extraction protocol were loaded on a 1% agarose gel and run for 30 min at 80V. The gel was stained with ethidium bromide.

markers to linkage group 4 covering the whole group. SSR marker mECIR3275 is the closest marker to the *Sh* gene. SSR marker mEgCIR1917 showed an allele pattern corresponding to a genotype configuration defined by Ritter et al. (1990) for one with four alleles segregating in the cross between heterozygous parents, whereas for the markers mEgCIR0059, mEGCIR3275, mEgCIR3260, mEgCIR3275, mEgCIR3533, and mEgCIR3557 the SSR allele patterns can be explained from genotype configurations resulting from three alleles segregating in the full-sib cross (Table 2).

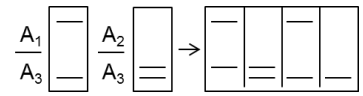
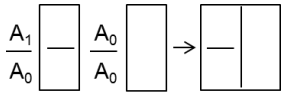
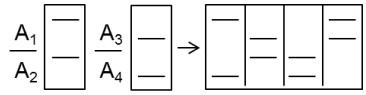
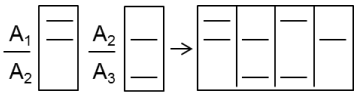
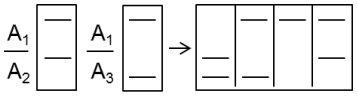
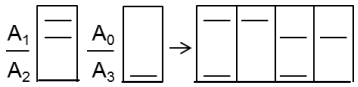
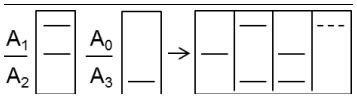
Marker mEgCIR0074 showed a segregation ratio of 1:1 corresponding to the segregation of one allele and a null allele in the second parent. Four SSR markers mEgCIR0059, mEgCIR3260, mEgCIR3533 and mEgCIR3557 showed a distorted segregation ($p < 0.05$). For mEgCIR3557, one allele pattern is missing indicating that the combination A_1A_0 may be selected against or is deleterious.

Screening progeny 12 with these SSR markers also proved that one individual showed a different SSR pattern as marked by 'X' for SSR marker mEgCIR1917 (Figure 3C). This was supported by data for mEgCIR3275 and mEgCIR0059 (data not shown). This palm tree is not part of this progeny and will be excluded from future segregation analyses. The results show that the SSR markers developed for Billotte et al. (2005) can be successfully transferred and used for marker-assisted selection in progeny 12 of the NIFOR breeding program.

DISCUSSION

Due to their long reproductive cycles and the time until traits of the mature tree can be evaluated marker-assisted selection is particularly attractive for tree breeding (Kumar et al., 2012; Thavamanikumar et al., 2013). In outcrossing crops like oil palm the use of markers is challenging as the segregation patterns observed in he progenies are more complex than in inbreeding species (Sewell and Neale, 2000; van Ooijen, 2011). An additional challenge has to be handled if in a breeding program the parents of the progeny are not available anymore for example, got lost due to diseases during the long time of cultivation. In this case, the constitution of the parents has to be reconstructed from the observed segregation pattern in the progeny (Billotte et al., 2005; Lespinasse et al., 2000) to allow mapping of the markers using mapping programs like JoinMap 4.0 (van Ooijen and Voorrips, 2001) and MapQTL 5 (van Ooijen, 2004). Markers that allow this are called informative markers (van Ooijen, 2011). In this study, we showed that for the progeny 12 (13.386 T x 26.1074 D) of the NIFOR oil palm breeding program that this situation could be successfully handled by identifying the segregation patterns for individual SSR markers in the progeny and deducting from this the genetic constitution of the parents at the locus (Table 2). The SSR analysis presented for the progeny 12 showed that the number of segregating alleles per locus in this full-sib family ranged between one

Table 2. Number of alleles segregating in progeny 12 and the corresponding segregation ratios.

Primer no.	Progeny 12 (*Tenera* x *Deli Dura*)							
	Lg	No Of Alleles Segregating	Parents P1 P2	Allelic pattern	Segregation ratio	DF	χ^2 value	P value
mEgCIR0059	4	3	$\frac{A_1}{A_3}$ $\frac{A_2}{A_3}$		1:1:1:1	3	22.091*	0.0
mEgCIR0074	4	1	$\frac{A_1}{A_0}$ $\frac{A_0}{A_0}$		1:1	1	1.103	0.294
mEgCIR1917	4	4	$\frac{A_1}{A_2}$ $\frac{A_3}{A_4}$		1:1:1:1	3	1.515	0.679
mEgCIR3260	3	3	$\frac{A_1}{A_2}$ $\frac{A_2}{A_3}$		1:1:1:1	3	8.378*	0.039
mEgCIR3275	4	3	$\frac{A_1}{A_2}$ $\frac{A_1}{A_3}$		1:1:1:1	3	6.205	0.102
mEgCIR3439	4	Monomorph						
mEgCIR3533	4	3	$\frac{A_1}{A_2}$ $\frac{A_0}{A_3}$		1:1:1:1	3	58.910*	0.0
mEgCIR3535	4	Monomorph						
mEgCIR3557	4	3	$\frac{A_1}{A_2}$ $\frac{A_0}{A_3}$		1:1:1:1	3	61.587*	0.0

*Significant at $p < 0.05$

and four, varying from locus to locus. Six SSR markers mEgCIR1917, mEgCIR0059, mEgCIR3260, mEgCIR3275, mEgCIR3533 and mEgCIR3557 proved to be fully informative markers following a segregation ratio of 1:1:1:1. However, SSR marker mEgCIR0074 belongs to a genotype configuration where in one parent only one allele was amplified, the other being a null allele with no amplification occurring in this parent (Table 2). Such markers segregate in either 1:1 or 3:1 ratios (Billotte et al., 2005; Lespinasse et al., 2000). Also, such markers provide complete information about one of the parents but no information on the other parent (van Ooijen 2011).

The efficiency of molecular markers lies in the fact that they can be used as a tool to detect sequence variation that exist between and within species (Doveri et al., 2008) and also to identify inbred lines (Shehata et al., 2009). SSR markers are particularly interesting in this regard and have been extensively used in oil crops (Cloutier et al., 2011; Rotondi et al., 2011; Xie et al., 2012). From the findings observed in the present study, the SSR markers segregated in the breeding population with up to four alleles. No immediate reason could be given why not all the SSR markers, which were used for the study, produced amplification products other than the fact that the primers (forward and/ or reverse) did not anneal due to sequence differences between the populations. Progeny 12 is quite different from the population used to develop the markers by Billotte et al. (2005). However, 22% of the SSR markers were polymorph in the NIFOR population. This is about in the

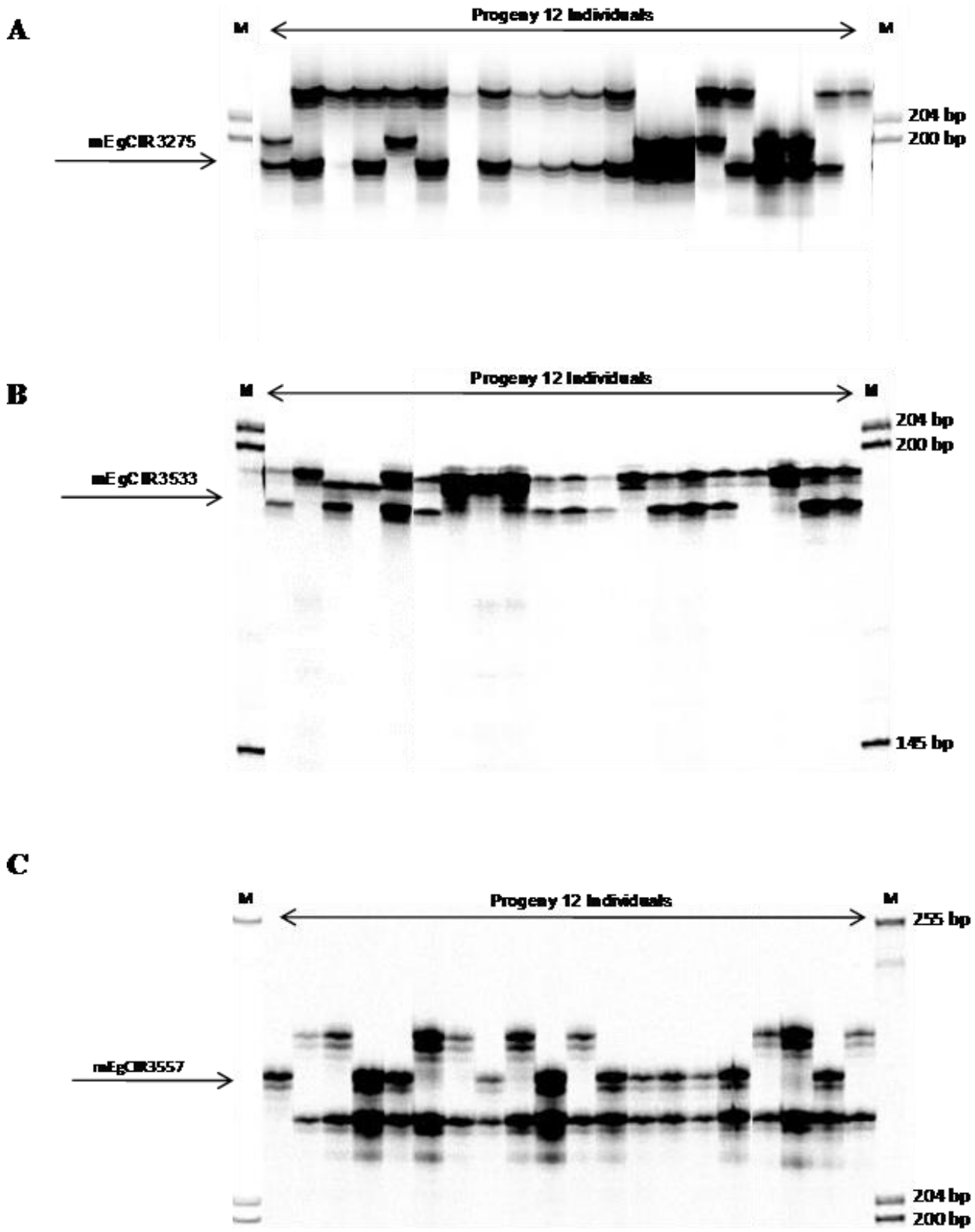


Figure 2. SSR marker analyses in progeny 12 using: **(A)** marker mEgCIR3275, **(B)** marker mEgCIR3533 and **(C)** marker mEgCIR3557. M: IRDye® 800 sizing standard 50-700 bp (LI-COR Biosciences) as marker. Twenty individuals of the segregating population are shown, which represent the observed segregation patterns in the progeny (Table 2), respectively. Samples were separated on a 6% polyacrylamide gel using a DNA analyzer 4300.

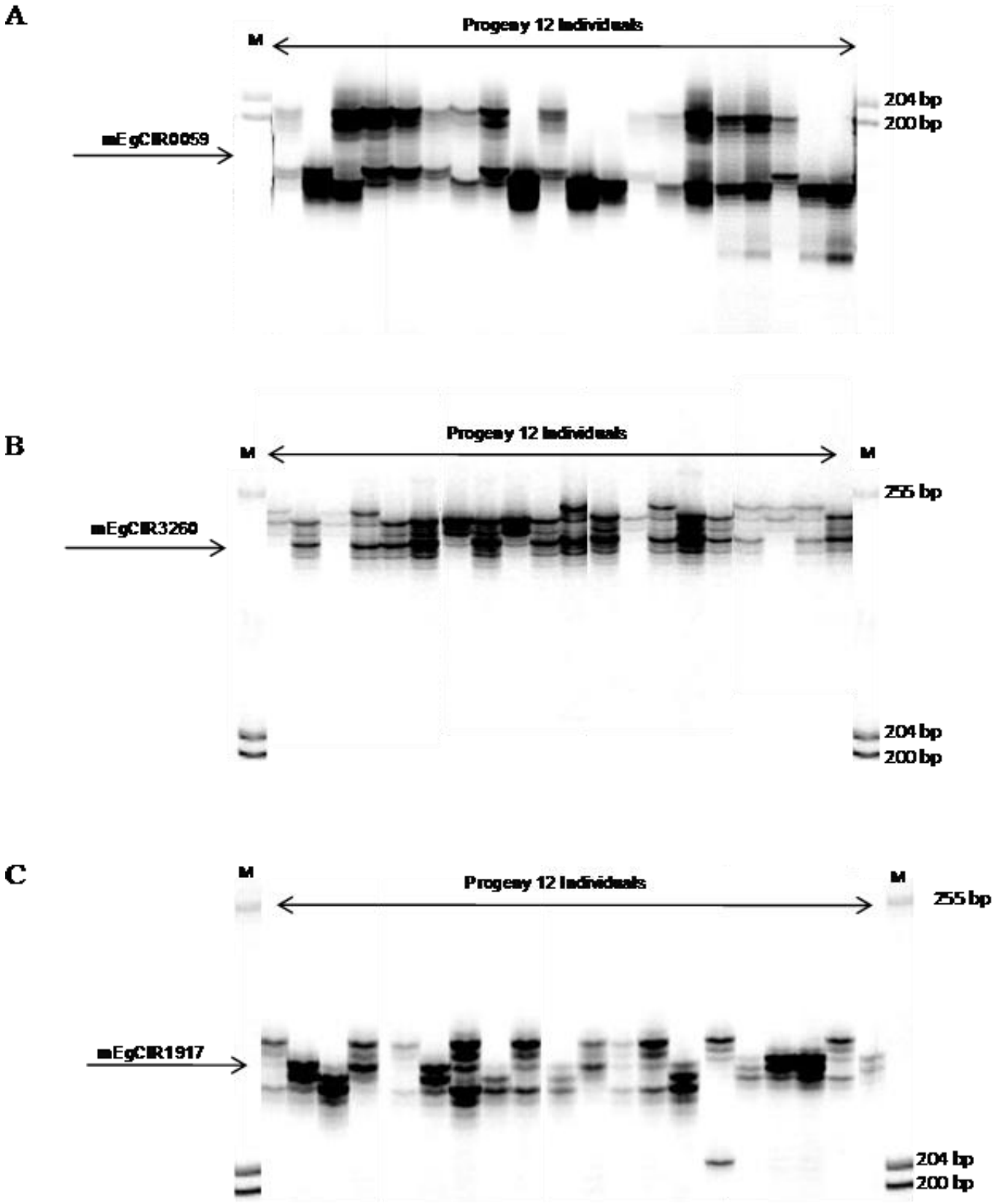


Figure 3. SSR marker analyses in progeny 12 individuals using (A) marker mEgCIR0059, (B) marker mEgCIR3260 and (C) marker mEgCIR1917. 'X' represents banding pattern for an individual, which does not belong to the population. M: IRDye® 800 sizing standard 50-700 bp (LI-COR Biosciences) as marker. Twenty individuals of the segregating population are shown, which represent the observed segregation patterns in the progeny (Table 2), respectively. Samples were separated on a 6% polyacrylamide gel using a DNA analyzer 4300.

range for transferability of SSR markers observed in other families for example, Rosaceae (Sajer et al., 2012), where 25% of the tested SSR markers proved on average to be polymorph in another mapping population.

Segregation distortion describes a phenomenon that observed genotypic frequencies deviate from the expected Mendelian frequencies (Sandler and Novitski, 1957). In progeny 12, distortions were observed for some of the SSR markers at $p > 0.05$. This was also reported by Billotte et al. (2005), however, for different markers, indicating in our case the detection of population-specific or population dependent distorted markers. In rice, Xu et al. (1997) investigated segregation distortion of markers on a large scale with six mapping populations and 1558 markers. A total of 17 chromosomal regions with distorted markers distributed over all 12 chromosomes were detected. Marker-assisted selection in distorted regions can be extremely useful to increase the frequency of the favorable allele by selecting for recombinants in the distorted region (Xu et al., 1997). Distorted markers are usually discarded prior to QTL mapping because unexpected consequences are feared (Zhan and Xu, 2011; Montoya et al., 2013). However, the former authors claim that segregation distortion can be actually helpful in the detection of QTLs (Xu, 2008; Xu and Hu, 2010) and developed a generalized linear mixed model for segregation distortion analyses (Zhan and Xu, 2011).

The application of molecular markers to the existing breeding programs at NIFOR has been considered. It is expected that with molecular markers higher accuracy, precision and earlier release of improved planting materials can be achieved within the shortest possible time when compared with the existing approach that is based on phenotypic observation. For the successful application of molecular markers for marker-assisted selection, high levels of variation should exist in the chosen breeding population. From the result of this study, it is clear that the progeny 12 can be used for the application of markers in crop improvement programs. The result of this study indicates that SSR markers can be of great benefit for breeding purposes at NIFOR if fully exploited. Even with the genome sequence (Singh et al., 2013b) and several transcriptome resources for fruit mesocarp maturation and ripening and other traits available (Bourgis et al., 2011; Tranbarger et al., 2011; Shearman et al., 2013), traits of interest still need to be localized on the genome using markers like for example, SSRs. In addition, breeding programs require markers for marker-assisted selection. Cost efficiencies of markers in breeding programs have been estimated for some crops and proved to be very efficient when integrated at the right generation into the breeding program (Dreher et al., 2003; Kuchel et al., 2005; Miah et al., 2013; Morris et al., 2003; Slater et al., 2013). Progeny 12 (13.386 T x 26.1074 D) was used in this first assessment of SSR markers for marker-assisted selection in the NIFOR breeding program. In addition, 14 additional mapping populations of the same size are available from the NIFOR breeding program (Okwuagwu 1989, NIFOR 2008). Shared parents in some of these populations will even allow multi-parent QTL analysis as performed by Billotte et al. (2010).

This is the first report of successfully applying SSR markers developed for the cross La Mé x Deli Dura (Bilotte et al., 2005) to the NIFOR oil palm breeding program opening the field to future breeding strategies applying marker-assisted selection.

Conflict of Interests

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

REFERENCES

Akpanabiatu MI, Ekpa OD, Mauro A, Rizzo R (2001) Nutrient composition of Nigerian palm kernel from the *dura* and *tenera* varieties of the oil palm (*Elaeis guineensis*). Food Chem. 72:173-177.

Asemota O, San CT, Shah FH (2004). Isolation of a kernel oleoyl-ACP thioesterase gene from the oil palm *Elaeis guineensis* Jacq. Afr. J. Biotechnol. 3:199-201.

Baisakh N, Subudhi PK, Arumuganathan K, Parco AP, Harrison SH, Knott CA, Materne MD (2009). Development and interspecific transferability of genic microsatellite markers in *Spartina* spp. with different genome size. Aquat. Bot. 91:262-266.

Beirnaert A, Vanderweyen R (1941). Contribution a l'étude génétique et biométrique des variétés d'*Elaeis guineensis* Jacq. Publ. Inst. Nat. Etude agron. Congo Belge Ser. Sci. 27:1-101.

Billotte N, Jourjon MF, Marseillac N, Berger A, Flori A, Asmady H, Adon B, Singh R, Nouy B, Potier F, Cheah SC, Rohde W, Ritter E, Courtois B, Charrier A, Mangin B (2010). QTL detection by multi-parent linkage mapping in oil palm (*Elaeis guineensis* Jacq.). Theor. Appl. Genet. 120:1673-1687.

Billotte N, Marseillac N, Risterucci AM, Adon B, Brottier P, Baurens FC, Singh R, Herran A, Asmady H, Billotte C, Amblard P, Durand-Gasselin T, Courtois B, Asmono D, Cheah SC, Rohde W, Ritter E, Charrier A (2005). Microsatellite-based high density linkage map in oil palm (*Elaeis guineensis* Jacq.). Theor. Appl. Genet. 110:754-765.

Bourgis F, Kilaru A, Cao X, Ngando-Ebongue GF, Drira N, Ohlrogge JB, Arondel V (2011). Comparative transcriptome and metabolite analysis of oil palm and date palm mesocarp that differ dramatically in carbon partitioning. Proc. Natl. Acad. Sci. USA 108:12527-12532.

Burri BJ (2012). Evaluating global barriers to the use of red palm oil as an intervention food to prevent vitamin A deficiency. Compr. Rev. Food Sci. Food Saf. 11:221-232.

Cloutier S, Ragupathy R, Niu Z, Duguid S (2011). SSR-based linkage map of flax (*Linum usitatissimum* L.) and mapping of QTLs underlying fatty acid composition traits. Mol. Breed. 28:437-451.

Collard BCY, Mackill DJ (2008). Marker-assisted selection: an approach for precision plant breeding in the twenty-first century. Phil. Trans. R. Soc. Lond. B 363:557-572.

Corley RHV, Tinker PB (2003). The oil palm. Fourth Edition. Blackwell Science Ltd, Oxford, U. K.

Doveri S, Lee D, Maheswaran M, Powell W (2008). Molecular markers – history, features and application. *In:* Kole C, Abbott AG (Eds) Principles and Practices of Plant Genomics. Volume 1: Genome Mapping. Science Publishers, U.S.A.

Dreher K., Khairallah M, Ribaut JM, Morris M (2003). Money matters (I): costs of field and laboratory procedures associated with conventional and marker-assisted maize breeding at CIMMYT. Mol. Breed. 11:221-234.

Gethi JG, Labate JA, Lamkey KR, Smith ME, Kresovich S (2002). SSR variation in important U. S. maize inbred lines. Crop Sci. 42:951-957.

Hartley CWS (1988). The Oil Palm (*Elaeis guineensis* Jacq.). Third

Edition. Longman Scientific and Technical, Longman Group, U. K.
Hu J, Zhou X, Li J (2010). Development of novel EST-SSR markers for cucumber (*Cucumis sativus*) and their transferability to related species. Sci. Hortic. 125(3):534-538.
Kapoor R, Banga SS, Banga SK (2009). A microsatellite (SSR) based map of *Brassica rapa*. New Biotechnol. 26:239-243.
Kok S, Ong-Abdullah M, Ee GC, Namasivayam P (2011). Comparison of nutrient composition in kernel of tenera and clonal materials of oil palm (*Elaeis guineensis* Jacq.). Food Chem. 129:1343-1347.
Kubo N, Hirai M, Kaneko A, Tanaka D, Kasumi K (2009). Development and characterization of simple sequence repeat (SSR) markers in the water lotus (*Nelumbo nucifera*). Aquat. Bot. 90:191-194.
Kuchel H, Ye G, Fox R, Jefferies S (2005). Genetic and economic analysis of a targeted marker-assisted wheat breeding strategy. Mol. Breed. 16:67-78.
Kumar S, Bink MC, Volz RK, Bus VG, Chagné D (2012). Towards genomic selection in apple (*Malus* x *domestica* Borkh.) breeding programmes: Prospects, challenges and strategies. Tree Genet. Genomes 8:1-14.
Lespinasse D, Rodier-Goud M, Grivet L, Leconte A, Legnate H, Seguin M (2000). A saturated genetic linkage map of rubber tree (*Hevea* spp.) based on RFLP, AFLP, microsatellites and isozyme markers. Theor. Appl. Genet. 100:127-138.
Li L, Wang J, Guo Y, Jiang F, Xu Y, Wang Y, Pan H, Han G, Li R, Li S (2008). Development of SSR markers from ESTs of gramineous species and their chromosome location on wheat. Proc. Natl. Acad. Sci. USA 18:1485-1490.
Low EL, Alias H, Boon S, Shariff EM, Tan CA, Ooi LC, Cheah S, Raha A, Wan K, Singh R (2008). Oil palm (*Elaeis guineensis* Jacq.) tissue culture ESTs: identifying genes associated with callogenesis and embryogenesis. BMC Plant Biol. 8:62.
Madon M, Clyde MM, Cheah SC (1998). Cytological analysis of *Elaeis guineensis* and *Elaeis oleifera* chromosomes. J. Oil Palm Res. 10:68-91.
Matthäus B (2007). Use of palm oil for frying in comparison with other high-stability oils. Eur. J. Lip. Sci. Technol. 109: 400-409.
McCouch SR, Teytelman L, Xu Y, Lobos KB, Clare K, Walton M, Fu B, Maghirang R, Li Z, Xing Y, Zhang Q, Kono I, Yano M, Jellstrom R, DeClerck G, Schneider D, Cartinhour S, Ware D, Stein L (2002). Development and mapping of 2240 new SSR markers for rice (*Oryza sativa* L). DNA Res. 9:199-207.
Miah G, Rafii M, Ismail MR, Puteh AB, Rahim HA, Islam KN, Latif MA (2013). A review of microsatellite markers and their applications in rice breeding programs to improve blast disease resistance. Int. J. Mol. Sci. 14:22499-22528.
Montoya C, Lopes R, Flori A, Cros D, Cuellar T, Summo M, Espeout S, Rivallan R, Risterucci A-M, Bittencourt D, Zambrano JR, Alarcon G WH, Villeneuve P, Pina M, Nouy B, Amblard P, Ritter E, Leroy T, Billotte N (2013). Quantitative trait loci (QTLs) analysis of palm oil fatty acid composition in an interspecific pseudo-backcross from *Elaeis oleifera* (H.B.K.) Cortés and oil palm (*Elaeis guineensis* Jacq.). Tree Genet. Genomes 9:1207-1225.
Morris M, Dreher K., Ribaut J-M, Khairallah M (2003). Money matters (II): costs of maize inbred line conversion schemes at CIMMYT using conventional and marker-assisted selection. Mol. Breed. 11:235-247.
NIFOR (2008). NIFOR in house research review. Progress report 2001-2008.
Oetting WS, Lee HK, Flanders DJ, Wiesner GL, Sellers TA, King RA (1995). Linkage analysis with multiplexed short tandem repeat polymorphisms using infrared fluorescence and M13 tailed primers. Genomics 30:450-458.
Oguntibeju OO, Esterhuyse AJ, Truter EJ (2009). Red palm oil: nutritional, physiological and therapeutic roles in improving human well-being and quality of life. Br. J. Biomed. Sci. 66:216-222.
Okwuagwu CO (1989). The developments from the comprehensive oil palm breeding programme of the Nigerian Institute for Oil palm Research (NIFOR) and future breeding strategy. Paper presented at Int. Conf. palms and palm products, Nigerian Institute for Oil palm Research, Benin City, November 21-25.
Ong AS, Goh SH (2002). Palm oil: a healthful and cost-effective dietary component. Food Nutr. Bull. 23(1):11-22.
Pan L, Quan Z, Li S, Liu H, Huang X, Ke W, Ding Y (2007). Isolation and characterization of microsatellite markers in the sacred lotus (*Nelumbo nucifera* Gaertn.). Mol. Ecol. Notes 7:1054-1056.
Ritter E, Gebhardt C, Salamini F (1990). Estimation of recombination frequencies and construction of RFLP linkage maps in plants from crosses between heterozygous parents. Genetics 125:645-654.
Rotondi A, Beghè D, Fabbri A, Ganino T (2011). Olive oil traceability by means of chemical and sensory analyses: A comparison with SSR biomolar profiles. Food Chem. 129:1825-1831.
Sajer O, Scorza R, Dardick C, Zhebentyayeva T, Abbott A, Horn R (2012). Development of sequence-tagged site markers linked to the pillar growth type in peach (*Prunus persica*). Plant Breed. 131:186-192.
Sambanthamurthi R, Sundram K, Tan YA (2000). Chemistry and biochemistry of palm oil. Prog. Lipid Res. 39(6):507-558.
Sandler L, Novitski E (1957). Meiotic drive as an evolutionary force. Am. Nat. 41:105-110.
Sewell MM, Neale DB (2000). Mapping quantitative traits in forrest trees. In Jain SM, Minocha SC (eds), Molecular biology of woody plants, Vol. I, Kluwer Academics Publishers, the Netherlands, pp. 407-423.
Seng T-Y, Mohamed Saad SH, Chin C-W, Ting N-C, Singh R, Singh H, Zaman FQ, Tan S-G, Syed Alwee SSR (2011). Genetic linkage map of a high yielding FELDA Deli x Yangambi oil palm cross. Plos One 6(11):e26593.
Shehata AI, Al-Ghethar HA, Al-Homaidan AA (2009). Application of simple sequence repeat (SSR) markers for molecular diversity and heterozygosity analysis in maize inbred lines. Saudi J. Biol. Sci. 16: 57-62.
Shearman JR, Jantasuriyarat C, Sangsrakru D, Yoocha T, Vannavichit A, Tragoonrung S, Tangphatsornruang S (2013). Transcriptome analysis of normal and mantled developing oil palm flower and fruit. Genomics 101:306-312.
Shen L, Courtois B, McNally KL, Robin S, Li Z (2001). Evaluation of near-isogenic lines of rice introgressed with QTLs for root depth through marker-aided selection. Theor. Appl. Genet. 103:75-83.
Slater AT, Cogan NOI, Forster JW (2013). Cost analysis of the application of marker-assisted selection in potato breeding. Mol. Breed. 32:299-310.
Singh R, Tan SG, Panandam JM, Rahman RA, Ooi LCL, Low EL, Sharma M, Jansen J, Cheah S (2009). Mapping quantitative trait loci (QTLs) for fatty acid composition in an interspecific cross of oil palm. BMC Plant Biol. 9:114.
Singh R, Low ETL, Ooi LCL, Ong-Abdullah M, Ting NC, Nagappan J, Nookiah R, Amiruddin MD, Rosli R, Manai MAA, Chan KL, HGamlim MA, Azizi N, Lakey N, Smith SW, Budiman MA, Hogan M, Bacher B, Van Brunt A, Wang C, Ordway JM, Sambanthamurthi R, Martienssen RA (2013a). The oil palm *SHELL* gene controls oil yield and encodes a homologue of *SEEDSTICK*. Nature 500:340-344.
Singh R, Ong-Abdullah M, Low ETL, Manai MAA, Rosli R, Nookiah R, Ooi LCL, Ooi SE, Chan KL, Halim MA, Azizi N, Nagappan J, Bacher B, Lakey N, Smith SW, He D, Hogan M, Budiman MA, Lee EK, DeSalle R, Kudrna D, Goicoechea JL, Wing RA, Wilson RK, Fulton RS, Ordway JM, Martienssen RA, Sambanthamurthi R (2013b). Oil palm genome sequence revelas divergence of interfertile species in old and new world. Nature 500:335-339.
Tang SX, Kishore VK, Knapp SJ (2003). PCR-multiplexes for a genome-wide framework of simple sequence repeat marker loci in cultivated sunflower. Theor. Appl. Genet. 107:6-19.
Thavamanikumar S, Southerton SG, Bossinger G, Thumma BR (2013). Dissection of complex traits in forest trees – opportunities for marker-assisted selection. Tree Genet. Genomes 9:627-639.
Ting NC, Jansen J, Nagappan J, Ishak Z, Chin CW, Tan SG, Cheah SC, Singh R (2013). Identification of QTLs associated with callogenesis and embryogenesis in oil palm using genetic linkage maps improved with SSR markers. PLOS 8(1):e53076.
Tranbarger TJ, Dussert S, Joet T, Argout X, Summo M, Champion A, Cros D, Omore A, Nouy B, Morcillo F (2011). Regulatory mechanisms underlying oil palm fruit mesocarp maturation, ripening and functional specialization in lipid and carotenoid metabolism. Plant Physiol. 156:564-584.
Van Ooijen JW (2004). MapQTL5, software for mapping of quantitative loci in experimental populations. Kyazma BV. Wageningen,

Netherlands.
Van Ooijen JW (2011). Multipoint maximum likelihood mapping in a full-sib family of an outbreeding species. Genet. Res. 93:343-349.
Van Ooijen JW, Voorrips RE (2001). JoinMap 4.0 Software for calculation of genetic linkage maps. Kyazma BV. Wageningen, Netherlands.
Xia Z, Tsubokura Y, Hoshi M, Hanawa M, Yano C, Okamura K, Ahmed TA, Anai T, Watanabe S, Hayashi M, Kawai T, Hossain KG, Masaki H, Asai K, Yamanaka N, Kubo N, Kadowaki K, Nagamura Y, Yano M, Sasaki T, Harada K (2007). An integrated high-density linkage map of soybean with RFLP, SSR, STS, and AFLP markers using a single F_2 population. DNA Res. 14:257-269.
Xie D, Han Y, Zeng Y, Chang W, Teng W, Li W (2012). SSR- and SNP-related QTL underlying linolenic acid and other fatty acid contents in soybean seeds across multiple environments. Mol. Breed. 30:169-179.
Xu S (2008). Quantitative trait locus mapping can benefit from segregation distortion. Genetics 180:2201-2208.
Xu S, Hu Z (2010). Mapping quantitative trait loci using distorted markers. Int. J. Plant Genomics 12:1-11.
Xu Y, Zhu L, Xiao J, Huang N, McCouch SR (1997). Chromosomal regions associated with segregation distortion of molecular markers in F_2, backcrosses, doubled haploid, and recombinant inbred populations in rice (*Oryza sativa* L.). Mol. Gen. Genet. 253:535-545.
Zhan H, Xu S (2011). Generalized linear mixed model for segregation distortion analysis. BMC Genetics 12:97-110.
Zhang R, Hu D, Zhang J, Zuo X, Jiang R, Wang H, Ng TB (2010). Development and characterization of simple sequence repeat (SSR) markers for the mushroom *Flammulina velutipes*. J. Biosci. Bioeng. 110(3):273-275.
Zheng Y, Zhang G, Lin F, Wang Z, Jin G, Yang L, Wang Y, Chen X, Xu Z, Zhao X, Wang H, Lu J, Lu G, Wu W (2008). Development of microsatellite markers and construction of genetic map in rice blast pathogen *Magnaporthe grisea*. Fungal Genet. Biol. 45:1340-1347.

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The suitability evaluation of lignocellulosic substrate as growing media substitute

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The use of peat moss (PM) as a growing media is decreasing due to high costs and environmental considerations. Therefore, diverse waste products are being used as organic amendments in certain soils before afforestation. The main objective of this work was to identify and evaluate possible substrate alternatives or amendments to peat moss. This study involves the physical and chemical characterization and growth test of lignocellulosic substrate (waste paper (WP), oakwood sawdust, hinoki wood sawdust and rice hull) in order to evaluate their use as components of growing media. Lignocellulosic substrates showed adequate physical and chemical properties compared to peat moss for their use as growing media. The mixtures growing media were prepared using different proportions as substrate to grow Chinese cabbage (Brassica rapa var. glabra) in a greenhouse. Different lignocellulosic substrates were compared with a commercially available substrate. The highest values for seed germination were obtained for 30% peat moss + 10% perlite + 60% waste paper substrate. The stem height and leaf area of the WP (containing 30% peat moss, 10% perlite and 60% waste paper) was higher than that of PM. Utilization of waste paper can be considered as an alternative media component to substitute the widely using expensive peat moss.

Key words: Growing media, waste paper, waste sawdust, rice hull, lignocellulosic substrate.

INTRODUCTION

Today, various organic substrate and inorganic substrate are used as growth media. Organic materials such as peat moss or pine bark and inorganic materials such as sand, calcined clay, perlite and vermiculite are used to formulate growth media to produce woody landscape plants in containers. Most of them are combined with various materials, such as a mixture of peat moss and pine bark, or a mixture of peat moss and pine bark with mineral materials like perlite or vermiculite. All over the world, stone wool and other materials like perlite, pumice, polyortanphome, zeolite, coco peat and sawdust are used as growth media in soilless culture (Ghehsarehet al., 2011, Tehranifaret al., 2012). The selection of media components in which grown plants can be successfully grown is usually based on freedom from soil pests and harmful chemicals, cost, shipping weight and local availability.

Peat moss is the most commonly used growing media. However, peatmoss is obtained from wetlands, which are being rapidly depleted, causing environmental concerns

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that have led to many individual countries to limit the extent of peat moss mining, and prices are increasing as a result. Research on peat moss alternatives is of great interest in the future. In this context, different authors have suggested that some organic materials such as well-composted municipal solid waste and biosolid composts could be feasible materials for a partial peat moss substitution. Also, alternative growing media are being sought in order to improve cost and sustainability (Hernandez et al., 2005). Alternative growing media were generally residual materials such as wood waste, coconut coir, rice hull (Bugbee, 2002; Wrigth et al., 2006; Salifu et al., 2006). Partially, composted pine bark is one of the most widely used substitutes for peatmoss and has been used for decades as a growing media. Currently, the use of other crop residues and by-product were increased.

A number of studies have shown that organic residues such as urban solid wastes, sewage sludge, paper waste, pruning waste, spent mushroom and even green wastes, after proper composting, can be used with very good results as growth media instead of peat moss (Ostos et al., 2008; Bustamante et al., 2008). The increasing interest in waste recycling is another cause to advocate the recycling and use of organic wastes and composts as soil or potting amendments; it could be one of the most attractive methods of solving the problem of waste disposal.

Waste paper, waste wood, agriculture residue may have potential as an organic component of artificial growth media. Bellamy et al. (1995) demonstrated the short-term benefits of amending agricultural soils with waste paper; however, they recognized a need to study the effects of repeated annual additions of waste paper on soil properties. Wood wastes, such as sawdust, are often composted with manure or supplemented with fertilizer to supply the needed nitrogen. Because of the inherent differences in chemical properties between different woods, however, the suitability of sawdust as an organic growing media component is extremely variable.

Rice hull is a product of rice milling process. It is mainly used in Korea generally as a mulching material (Roh and Pyon, 2004) and for water absorption (Kim et al., 2004). In a forest nursery in Greece (Tsakaldimi, 2006), rice hulls in a 1:3 mixture with peat moss were an effective substitute for growing *Pinus halepensis* seedlings. The properties of different material used as growing media exhibit direct and indirect effects on plant growth and production. The selection of a particular material depends on its availability, cost and local experience of its use (Klute, 1986). Physical and chemical features of alternative growing media such as degree of dispersion, pH, porosity, water holding capacity, must be considered for choosing the materials (Ezure and Wilson, 1983). To provide optimal characteristics, substrates are generally composed of a mixture of different materials in varying proportions. Based on the fact that it is not possible to predict with precision the performance of a mixture is based on its components, crop response to various mixtures must be tested and classified. Thus in evaluating the aptitude of a material for use as a new substrate for the cultivation of a variety of species, its physical, chemical and biological characteristics must be determined as well as its fertilization and water needs. Growth performance must also be evaluated to determine the quality of a substrate.

The objective of this study was to determine the physical and chemical characteristics of the various lignocellulosic substances and evaluate their potential for use as substrate components. This study was performed under greenhouse conditions, with peat moss, perlite and lignocellulosic substrate (waste paper, oak wood sawdust, hinoki wood sawdust and rice hull) as the main components of the growing media. The performance of the mixtures growing media was evaluated through the germination behavior of the Chinese cabbage (*Brassica rapa* var. *glabra*).

MATERIALS and METHODS

Growing media preparation

The commercial growing media used routinely at nursery was used as control (peat moss). The lignocellulosic substrate included wastepaper, oakwood sawdust, hinoki wood sawdust, rice hull. The lignocellulosic substrate was air-dried and hammer-milled to a particle size of -20 mesh/+80 mesh, and then stored in sealed plastic bags at 4°C until used. The mixtures growing media were prepared by mixing 30% of peat moss, 10% of perlite and 60% of waste paper, oakwood sawdust, hinoki wood sawdust and rice hull. Ratios of each component in each substrate are shown in Table 1.

Physical and chemical properties of growing media

The carbohydrate content of lignocellulosic substrate and mixture growing media were analyzed by gas chromatography (YL-6100, Young Lin Ins. Co., Ltd. Korea) with a DB-225 capillary column (15 m, 0.25 mm ID, 0.25 mm film thickness) (J&W Scientific, Folsom, CA). Injection samples were prepared according to ASTM 1821 - 96. This method describes a procedure for derivatizing monomers to their respective alditol acetates and tests for the sugars arabinose, xylose, mannose, galactose and glucose. Alditol acetates were identified by their retention time in comparison to a mixture of authentic monosaccharides. Monosaccharides were quantified on the basis of the peak area of the internal standard (*myo*-inositol added in known concentration) and normalized to the sugar content of the samples. Total carbohydrate content was calculated as the sum of the individual sugar. The pH was measured in water extracts of all lignocellulosic substrate (sample : distilled water ratio of 1:5) using an Orion (Cambridge, Mass.) pH meter. The ash content of lignocellulosic substrate was determined by a muffle furnace at 550°C according to ISO 1171-1981. Carbon (C) and nitrogen (N) content in lignocellulosic substrate were determined by using CN analyzer (Micro coder JM 10; G-Science Laboratory, Tokyo, Japan). The Mineral elements (K, Ca, Mg, Na, Zn, Fe, Mn and P) of lignocellulosic substrate were measured using inductively coupled plasma atomic emission spectrometry (ICP-AES, OPTIMA 3300 DV) to quantify aqueous constituents following microwave digestion with HNO_3- H_2SO_4-$HClO_4$ solution.

Table 1. Substrate mixtures used in the study.

Treatment	Formulation	Designation
1	100 % Peatmoss (commercial substrate)	PM
2	30 % Peatmoss + 10 % perlite + 60 % waste paper	WP
3	30 % Peatmoss + 10 % perlite + 60 % oakwood sawdust	OS
4	30 % Peatmoss + 10 % perlite + 60 % hinoki wood sawdust	HS
5	30 % Peatmoss + 10 % perlite + 60 % rice hull	RH

Table 2. Carbohydrate content of lignocellulosic substrate.

Growing media component	Dry weight (g / 100 gdry mass)						
	Arabinose	Xylose	Mannose	Galactose	Glucose	Total carbohydrate	Optimal range
Peatmoss	0.8	1.6	2.2	2.9	16.4	23.9^{e}	
Wastepaper	1.7	5.0	3.1	0.0	57.1	66.9^{a}	
Oakwood sawdust	0.9	10.5	3.2	1.8	42.5	58.9^{b}	> 80
Hinoki wood sawdust	0.6	7.1	0.8	0.8	36.6	45.9^{d}	
Rice hull	3.2	8.9	0.0	1.3	37.1	50.5^{c}	

In each column, values with different letters indicate statistically significant differences ($p < 0.05$) by Duncan's test. Optimal range in growing media according to Abad et al. (2001).

The porosity and water holding capacity of lignocellulosic substrates were measured by Verdonck and Gabriels (Verdonck and Gabriels, 1992) methods. The measurements were carried out for three replicates, and values are an average of three replicates. The results are expressed as mean values ± standard deviation (SD).

Growth test

The plant species used to evaluate the suitability of the growing media was Chinese cabbage (*Brassica rapa* var. *glabra*). Twenty seeds of Chinese cabbage were placed in Petri dishes (three dishes per substrate) containing 10 g of each mixture substrate. The seed was cultivated in the greenhouse at 25°C for 5 days duration in light/ 8 h dark and 70% relative air humidity. Seed germination was recorded every day. The percentage of seed germination was calculated as follows:

$$\text{Seed germination (\%)} = \frac{\text{Total number of seeds germinated}}{\text{Total number of seeds in all replicates}} \times 100$$

Statistical analysis

Obtained data were subjected to analysis of variance to determine the growing media effects. Statistical analysis had been carried out with SAS statistical software and according to analyzing from ANOVA test and comparing data mean to Duncan test. Duncan's multiple comparison range test was used to determine significant differences between the means.

RESULTS AND DISCUSSION

Chemical and physical properties of growing media components

The physico-chemical characteristics of substrates were shown in Tables 2 to 5. The four lignocellulosic substrates used as growing media were tested (Peat moss was a commercial growing media used as the control). The carbohydrate of lignocellulosic substrate was indices for the organic material. The carbohydrate content of lingo-cellulosic substrates was presented in Table 2. The total carbohydrate content differed significantly among sub-strates, with total carbohydrate ranging from 23.9 to 66.9 g / 100 g. The highest carbohydrate content was observed in waste paper. Glucose, together with xylose, represented a significant part of the total carbohydrate. Glucose modulates many vital processes in photosyn-thetic plants.

Organic material is one of the most important constituents of soils due to its capacity in affecting plant growth indirectly and directly. Indirectly, organic material improves the physical conditions of soils by enhancing aggregation, aeration and water retention, thereby creating a suitable environment for root growth (Senesi and Loffredo, 1999). Recently, various organic material such as mulch, manure and compost, have been investigated for their effectiveness in soil remediation. It has been demonstrated that the application of organic material to

Table 3. Ashand C/N ratio of lignocellulosic substrate.

Growing media component	Ash (g / 100 g)	C (%)	N (%)	C/N ratio
Peat moss	6.1 ± 0.4^{c}	45.0^{b}	0.8^{a}	56.3^{e}
Wastepaper	15.6 ± 0.1^{a}	42.3^{d}	0.2^{c}	211.5^{c}
Oakwood sawdust	0.7 ± 0.0^{d}	48.0^{a}	0.2^{c}	240.0^{b}
Hinoki wood sawdust	7.8 ± 0.0^{b}	43.4^{c}	0.1^{c}	434.0^{a}
Rice hull	15.6 ± 0.1^{a}	39.4^{e}	0.5^{b}	78.8^{d}
Optimal range				20 - 40

In each column, values with different letters indicate statistically significant differences ($p < 0.05$) by Duncan's test. Optimal range in growing media according to Abad et al. (2001).

Table 4. Mineral elements of lignocellulosic substrate.

Growing media component	Dry weight (g / 100 gdry mass)							
	K	Ca	Mg	Na	Zn	Fe	Mn	P
Peatmoss	0.02 ± 0.0^{e}	0.57 ± 0.1^{b}	0.13 ± 0.0^{b}	0.00 ± 0.0^{c}	0.0 ± 0.0^{a}	0.02 ± 0.0^{b}	0.00 ± 0.0^{a}	0.00± 0.0^{a}
Waste paper	0.12 ± 0.2^{c}	2.55 ± 0.7^{a}	0.12 ± 0.0^{b}	0.08 ± 0.1^{b}	0.0 ± 0.0^{a}	0.06 ± 0.0^{a}	0.00 ± 0.0^{a}	0.01± 0.0^{a}
Oakwood sawdust	0.04 ± 0.0^{d}	0.07 ± 0.0^{c}	0.00 ± 0.0^{c}	0.00 ± 0.0^{c}	0.00 ± 0.0^{a}	0.00 ± 0.0^{c}	0.00 ± 0.0^{a}	0.00± 0.0^{a}
Hinoki wood sawdust	0.15 ± 0.0^{b}	0.00 ± 0.0^{d}	0.24 ± 0.0^{a}	0.81 ± 0.0^{a}	0.00 ± 0.0^{a}	0.01 ± 0.0^{bc}	0.00 ± 0.0^{a}	0.00 ± 0.0^{a}
Rice hull	0.24 ± 0.0^{a}	0.00 ± 0.0^{d}	0.00 ± 0.0^{c}	0.00 ± 0.0^{c}	0.00 ± 0.0^{a}	0.00 ± 0.0^{c}	0.00 ± 0.0^{a}	0.01± 0.0^{a}

In each column, values with different letters indicate statistically significant differences ($p < 0.05$) by Duncan's test.

Table 5. Comparison of physical properties of lignocellulosic substrate.

Growing media component	pH	Total porosity (%)	Water holding capacity (%)
Peatmoss	5.1 ± 0.0d	89.9a	47.0a
Waste paper	7.5 ± 0.2a	86.2b	30.8d
Oakwood sawdust	4.5 ± 0.0e	65.3d	35.3c
Hinoki wood sawdust	5.3 ± 0.0c	82.2c	42.9b
Rice hull	7.0 ± 0.1b	82.3c	18.9e
Optimal range	5.2 - 6.5	>85	20-30

In each column, values with different letters indicate statistically significant differences ($p < 0.05$) by Duncan's test. Optimal range in growing media according to Abad et al. (2001).

saline soils can accelerate Na leaching, decrease the exchangeable sodium percentage and electrical conductivity and increase water infiltration, water holding capacity and aggregate stability (El-Shakweer et al., 1998).

The ash, C, N and C/N ratio of substrate were presented in Table 3. The highest ash content (15.6 g / 100 g) was obtained from waste paper and rice hull, while the lowest ash content was obtained from oak wood sawdust (0.7 g / 100 g). Ash of lignocellulosic substrate was essentially a direct source of other major elements, notably P, Ca, Mg and especially K in soils. Since ash of lignocellulosic substrate generally contains very little carbon and nitrogen, its application to the soil may reduce the total contents in C and N, by increasing the solubility of organic carbon (Kahl et al., 1996) and the nitrification rate (Meiwes, 1995). The ratio of carbon to nitrogen is that the parameter most often considered in the growing media. Various lignocellulosic substrate showed differences in the C/N ratio. The trend in C/N ratio was hinoki wood sawdust (434.0) >oakwood sawdust (240.0) > waste paper (211.5) > rice hull (78.8). The C/N ratio was very high in waste paper, oakwood sawdust and especially in hinoki wood sawdust, which causes immobilization of N. The C/N ratio is important after composting because it determines the value of the mature compost as a soil amender material for plants. Some authors suggest that the C/N ratio is an extremely important property in the decomposition of organic matter by microorganisms (Marin, 2004), and for this reason, the

organic matter added to saline soils plays an important role in the positive effect observed in microbial activity and enzymatic activities such as urease, alkaline phosphatase and dehydrogenase (Bellamy et al., 1995). Tejada and Gonzalez (2005) demonstrated that an increase in the organic matter content of saline soils increases soil structural stability, soil bulk density and, therefore, soil microbial biomass.

The results of comparison means for the concentration of mineral element in the lignocellulosic substrates are shown in Table 4. The levels of P, Mn, Zn and Fe were 0.00 to 0.06 g / 100 g, respectively, and were similar for all substrates. K, Ca, Mg and Na (g / 100 g) differed significantly among sources, ranging from 0.02 to 0.24, 0.00 to 2.55, 0.00 to 0.24 and 0.00 to 0.81, respectively. Generally, peat moss was poor in nutrients (K, Ca, Mg). It would require frequent or regular use of fertilizer if used as substrate. However, waste paper provides and contains a good reserve of nutrients: it is high in K, Ca and Mg. All physical properties tested differed significantly among substrates. Peat moss, waste paper, oakwood sawdust, hinoki wood sawdust and rice hull substrates, used in a growing media, can differ in their physical properties. Physical properties affect the air content and retain the volume of available water. It also adsorbs the rate of nutrients in substrate. Some physical properties of substrates are presented in Table 5. Depending on lignocellulosic substrate pH ranged from 4.5 to 7.5. In general, all the substrates, including peat moss, showed pH values within the established optimal range (5.2 to 6.5) suggested by different authors (Sanchez-Monederoet al., 2004; Herrera et al., 2008; Noguera et al., 2003). The pH of the waste paper, hinoki wood sawdust and rice hull should not interfere with nutrient assimilation, oakwood sawdust pH was highly acidic, which would negatively influence nutrient availability. Lignocellulosic substrates also maintain a more constant media pH, and in some cases contribute to acidification of the soil (Karp et al., 2006). One of the most influential factors determining the growth and composition of soil bacterial communities was pH. However, pH was often correlated with many other factors, including nutrient availability and plant community, and causality among factors is not easily determined. The pH was often correlated with important environmental factors influencing the microbial community, including nutrient availability, heavy metal availability and toxicity (Degryse et al., 2009; Fernandez et al., 2009) and plant community structure.

The total porosity space was expressed as a percentage, and can be divided into large pores (“macropores”) that provide for gaseous exchange and root growth, and small pores (“micropores”) that control the water holding capacity. Total porosity percentage that is, an index for root media aeration was high for waste paper (86.2%) and it was low for oakwood sawdust (65.3%). When root media aeration is sufficient, supply of water and nutrient elements for plants is easy. The total porosity of the media was important, probably more crucial than the portion of water holding capacity. On average, 10 to 30% of the media volume should be composed of air space, while 45 to 65% should be composed of water (Altland, 2006). The amount of water holding capacity was between 18.9 and 42.9% of the volume for the lignocellulosic substrates. These results showed that the particle size of perlite was greater than that of the coco peat, because by decreasing the particle size, the water holding capacity will increase. The same trend was reported by other researchers (Wada, 2005). Hence, the percentages of organic matter and fine granules in the substrate were increased, which increases the maximum water holding capacity and therefore potentially increases saturated weight and reduces air content at the maximum water capacity.

Physical and chemical properties of the mixtures growing media

Mixtures of varying proportions can be designed to take advantage of the positive characteristics of each substance and their interactions, in order to create optimal characteristics for plant growth (best water retention, pH levels, non-limiting salts, etc.). The mixtures substrate assayed were: peat moss (100%), peat moss (30%) + perlite (10%) + waste paper (60%), peat moss (30%) + perlite (10%) + oakwood sawdust (60%), peat moss (30%) + perlite (10%) + hinoki wood sawdust (60%), peat moss (30%) + perlite (10%) +rice hull (60%). Table 6 and 7 describe some of the characteristics of the various mixtures, along with the commercial substrate used as control (peat moss). Most of the mixtures growing media present pH levels close to optimal range. The pH of WP was slightly alkaline and was, therefore, not considered a limiting factor (Miller, 2004). The main physical characteristics of mixture growing media included increase of porosity and water holding capacity.

Substrates containing only organic components often lose macroporosity over time. Decomposition of organics creates an over abundance of small particles that hold excessive water and reduce air porosity. A mixture of organic and inorganic components, such as perlite, can help maintain the percentage of large pores later in the growing season (Warren and Bilderback, 2005). The mineral elements of mixtures substrate were presented in Table 7. The K, Ca, Mg and Na contents were high in mixture substrates. The WP was especially rich in Ca when compared to the other test mixtures growing media. Calcium is well known to have regulatory roles in metabolism, and sodium ions may compete with calcium ions for membrane-binding sites. Therefore, it has been hypothesized that high calcium levels can protect the cell membrane from the adverse effects of salinity (Cramer et al., 1986).

Table 6. Comparison of physical properties of mixtures substrate.

Mixtures substrate	pH	Total porosity (%)	Water holding capacity (%)
Peatmoss	5.1[c]	89.9[a]	47.0[b]
Waste paper	7.9[a]	88.1[b]	50.5[a]
Oakwood sawdust	5.0[cd]	72.6[e]	46.2[c]
Hinoki wood sawdust	4.9[d]	82.9[d]	44.6[d]
Rice hull	6.1[b]	84.9[c]	19.8[e]

In each column, values with different letters indicate statistically significant differences ($p < 0.05$) by Duncan's test.

Table 7. Mineral elements of mixtures substrate.

Mixtures substrate	Dry weight (g / 100 g)							
	K	Ca	Mg	Na	Zn	Fe	Mn	P
Peatmoss	0.02 ± 0.0[d]	0.57 ± 0.0[c]	0.01 ± 0.0[d]	0.00 ± 0.0[d]	0.00 ± 0.0[a]	0.00 ± 0.0[c]	0.00 ± 0.0[a]	0.00 ± 0.0[a]
Waste paper	0.12 ± 0.0[c]	3.39 ± 0.0[a]	0.14 ± 0.0[b]	0.20 ± 0.0[b]	0.01 ± 0.0[a]	0.07 ± 0.0[a]	0.00 ± 0.0[a]	0.00 ± 0.0[a]
Oakwood sawdust	0.17 ± 0.0[b]	0.20 ± 0.0[d]	0.05 ± 0.0[c]	0.09 ± 0.0[c]	0.00 ± 0.0[a]	0.02 ± 0.0[b]	0.00 ± 0.0[a]	0.00 ± 0.0[a]
Hinoki wood sawdust	0.32 ± 0.0[a]	0.14 ± 0.0[e]	0.24 ± 0.0[a]	0.72 ± 0.0[a]	0.00 ± 0.0[a]	0.02 ± 0.0[b]	0.00 ± 0.0[a]	0.00 ± 0.0[a]
Rice hull	0.12 ± 0.0[c]	1.40 ± 0.0[b]	0.14 ± 0.0[b]	0.20 ± 0.0[b]	0.01 ± 0.0[a]	0.07 ± 0.0[a]	0.00 ± 0.0[a]	0.00 ± 0.0[a]

Mixtures substrate	Dry weight (g / 100 g)							
	K	Ca	Mg	Na	Zn	Fe	Mn	P
Peatmoss	0.02 ± 0.0[d]	0.57 ± 0.0[c]	0.01 ± 0.0[d]	0.00 ± 0.0[d]	0.00 ± 0.0[a]	0.00 ± 0.0[c]	0.00 ± 0.0[a]	0.00 ± 0.0[a]
Waste paper	0.12 ± 0.0[c]	3.39 ± 0.0[a]	0.14 ± 0.0[b]	0.20 ± 0.0[b]	0.01 ± 0.0[a]	0.07 ± 0.0[a]	0.00 ± 0.0[a]	0.00 ± 0.0a
Oakwood sawdust	0.17 ± 0.0[b]	0.20 ± 0.0[d]	0.05 ± 0.0[c]	0.09 ± 0.0[c]	0.00 ± 0.0[a]	0.02 ± 0.0[b]	0.00 ± 0.0[a]	0.00 ± 0.0[a]
Hinoki wood sawdust	0.32 ± 0.0[a]	0.14 ± 0.0[e]	0.24 ± 0.0[a]	0.72 ± 0.0[a]	0.00 ± 0.0[a]	0.02 ± 0.0[b]	0.00 ± 0.0[a]	0.00 ± 0.0[a]
Rice hull	0.12 ± 0.0[c]	1.40 ± 0.0[b]	0.14 ± 0.0[b]	0.20 ± 0.0[b]	0.01 ± 0.0[a]	0.07 ± 0.0[a]	0.00 ± 0.0[a]	0.00 ± 0.0[a]

In each column, values with different letters indicate statistically significant differences ($p < 0.05$) by Duncan's test

Growth test

The objective was to test different ternary mixtures of the residual components as a substrate for growing Chinese cabbage (*Brassica rapa* var. *glabra*). Figure 1 shows that WP (containing 30% peat moss, 10% perlite and 60% waste paper), performed best, while those that performed most poorly contained 60% hinoki wood sawdust mixed with 10% perlite (HS) or 60% oakwood sawdust with 10% perlite (OS). The growth of the Chinese cabbage cultivated in the wood waste mixtures (oakwood sawdust, hinoki wood sawdust) was clearly lower than that of Chinese cabbage cultivated with the commercial substrate (100% peat moss). In growing media consisting of a mixture of rice hull with perite, the seed germination was similar to that observed in the PM (control). The inherent differences in chemical properties between different wood species, however, the suitability of sawdust as an organic growing media component was extremely variable. Mastalerz (1977) stated that sawdust from incense-cedar (*Libocedrus decurrens*), walnuts (*Juglans* spp.), or redwood (*Sequoia sempervirens*) is known to have direct phytotoxic effects, and sawdust from western red cedar (*Thuja plicata*) is toxic to many horticultural plants (Schaefer, 2009). The plant height and leaf area of Chinese cabbage grown in PM (control) and WP (containing 30% peat moss, 10% perlite and 60% waste paper) was presented in Figure 2. After 7 day, stem height and leaf area of the WP (containing 30% peat moss, 10% perlite and 60% waste paper) was much higher than that of the PM (control) (Figure 3). Results of this study confirm that waste paper can be used effectively in growing media.

Conclusion

Waste materials are not always adequately used in current commercial, agricultural practices, such as nurseries, despite the possible immediate benefits from using them, especially if they are readily available and less expensive than traditional substrates like peat moss. This work shows that the utilization of lignocellulosic substrate (waste paper, waste wood and agriculture residue)

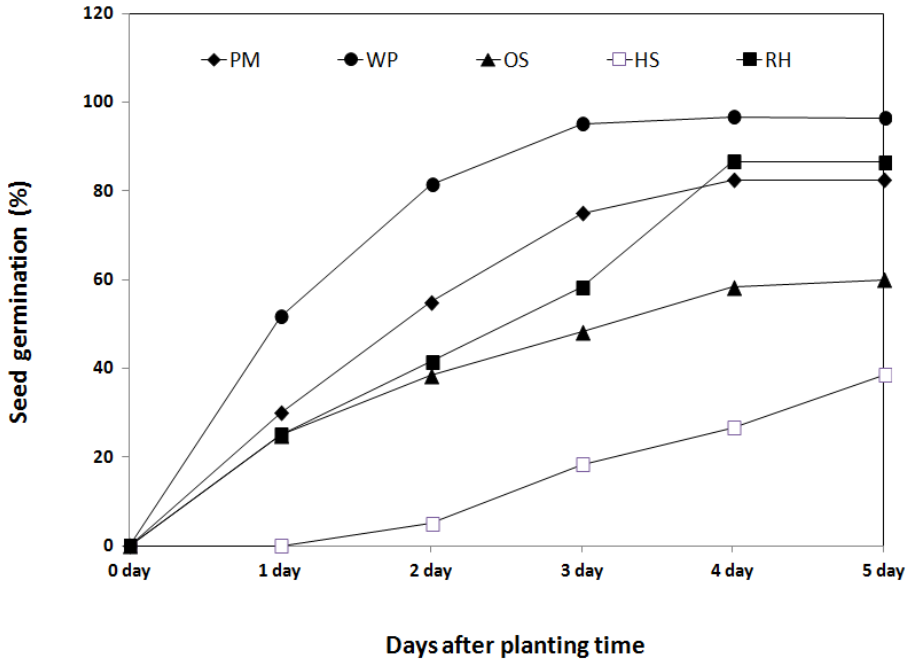

Figure 1. Seed germination (%) of Chinese cabbage grown in different mixtures.

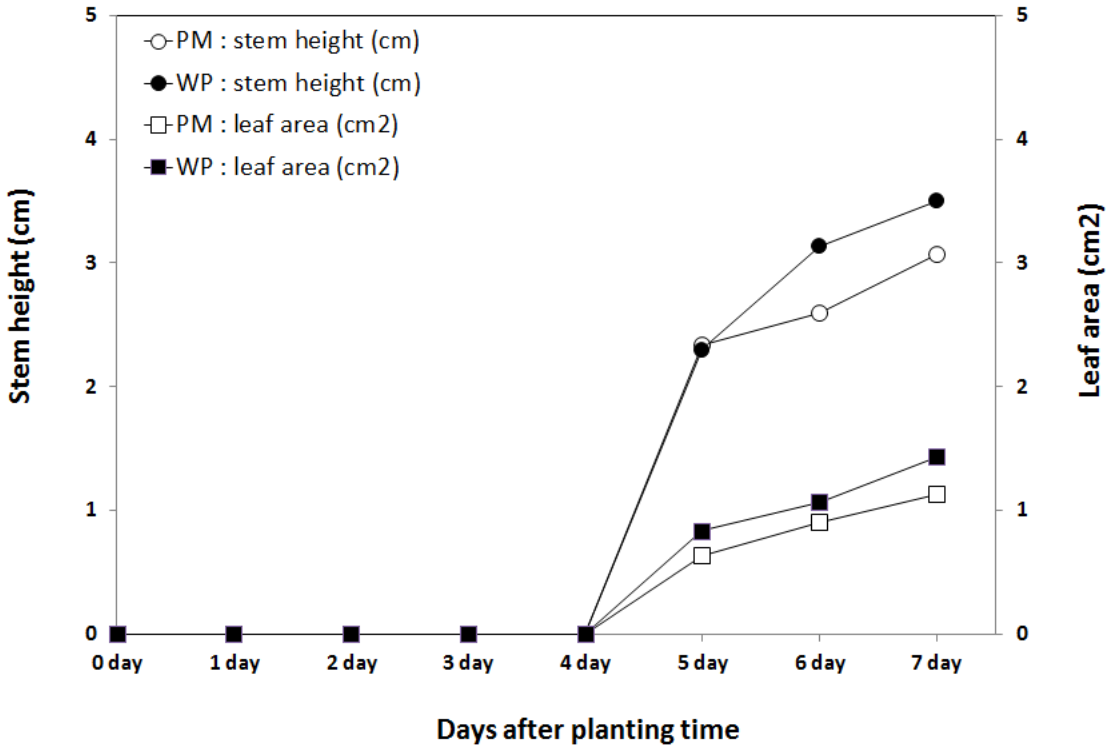

Figure 2. Plant height and leaf area of Chinese cabbage grown in PM (control) and WP (containing 30% peat moss, 10% perlite and 60% waste paper).

for peat moss substitution has proven to be a useful procedure to obtain suitable growing media. Moreover, due to physical and chemical characteristics of the media developed by mixtures growing media can be considered

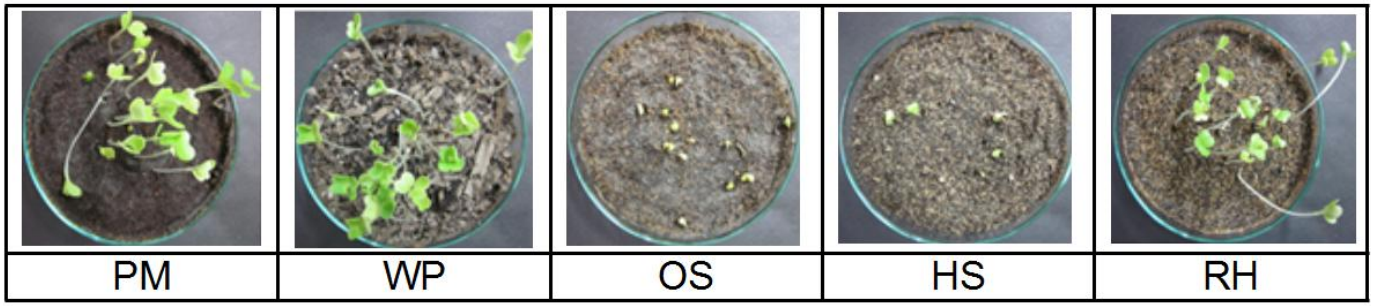

Figure 3. Chinese cabbage growth in different mixtures.

as valuable partial peat moss substitutes for Chinese cabbage, especially at the rates of 30% peat moss + 10% perlite + 60% waste paper substrate, which gave the maximum seed germination when compared to peat moss. In general, chemical properties and seed germination were enhanced by using the lignocellulosic substrate: the waste paper seemed to be more effective than the waste wood and agriculture residue for this purpose. The use of this kind of domestic refuse could contribute to solve two important problems: waste disposal and limit peat moss extraction. Also, this research indicates that waste paper may be utilized as a suitable replacement for peat moss in restoration of damaged land.

Conflict of Interests

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

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REFERENCES

Abad M, Noguera P, Bures S (2001). National inventory of organicwastes for use as growing media for ornamental potted plantproduction: case study in Spain. Bioresour. Technol. 77: 197-200.

Altland J (2006). Physical Properties of Container Media. <http://oregonstate.edu/dept/nurseryweeds/feature_articles/physical_properties/physical_properties.html>.

Bellamy KL, Chong C, Cline RA (1995). Paper sludge utilization in agriculture and container nursery culture. J. Environ. Qual. 24:1074-1082.

Bugbee JG (2002). Growth of ornamental plants in container media amended with biosolids compost. Compost Sci. Util. 10: 92-98.

Bustamante MA, Paredes C, Moral R, Agullo E, Perez-Murcia MD, Abad M (2008). Composts from distillery wastes as peat substitutes for transplant production. Resour. Conserv. Recycl. 52: 792-799.

Cramer GR, Lauchli A, Epstein E (1986). Effects of NaCl and $CaCl_2$on ion activities in complex nutrient solutions and root growth in cotton. Plant Physiol. 81: 792-797.

Degryse F, Smolders E, Parker DR (2009). Partitioning of metals (Cd, Co, Cu, Ni, Pb, Zn) in soils: concepts, methodologies, prediction and applications- a review. Eur. J. Soil Sci. 60: 590-612.

El-Shakweer MHA, El-Sayad EA, Ewees MSA (1998). Soil and plant analysis as a guide for interpretation of the improvement efficiency of organic conditioners added to different soils in Egypt. Commun. Soil Sci. Plant Anal. 29: 2067-2088.

Ezure K, Wilson VJ (1983).Dynamics of neck-to-forelimb reflexes in the decerebrate cat. J. Neurophysiol. 50: 688-695.

Fernandez-Calvino D, Perez-Novo C, Novoa-Munoz JC, Arias-Estevez M (2009).Copper fractionation and release from soils devoted to different crops. J. Hazardous Materials. 167: 797-802.

Ghehsareh AM, SamadiN, BorjiH (2011). Comparison of date-palm wastes and perlite as growth substrates on some tomato growing indexes. Afr. J. Biotechnol. 10(24): 4871-4878.

Hernandez-Apaloaza L, Gasco AM, Gasco JM, Guerrero F (2005). Reuse of waste materials as growing media for ornamental plants. Bioresour. Technol. 96(1): 125-131.

Herrera F, Castillo JE, Chica AF, Bellido LL (2008). Use of municipal solid waste compost (MSWC) as a growing medium in the nursery production of tomato plants.Bioresour. Technol. 99: 287-296.

Kahl JS, Fernandez IJ, Rustad LE, Peckenham J (1996). Threshold application rates of wood ash to an acidic forest soil. J. Environ. Qual. 25: 220-227.

Karp K, Noormets M, Starast M, Paal T (2006). The influence of mulching on nutrition and yield of ´Northblue´ blueberry.Acta Hort. 715: 301-306.

Kim TI, Jeung JW, Hong EC, Bang WR, Yoo YH, Yang CB (2004). Effects of rice hull addition and bin wall characteristics on pig slurry composting properties. J. Lives. Hous. Environ. 10(1): 47-58.

Klute A (1986). Methods of Soil Analysis. Part 1.Physical and Mineralogical Methods.Agron.Dep. Colorado State Univ. Fort Collins, Colorado, USA.

Marin JA (2004). Biorremediacio´n, mediantete´cnicasbiolo´gicas, de hidrocarburoscontenidos en lodos de refineri´a. Experiencias en ClimaSemia´rido. PhD Thesis, Murcia University.

Mastalerz JW (1977). The greenhouse environment. New York (NY): John Wiley and Sons. 629 p

McGuire JJ (1972). Growing ornamental plants in containers: ahandbook for the nurseryman. University of Rhode Island, Coop. Ext. Ser., Bull.197.

Meiwes KJ (1995). Application of lime and wood ash to decrease acidification of forest soils.Water Air Soil Pollut. 85(1): 143-152.

Miller M (2004). Using compost successfully in propagation systems. International Plant Propagators’ Society Combined Proceedings 53: 412-415.

Noguera P, Abad M, Puchades R, Maquieira A, Noguera V (2003). Influence of particle size on physical and chemical properties of coconut coir dust as a container medium. Commun. Soil Sci. Plant

Anal. 34: 593-605.
Ostos JC, Lopez-Garrido R, Murillo JM, Lopez R (2008). Substitution of peat for municipal solid waste and sewage sludge based composts in nursery growing media: effects on growth and nutrition of the native shrub *Pistacialentiscus* L. Bioresour. Technol. 99: 1793-1800.
Roh SE, Pyon JY (2004). Control of weeds in ginseng gardens by herbicides and mulching practices. Kor. J. Weed Sci. 24:14-20.
Salifu KF, Nicodemus MA, Douglas FJ, Davis AS (2006). Evaluating chemical indices of growing media for nursery production of *Quercusrubra* seedlings.Hortic.Science. 41(5): 1342-1346.
Sanchez-Monedero MA, Roig A, Cegarra J, Bernal MP, Noguera P, Abad M, Anton A (2004). Composts as media constituents for vegetable transplant production, Compost Sci. Util. 12: 161-168.
Schaefer JK (2009). Growing reforestation conifer stock: Utilizing peat/sawdust medium. In: Dumroese, R.K.; Riley, L.E., tech. coords. 2009. National proceedings: Forest and Conservation Nursery Associations.2008. Proc. RMRS-P-58. Fort Collins, CO: U.S. Department of Agriculture, Forest Service, Rocky Mountain Research Station: 35-36.
Senesi N, Loffredo E (1999). The chemistry of soil organic matter. In: Spark, D.L. (Ed.), Soil Physical Chemistry. CRC Press, Boca Raton, FL, pp. 239-370.
Tehranifar A, Ameri A, Shoor M, Davarynejad GH (2012). Effect of substrate and cultivar on growth characteristic of strawberry in soilless culture system. Afr. J. Biotechnol. 11(56): 11960-11966.
Tejada M, Gonzalez JL (2005). Beet vinasse applied to wheat under dryland conditions affects soil properties and yield. Europe. J. Agronomy. 23: 336-347.
Tsakaldimi M (2006). Use of inorganic and organic solid wastes for container-seedling production. In: Protection and restoration of the environment VII. Selected proceedings of the seventh biennial protection and restoration of the environment international conference. J. Hazardous Materials 136(1): 8.
Verdonck O, Gabriels R (1992). Reference method for the determination of physical and chemical properties of plant substrates.Acta Hort. 302: 169-179.
Wada S (2005). Nursery Container Weeds Respons to Modification of Substrate pH, Substrate Particle Size and Applied Nitrogen Form. Master of Science Thesis.Oregan State University.
Warren SL, Bilderback TE (2005). More plant per gallon:getting more out of your water. Hort Technol. 15: 14-18.
Wrigth RD, Browder JF, Jackson BJ (2006). Ground pine chips as a substrate for container-grown woody nursery crops. J. Environ. Hort. 24(4): 181-184.

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Full Length Research Paper

Acute toxicity of ammonia to blue tilapia, *Oreochromis aureus* in saline water

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The acute toxicity of blue tilapia, *Oreochromis aureus* (3.28 ± 0.36 g in body weight, 61.84 ± 2.08 mm in body length) exposed to environmental un-ionized ammonia at different salinities (1, 8, 12, 16 and 20 ppt) was assessed via a series of static exposure trials. Median lethal concentrations of 24 h of exposure were calculated for each salinity. Tolerance of blue tilapia to acute un-ionized ammonia exposure was not influenced by salinity. Median lethal concentrations (24-h LC_{50}) values were found to be 2.83, 2.26, 3.14, 3.11 and 1.93 mg/l NH_3 at 1, 8, 12, 16 and 20 ppt of salinities, respectively. The results of this study indicate that using brackish water for blue tilapia culture may not be a single factor to reduce the toxicity of high ammonia.

Key words: *Oreochromis aureus*, blue tilapia, ammonia, salinity.

INTRODUCTION

All tilapia species can live in saline waters. *Oreochromis mossambicus* is the most tolerant species and *Oreochromis aureus* is more tolerant than *Oreochromis hornorum* but red tilapia can survive even marine water.

There are essentially two liming water quality factors, that is, dissolved oxygen (DO) and ammonia in aqua-culture practices. These factors limit feeding rate and stocking rate of fishes in ponds or raceway systems (Knud-Hansen et al., 1991). Ammomia is excreted through gills of most teleostean fish and they are secreted as a waste product of catabolic process taking place in the liver. If they are accumulated in the body, they are toxic to fishes. Ammonia consists of two forms, one is un-ionized ammonia (NH_3), which is toxic to fishes and ionized ammonia (NH_4^+), which is comperatively nontoxic. These two ammonia forms are in balance in aquaculture systems depending on the pH and temperature (Emerson et al., 1975). Morever, the ammonia toxicity may also rely on salinity and DO (Alabaster et al., 1979). Previous studies on Atlantic salmon showed that ammonia toxicity could be decreased with increase in DO and salinity (Alabaster et al., 1979). Another study showed that the increase in temperature reduced the acute ammonia toxicity (96-h LC_{50}) in fathead minnows (Thurston et al., 1983). Another study showed that the un-ionized ammonia concentration could increase in pH (Tomasso et al., 1980). The choice of fish species in culture, size of fish, acclimation and type of culture system could also influence toxicity level (El-Sayed, 2006). The salinity has often shown beneficial effect on growth rate and effective in the reduction of un-ionized ammonia toxicity in many fishes. Researchers

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Table 1. Water quality parameters in fresh and salt water.

Parameter	Fresh water	Saltwater
EC (mmhos/cm)	147.80	842.20
Hardness (mg/l $CaCO_3$)	109.80	732.50
Alkalinity (mg/l $CaCO_3$)	90.00	600.00
Bicarbonate (mg/l)	71.40	348.90
Calcium (mg/l)	0.02	89.80
Magnesium (mg/l)	4.37	12.40
Total Ammonia (mg/l)	0.10	0.22
Nitrite (mg/l)	0.00	0.007
pH	7.94	8.18

have earlier reported that lowering the salinity concentration enhanced the growth rate in turbot (Imsland et al., 2001), European sea bass (Rubio et al., 2005), cobia (Resley et al., 2006), Atlantic halibut (Imsland et al., 2008) and Senegalese sole (Arjona et al., 2008). Nevertheless, elevating the salinity level also resulted in the increase in growth rate of fish species like, tilapia (Kangombe and Brown, 2008), Eurasian perch (Overton et al., 2008) and goldfish (Luz et al., 2008). In addition, increasing the salinity elevated the tolerance limit of ammonia toxicity in rainbow trout (Herbert and Shurben, 1965) and chinook salmon (Harader and Allen, 1983).

Hence, the present study was designed to evaluate the influence of different salinity levels of saline waters (1, 8, 12, 16 and 20 ppt) on un-ionized ammonia toxicity level in the fingerlings of blue tilapia, *O. aureus*.

MATERIALS AND METHODS

The toxicity of un-ionized ammonia in tilapia was investigated via 24-h static acute bioassays at different salinity levels (1, 8, 12, 16 and 20 ppt). Bioassays were performed according to the techniques by Boyd and Tucker (1998). Ten (10) fingerlings of blue tilapia (3.28 ± 0.36 g in body weight an 61.84 ± 2.08 mm in body length) were placed into 10-L aquarium with two replicates (2 x 5 x 10, 100 fish). In order to optimize the desired salinity for the experiment, the artificial marine salt (NaCl) was added to fresh water, where the salinity was 1 ppt; and salt water, where the salinity was 8 ppt. The criteria for the two water sources are given in Table 1. Ammonia concentrations for four different treatments were prepared by using ammonium solution (Merck 25%, cat no: 105432) following fundamental laboratory techniques. Ammonia stock solution prepared was 0.91 kg/l. Before the commencement of each experiment, the fish were acclimatized for a week under saltwater source (8 ppt) in laboratory conditions. Acclimation was followed by methods suggested by Boyd and Tucker (1998). The experimental fishes were measured for length and weight and then they were placed into the desired ammonia concentration and salinity for the next 24-h period. During the experimental trial, feeding was stopped. Room temperature (24 to 25°C) was controlled by using an air conditioner, which maintained the temperature at a constant range. Fish mortality, water pH and temperature were recorded at 4 h interval. Approximately, 90% of mortality was seen within the first 8 h in all the four treatments. Un-ionized ammonia concentrations for each salinity and total ammonia treatments were calculated (Emerson et al., 1975). Median lethal concentrations were estimated by the graphical method used by Finney (1971). During the exposure trial, ammonia concentration of water source and that of excretion by fish were ignored. Hazel et al. (1971) reported that ammonia excretion by test concentrations used for treatments.

RESULTS AND DISCUSSION

Median lethal concentrations of un-ionized ammonia in fingerlings of blue tilapia at salinities of 1, 8, 12, 16 and 20 ppt are presented in Table 2. There were no significant differences ($p < 0.05$) among the 24-h LC_{50} values at different salinities. Mean 24-h LC_{50} values were 2.83, 2.26, 3.14, 3.11 and 1.93 mg/l NH_3 at 1, 8, 12 16 and 20 ppt of salinities, respectively. Mean LC_{50} values slightly fluctuated among different salinities (Figure 1). Fresh water hardness and alkalinity were much more diffferent from that of saltwater and this did not have any influence on 24-h LC_{50} of un-ionized ammonia at different salinities.

Median lethal concentrations of un-ionized ammonia in fingerlings of blue tilapia were not influenced by salinity and showed only minimum changes with different salinities. The results from this exposure trial showed similar pattern with many other earlier studies. Weirich and Riche (2006a) found that the ammonia tolerance of Florida pompano, *Trachinotus carolinus* was not affected by salinity. Similarly, Weirich and Riche (2006b) also reported that 24 and 96-h LC_{50} values of black sea bass were not influenced by salinity increase. Atwood et al. (2004) also examined ammonia toxicity in black sea bass and reported similar trend. Sunshine Bass (Weirich et al., 1993) and golden shiner (Sink, 2010) also did not show any difference in toxicity of ammonia at different Salinities. In this study, mean 24-h LC_{50} was found to have 2.83 mg/NH_3 in fresh water (1 ppt). However, a study by Redner and Stickney (1979) estimated comparatively lesser level (2.4 mg/l NH_3) of LC_{50} 48-h in blue tilapia (7 to 8 cm long).

Toxicity trials are classified as short-term tests (lethal or acute) and long-term tests (sublethal). Acute toxicity (median lethal concentration, LC_{50}) is defined as the

Table 2. Chemical characteristics of bioassay waters and associated median lethal concentrations (LC_{50s}) of un-ionized ammonia in tests with tilapia.

Salinity (ppt)	Mean fish weight (g)	Mean fish Length (mm)	Temperature (°C)	pH	Hardness ($CaCO_3$) (mg/l)	Alkalinity ($CaCO_3$) (mg/l)	TAN (mg/l)	24-h LC_{50} (mg/l) NH_3
1	3.19±0.40	61.90±2.25	25.34±0.17	7.94±1.13 (7.14-9.56)	4.4	71.4	14.5	2.83
8	3.41±0.50	62.26±2.92	24.84±0.19	8.13±0.33 (7.85-8.97)	732.5	600.0	25.5	2.26
12	3.36±0.26	61.98±1.64	25.10±0.18	8.15±0.46 (7.77-8.94)	732.5	600.0	30.5	3.14
16	3.11±0.40	60.70±2.22	25.16±0.22	8.15±0.42 (7.73-8.89)	732.5	600.0	32.0	3.11
20	3.30±0.18	62.25±1.49	24.86±0.13	8.31±0.64 (7.79-9.00)	732.5	600.0	24.0	1.93

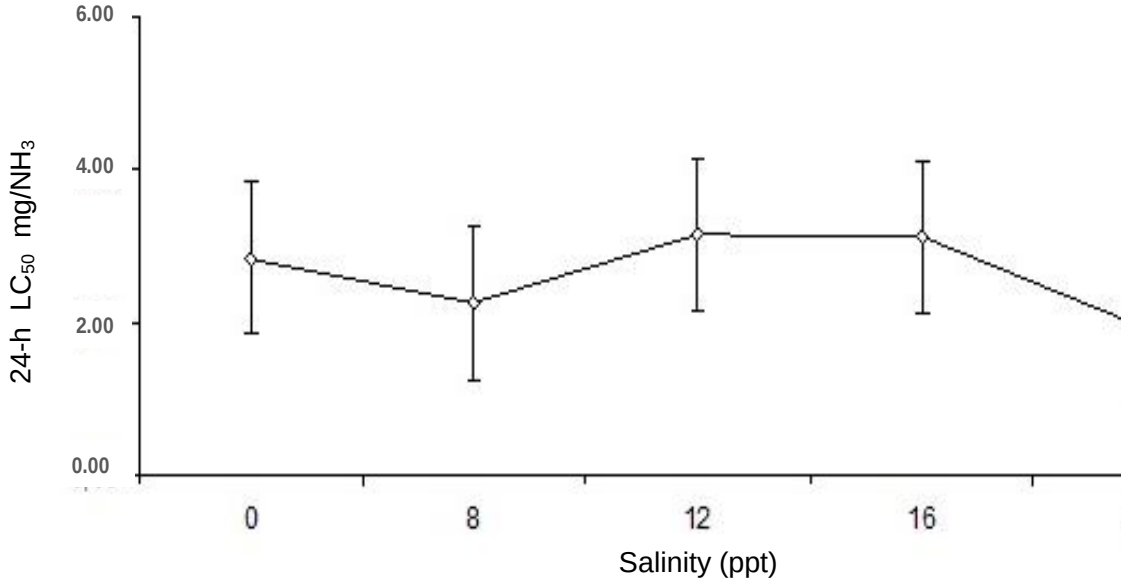

Figure 1. Median lethal concentrations of ammonia in blue tilapia at salinities of 1, 8, 12, 16 and 20 ppt.

concentration in which 50% of fishes show mortality. Various test durations such as 24, 48 and 96 h are used for toxicity studies. Actually, acute ammonia concentration is rarely been encountered in aquaculture areas but it is used to find out the maximum allowable toxicant concentration (MATC) for a particular species. Toxicity of un-ionized ammonia to fingerlings of blue tilapia in saline waters has not been studied in detail yet. Therefore, this study was undertaken to examine the 24-h UIA toxicity to fingerlings of blue tilapia in various salinities.

Most of the recent studies examined the effects of sublethal ammonia on various species (Kawamoto, 1961; Robinette, 1976; Colt and Tchobanoglous 1978; El-Shafai et al., 2004; Redner and Stickney 1979). Long-term ammonia exposure causes growth reduction and physiological

disabilities in fishes. Kawamoto (1961) reported that higher ammonia level reduced growth in common carp. Robinette (1976) indicated that ammonia exposure at 0.12 to 0.40 mg/l NH_3 decreased growth rate in channel catfish. Colt and Tchobanoglous (1978) observed that channel catfish grew 50% less when exposed to 0.517 mg/l NH_3 and showed no growth when exposed to 0.967 mg/l NH_3. El-Shafai et al. (2004) estimated the effect of chronic ammonia exposure to Nile tilapia fed with duckweed. Chronic ammonia ranging from 0.07 to 0.14 mg/l NH_3 caused a negative effect on tilapia growth. They recommended chronic ammonia concentration to be less than 0.1 mg/l NH_3. Redner and Stickney (1979) also indicated that acute and sublethal ammonia exposure caused histological effects (capillary cogestion, hemorrhaging and telangiectasis) on gills of *Tilapia aurea*.

In conclusion, the result of this study demonstrates that manipulation of salinity may not mitigate the acute toxicity of un-ionized ammonia to fingerlings of blue tilapia.

Conflict of Interests

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

REFERENCES

Alabaster JS, Shurben DG, Knowles G (1979). The effect of dissolved oxygen and salinity on the toxicity of ammonia to smolts of salmon, *Salmo salar* L. J. Fish Biol. 15:705-712.

Arjona FJ, Vargas-Chacoff L, Ruiz-Jarabo I, Gonçalves O, Pascoa I, Martin Del Rio MP, Mancera JM (2008). Tertiary stress responses in Senegalese sole (*Solea senegalensis* Kaup, 1858) to osmotic challenge: Implications for osmoregulation, energy metabolism and growth. Aquaculture 287:419-426.

Atwood HL, Young SP, Tomasso JR, Smith TIJ (2004). Information on selected water quality characteristics for the production of sea bass, *Centropristis striata*, juveniles. J. Appl. Aquacult. 15:183-190.

Boyd CE, Tucker CS (1998). Pond Aquaculture Water Quality Management, Kluwer, Norwell. pp. 472-478.

Colt J, Tchobanoglous G (1978). Chronic exposure of channel catfish, *Ictalurus punctatus*, to ammonia: effects on growth and survival. Aquaculture 15:353-372.

El-Sayed AFM (2006). Tilapia Culture, Cabi, Wallingford. pp 42-43.

El-Shafai SA, El-Gohary FA, Nasr FA, van der Steen NP, Gijzen HJ (2004). Chronic ammonia toxicity to duckweed-fed tilapia (*Oreochromis niloticus*). Aquaculture 232:117-127.

Emerson K, Russo RC, Lund RE, Thurston RV (1975). Aqueous ammonia equilibrium calculations: Effects of pH and temperature. J. Fish. Res. Board Can. 32:2379-2383.

Finney DJ (1971). Probit analysis. Cambridge University Press, New York, New York, USA.

Harader RR, Allen GH (1983). Ammonia toxicity to chinook salmon parr: reduction in saline water, T. Am. Fish. Soc. 112:834-837.

Hazel CR, Thompson W, Meith SJ (1971). Sensitivity of striped bass and stickleback to ammonia in relation to temperature and salinity. Calif. Fish Game 57:154-161.

Herbert DWM, Shurben DS (1965). The susceptibility of salmonid fish to poisons under estuarine conditions II. Ammonium chloride. Int. J. Air Water Pollut. 9:89-91.

Imsland AK, Foss A, Gunnarsson S, Berntssen MHG, FitzGerald R, Bonga SW, Ham EV, Naevdal G, Stefansson SO (2001). The interaction of temperature and salinity on growth and food conversion in juvenile turbot (*Scophthalmus maximus*). Aquaculture 198:353-367.

Imsland AK, Gustavsson A, Gunnarsson S, Foss A, Arnason J, Arnarson I, Jonsson AF, Smaradottir H, Thorarensen H (2008). Effects of reduced salinities on growth, feed conversion efficiency and blood physiology of juvenile Atlantic halibut (*Hippoglossus hippoglossus* L.). Aquaculture, 274:254-259.

Kangombe J, Brown JA (2008). Effect of salinity on growth, feed utilization and survival of *Tilapia rendalli* under laboratory conditions. J. Appl. Aquacult. 20:256-271.

Kawamoto NY (1961). The influence of excretory substances of fishes on their own growth. Prog. Fish Cult. 34:70-75.

Knud-Hansen CF, McNabb CD, Batterson TR (1991). Application of limnology for efficient nutrient utilization in tropical pond aquaculture. Verhandlungen Internationale Vereinigung fuür Theoretische und Angewandte Limnologie 24:2541-2543.

Luz RK, Martinez-Alvarez RM, DePedro N, Delgado MJ (2008). Growth, food intake regulation and metabolic adaptations in goldfish (*Carassius auratus*) exposed to different salinities. Aquaculture 276: 171-178.

Overton JL, Bayley M, Paulsen H, Wang T (2008). Salinity tolerance of cultured Eurasian perch, *Perca fluviatilis* L.: effects on growth and survival as a function of temperature. Aquaculture 277:282-286.

Redner BD, Stickney RR (1979). Acclimation to ammonia by *Tilapia aurea*. T. Am. Fish. Soc. 108:383-388.

Resley MJ, Webb KA, Holt GJ (2006). Growth and survival of juvenile cobia, *Rachycentron canadum*, at different salinities in a recirculating aquaculture system. Aquaculture 253: 398-407.

Robinette HR (1976). Effect of selected sublethal levels of ammonia on growth of channle catfish, *Ictalurus punctatus*. Prog. Fish Cult. 38: 26-29.

Rubio VC, Sanchez-Vazquez FJ, Madrid JA (2005). Effects of salinity on food intake and macronutrient selection in European sea bass. Physiol. Behav. 85:333-339.

Sink TD (2010). Influence of pH, salinity, calcium and ammonia source on acute ammonia toxicity to Golden Shiners, *Notemigonus crysoleucas*. J. World Aquacult. Soc. 41:411-420.

Tomasso JR, Goudie CA, Simco BA, Davis KB (1980). Effects of environmental pH and calcium on ammonia toxicity in channel catfish. T. Am. Fish. Soc. 109:229-234.

Thurston RV, Russo RC, Phillips GR (1983). Acute toxicity to fathead minnows. T. Am. Fish. Soc. 112:705-711.

Weirich CR, Tomasso JR, Smith TIJ (1993). Toxicity of ammonia and nitrite to sunshine bass in selected environments. J. Aquat. Anim. Health 5:64-72.

Weirich CR, Riche M (2006a). Acute tolerance of juvenile Florida pompano, *Trachinotus carolinus* L., ammonia and nitrite at various salinities. Aquaculture Res. 37:855-861.

Weirich CR, Riche M (2006b). Tolerance of juvenile black sea bass *Centropristis striata* to acute ammonia and nitrite exposure at various salinities. Fish. Sci. 72:915-921.

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Full Length Research Paper

Fluorene biodegradation potentials of *Bacillus* strains isolated from polluted tropical hydrocarbon-contaminated soils

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Two fluorene-degrading Gram-positive *Bacillus* strains, putatively identified as *Bacillus subtilis* BM1 and *Bacillus amyloliquefaciens* BR1 were isolated from hydrocarbon- and asphalt-contaminated soils in Lagos, Nigeria. The polluted soils have a relatively high total hydrocarbon content (16888.9 and 9923.1 mg/kg, respectively), very low concentrations of macronutrients and the total organic carbon was less than 4%. The two strains tolerated NaCl concentration of up to 7% while strain BR1 exhibited moderate growth at 10%. Shared resistance to ceftriazone and cotrimozaxole were exhibited by both strains while only strain BM1 was resistant to both amoxycilin and streptomycin. The rate of degradation of fluorene (50 mg/L) by the two isolates, after 30 days of incubation were 0.09 and 0.08 mg/L/h for strains BM1 and BR1, respectively. Gas chromatographic analyses of residual fluorene, revealed that 56.9 and 46.8% of 50 mg/L fluorene was degraded in 12 days by strains BM1 and BR1. However, after 21 days on incubation, 86 and 82% of 50 mg/L fluorene were degraded by strains BM1 and BR1, respectively. To the best of our knowledge, this is the first report highlighting flourene degradation potential of *Bacillus* strains isolated from tropical African environment.

Key words: Biodegradation, fluorene, hydrocarbon-contaminated soils, *Bacillus* spp.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are organic compounds composed of two or more fused benzene rings. They are found in fossil fuel and result from incomplete combustion of organic compounds and other forms of pyrolysis and pyrosynthesis (Kanaly and Harayama, 2010). They have low aqueous solubility, are highly lipophilic and often persist in soil and sediments. PAHs are of concern because of their genotoxic effect for humans and environmental persistence.

Fluorene is a non-alternant PAH composed of two benzene rings between which is tucked a five-membered ring. It is sparingly soluble in water (1.992 mg/L) and has been found to persist in ground water and sediments at coal and oil gasification sites. It is found as constituent of refined coal derivatives such as creosotes as well as in vehicle exhausts. It is classified by the United States Environmental Protection Agency (USEPA) as a priority pollutant (Keith and Telliard, 1979).

The mutagenicity of its derivatives, which are used in pharmaceutical industry and as dyestuff, has been established (Shibutani et al., 1998). The interest in the biodegradation of fluorene is due in part to its persistence

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and genotoxicity as well as its potential for use as model for study of other rather carcinogenic non-alternant PAHs.

Unlike many of the other lower molecular weight PAHs such as naphthalene, phenanthrene and anthracene, fluorene degraders are not as readily isolated from the environment. However, a wide range of bacteria spanning both Gram-positive and Gram-negative genera including *Pseudomonas, Sphinghomonas, Burkholderia, Brevibacterium, Micrococcus*, *Arthrobacter* and *Terrabacter* have been reported (Grifoll et al., 1992; Grifoll et al., 1994; Grifoll et al., 1995; Monna et al., 1993; Trenz et al., 1994; Wattiau et al., 2001; Mukesh et al., 2012). Equally, there have been reports on degradation of fluorene in mixed cultures or by consortia (Gomes et al., 2006; Arulazhagan et al., 2010).

The non-alternant structure of fluorene offers a variety of routes for the initiation of its aerobic degradation by bacteria. Essentially, the pathways for its degradation to the intermediates of tricarboxylic acid (TCA) cycle have been described (Kasuga et al., 1997; Habe et al., 2004; Habe et al., 2005). It usually proceeds by initial di-oxygenation at the 1, 2 or 3, 4 positions with consequent dehydrogenation of the resulting cis-dihydrodiol and subsequent meta cleavage. Alternatively, there is mono-oxygenation at the 9-carbon position to 9-hydroxyfluorene and dehydrogenation to 9-fluorenone (Schuler et al., 2008).

Although there is a considerable information in the literature on the metabolism and genetics of fluorene degradation, there is virtually no report of study of fluorene degradation in the tropical African environment and in Nigeria especially where gas flaring, unabated release by automobiles and at times deliberate sabotage of oil pipelines are loading the environment with plethora of PAHs. Improving on the available bank of microbial resources (isolates) and information is crucial to the proper management of petroleum-polluted sites. In this paper, we report the degradation of fluorene by pure isolates of two strains of *Bacillus* species from hydrocarbon-polluted sites in Lagos, Nigeria.

MATERIALS AND METHODS

Sampling

Soil samples were collected from contaminated sites in Lagos, Nigeria, namely MWL (a mechanic workshop at Mebamu, Badagry) and APS (an asphalt-polluted soil along Lagos-Ibadan Highway). Soil samples were collected at a depth of 10-12 cm with sterile trowel after clearing debris from the soil surface. Samples for physicochemical analyses were collected in polyethylene bags, while those for microbiological analyses were collected in sterile screw-capped bottles. Samples were analyzed immediately upon arrival in the laboratory. Leftover samples were refrigerated at 4°C.

Physicochemical analysis of soil samples

The pH of the soil samples was determined with a pH meter (Jenway, 3051) in 1:1 soil solution in distilled water. The moisture content, organic carbon content, total nitrogen content, potassium content and available phosphorous were determined as described previously (Bray and Kurtz, 1945; Black, 1965; Chopra and Kanwar, 1998). Conductivity and total hydrocarbon content of the soil were determined as described by Salam et al. (2014). The heavy metal content of the soils was determined using atomic absorption spectrophotometer (Alpha 4, AAS) following mixed acid digestion and extraction of the soil samples.

Enrichment and isolation of fluorene-degrading bacteria

Bacteria able to degrade fluorene were isolated on fluorene mineral salts medium (MSM) by continual enrichment method. The mineral salts medium described by Kästner et al. (1994) was used. The medium contained in liter of distilled water: Na_2HPO_4, 2.13 g; KH_2PO_4, 1.30 g; NH_4Cl, 0.50 g and $MgSO_4.7H_2O$, 0.20 g. It was supplemented with yeast extract (0.005 g/L) as source of growth factors. After adjusting the pH to 7.2, the medium was fortified with 50 µg/mL of nystatin to suppress fungal growth. Sterile trace elements solution (1.0 mL/L) described by Bauchop and Elsden (1960) was aseptically added to the medium after sterilization. Contaminated soil (5 g) was added to 45 ml of MSM containing 50 mg/L of fluorene. Enrichment was carried out by incubation with shaking (180 rpm) at room temperature (28 ± 2°C) in the dark for four to five weeks until there was turbidity. After five consecutive transfers, fluorene degraders were isolated by plating out dilutions from the final flasks on Luria-Bertani (LB) agar. The colonies that appeared were further purified by sub culturing once onto LB agar. Ability to degrade fluorene was confirmed by inoculating washed LB broth grown culture in fresh MSM flask supplemented with 50 mg/L fluorene as sole carbon source.

Maintenance and identification of isolates

Pure bacterial isolates were maintained in glycerol/LB broth medium (1:1, v/v). Pure colonies of fluorene degraders sub cultured on LB agar supplemented with low percentage of fluorene (0.005%) were harvested with sterile inoculating loop, pooled and transferred to the medium. The mixture was shaken to homogenize and kept at -20°C.

Pure identification of fluorene-degrading isolates was carried out based on their colonial morphology, cellular morphology and biochemical characteristics according to the identification scheme of Bergey's Manual of Determinative Bacteriology (Holt et al., 1994). Antibiotic sensitivities of the isolates were determined using standard multidisc.

Salt tolerance test of the pure isolates was conducted in LB broth supplemented with varying concentrations (1%-10%, w/v) of NaCl. Incubation was carried out with intermittent shaking at room temperature (28 ± 2°C) for two weeks.

Evaluation of fluorene biodegradation

Replicate 250 ml flasks containing 50 ml of MSM with 50 mg/L of fluorene as sole source of carbon were prepared. Flasks were inoculated with 0.5 ml of MSM-washed 18 - 24 h LB agar-grown cells to achieve an initial cell concentration of about 3.2×10^6 cfu/ml and subsequently incubated at 180 rpm in the dark for 21 days at room temperature (28 ± 2°C). Flasks containing fluorene as described above but inoculated with heat-killed cells were used as controls. Samples were withdrawn from each flask at 3 days interval and aliquots of appropriate dilutions were plated (in triplicates) onto nutrient agar for total viable counts (TVC).

Table 1. Physicochemical properties of hydrocarbon-contaminated sampling sites.

Parameter	MWL	APS
pH	5.01	6.12
Moisture (%)	7.49	9.64
Conductivity (µs/cm)	62.6	64.9
Total organic carbon (%)	1.94	3.14
Total hydrocarbon content (mg/kg)	16888.9	9923.1
Potassium (mg/kg)	9.40	8.10
Nitrogen (%)	0.10	0.15
Phosphorus (mg/kg)	1.52	0.08
Lead (mg/kg)	0.001	0.003

MWL, A mechanic workshop at Mebamu, Badagry; **APS**, An asphalt-polluted soil along Lagos-Ibadan Highway in Nigeria.

Extraction of residual fluorene

Residual fluorene was extracted by liquid-liquid extraction. Briefly, broth culture (50 ml) was extracted twice with an equal volume of hexane. After removing the aqueous phase with separating funnel, the organic fraction was concentrated to 1 ml and the residual fluorene concentration was determined by gas chromatography. Control flasks were also extracted similarly.

Analytical method

Hexane extracts (1.0 µl) of residual fluorene were analyzed with Hewlett Packard 5890 Series II gas chromatograph equipped with flame ionization detector (FID) and 30 m long HP-5 column (internal diameter, 0.25 mm; film thickness, 0.25 µm). The carrier gas was nitrogen. The injector and detector temperatures were maintained at 300 and 320°C, respectively. The column temperature was programmed from 60 to 500°C for 27 min. The gas chromatograph column was programmed at an initial temperature of 60°C; this was held for 2 min, and then ramped at 12°C/min to 205°C and held for 16 min. Nitrogen column pressure was 37 psi, the hydrogen pressure was 9 psi and compressed air pressure was 13 psi. The software was Chem Station. Rev. A. 05. 01.

Statistical analysis

Mean generation times (T_d) and growth rate (K) of the isolate on fluorene was calculated using Prism version 5.0 (Graphpad software, San Diego, CA).

RESULTS AND DISCUSSION

Table 1 shows the physicochemical properties of the soils used in this study. The pH of the soils was acidic with a moisture content of 7.49 and 9.64%, respectively. The total hydrocarbon content of MWL (a mechanic workshop at Mebamu, Badagry) site is relatively higher (16888.89 mg/kg) compared to APS (an asphalt-polluted soil along Lagos-Ibadan Highway) site (9923.08 mg/kg). Concentrations of macronutrients such as nitrogen, phosphorus and potassium at the two sites were very low while the total organic carbon of the two sites was less than 4%. These results are not surprising as hydrocarbon contamination in soil have been reported to induce reduction in water holding capacity as well as a shift to acidic pH (Dibble and Bartha, 1979; Chikere and Okpokwasili, 2002).

Indigenous bacteria inhabiting hydrocarbon-contaminated niches have been widely employed with outstanding successes in the degradation of PAHs. This is because previous exposure to the compounds often results in evolution of adapted microflora that have acquired the necessary degradative genes and capable of transforming and mineralizing the compounds after a long period (Wackett and Hershberger, 2001). In this study, continual enrichment resulted in the isolation of several fluorene degraders. The best fluorene degraders from each of the two-soil sample were used. The two isolates were Gram-positive, endospore-forming, motile rods that are oxidase and catalase positive. They showed negative reaction to indole, urease and methyl red and failed to ferment xylose, galactose and raffinose. Colonial morphology of strain BM1 obtained from MWL indicated that it was circular in shape, dull cream in color, opaque, flat in elevation with round smooth edges and an entire margin. It was positive for citrate utilization, Voges-Proskauer, casein, gelatin and starch hydrolysis, failed to ferment lactose and arabinose, and reduces nitrate to nitrite. It was thus putatively identified as *Bacillus subtilis*. The colonies and biochemical characteristics of strain BR1 obtained from APS were quite similar to strain BM1. However, it had irregular rough edges, cream in color, ferment lactose and arabinose, reduced nitrate to nitrite and failed to hydrolyze casein. Based on these characteristics, it was putatively identified as *Bacillus amyloliquefaciens*. It is noteworthy that though, previous reports have established the propensity of PAHs degraders from the Nigerian environment (Ilori and Amund, 2000; Igwo-Ezikpe et al., 2006; Obayori et al., 2008; Salam et al., 2014), to the best of our knowledge, this is the first report of fluorene degraders from the Nigerian environment.

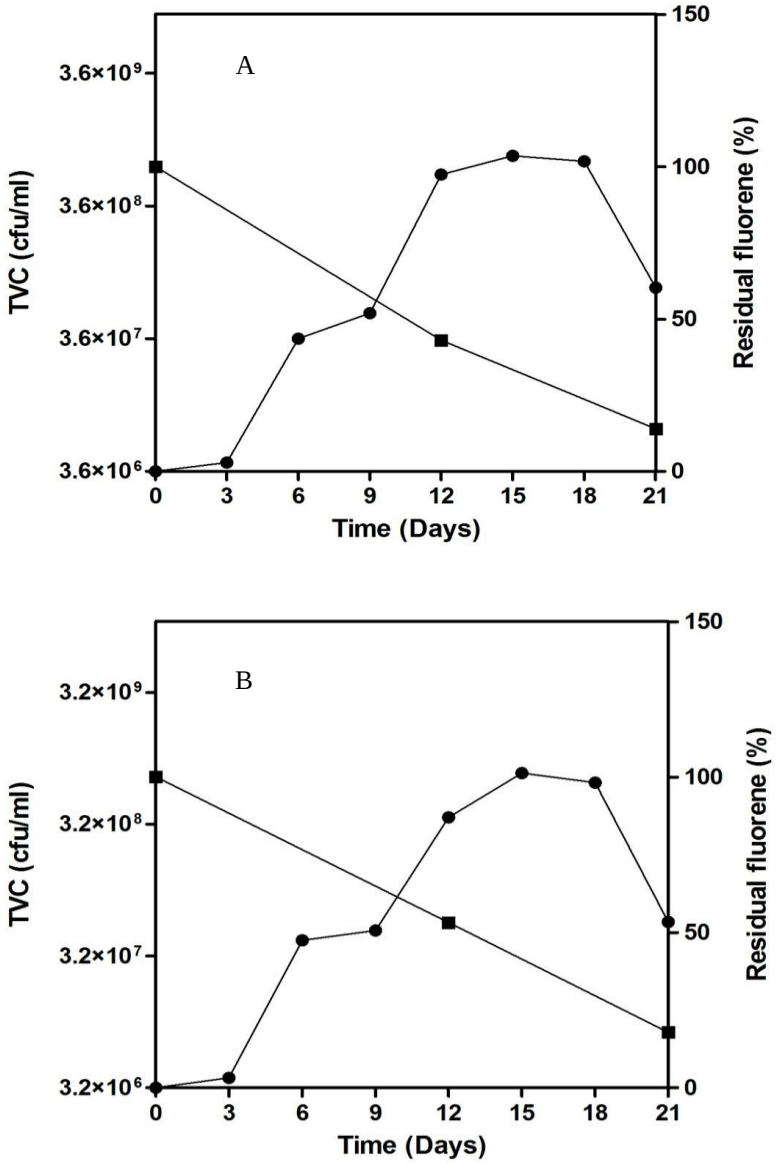

Figure 1. Growth dynamics of BM1 (A) and BR1 (B) strains in minimal medium amended with 50 mg L^{-1} fluorene showing total viable count, TVC (●) and residual fluorene (■). Data points represent the mean of three replicate flasks. In the case of population counts, error bars that represent standard deviation were removed for clarity. Residual fluorene were determined with reference to fluorene recovered from heat-killed controls.

The two isolates tolerated NaCl concentration of 7% with good and moderate growth exhibited by strain BR1 and BM1, respectively. However, at 10% NaCl concentration, only strain BR1 showed moderate growth. This physiological property favours the use of these strains as possible candidates for bioaugmentation purpose. Previous reports have shown that salinity could be a critical factor that determines the survival of allochthonous bacterial strains during bioremediation (Kästner et al., 1998; Obayori et al., 2008). In addition, the two strains resisted ceftriazone and cotrimozaxole but were susceptible to ofloxacin, gentamicin, and ciprofloxacin. Only strain BM1 was resistant to both amoxycilin and streptomycin. Shared resistance of strains BM1 and BR1 to ceftriazone and cotrimozaxole may be attributed to acquisition of resistant genes to these antibiotics through gene transfer, as soil environments are replete with these antibiotics, which could allow evolution of resistance by indigenous strains (Obayori et al., 2008).

The growth kinetics of the *Bacillus* strains on fluorene is illustrated in Figure 1 and Table 2. The two strains

Table 2. Growth kinetics of fluorene-degrading isolates.

Isolate	Growth rate, K (h^{-1})	Mean generation time, ΔT_d (h)	Percentage (%) degradation[1] (day 12)	Percentage (%) degradation[1] (day 21)	Degradation rate (%/h)	Rate of degradation (mg $L^{-1}h^{-1}$)
BM1	0.016	44.9	56.9	86	0.17	0.09
BRI	0.015	45.2	46.8	82	0.16	0.08

[1]Percent degradation values represent the net decrease (FID area counts) calculated with reference to the amount recovered from heat-killed control flasks.

exhibited slight lag phases followed by gradual population increase with concomitant decrease in fluorene concentration. Strain BM1 grew from an initial population density of 3.6×10^6 cfu/ml to peak at 8.6×10^8 cfu/ml, resulting in over 200-fold increase in 15 days. It thereafter maintained a decreasing trend. During the exponential growth of the isolate on fluorene, it exhibited a growth rate and doubling time of 0.016 h^{-1} and 44.9 h, respectively. Similar growth pattern on fluorene was obtained with strain BR1, which increase from an initial cell density of 3.2×10^6 cfu/ml to 7.8×10^8 cfu/ml in 15 days. It also exhibited a growth rate and doubling time of 0.015 h^{-1} and 45.2 h during exponential growth on fluorene.

The rates of fluorene utilization as quantified by gas chromatographic (GC) analysis were 0.09 and 0.08 mg/L/h respectively for strains BM1 and BR1. These rates of degradation were lower than 0.8 ± 0.07 mg/L/h reported for fluorene-degrading *Pseudomonas putida* ATCC 17514 in 10 to 15 days (Rodrigues et al., 2005). Fluorene transformation by strains BM1 and BR1 were studied at 72 h intervals in MSM containing 50 mg/L fluorene. After 12 days of incubation, the residual fluorene content for strains BM1 and BR1 decreases to 43.06% (21.53 mg/L) and 53.22% (26.61 mg/L) corresponding to uptake of 56.94% (28.47 mg/L) and 46.78% (23.39 mg/L) fluorene, respectively. At the end of 21 days incubation, the residual fluorene content decreases further to 14% (7.0 mg/L) and 17.82% (8.90 mg/L) corresponding to uptake of 86% (43 mg/L) and 82.2% (41.1 mg/L) fluorene, respectively (Table 2). In the heat-killed control flasks, no apparent decrease of the substrate was observed, thus confirming that fluorene depletion from the MSM was due to biodegradation by the isolates rather than to non-specific abiotic losses such as substrate volatility or absorption to the glass tubes.

The percentages of fluorene degraded by these strains were lower than the 97% recorded for *Pseudomonas* sp. PSS6 (Mukesh et al., 2012). However, the difference is rather insignificant since the authors challenged the *Pseudomonas* strain with only 3 mg/L of fluorene compared to our 50 mg/L. Furthermore, fluorene degradation rates of the two strains is higher than 40.6% in 20 days reported for enteric bacterium *Leclercia adecarboxylata* isolated from oil sludge contaminated soil (Sarma et al., 2004). It is equally higher than the 81.87% in 50 days reported for *Rhodococcus ruber* ISO-2 isolated from automobile workshop sediments (Srujana and Khan, 2012).

The ability of the two strains to degrade fluorene may not be unconnected with the fact that they were isolated from sites where, non-volatile hydrocarbons, spent oils with its attendant PAHs, were indiscriminately dumped. This may have allowed autochthonous organisms to adapt and evolve necessary gene battery to degrade pollutants.

Previous reports have established the importance of prior exposure and adaptation to acquisition of degradative genes by autochthonous microorganisms. The mechanisms of adaptation include synthesis of inducible enzymes, mutations such as single nucleotide change or DNA re-arrangement that results in degradation of the compound and acquisition of genetic information from closely related or phylogenetically distinct population within the hydrocarbon-impacted community through horizontal gene transfer (Top and Springael, 2003; Obayori and Salam, 2010).

Though, several researchers have highlighted degradative abilities of *Bacillus* strains on PAHs (Das and Mukherjee 2007; Lily et al. 2009; Yuliani et al. 2012), globally, only one report exists on fluorene utilization by *Bacillus* species. Hidayati et al. (2011) reported fluorene removal by a biosurfactant-producing *Bacillus megaterium*.

The presence of the amended crude biosurfactant increase fluorene removal by the organism. In this study, two species of *Bacillus* putatively identified as *Bacillus subtilis* BM1 and *Bacillus amyloliquefaciens* BR1 displayed extensive degradation on fluorene and to the best of our knowledge, this is the first report highlighting the fluorene degradative potentials of these two species of *Bacillus*.

Further works will focus on the environmental factors favorable for the application of these organisms, the metabolites produced and the degradative genes involved in the process.

Conflict of Interests

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

REFERENCES

Arulazhagan P, Vasudevan N, Yeom T (2010). Biodegradation of polycyclic aromatic hydrocarbon by a halotolerant bacterial consortium isolated from marine environment. Int. J. Environ. Sci. Tech. 7:639-652.

Bauchop T, Elsden SR (1960). The growth of microorganisms in relation to their energy. J. Gen. Microbiol. 23:457-459.

Black CA (1965). Methods of soil analysis. No. 9, Part 2. American Society of Agronomy, Madison, Wisconsin.

Bray RH, Kurtz LT (1945). Determination of total organic and available forms of phosphorus in soils. Soil Sci. 59:39-45.

Chikere BO, Okpokwasili GC (2002). Seasonal dynamics of the pollution in a Niger Delta river receiving petrochemical effluents. Trop. Freshw. Bio. 11:11-22.

Chopra SL, Kanwar JS (1998). Analytical Agricultural Chemistry, MacMillian Press, London.

Das K, Mukherjee AK (2007). Differential utilization of pyrene as the sole source of carbon by *Bacillus subtilis* and *Pseudomonas aeruginosa* strains: Role of biosurfactants in enhancing bioavailability. J. Appl. Microbiol. 102:195-203.

Dibble JT, Bartha R (1979). Effect of environmental parameters on the biodegradation of oily sludge. Appl. Environ. Microbiol. 37:729-739.

Gomes RCB, Nogueira R, Oliveira JM, Peixoto J, Brito AG (2006). Kinetics of fluorene biodegradation by a mixed culture, Proceedings of the Second IASTED International Conference Advanced Technology in the Environmental Field, Lanzarote, Canary Island, Spain, pp. 84-87.

Grifoll M, Casellas M, Bayona JM, Solanas AM (1992). Isolation and characterization of a fluorene-degrading bacterium: identification of ring oxidation and ring fission products. Appl. Environ. Microbiol. 58:2910-2917.

Grifoll M, Selifonov SA, Chapman PJ (1994). Evidence for a novel pathway in the degradation of fluorene by *Pseudomonas* sp. strain F274. Appl. Environ. Microbiol. 60:2438-2449.

Grifoll M, Selifonov SA, Gatlin CV, Chapman PJ (1995). Actions of a versatile fluorene-degrading bacterial isolate on polycyclic aromatic compounds. Appl. Environ. Microbiol. 61:3711-3723.

Habe H, Chung J-S, Ishida A, Kasuga K, Ide K, Takemura T, Nojiri H, Yamane H, Omori T (2005). The fluorene catabolic linear plasmid in *Terrabacter* sp. strain DBF63 carries the beta-ketoadipate pathway genes, *pcaRHGBDCFIJ*, also found in proteobacteria. Microbiology 151:3713-3722.

Habe H, Chung J-S, Kato H, Ayabe Y, Kasuga K, Yoshida T, Nojiri H, Yamane H et al (2004). Characterization of the upper pathway genes for fluorene metabolism in *Terrabacter sp*. strain DBF63. J. Bacteriol. 186:5938-5944.

Hidayati NV, Hilmi E, Haris A, Effendi H, Guiliano M, Doumenq P, Syaki AD (2011). Fluorene removal by biosurfactants producing *Bacillus megaterium*. Waste Biomass Valor. 2(4):415-422.

Holt JG, Krieg NR, Sneath PHA (1994). Bergey's Manual of Determinative Bacteriology. William and Wilkins, Baltimore, Maryland. 10:112-125.

Igwo-Ezikpe MN, Gbenle OG, Ilori MO (2006). Growth study on chrysene degraders isolated from polycyclic aromatic hydrocarbon polluted soils in Nigeria. Afr. J. Biotechnol. 5(10):823-828.

Ilori MON, Amund OO (2000). Degradation of anthracene by bacteria isolated from oil polluted tropical soils. Z. Naturforsch [C] 55:890-897.

Kanaly RA, Harayama S (2010). Advances in the field of high molecular-weight polycyclic aromatic hydrocarbon biodegradation by bacteria. Microbiol. Biotechnol. 3:132-164.

Kästner M, Breuer-Jammali M, Mahro B (1994). Enumeration and characterisation of the soil microflora from hydrocarbon-contaminated soil sites able to mineralise polycyclic aromatic hydrocarbons. Appl. Microbiol. Biotechnol. 41:267-273.

Kästner M, Breuer-Jammali M, Mahro B (1998). Impact of inoculation protocols, salinity, and pH on the degradation of polycyclic aromatic hydrocarbons (PAHs) and survival of PAH-degrading bacteria introduced into soil. Appl. Environ. Microbiol. 64:359-362.

Kasuga K, Nojiri H, Yamane H (1997) Genes of enzymes involved in the biodegradation of carbazole, dibenzofuran, fluorene, and dibenzo-*p*-dioxin by bacteria. Water Sci. Technol. 36:9-16.

Keith LH, Telliard WA (1979) Priority pollutants : A perspective view. Environ. Sci. Technol. 13:416-423.

Lily MK, Bahuguna A, Dangwal K, Garg K (2009) Degradation of benzo-a-pyrene by a novel strain *Bacillus subtilis* BMT4i (MTCC 9447). Braz. J. Microbiol. 40:884-892.

Monna L, Omori T, Kodama T (1993) Microbial degradation of dibenzofuran, fluorene, and dibenzo-*p*-dioxin by *Staphylococcus auriculans* DBF63. Appl. Environ. Microbiol. 59:285-289.

Mukesh KDJ, Amutha MB, Devika S, Balakumaran MD, Kalaichevlan PT (2012) Biodegradation of polycyclic aromatic hydrocarbons by *Pseudomonas* sp. PSS isolated from municipal wastes sediment. Der Chemica Sinica 3:543-547.

Obayori OS, Salam LB (2010). Degradation of polycyclic aromatic hydrocarbons: role of plasmids. Sci. Res. Essays 5(25):4093-4106.

Obayori OS, Ilori MO, Adebusoye SA, Oyetibo GO, Amund OO (2008) Pyrene-degradation potentials of *Pseudomonas* species isolated from polluted tropical soils. World J. Microbiol. Biotechnol. 24:2639-2646.

Rodrigues AC, Wuertz S, Brito AG, Melo LF (2005) Fluorene and phenanthrene uptake by *Pseudomonas putida* ATCC 17514: kinetic and physiological aspects. Biotechnol. Bioeng. 90(3):281-289.

Salam LB, Obayori OS, Olatoye NO (2014). Biodegradation of anthracene by a novel actinomycete, *Microbacterium* sp. isolated from hydrocarbon-contaminated soil. World J. Microbiol. Biotechnol. 30:333-341.

Sarma PM, Bhattacharya D, Krishnan S, Lal B (2004). Degradation of polycyclic aromatic hydrocarbons by a newly discovered enteric bacterium *Leclercia adecarboxylata*. Appl. Environ. Microbiol. 70(5):3163-3166.

Schuler L, Ni Chadhain SM, Jouanneau Y, Meyer C, Zylstra GL, Hols P, Agathos SN (2008). Characterization of a novel angular dioxygenase from fluorene-degrading *Sphingomonas* sp. Strain LB126. Appl. Environ. Microbiol. 74:1050-1057.

Shibutani S, Suzuki N, Grollman AP (1998). Mutagenic specificity of (acetylamino)fluorene-derived DNA adducts in mammalian cells. Biochemistry 37(35):12034-12041.

Srujana K, Khan AB (2012). Isolation and characterization of polycyclic aromatic hydrocarbon degrading soil microbes from automobile workshop sediments. J. Environ. Sci. Technol. 5(1):74-83.

Top EM, Springael D (2003). The role of mobile genetic elements in bacterial adaptation to xenobiotic organic compounds. Curr. Opin. Biotechnol. 14:262-269.

Trenz SP, Engesser KH, Fischer P, Knackmuss HJ (1994). Degradation of fluorene by *Brevibacterium* sp. strain DPO 1361: a novel C-C bond cleavage mechanism via 1, 10-dihydro-1,10-dihydroxyfluoren-9-one. J. Bacteriol. 176:789-795.

Wackett LP, Hershberger LCD (2001). Biocatalysis and Biodegradation: Microbial Transformation of Organic Compounds. ASM Press, Washington.

Wattiau P, Bastiaens L, van Herwijnen R, Daal L, Parsons JR, Renard ME, Springael D, Cornelis GR (2001). Fluorene degradation by *Sphingomonas* sp. LB126 proceeds through protocatechuic acid: a genetic analysis. Res. Microbiol. 152:861-872.

Yuliani H, Hermansyah H, Sahlan M, Wijanarko A (2012). Dioxygenase gene of PAHs degrading *Bacillus* strains isolated from marine Indonesian environment and its biosurfactant production ability. Int. J. Pharm. Bio. Sci. 3(4) (B):380-390.

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Full Length Research Paper

Isolation and characterization of a new *Pseudomonas*-related strain capable of degradation of phenol from oil contaminated soil

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A novel phenol-degrading bacterium named as SKDP-1 was isolated from crude oil contaminated soil, Gudao oil field in the Northeast of Shandong Dongying, East China. The biochemical tests indicated that strain SKDP-1 was Gram-negative, and glucose and citrate could be utilized and starch not gelatin. Both Voges-Proskauer and H_2O_2 enzyme tests were positive. The activities of catechol 1,2-dioxygenase (C12O) and catechol 2,3-dioxygenase (C23O) in free cells were measured to be 8 and 91 IU, respectively. 16S rDNA gene sequences of strain SKDP-1 analysis showed the similarity of 98% with *Pseudomonas putida* (AB680847). The phylogenetic tree formed by 16S rDNA sequences from both strain SKDP-1 and its most related bacteria also proved strain SKDP-1 to be one member of the genus *Pseudomonas*. Strain SKDP-1 showed the high phenol-degrading rates from 30 to 99% cultured by mineral salts medium (MSM), which was added with phenol from 100 to 1600 mg/L, respectively. The optimum pH and growth temperature for strain SKDP-1 to remove phenol were about 7.0 and 30°C, respectively. Based on its biochemical properties and high capability of degrading phenol, strain SKDP-1 provided the possibilities of treating phenol contaminated environment in the future.

Key words: Phenol degradation, *Pseudomonas*, *ortho*-cleavage pathway.

INTRODUCTION

Phenol, as an air pollutant, could be emitted from wood and solid waste combustion as well as automobile exhaust and cigarette smoke. It is also responsible for the malodors during the course of decomposition of animal wastes (Zahn et al., 2001). The global production of phenol has been estimated to be about 8.5 million metric tons per annum, and its environmental risk assessments have concluded that phenol has the potential of causing harm both to marine aquatic life and terrestrial biota (Jiang et al., 2002).

Biodegradation of phenol has been extensively studied due to the widespread distribution of phenol as a pollutant in water and soil (Jiang et al., 2005). The biological degradation is accomplished through benzene ring cleavage mediated by intracellular enzymatic reaction (Kumar et al., 2004). In addition, studies on phenol toxicity to bacteria have shown that bacteria can adapt a low level of phenol concentrations, but increasing

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phenol concentrations appeared to decrease overall phenol degradation (Dean and Rahimi, 1995). A range of phenol-degrading microorganisms have been identified, including *Acinetobacter* (Abd et al., 2002), *Bacillus* (Arutchelvan et al., 2005), *Burkholderia* (El et al., 2003), *Pseudomonas* (Whiteley et al., 2001), *Valivorax* (Watanabe et al., 1998), mesophilic and thermophilic *methanogens* (Chen et al., 2008), and the yeast *Candida tropicalis* (Jiang et al., 2005). Many studies proved that phenol-degrading bacteria have been isolated from natural soils (De et al., 2005), plants roots (Wang et al., 2007), root nodules (Wei et al., 2008), rivers (Parvanov and Topalova, 2008), and marine ecosystems (Shashirekha et al., 1997). Oil contaminated soil as the resource of isolating phenol-degrading bacteria has only been reported by Bhavna et al. (2011) until now. The detailed information on those phenol-degrading bacteria in that study such as their phylogenetic analysis was very limited.

Gudao oil field (133.1 km^2) located in the Northeast of Shandong Dongying (37°86'N, 118°78'E), East China, is the biggest production plant of Sinopec Group. Unavoidable oil leak in the processes of oil extraction and transport caused the serious contamination on the soil. The objectives of the present study were: (1) to isolate the bacterium with high phenol-degrading ability from oil contaminated soil in Gudao oil field, (2) to identify the phylogenetic position of this bacterium, (3) to test the isolate on its degrading-phenol capability with the increasing phenol concentration, and (4) to prove the pathway for phenol metabolism of this isolate.

MATERIALS AND METHODS

Isolation of phenol-degrading bacteria

Surface soil (area of 13×13 cm; depth of 0 to 8 cm) in Gudao oil field as the sample, was collected. The enrichment operation of bacteria was described as follows: in brief, 1 ml of suspension from 5 g soil sample was incubated in 100 ml of mineral salt medium (MSM). The culture condition was at 30 on rotary shaking incubator at 180 rpm for four weeks. MSM medium contained the following ingredients (L^{-1}): K_2HPO_4 2.75 g, KH_2PO_4 2.25 g, $(NH_4)_2SO_4$ 1 g, $MgCl_2.6H_2O$ 0.2 g, NaCl 0.1 g, $FeCl_3.6H_2O$ 0.02 g and $CaCl_2$ 0.01 g, pH was adjusted to 7.0 (Watanabe, 1998). The medium was autoclaved at 121°C for 15 min for sterilization. Phenol, as the sole carbon source, was separately sterilized and aseptically added into the sterile medium with the concentration of 300 mg/ L.

The purification of strains were summarized as follows: 0.2 ml of the enriched culture obtained from the above steps was streaked onto plates with MSM medium containing 2% agar and incubated at 30°C for one week. The separated colonies were chosen and streaked onto plates for incubation. After four replicates of streaking incubation, the purified isolates were maintained in semi-solid MSM stab cultures supplemented with phenol as sole carbon source at 4°C until further use. One isolate was chosen as the representative bacteria named strain SKDP-1 for the future study.

Phenotypic characterization of isolate

The cell morphology was verified by scanning electron microscope (SEM) (Hitachi, S-4800) and Gram-stain was examined as previously described (Holt et al., 2010).

Physiological and biochemical tests

The physiological and chemical properties were carried out according to Bergey´s Manual of Determinative Bacteriology (Holt et al., 2010).

Phenol removal tests

Strain SKDP-1 was pre-cultured in Luria Bertani (LB) medium (L^{-1}): yeast extract 5.0 g, peptone 10.0 g, sodium chloride 10.0 g, at 30°C with shaking (180 rpm) till logarithmic phase. 3% (v/v) of the above pre-culture was inoculated to MSM medium containing phenol from 100 to 1600 mg/L as the sole carbon source. The growth condition was also at 30°C with shaking (180 rpm). The reaction mixture containing all components but devoid of bacterial inoculums was used as control. The residual phenol concentration present in culture at different incubation period was measured using UV visible spectrophotometer by colorimetric assay 4-amino antipyrene method (Lacoste et al., 1959). In order to test the influences of both pH and temperature on phenol removal for the isolate, series of pH values ranging from 3.0 to 9.0 (intervals of 2) and temperatures from 20 to 40°C (intervals of 5) were made for culturing strain SKDP-1 with 400 mg/L of phenol (according to the above experiments results) contained in MSM medium, respectively. The method of measuring residual phenol in medium was the same as described previously.

Enzyme assays

Strain SKDP-1 was grown on MSM liquid medium added with 400 mg/L of phenol as the sole carbon source for 100 h at 30°C. The above culture conditions were based on the previous experiments (phenol removal tests). Then the cells (strain SKDP-1) were harvested by the ultra filtration method (filter membrane diameter, 0.22 μm) and then washed with phosphate potassium buffer for the following steps (Zhang et al., 2008; Ren et al., 2005).

The cells obtained from the above steps were ruptured for 30 s with ultrasonic processor. The cell debris and undisrupted cells were removed away by centrifuging at 10000 rpm for 20 min. The supernatant obtained was used as free cell extracts (crude enzyme) for enzyme assay. Total protein content in the crude enzyme was measured by the Bradford method using bovine serum albumin as the standard (Ausubel et al., 1995). The reaction mixture of 1.0 mL contained 50 mM sodium phosphate buffer, 50 μg enzyme and 75 μmol of substrate (catechol), and pH was adjusted to 7.0. The activities of catechol 1,2-dioxygenase (C12O) and catechol 2,3-dioxygenase (C23O) for strain SKDP-1 were measured by spectrophotometric method. C12O activity was measured as an increase of absorbance at 260 nm by the formation of *cis, cis*-muconic acid (Hegeman, 1966). C23O activity was measured at 375 nm by determining the accumulation of 2-hydroxymuconic semialdehyde (2-HMS) (Feist and Hegeman, 1969). Control experiments (without catechol) were carried out for each assay. Triplicates were done for determining the quantity of *cis, cis*-muconic acid and 2-HMS. The enzyme activities were expressed as μmoles of product formed per min per mg of protein at 30°C.

PCR amplification of 16S rDNA from the isolates

Genomic DNA of the isolate cultured in MSM medium was extracted with TIANamp Bacteria DNA DP302 Kit (Beijing Tiangen Biotech)

Table 1. Morphological and biochemical characteristics of bacteria SKDP-1able to grow on MSM medium with phenol as sole carbon source.

Characteristic	Bacteria strain SKDP-1	Characteristic	Bacteria strain SKDP-1
Gram staining	negative	Citrate	Positive
Motility	Positive	Glucose	Positive
Colony	White slimy	Starch	Negative
Catalase	Positive	Voges-Proskauer test	Positive
Urease	Negative	M.R	Negative
H_2S production	Negative	Aerobic test	Negative
Nitrate reduction	Positive	Starch hydrolyzation	Negative
Indole production	Positive	Gelatin liquefaction test	Negative
Maltose	Negative	Optimum pH	7.0

according to its instruction. PCR amplification of 16S rRNA for the isolate was performed using the primers PF5′-AGA GTT TGA TCC TGG CTC AG-3′and PR 5′-GGY TAC CTT GTT ACG ACT T-3′. PCR reactions contained 100 ng of genomic DNA, each primer at a concentration of 0.4 µM, each dNTP at a concentration of 200 µM. PCR reaction conditions were an initial denaturation step of 3 min at 94°C, 32 cycles of 45 s at 95°C, 45 s at 55°C, with a final extension for 10 min at 72°C. The electrophoresis, the purification of PCR products were essential as described previously (Lu et al., 2008).

Sequence alignment and phylogenetic analysis

The 16S rRNA sequences were determined by the automatic DNA sequencer (ABI Prism Model 3700, CA, USA). The primer used for sequencing was PF as mentioned above. The sequences determined from the isolate were compared with the similar sequences retrieved from DDBJ /EMBAL/GenBank database using the BLAST program. All the obtained sequences were aligned using the CLUSTAL X program (Thompson et al., 1997) and the phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei, 1987), using the Mega.5 program(Sharma et al., 2002). Bootstrap analysis was performed 1000 times using the same program as above.

Nucleotide sequence accession number

The 16S rRNA sequence for the isolate has been deposited in DDBJ under accession number AB773822.

RESULTS

Isolation and identification of phenol-degrading strain

Soil contaminated with oil was chosen as the source of indigen bacteria isolation in this study. Up to four weeks of treating with phenol as sole carbon source in MSM medium, only phenol resistant strains were left in culture. After four replicates of purification treatments, one isolate was selected as the representative strain and named SKDP-1. Electron scanning micrograph indicated that strain SKDP-1 cultured in MSM medium was slightly curved with rods (sizes, 0.2-0.4 µm wide and 1.0-1.45µm long). The morphological and chemical properties of strain SKDP-1 were listed in Table 1. Comparing with the characteristics of the microorganisms with the ability of degrading phenol mentioned in Bergey′s manual of systematic Bacteriology, strain SKDP-1 was seemed to be one member of the genus *Pseudomonas* sp.

Degradation of phenol by strain SKDP-1

Acclimatization has been regarded as the useful way for microorganisms to obtain the highest resistant capability of phenol concentrations (Lob and Tar，2000). The experiment in the present study aimed to find the highest tolerance of phenol concentration for strain SKDP-1. A series of phenol concentration (as the sole carbon source, 100 to 1600 mg/L) were prepared for culturing strain SKDP-1. The residual of phenol with time changes by strain SKDP-1 under different concentrations phenol in MSM media were shown in Figure 1. Strain SKDP-1 showed the similar capabilities of degrading phenol almost completely (about 94 - 99%) within a relatively short time of 100 h with phenol concentration of 100 to 400 mg/L in MSM medium. Strain SKDP-1 showed the very low removal rates of about 40 and 30% at the time of 100 h when phenol was added up to the concentration of 800 and 1600 mg/L in MSM medium, respectively. The results (Figure 1) prove that 400 mg/L of phenol in MSM medium seemed to be the maximum concentration for strain SKDP-1 to endure and remove. Cells growing at high phenol concentration (800 and 1600 mg/L) showed the longer lag time compared to those growing at low concentration of phenol (data not shown). The same stationary phase at about 100 h for strain SKDP-1 growing at the whole range concentration of phenol (100 to 1600 mg/L) could also be proved by Figure 1.

Based on the previous experiment, phenol concentration of 400 mg/L in MSM medium and 100 h incubation time were used for evaluating the influences of pH and culture temperature on phenol removal for strain SKDP-1.

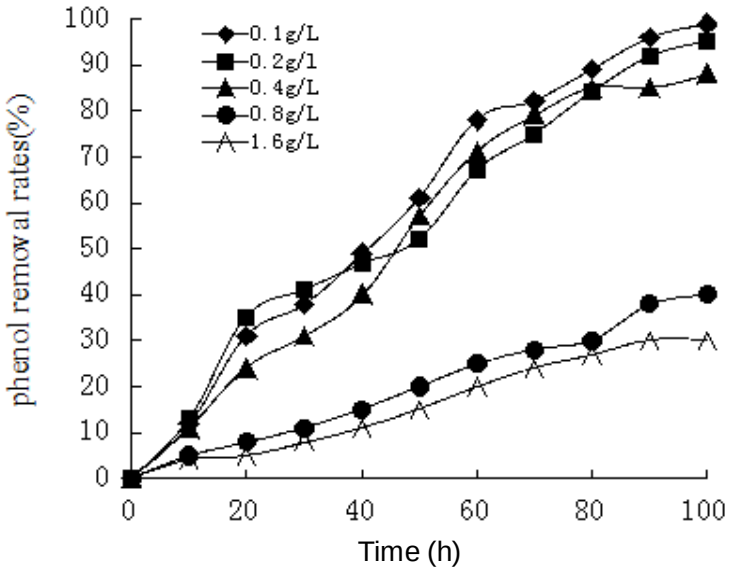

Figure 1. Phenol removal rates of strain SKDP-1 with phenol concentrations of 0.1, 0.2, 0.4, 0.8, 1.6 g/L in MSM medium at 100 h incubation, respectively.

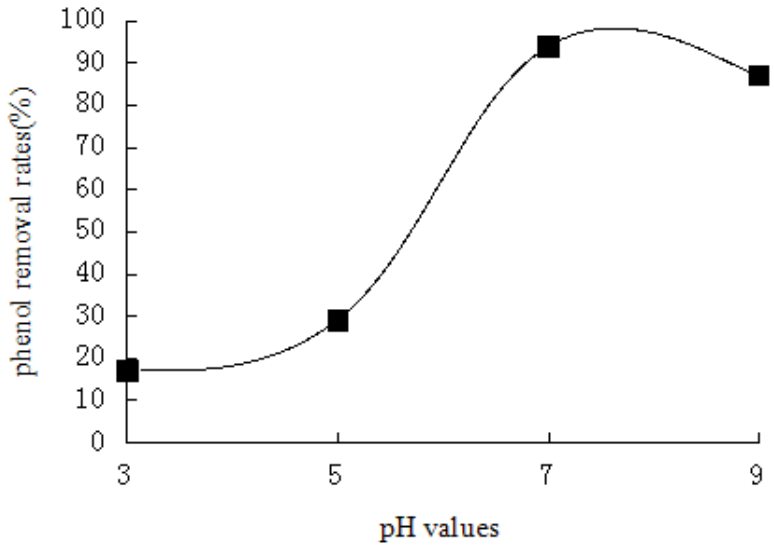

Figure 2. Phenol removal rates of strain SKDP-1 at 100 h incubation in MSM medium with different pH of 3, 5, 7 and 9, respectively.

The residual phenol was tested at 100 h incubation time with the different pH values (3.0, 5.0, 7.0 and 9.0) and culture temperatures (20 to 40°C) in MSM medium, respectively. The phenol removal dependant changes of pH values and temperatures in MSM medium were shown as Figures 2 and 3, respectively. The highest removal rates of about 99% for strain SKDP-1 with pH value of 7.0 and temperature of 30°C in MSM medium were shown in Figure 2 and Figure 3, respectively.

Figure 1 shows the degradation profile by strain SKDP-1 with phenol concentrations of 100, 200, 400, 800, 1600 mg/L in MSM medium, respectively. The results are shown as average of triplicate independent experiments and the bars indicate the standard deviation less than 5%. Figure 2 shows degradation profile by strain SKDP-1 at 100 h incubation with different pH of 3, 5, 7, 9, respectively. The results are shown as average of triplicate independent experiments and the bars indicate the

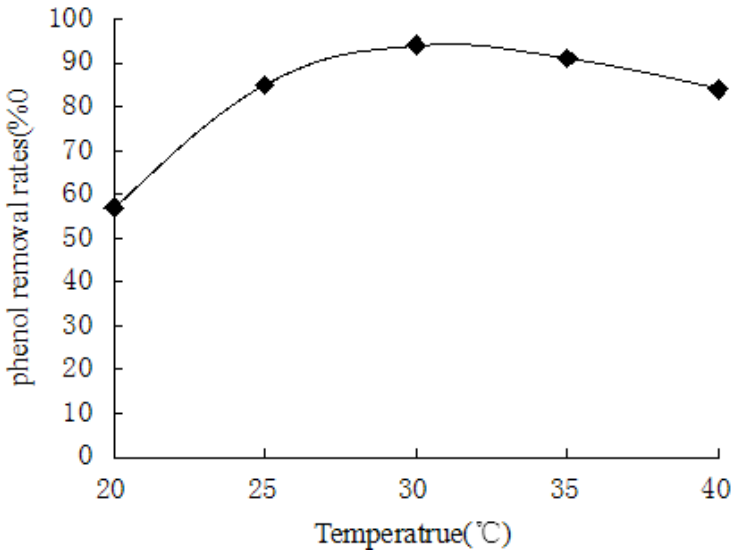

Figure 3. Phenol removal rates of strain SKDP-1 at 100 h incubation in MSM medium with different temperature of 20, 25, 30, 35 and 40°C, respectively.

standard deviation less than 5%. Figure 3 shows the degradation profile by strain SKDP-1 of 400 mg/L at 100 hr incubation with different temperature of 20, 25, 30, 35 and 40°C, respectively. The results are shown as average of triplicate independent experiments and the bars indicate the standard deviation less than 5%.

Enzyme studies

There are two main metabolic pathways (*ortho*- and *meta*-cleavage) for phenol biodegradation which were initiated either by catechol 1,2-dioxygenase (C120) or catechol 2,3-dioxygenase (C230), respectively. In this study, the activities of C120 and C230 in free cells of strain SKDP-1 were found to be 8 and 91 IU, respectively. Therefore, the main way of metabolizing phenol for strain SKDP-1 was *meta*-pathway.

Phylogenetic analysis

The DDBJ database was used to search for 16S rRNA sequences homologous of all the sequences used in constructing phylogenetic tree (Figure 4). Phylogenetic tree revealed that strain SKDP-1 exhibited a highest similarity (98%) with *Pseudomonas putida* (AB680847) and was belonged to genus *Pseudomonas.*

Figure 4 shows the phylogenetic analysis based on the 16S rDNA sequences of strain SKDP-1 and related species. Neighbour-joining method is used to construct this tree. Bootstrap values obtained with 1000 repetitions are indicated at the nodes. Bar 0.02 substitutions per nucleotide position. Accession numbers from GenBank are in parentheses.

DISCUSSION

It has been found that some bacteria withstand the toxicity from phenol by secreting monocyclic aromatic compounds (MACs) or polycyclic aromatic compounds (PACs) (Hearn et al., 2003; Jiang et al., 2005; Lacoste et al., 1959; Leung et al., 1997). Considering this fact, the residual phenol in culture and the enzyme activities (C12O and C23O) were determined in our present work to find out how strain SKDP-1 tolerate and grow in medium added with phenol as its sole carbon source. The time dependent decrease of residual phenol in MSM medium and the activities of C12O and C23O could prove that the tolerance of the phenol toxicity for strain SKDP-1 was from its high phenol-degrading, not by secreting MACs or PACs (Figures 1, 2 and 3).

In the study of Bhavna et al. (2011), two aerobic bacterial strains OCS-A and OCS-C were also isolated from the oil contaminated soil. The phenol-tolerating and -degrading abilities for strains OCS-A and OCS-C were also tested in that study. Strains OCS-A and OCS-C could only tolerate phenol up to concentrations of 100 mg/L, while strain SKDP-1 in our work showed the much higher phenol tolerance of 400 mg/L (Figure 1). In the same phenol concentration of 100 mg/L in medium, strains OCS-A and OCS-C were only able to degrade about 90% of phenol, while strain SKDP-1 could degrade phenol almost completely (99%) (Figure 1). In addition, comparing strains AT2 and PW3 isolated by Wael et al., (2003) with strain SKDP-1, their optimal temperature (30°C) and pH (7.0) for phenol-degrading were identical.

There are two different pathways for phenol metabolism under aerobic condition, either the *ortho*- or *meta* pathway from catechol in phenol-degrading bacteria, in

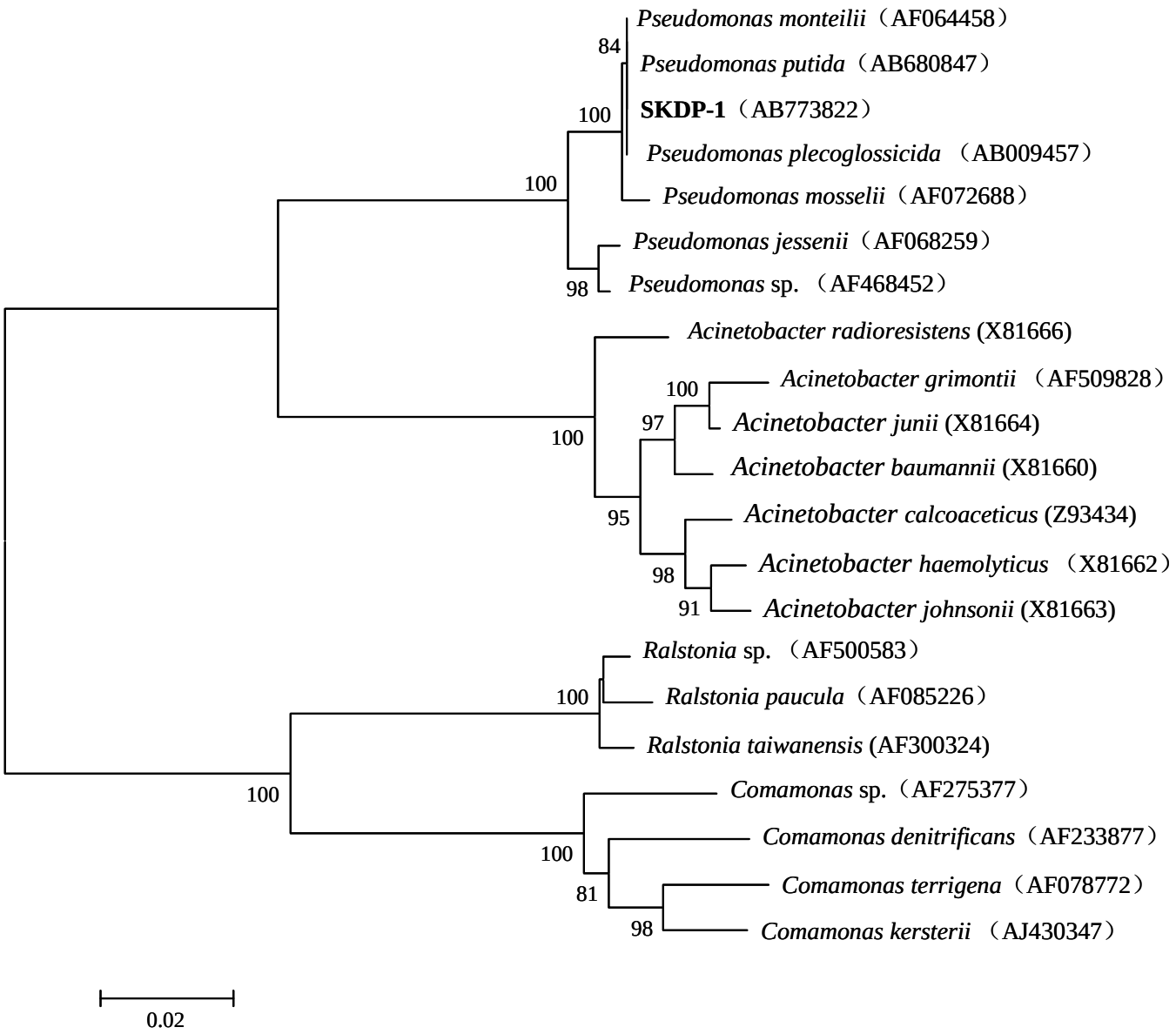

Figure 4. Phylogenetic analysis based on the 16S rDNA sequences of strain SKDP-1 and related species. Neighbour-joining method is used to construct this tree. Bootstrap values obtained with 1000 repetitions are indicated at the nodes. Bar 0.02 substitutions per nucleotide position. Accession numbers from GenBank are in parentheses.

which *meta* cleavage of catechol for phenol metabolism was found to be predominant in nature. Our results also reveal that phenol was mainly degraded by strain SKDP-1 through *meta* pathway and was consistent with the report by Dong et al. (2008).

The microorganism isolated from oil contaminated site used phenol as sole carbon source. This was identified and characterized as strain SKDP-1 in our study. These mesophilic bacteria showed optimal growth at 30°C and at pH of 7.0. According to Bergey's manual, strain SKDP-1 seemed to be one member of *Pseudomonas* genus with its morphological and biochemical properties.

Further identification of phylogenetic position (Figure 4) indicated that strain SKDP-1 was most related to *Pseudomonas putida* (AB680847) with a high similarity of 98%, therefore strain SKDP-1 was certainly one member of *Pseudomonas* genus. Further characterization including DNA-DNA homology assay, G+C contents determination and fat acids composition tests will be needed for strain SKDP-1(AB773822). Additionally, an on-site determination of phenol-degrading capability for strain SKDP-1 (AB773822) in the oil contaminated site will also be needed for obtaining the more exact reflection of its phenol degrading properties.

The utilization of biological systems on bioremediation is more cost-effective than traditional cleaning techniques such as waste incineration; possible savings have been estimated to 65-85%. This work could provide a useful guideline in evaluating potential phenol microorganisms living at the polluted environments and a possibility for using the bacterium to decontaminate phenolic and hydrocarbon wastes.

Conflict of Interests

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

ACKNOWLEDGEMENTS

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REFERENCES

Abd-El-Haleem D, Moawad H, Zaki EA, Zaki S (2002). Molecular characterization of phenol-degrading bacteria isolated from different Egyptian ecosystems. Microb. Ecol. 43(2):217-224.

Arutchelvan V, Kanakasabai V, Nagarajan S, Muralikrishnan V (2005). Isolation and identification of novel high strength phenol degrading bacterial strains from phenol-formaldehyde resin manufacturing industrial wastewater. J. Hazard. Mater. B127:238-243.

Ausubel FM , Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K(1995). Current Protocols in Molecular Biology. Greene Publishing Associates/Wiley Interscience, New York.

Bhavna VM, Shraddha PP, Ankush M (2011). Isolation,selection and biodegradation profile of phenol degrading bacteria from oil contaminated soil. Bull. Environ. Contam. Toxicol. 87(2):143-146.

Chen CL, Wu JH, Liu WT (2008). Identification of important microbial populations in the mesophilic and thermophilic phenol degrading methanogenic consortia. Water Res. 42(9):1963-1976.

De RCM, Pumphrey GM, Madsen EL (2005). Use of field-based stable isotope probing to identify adapted populations and track carbon flow through a phenol-degrading soil microbial community. Appl. Environ. Microbiol. 71(12):7858-7865.

Dean RD, Rahimi M(1995). Toxicity of phenolic compounds to sediment bacteria. Bull. Environ. Contam. Toxicol. 55(2):245-250.

Dong XJ, Hong Q, He LJ, Jiang X, Li SP (2008). Characterization of phenol-degrading bacterial strains isolated from natural soil. Int. Biodeter. Biodegr. 62(3):257-262.

El SWS, Ibrahim MK, Shady MA, Beih FE, Ohmura N, Saiki H, Ando A (2003). Isolation and characterization of phenol-catabolizing bacteria from a coking plant. Biosci. Biotechnol. Biochem. 67(9):2026-2029.

Feist CF, Hegeman GD (1969). Phenol and benzoate metabolism by *Pseudomonas putida*: regulation of tangential pathways. J. Bacteriol. 100(2):869-877.

Hearn EM, Dennis JJ, Gray MR, Foght JM(2003). Identification and characterization of the emhABC efflux system for polycyclic aromatic hydrocarbons in *Pseudomonas fluorescens* cLP6a. J. Bacteriol. 185(21):6233-6240.

Hegeman GD (1966). Synthesis of the enzymes of the mandelate pathway by *Pseudomonrs putida*. I. Synthesis of enzymes of the wild type. J. Bacteriol. 91(3):1140-1154.

Holt SG, Kriey NR, Sneath PHA, Staley JT, Williams ST(2010). Bergey's Mannual of Determinative Bacteriology. Williams and Wilkins, New York.

Jiang Y, Wen JP, Li HM, Yang SL, Hu ZD (2005). The biodegradation of phenol at high initial concentration by the yeast *Candida tropicalis*. Biochem. Eng. J. 24(3):243-247.

Jiang, HL, Tay JH, Tay STL (2002).Aggregation of immobilized activated sludge cells into aerobically grown microbial granules for the aerobic biodegradation of phenol. Lett. Appl. Microbiol. 35(5):439-445.

Kumar A, Kumar S, Kumar S (2004). Biodegradation kinetics of phenol and cathecol using *Pseudomonas putida* MTCC1194. Biochem. Eng. J. 22 (2):151-159.

Lacoste RJ, Venable SH, Stone JC (1959). Modified 4-aminoantipyrene colorimetric method for phenols. Applications to an acrylic monomer. Anal. Chem. 31(7):1246-1249.

Leung KI, Tresse O, Errampalli D, Lee H, Trevors JT(1997). Mineralization of p-nitrophenol by pentachlorophenol-degrading. Sphingomonas spp. FEMS. Microbiol. Lett. 155(1):107-114.

Lob KC, Tar PP (2000). Effect of additional carbon Source on biodegradation of phenol. Bull. Environ. Contam. Toxicol. 64(6):756-763.

Lu HS, Fumimura R, SatoY, Nanba K, Kamijo T, Ohta H (2008). Characterization of *Herbaspirillum*- and *Limnobacter*-related strains isolated from young volcanic deposits in Miyake-jima island, Japan. Microbes Environ. 23(1):66-72.

Parvanov D, Topalova Y (2008). Biodegradation potential of phenol-resistant bacteria localized in different stream habitats. Biotechnol. Biotechnol. Equip. 22(2):709-715.

Ren YL, Xing XH, Zhang C, Gou ZX (2005). A simplified method for assay of hydrogenase activities of H_2 evolution and uptake in *Enterobacter aerogenes*. Biotechnol. Lett. 27(14):1029-1033.

Saitou N, Nei M (1987).The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4(4):406-425.

Sharma A, Kachroo D, Kumar R (2002). Time dependent influx and efflux of phenol by immobilized microbial consortium. Environ. Monit. Assess. 76(2):195-211.

Shashirekha S, Uma L, Subramanian G (1997). Phenol degradation by the marine cyanobacterium *Phormidium valderianum* BDU 30501. J. Ind. Microbiol. Biotechnol. 19(2):130-133.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997). The clustal-X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acid Res. 24(25):4876-4882.

Wael SES, Mohamed KI, Mohamed AS, Fawkia EB, Naoya O, Hiroshi S, Akikazu A(2003). Isolation and characterization of phenol-catabolizing bacteria from a coking plant. Biosci. Biotechnol. Biochem. 69(9):2026-2029.

Wang YJ, Xiao M, Geng XL, Liu JY, Chen J (2007). Horizontal transfer of genetic determinants for degradation of phenol between the bacteria living in plant and its rhizosphere. Appl. Microbiol. Biotechnol. 77(3):733-739.

Watanabe K, Teramoto M, Futamata H, Harayama S (1998). Molecular detection, isolation, and physiological characterization of functionally dominant phenol-degrading bacteria in activated sludge. Appl. Environ. Microbiol. 64(11):4396-4402.

Wei GH, Yu JF, Zhu YH, Chen WM, Wang L (2008). Characterization of phenol degradation by Rhizobium sp CCNWTB 701 isolated from Astragalus chrysopteru in mining tailing region. J. Hazard. Mater. 151(1):111-117.

Whiteley AS, Wiles S, Lilley AK, Philp J, Bailey MJ (2001). Ecological and physiological analyses of *Pseudomonad* species within a phenol remediation system. J. Microbiol. Methods 44(1):79-88.

Zahn JA, DiSpirito AA, Do YS, Brooks BE, Cooper EE, Hatfield JL (2001). Correlation of human olfactory responses to airborne concentrations of malodorous volatile organic compounds emitted from swine effluent. J. Environ. Qual. 30(2):624-634.

Zhang JF, Fengyi S, Xinhui X (2008). A simple assay of catechol 2,3-dioxygenase activity in whole-cell catalysis of Pseudomonas putida mt-2. J. Chem. Ind. Eng. (China) 59(2):450-455.

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Full Length Research Paper

Production, optimization, characterization and antifungal activity of chitinase produced by *Aspergillus terrus*

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Chitin is one of the most abundant biopolymers widely distributed in the marine and terrestrial environments. Chitinase enzyme has received increased attention due to its wide range of biotechnological applications. *Aspergillus terrus* was found to be a good chitinase producer among the five fungi isolated from different soil samples from Al-Jouf city, Saudi Arabia. Maximum production of chitinase was obtained when using 2% of Shrimp-shell powder as a sole carbon source in the fermentation medium. The high level of chitinase production was observed in the culture medium with pH 5 at 30°C for five days at shaking conditions. Some properties of the crude chitinase were studied. In the present study, the antifungal activity of crude *A. terrus* chitinase was investigated against *Apergillus niger*, *Aspergillus oryzae, Pencillum oxysporium*, *Rhizoctonia solan*i, *Fusarium oxysporium*, *Rhizopus sp.* and *Mucor* sp. and also estimated. The chitinase was found to inhibit the growth of some phytopathogenic fungi tested. The present work provides a suitable medium composition for enhancement of chitinase production by *A. terrus* and some properties of crude enzyme. Moreover, the study reflects the potential of *A. terrus* chitinase for biotechnological application.

Key words: Chitinase, *Aspergillus terrus*, optimization, antifungal activity.

INTRODUCTION

Chitin, a (β-1,4)-linked homopolymer of N-acetylglucosamine is the second most abundant biodegradable polymer, can be found as a part of fungi, plants, crustaceans, insects, arthropods, and algae components (Gohel et al., 2006). Approximately 75% of the total weight of shellfish, such as shrimp, crabs and krill are considered as waste, and chitin comprises 20 to 58% of the dry weight of the said waste (Wang and Chang, 1997). Microbial degradation of insoluble macromolecules such as lignin, cellulose, keratin and chitin depends on the production of extracellular enzymes with the ability to act on compact substrate surface. The use of chitin as a carbon source by bacteria and fungi has been a subject of sporadic interest. The enzymatic degradation of chitin by microorganisms occurs in two steps: first the hydrolysis by chitinase poly (1.4(N-acetyl-

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β-D-glucosaminide) glycanohydro-ase; EC3.2.1.14) to oligomer ss, mainly dimers, followed by their degradation to free N-acetylglucoseaminidase; EC3.2.1.30). Chitinolytic enzymes are able to lyze the cell wall of many fungi. The microorganisms that produce these enzymes are able to destroy the cell wall of many fungi, and capable of eradicating fungal diseases that are a problem for global agricultural production (Budi et al., 2000). Also, chitinase find widespread applications in the field of agriculture, medicine, biochemical processing engineering, waste management, pesticide control, in food and feed, sweeteners and cell wall degrading enzyme. In viruses, chitinases are involved in pathogenesis (Dahiya et al., 2006). Other application of chitinases is bioconversions of chitin waste to single cell proteins and ethanol and fertilizers. Industrial applications of chitinases have been governed mainly by key factors such as cost production, self-life stabilities and improvement in enzyme properties by immobilization (Daizo, 2005). Due to multiple applications of chitinases, they become interesting enzymes for study.

Several microorganisms, including bacteria such as *Serratia marcescens Bacillus lichiniformis, Bacillus subitils, Bacillus thuringiensis*, and *Vibrio alginolyticus* (Joshi et al., 1989; Sowmeya et al., 2001; Wang et al., 2006; Merina et al., 2012) and many species of fungi such as: *Asergillus* sp*., Myrothecium anisopliae, Streptomyces sp., Trichoderma harzianum, Trichoderma viride,* and *Verticillium lecanii* (De Siqueira et al., 1997; Mathivanan et al., 1998; Liu et al., 2003; Nampoothiri, et al., 2004; Gunalan et al., 2012; Sowmya et al., 2012) have a chitinase producing ability. Chitinase activity in plant (Gomes et al., 1996), animals (Mana et al., 2009) and human serum has also been described recently (Mathivanan et al., 1998).

Optimization of culture media is very important to maximize the yield and productivity of enzyme, and minimize the product cost (Abdel-Fattah et al., 2005). The aim of this work was to isolate the locally prominent chitinolytic fungi from different soil samples with unique properties and optimize their fermentation conditions for maximum chitinase production.

MATERIALS AND METHODS

Shrimp and crab-shell powder

Shells of shrimp and crab were collected, washed several times in warm tap water, then distilled water and dried in an air dried oven at 60°C for 24 h. After drying, the shells were hammer milled to fine particles.

Chitin and colloidal chitin

Demineralization of the chitinous wastes was done. They were treated with 1.75 M acetic acid at room temperature for about 12 to 15 h. The ratio of waste to solvents was maintained (1:15 w/v). The dematerialized material obtained was recovered by filtration, rinsed with de-ionized water and dried in forced hot air oven at 65°C. The dematerialized material was then deproteinized with 21 ml of 5% NaOH solution for 2 h at 100°C, and rinsed with cold water. Chitin was collected on a coarse sintered glass funnel and washed with deionized water to pH 7.0. Dried material (chitin) was powdered and sieved to fine size. Colloidal chitin was prepared as follows: 5 g of the chitin powder were homogenized in 100 ml of 12 M HCl and stirred at 40°C for 2 h. The chitin was added by NaOH and collected by centrifugation. The pellet was washed twice with distilled water and lyophilized (Trachuk et al., 1996).

Sample collection and isolation of chitinolytic fungi

Sediment samples were collected from different areas of Al-Jouf in Saudi Arabia. The samples were taken from 2 to 3 cm depth with the help of sterile spatula and put in sterile bags for further processing. For isolation of chitinase producing fungi, the agar medium amended with colloidal chitin was used. The mineral synthetic medium consists of (g/L) $(NH_4)_2SO_4$, 1: K_2HPO_4, 1; $MgSO_4.H_2O$, 0.5; KCl, 0.5; NaCl, 5.0; $CaCl_2$, 0.02; $FeSO_4.7H_2O$, traces; shrimp-shell powder, 20, agar, 15; and distilled water 1 L. The pH of the medium was adjusted to 5 with KOH and HCl. The medium were sterilized by autoclaving at 121°C for 20 min. Different types of fungi were isolated from the soil samples by enrichment method. The colonies showing clearance zones on creamish background were considered as chitinase-producing fungi.

Screening of chitinase producing fungi

Fungal isolates were selected on the basis of larger hydrolysis zones after three days of incubation and further screened for maximum enzyme production in MSM. The cultures were centrifuged at 6000 rpm for 15 min at 4°C and the supernatant was used for chitinase assay.

Preparation of the crude enzyme

At the end of incubation period, the fungal mat was separated by filtration in sintered glass filter (G3). The culture filtrates of several batches were collected and pooled. The culture filtrate was centrifuged at 6000 rpm for 15 min to remove any muddy material. The clear supernatant was considered as the crude enzyme source.

Assay of chitinase activity

Chitinase activity was determined spectrophotometrically by estimating the amount of free reducing groups formed after colloidal chitin hydrolysis. The reaction mixture was composed of 0.5 ml of 1% colloidal chitin suspended in 0.02 M phosphate buffer (pH 7.0) and 0.5 ml of the enzyme solution. After 30 min incubation at 40°C, 0.75 ml of 3,5-dinitrosalicylic acid reagent (DNSA) (Joshi et al., 1989) was added to stop the reaction. The suspension was heated for 10 min at 100°C and centrifuged at 8000 rpm for 10 min. The supernatant absorbance at 530 nm was measured. A standard curve was obtained using N-acetylglucosamine as a standard. One unit of the chitinase activity was defined as the amount of enzyme which yields 1 µmol of reducing sugar as N-acetyl-D-glucosamine (Glc NAC) equivalent per minute.

Protein estimation

The extracellular protein of the culture was determined by Lowry method (Lowry et al., 1951) using bovine serum albumin as the standard.

Optimization of enzyme production

Effect of using different sources of chitin

To find out the best substrate for highest enzyme production, the chitinase production was carried out by using different polysaccharides of marine origin, shrimp-shell powder (2%), crab-shell powder (2%), chitin powder (1%), colloidal chitin (1%), chitosan(1%), demineralized chitin (1%), deproteinized chitin (1%), agar (1%), alginate (1%), mannan (1%), K –carrageenan (1%).

Effect of using single and mixed carbon sources

The effect of using additional carbon source supplemented in media at a concentration of (1%) such as glucose, sucrose, lactose, maltose and N-acetyl-D-glucosamine (Glc NAC) for maximum enzyme production was also investigated. The supplemented media were inoculated with 2% inoculums and fermented at an optimized chitin source.

Effect of different concentration of chitin

50 ml of sterile production medium prepared with different substrate (shrimp-shell powder) concentration varied from 5 to 40 g/l, inoculated with 2 ml spore suspension of the culture and incubated at 30°C under shaking condition (120 rpm). The culture filtrate was harvested after five days and enzyme assay as well as the extracellular protein was measured.

Effect of incubation time on chitinase production

To determine the optimum incubation time for the chitinase production, inoculated flasks were incubated in a rotary shaker in 120 rpm at 30°C for about sseven days. Every 12 h the culture filtrate was collected and checked for the chitinase activity, dry weight as well as total extracellular protein.

Effect of nitrogen source on chitinase production

To investigate the influence of using various nitrogen sources on the production of chitinase, 50 ml of production medium with different nitrogen sources such as NH_4NO_3, $NaNO_3$, urea, KNO_3 and NH_4Cl was prepared, sterilized, inoculated and incubated at 30°C for optimum incubation period under the shaking conditions. The culture filtrate was harvested and the enzyme activity, dry weight as well as extracellular protein were measured.

Utilization of fungal biomass as a source of chitin for chitinase production

Pencillium chrysogenium, *F. oxysporium*, *A. oryzae A. niger* were grown in Czapek-Dox broth. After 15 days of incubation, the fungal mats were collected and sterilized by autoclave at 121°C for 20 min. The sterilized fungal mats were washed twice with sterile distilled water and dried in an oven at 80°C till constant weight (Goel et al., 2004). The dried fungal mat was powdered and used as chitin source (1 g/l) for the production of chitinase. Three sets of flasks were used: 1) MSM in which shrimp-shell powder was replaced by fungal mat (2 g/l), 2) MSM supplemented with fungal mat (2 g/l), and 3) control only with SMS without any fungal mat.

Characterization of crude chitinase enzyme

Some properties of the crude chitinase were studied. These include the enzyme concentration (varied from 0.198 to 1.980), substrate concentration (range from 1.0 to 20.0 mg), pH value of the reaction mixture (range from 3.6 to 8.0), and the temperature of the reaction (varied from 30 to 60°C) and substrate specificity.

Effect of crude chitinase enzyme on fungal cell walls

Test microorganisms

Different fungal strains were obtained from Fungi Center, Faculty of Science, Assuit University, Egypt. The cultures were subcultured, maintained on Sabouraud's dextrose agar (SDA) medium and stored in refrigerator at 4°C.

Inoculum preparation

Fungal inoculums were prepared by inoculating a looful of test organisms in 5 ml of Sabouraud's dextrose agar medium and incubated at 30°C for three days.

Antifungal activity by clearance zone

The antifungal activity was assayed *in vitro* by inhibiting the growth of fungus on Sabouraud's dextrose agar (SDA) medium. A spore suspension of pathogenic fungi was uniformly spread on plates of SDA medium. Discs that were soaked with the crude enzyme (50 U) were laid on the inoculated plates; the control was disc-soaked in boiled-enzyme extract. Fungal growth and the clearance zones were observed over two to five days of incubation at 30°C (Haggag and Abdallh, 2012).

Antifungal activity by measuring the libration of sugars

Fungal mycelia were homogenized by an ultraturrax blender for 15 s washed three times with 300 ml deionized water on filter paper, and then suspended in 15 to 20 ml of deionized water. These mycelia were used for estimation of sugar libration from whole cells (Beyer and Diekmann, 1985).

Statistical analysis

All results are made in triplicates, and the values are the mean values. An analysis of variance (ANOVA) for mean comparison was used.

RESULTS AND DISCUSSION

A total of 10 morphologically different chitinolytic fungi were isolated from different soil samples from the market of fishery in Al-Jouf, Saudi Arabia. On the basis of colloidal chitin degradation and zone of clearance, five isolates were selected for secondary screening in mineral synthetic broth medium and tested for chitinase activity. All isolates are identified by Faculty of Science, Assiut University, Egypt. Based on maximum chitinase production (4.82 U/mg protein) after 5 days of incubation, the *A. terrus* was selected for further and more detailed

Table 1. Chitinolytic activity of the tested isolates grown on shrimp-shell Powder as a sole carbon source.

Isolated strain	Final pH	Extracellular protein (mg/ml)	Chitinase activity (U/mg protein)
Aspergillus terrus	5.6	1.13	4.82±0.011
Aspergillus oryzae	5.8	1.10	4.27±0.011
Aspergillus niger	5.9	1.03	3.87±0.023
Pencillium chrysogenium	6.2	0.96	2.76±0.030
Fusarium sp.	6.0	0.90	2.05±0.050

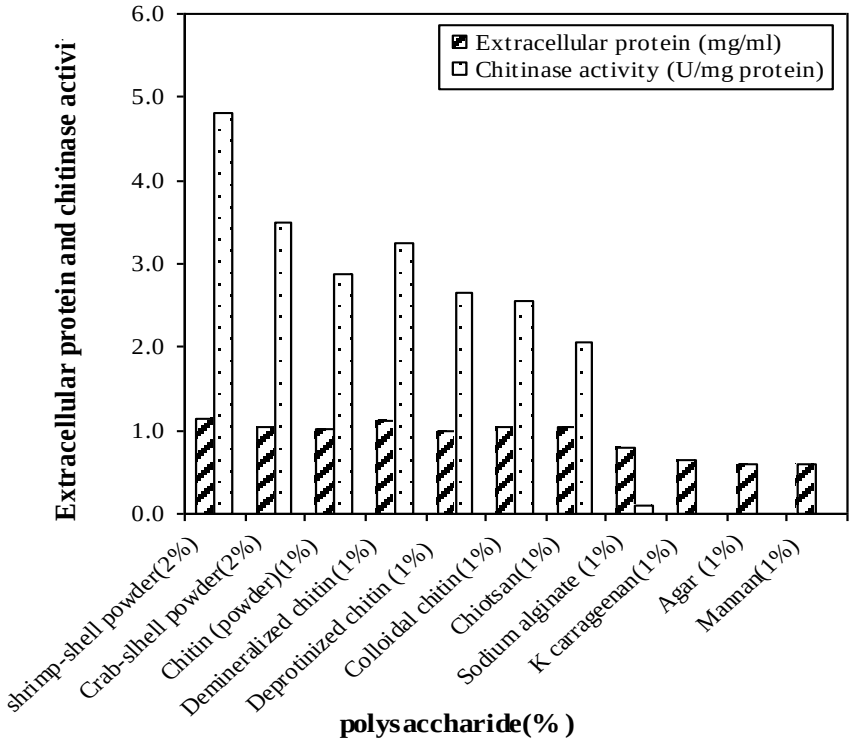

Figure 1. Extracellular cellular and chitinase activity of *Aspergillus terrus* as grown on some polysaccharides of marine origin.

(Table 1). Among the various substrates like shrimp-shell powder, crab-shell powder, chitin powder, colloidal chitin, chitosan, demineralized chitin, deproteinized chitin, sodium alginate, k carrageenan, agar, and mannan, the shrimp-shell powder (2%) was found to be the best substrate for maximum chitinase production (4.82 U/mg protein) (Figure 1). Similar observation has also been reported with *Bacillus amyloliquefaciens* (Sabry, 1992), *Beauveria brassiana* (Suresh and Chandrasekaron, 1999) and *Verticillum lecanii* (Matsumoto et al., 2004). On the other hand, Kuddus and Ahmed (2013) used colloidal chitin for the production of chitinase by *Aphanocladium.*

To optimize the shrimp-shell powder concentration, the effect of various levels was investigated and the results are shown in Figure 2. The activity obtained in the culture filtrate increased by 4.9-fold by increasing shrimp-shell powder concentration from 5 to 20 g/l. Higher shrimp-shell powder concentrations increased the viscosity of the medium and thus reduced the oxygen supply which yield lower growth and enzyme production. These results are in good agreement with many reported for the production of chitinase by other microbial strains (Trachuck et al., 1996; Wang and Chang, 1997).

To determine the effect of using single and mixed carbon sources on chitinase production, experiments were carried out with *A. terrus* in which chitin medium was supplemented with additional carbon source in a concentration of 1% (w /v). Data on the effect of several carbon sources on chitinase production by *A. terrus* are represented in the Table 2. Enzyme production was supported in the presence of all sugars in the medium alone or with the chitin as compared to control. Joo

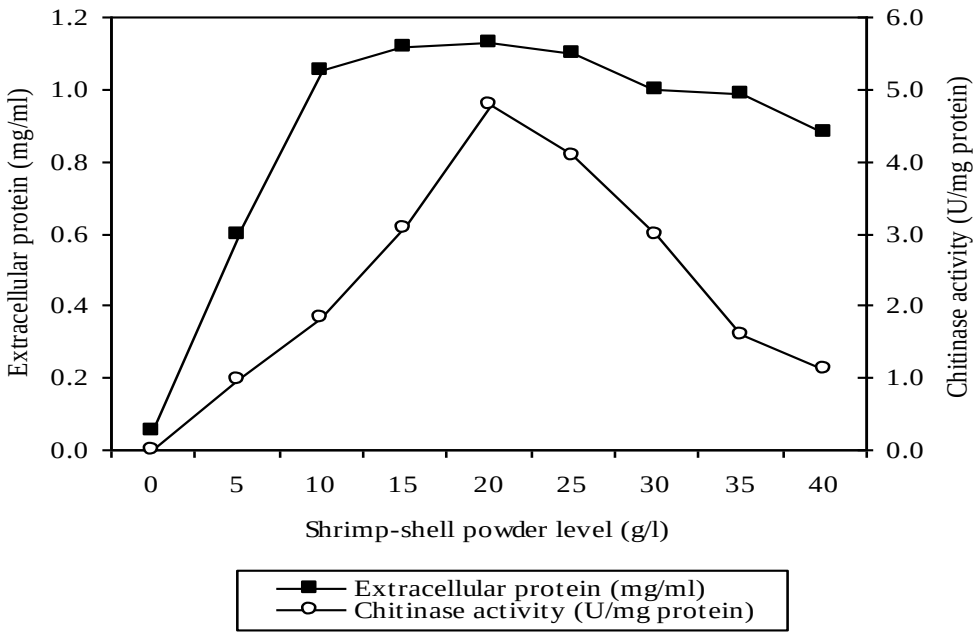

Figure 2. Chitinase activity and extracellular protein of *Aspergillus terrus* culture grown in different concentration of shrimp- shell powder.

Table 2. Effect of using single and mixed carbon sources on chitinase production *by A. terrus* cultures.

Carbon source	Final pH	Extracellular protein (mg/ml)	Chitinase activity (U/mg protein)
Glucose (1%)	6.2	2.25	0.86± 0.11
Shrimp-shell powder + glucose	6	2.55	2.98± 0.06
Sucrose	6.5	2.16	0.75± 0.04
Shrimp-shell powder + sucrose	6.2	2.52	2.55± 0.04
Lactose	6.4	1.85	0.5± 0.01
Shrimp-shell powder + lactose	5.9	2.11	1.98± 0.03
Maltose	5.8	1.65	0.45± 0.02
Shrimp-shell powder + maltose	5.8	1.89	1.55± 0.04
N-acetylglucosamine	5.8	0.99	0.35± 0.04
Shrimp-shell powder + Glc-NAGA	5.8	1.05	0.98±0.02

(2005) reported that glucose (0.4%) along with chitin induced high levels of chitinase by *Streptomyces halsteolii*. Narayana et al. (2007) found that amended of starch 0.2% to the colloidal chitin medium increased chitinase production by *Streptomyces* sp. ANU6277.

The effect of various nitrogen sources on chitinase production was studied. The nitrogen source constituent was replaced on equal nitrogen basis, by NH_4NO_3, $NaNO_3$, urea, KNO_3 and NH_4Cl. As shown in Figure 3. The production of chitinase was influenced by the nitrogen source then incorporated into the medium. The highest activity (6.28 U/mg protein) was recorded at ammonium sulphate at a concentration of 1%. Ammonium sulphate is a cheap nitrogen source and represented an advantage for the production of chitinase by low cost fermentation. These results are in good agreement with those reported for the production of chitinase by *Aspergillu*s sp. SI.13 (Rattanakit et al., 2002).

Role of fungal biomass on chitinase production

The cell wall of most fungi contains chitin as the major component, chitinase enzymes are well known to lyse the cell wall of both live and dead fungi (Ueno et al., 1990). Utilization of dead mass of *F. oxysporium*, *A.* oryzae, *A. niger* and *Pencillium citrinum* by *A. terrus* for chitinase

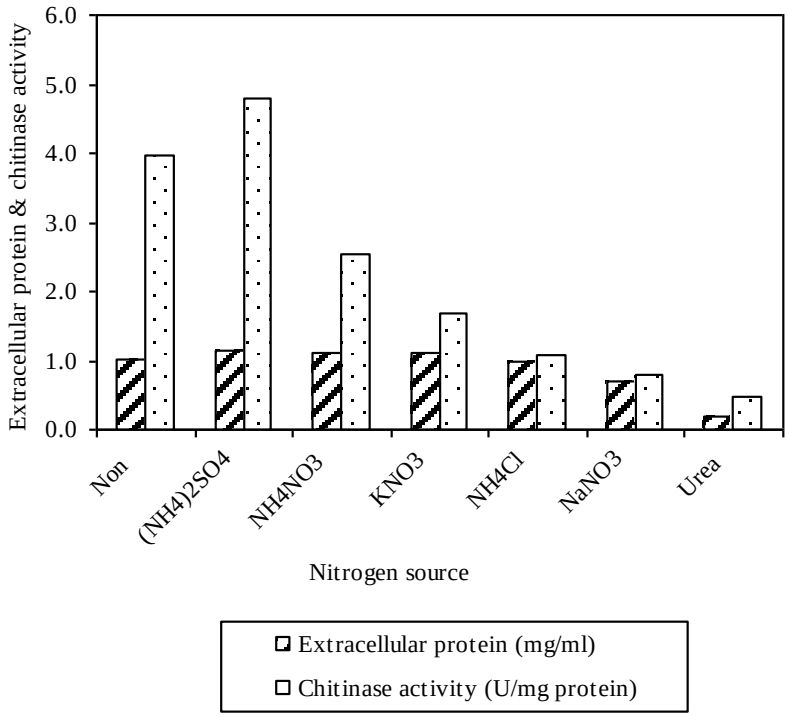

Figure 3. Chitinase activity and extracellular protein of *Aspergillus terrus* cultures grown in different nitrogen source.

production was studied (Figure 4). The production of chitinase enhanced in MSM amended with dried dead fungal mats over MSM. On the other hand, the enzyme production decreased when the fungal mats replaced shrimp-shell powder from MSM. An increase in chitinase production was also observed from *Streptomyces aureofaciens* and *Streptomyces halstedii* when cultured in a medium containing colloidal chitin supplemented with fungal cell wall preparations (Joo, 2005; Kumar and Gupta, 2006). Temperature affects various biological processes, therefore the growth of fungi and enzyme production are also affected with the change in incubation temperature. In the present investigation, the increased of incubation temperature from 25 to 35°C increased the growth and the production of the enzyme where maximum enzyme activity (14.88 U/mg protein) obtained at an optimum temperature of 30°C (Figure 5). These results are in good agreement with those reported by other workers (Sandhya et al., 2004, Sowmya et al., 2012). On the other hand, the highest production of chitinase by other fungi was carried out at optimum temperature ranging from 26 to 28°C (Sherief et al., 1991; Brzezinska and Jankiewicz, 2012).

The results showed that an initial pH range from 4 to 9 seems appropriate for both growth and chitinase production. However, pH 5 for *A. terrus* was the optimum value for the highest enzyme activity (Figure 6). Higher or lower pH values showed an adverse effect on the enzyme production. These results are in line with other chitinase production by *Aspergillus carneus* (Sherief et al., 1991). In contrast, the high level of *A. terrus* chitinase is observed in culture medium with pH 6.5 (Ghanem et al., 2010).

Some properties of crude chitinase enzyme

The effect of enzyme concentration on the activity of chitinase is shown in Figure 7, the maximum activity was obtained using 1.188 mg of the crude chitinase extract. The effect of colloidal chitin concentration on the chitinase enzyme (Figure 8) shows that maximum chitinase activity was obtained at 7.5 and 10.0 after which the activity falls slightly. A decrease in the chitinas activity 12.5 mg colloidal chitin may indicate accumulation of intermediates that results from chitin decomposition into the medium which make up a synthetic inhibitor of chitinase itself. The chitinase activity was determined using crude enzyme concentration (1.188 mg) and colloidal chitin as a substrate (7.5 mg) at different temperature ranging from 30 to 60°C is shown in Figure 9. The optimum temperature for chitinase activity was found at 40°C, above that the activity was decreased. A drop in the activity may due to heat inactivation of the enzyme. Similar temperature values were determined for chitinase from different microorganisms (Sherief et al.,

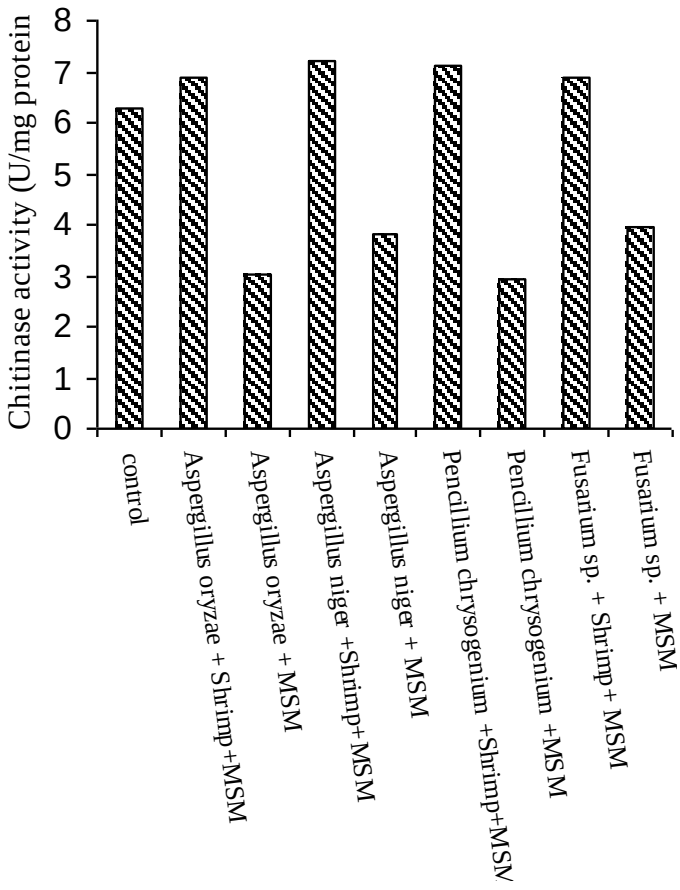

Figure 4. Utilization of fungal mate for chitinase production by *Aspergillus terrus* (values are means of three replicates + SD).

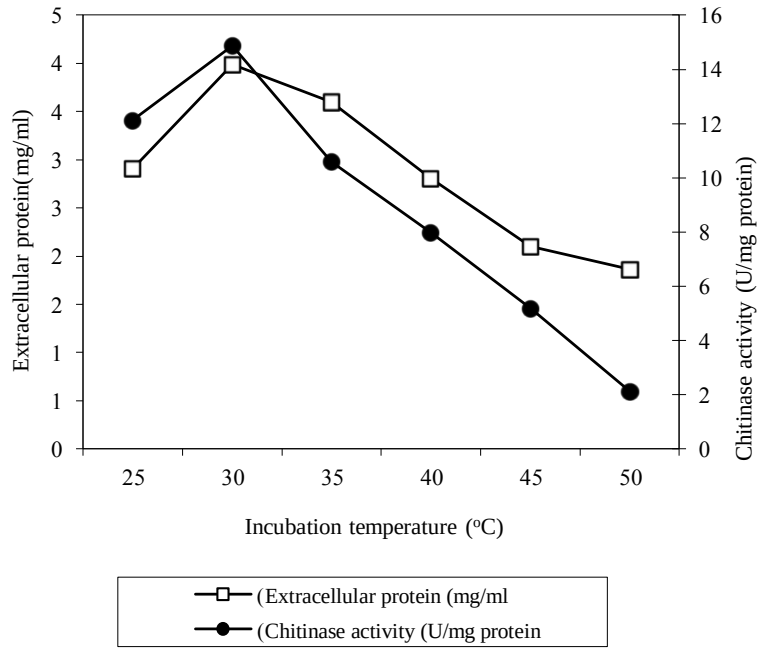

Figure 5. Chitinase activity and axtracellular protein of *Aspergillus terrus* cultures at different incubation temperature.

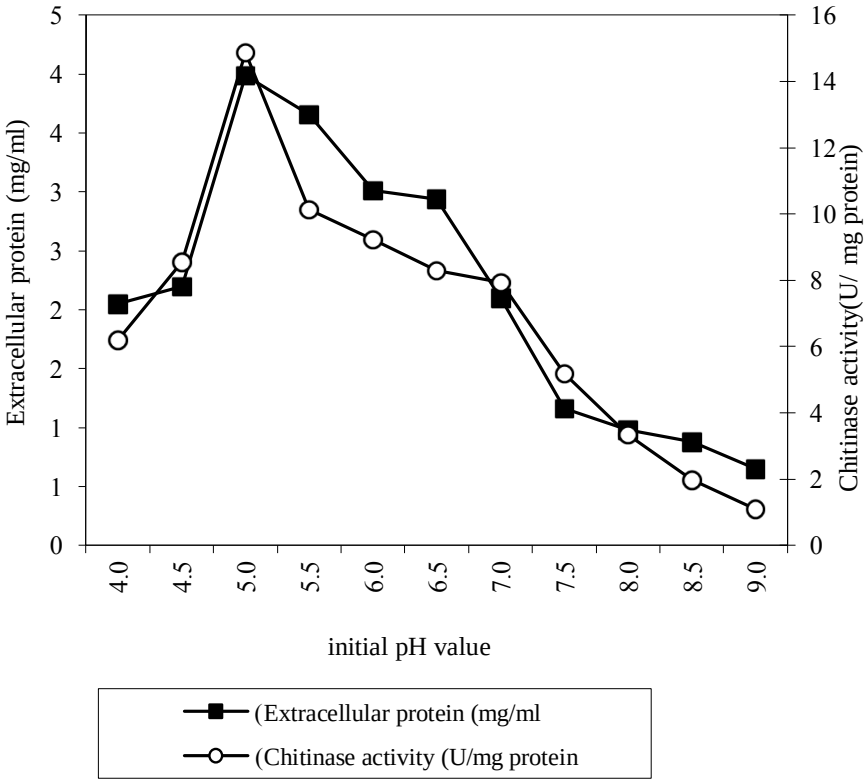

Figure 6. Chitinase activity and extracellular protein of *Aspergillus terrus* cultures at different incubation temperature**.**

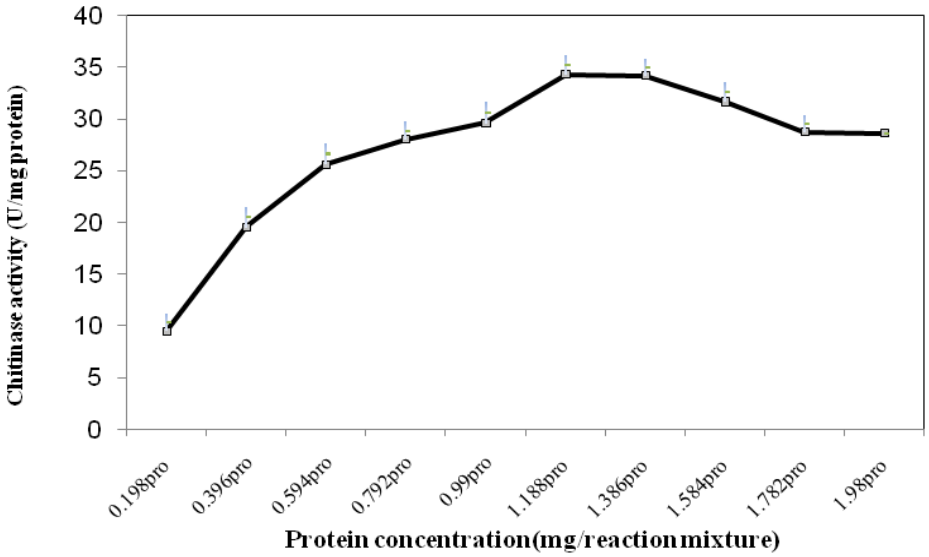

Figure 7. Effect of enzyme protein concentration on the activity. Each data point represents means ± standard deviation (n=3).

1991; Kinz et al., 1992, Brzezinka and Jankiewcz, 2012). The effect of pH of the reaction on the chitnase activity is shown in Figure 10. It shows that the maximum activity was detected at pH 5.2. These results are in good agreement with other microbial chitnases (Dahyia et al., 2005; Sandhya et al., 2004). On the other hand, the optimal pH for the crude chitinase produced by *A. niger* LOCK62 was found to be 6.5 and for *A. carneus* was 4.5

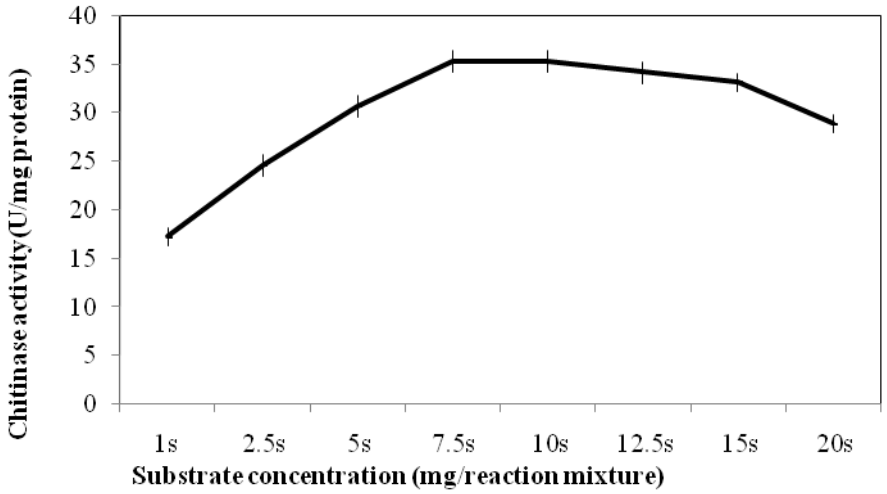

Figure 8. Effect of substrate concentration on the crude chitinase activity. Each data point represents means ± standard deviation (n=3).

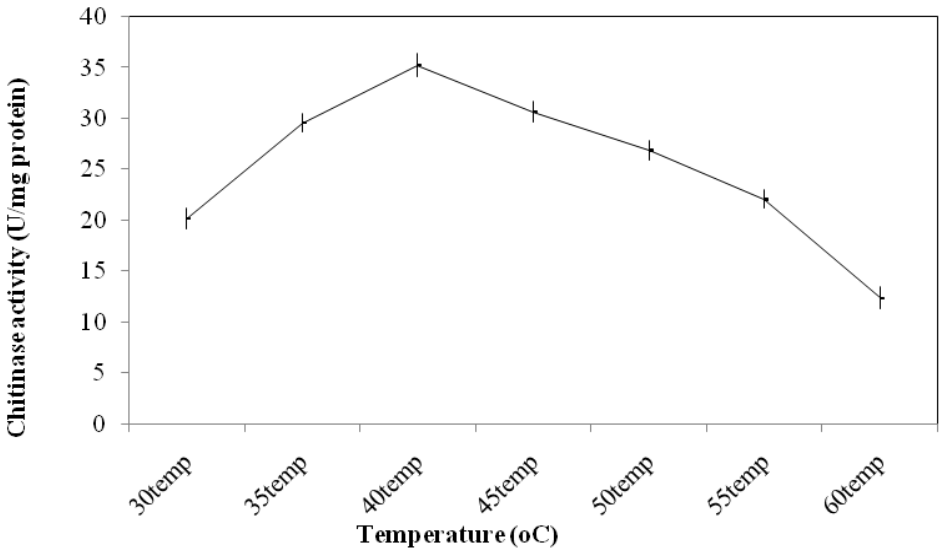

Figure 9. Effect of temperature of the reaction on the crude chitinase activity. Each data point represents means ± standard deviation (n=3).

(Brzezinka and Jankiewcz, 2012).

The ability to degrade several polysaccharides is an important criterion of chitinase potency. Table 3 compares the digestive capability of this chitinase on nine substrates. It is evident that the chitinase had better digestive ability on colloid chitin than other polysaccharides under the same assay condition. The relative activity of chitinase only reached 52.6% for chitin powder and 8.3% for chitosan substrate that when colloid chitin was used as the control. On the other hand, no any chitinase activity with n-carboxymethyl cellulose, cellulose, starch, sodium alginate, agar, and K-carragennan. From these results, *A. terrus* chitinase has high specificity which could only degrade glycosidic bond GlcNAc-GlcNAc. Similar results are reported from chitinase of thermophilic *Bacillus* sp. HU1 (Dai et al., 2011).

Antifungal activity of crude chitinase enzyme

In the present study, the antifungal activity of chitinase was tested against the seven different plant pathogenic fungi, *A. niger*, *A. oryzae, P. oxysporium*, *R. solan*i, *F.*

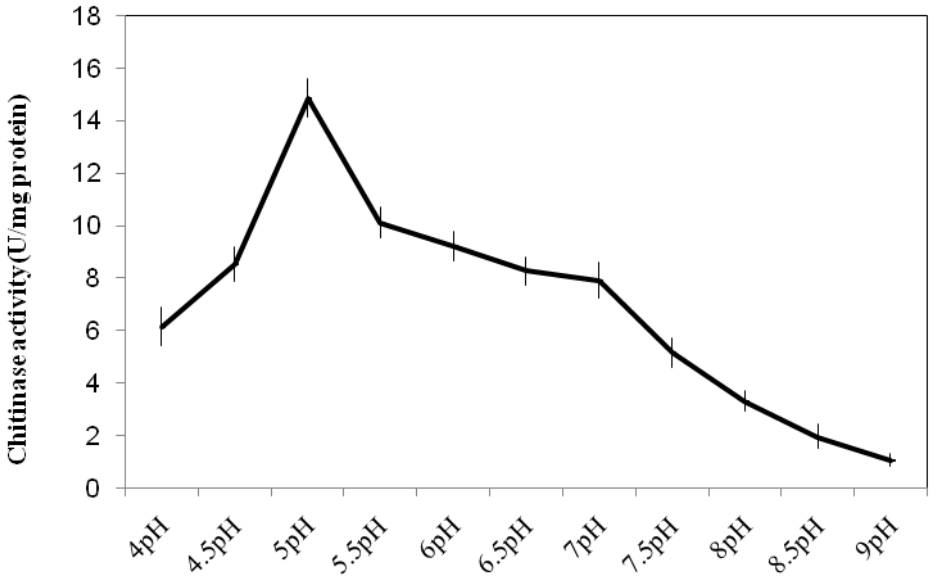

Figure 10. Effect of pH of the reaction on the crude chitinase produced by *Aspergillus terreus.* Each data point represents means ± standard deviation (n=3).

Table 3. Chitinase activity on various polysaccharide substrates.

Substrate	Relative activity (%)
Colloidal chitin	99.97±0.011
Powder chitin	52.8±0.015
Chitosan	8.3±0.050
Carboxymethyl cellulose	0
Cellulose	0
Starch soluble	0
Sodium alginate	0
Agar	0
K-carrengeen	0

Table 4. Antifungal activity of crude chitinase of *Aspergillus terrus.*

Fungi	Diameter of the zone of inhibition (mm)
Aspergillus niger	20±0.090
Aspergillus oryzae	15±0.050
Pencillum oxysporium	12±0.032
*Rhizoctonia solan*i	7±0.050
Fusarium oxysporium	5±0.035
Rhizopus sp.	0
Mucor sp.	0

oxysporium, Rhizopus sp and *Mucor* sp. Among them, the chitinase showed maximum inhibitory activity against *A. niger*. (20 mm in diameter), followed by *Aspergillus versicolor* (15 mm in diameter*) P. oxysporium* (12 mm in diameter) and *R. solan*i (7 mm in diameter). The fungus *F. oxysporium*. (5 mm in diameter) recorded the least inhibitory activity (Table 4). The growth of *Rhizopus* sp. and *Mucor* sp. was not inhibited. These results are in partial agreement with Brzezinska and Jankiewicz (2012) who found that the chitinase produced from *A. niger* LOCK 62 has an antifungal activity against many phytopathogens fungi. Many authors studied the antifungal activity of chitinase enzyme from different microorganisms (Howell, 2003; Kavitha et al., 2005; Kim and Chung, 2004; Patel et al., 2004; Zhao et al., 2010).

Another method for determination of the effectiveness of an enzyme preparation in degrading fungal cell walls was done by the estimation of the rate of sugar libration (Thomas et al., 1979). The libration of glucose and N-acetylglusoamine from the cell walls of 6 different fungi by the crude chitinase was determined as shown in Figure 11. The degradability of the cell wall polysaccharides can be compared by taking the rate of sugar libration; higher sugar libration activities were observed with ascomycetes and deuteromycetes , while no sugar libration with zygomycetes. Our results are in line with Farag (2004) who found that the *Bacillus brevis* chitinase could degrade the cell walls of filamentous ascomycetes and deteuromycetes, but was inactive with zygomycetes. Also, it was in a good agreement with the results observed by Beyer and Diekmann (1985).

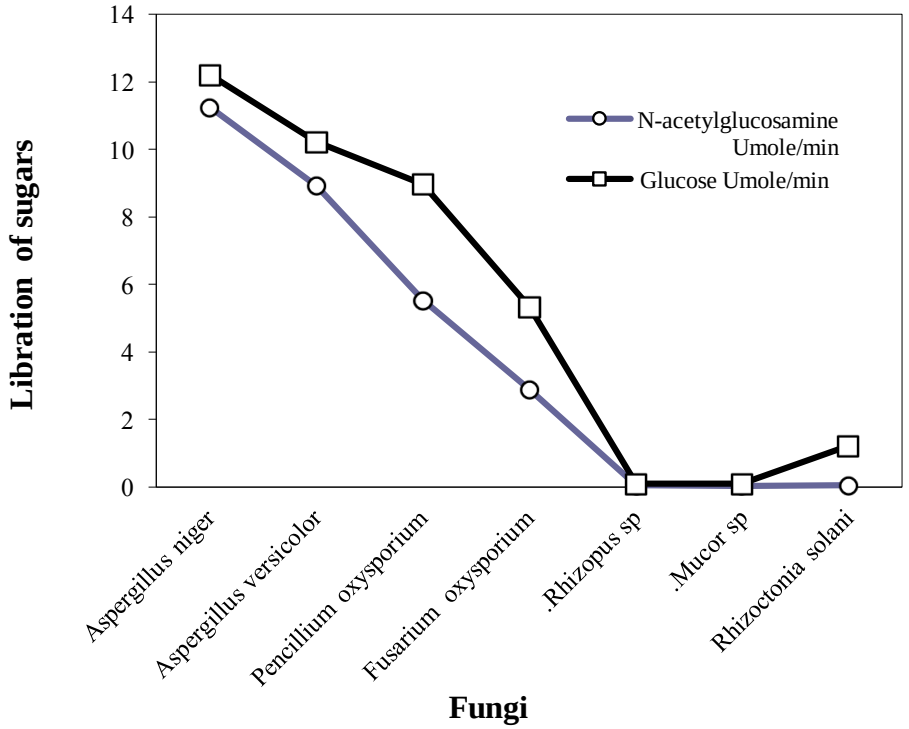

Figure 11. Sugar liberation from the hydrolysis of fungal cell walls.

Conclusion

From the present investigation, it is confirmed that strains isolated from the soil as *A. terrus* had chitinase activity and thus the enzyme extracted from these strains can be used as a catalyst for the degradation of chitin which is abundant in polysaccharides after cellulose. The result concluded that *A. terrus* chitinase has the ability to degrade the cell wall of many fungi, so it can be used as antifungal for phytopathogenic fungi.

Conflict of Interests

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

REFERENCES

Abdel-Fattah YR, Saeed HM, Gohar YM, El-Baz MA (2005). Process Biochem. 40:1707-1714.

Bayer M, Diekmann H (1985). The chitinases ystem of Streptomyces sp. ATTC 11238 and its significance for fungal cell wall degradation. Appl. Microbiol. Biotechnol. 23:140-146.

Brzezinska MS, Jankiewicz U (2012). Production of antifungal chitinase by Aspergillus niger LOCK62 and its potential role in the biological control. Curr. Micriol. 65:666-672.

Budi SW, van Tuien D, Arnould C, Dumas-Gaudut E, Gianinazzi-Pearson V, Gianinazzi S (2000). Hydrolytic enzyme activity of Paenibacillus sp. Strain B2 and effect of antagonistic bacterium on the cell wall integrity of two soil-borne pathogenic fungi. Appl. Soil Ecol. 15:191-199.

Dahiya N, Rupinder T, Gurinder Singh H (2006). Biotechnological aspects of chitinolytic enzymes a review. Appl. Microbiol. Biotechnol. 71:773-782.

Dahiya N, Tewari R, Tewari RM, Hoondal GS (2005). Chitinase production in solid state fermentation by Enterobacter sp. NRG4 using statistical experimental design. Curr. Mictobiol. 4:1-12.

Dai D, HW, Huang G, Li W (2011). Purification and characterization of a novel extracellular chitinase from thermophilic Bacillus sp. Hu1. Afr. J. Biotechnol. 10(13): 2476-2485.

Daizo K (2005). Application of chitinase in Agriculture. J. Metals Mater. Min. 15(1):33-36.

De Siqueira Pinto A, Barreto CC, Schrank A, Ulhoa CJ, Henning Vainstein M (1997). Purification and characterization of extracellular chitinase from the entomopathogen Metarhizium anisopliae. Can. J. Microbiol. 43:322-327.

Farag AM (2004). Chitinase production by free and entrapped halophilic Bacillus brevis cells grown on crab-shell powder. El-Minia Sci. Bull. 15(2): 365-388.

Ghanem KM, Al-Garni SM, Al-Makishah NH (2010). Statistical Optimization of cultural conditions for chitinase production from fish scales wastes by Aspergillus terrus. African J. Biotechnol. 9(32):5135-5146.

Goel V, Chaudhary T, Vyas P, Chhatpar H S (2004). Isolation and identification of chitinolytic bacteria and vtheir potential in antifungal biocontrol. Indian J. Exp. Biol. 42:7815-720.

Gohel V, Singh A, Vimal M, Ashwini P, Chatpar H S (2006). Bioprospecting and antifungal potential of chitinolytic microorganisms. Afr. Biotechnol. 5:54-72.

Gomes VM, Oliveira AEA, Xavier FJ (1996). A chitinase and B-1,3-glucanase isolated from the seeds of cowpea (Vigna unguiculata Walp) inhibit growth of fungi and insect estsof the seed. J. Sci. Food Agric. 72:86-90.

Gunalan G, Sadhana D, Rupa Ramya P (2012). Production, optimization of chitinase using Aspergillus flavus and its biocontrol against phytopathogenic fungi. J. Pharma. Res. 5(6):3151.

Haggag WM, Abdallh EG (2012). Purification and characterization of

chitinase produced by Endophytic Streptomyces hygroscopicus against some phytopathogens. J. Microbiol. Res. 2(5):154-151.
Howell CR (2003). Mechanisms employed by Trichoderma species in the biological control of plant diseases: the history and evolution of current concepts. Plant Dis. 87:4-10.
Joo JG (2005). Purification and characterization of an extracellular chitinase from the antifungal biocontrol from Streptomyces halstedii. Biotechnology 27:1483-1486
Joshi S, Kozlovski M, Richens S, Comberbech DM (1989). Chitinase and chitobiase production during fermentation of genetically imporved Serratia liquifaciens. Enzyme Microbiol. Technolo. 11:289-296.
Kavitha S, Senthilkumar S, Gnanamanickam S, Inayathullah M, Jayakumar R (2005) . Isolation and paritial characterization of antifungal protein from Bacillus polymyxa strain VLB16. Process Biochem. 40:3236-3243.
Kim PL, Chung KC (2004). Production of an antifungal protein for control of Colletotrichum lagenarium by Bacillus amyloliquefaciens MET0908. FEMS Microbiol. Lett. 234:177-183.
Kinz C, Sellam O, Bertheau Y (1992). Purification and characterization of chitinase from the hyperparasitic fungus Aphanocladium album. Physiol. Mol. Plant Pathol. 40:117131.
Kuddus SM, Ahmad RIZ (2013). Isolation of novel chitinolytic bacteria and production optimization of extracellular chitinase. J. Gene. Eng. Biotechnol. 11(1):39-46.
Kuma D, Gupta RA (2006). Biocontrol of wood rotting fungi. Indian J. Biotechnolol. 5: 20-25
LingappaY, Lockwood JL (1961). Chitin media for selective isolation and culture of actinomycetes. Phytopathology 52:317-323.
Liu BL, Kao PM, Tzeng YM, Feng KC (2003). Production of chitinase from Verticillium lecanii F091 using submerged fermentation. Enzyme Microbiol. Technol. 33: 410-415.
Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951). Protein measurement with the Folin Phenol reagent. J. Biol. Chem. 193:265-275.
Mahadevan B, Martinko J (2005). Brock Biology of Microorganisms. Pearson
Mana I, Kouji M, Atsushi M, Masahiro M (2009). Purification and characterization of chitinase from the stomach of sliver croaker Pennahia argentatus. Protein Exerssion Purif. 65:214-222.
Mathivana N, Kabian V, Murugesan K (1998). Purifican, characterization and antifungal activity of chitinase from Fusarium chlamydosporum, a mycoparasite to ground rust, Puccinia rachnids. Can. J. Microbiol. 44(7): 646-651. .
Matsumoto Y, Saucedo-Castaneda G, Revah S, Shirai K (2004). Production of b-N-acetylhexosaminidase of Verticillium lecanii by solid state and submerged fermentations utilitizing shrimp waste silage as substrate and inducer. Process Biochem. 39:665-671.
Merina PD, Rebecca LJ, Sharmila SA, Banerjee A, Kumar D (2012).Identification and optimization of cultural conditions for chitinase production by Bacillus amyloliquefaciens SM3. J. Chem. Pharma. Res. 4(11):4816-4821.
Nampoothiri KM, Baiju TV, Sandhya C, Sabu A, Szakacs G, Pandey A (2004). Process optimization for antifungal chitinase production by Trichoderma harzianum. Process Biochem. 39:1583-1590.
Narayana KJP, Prabhakar P, Vijayalakshmi M, Vekateswarlu Y, Krishna PSJ (2007). Biological activity of phenylpropionic acid from a terrestrial Streptomycetes. Folish J. Microbiol. 56:191-197.
Patel VJ, Tendulkar SR, Chattoo BB (2004). Bioprocess development for the production of an antifungal molecule by Bacillus licheniformis BC98. J. Biosci. Bioeng. 98:231-235.
Rattanakit NA, Plikomol S, Yano M, Wakayama T (2002). Utilization of shrimp shellfish waste as a substrate for solid-state cultivation of Aspergillus sp. S1–13: evaluation of a culture based on chitinase formation which is necessary for chitin-assimilation. J. Biosci. Bioeng. 93: 550–556.
Sabry SA (1992). Microbial degradation of Shrimp-shell waste. J. Basci Microbiol. 32(1):100-110.
Sandhya C, Adapa LK, Nampoothiri KM, Binod P, Szakacs G, Pandy A (2004). Extracellular chitinase production by Trichoderma harzianum in submerged fermentation. J. Basic Microbiol. 44(1):49-58.
Sherief AA, El-Sawah MMA, Abd-El-Naby MA (1991). Some properties of chitinase produced by potent Aspergillus carneus strain. Appl. Microbiol. Biotechnol. 35:226-230.
Sowmya B, Gomathi D, Kalaiselvi M, Ravikumar G, Arulraj C,Uma C (2012) . Production and purification of chitinase by streptomyces sp. From soil. J. Adv. Sci. Res. 3(3): 25-29.
Suresh PV, Chandrasekaran M (1999). Impact of process parameters on chitinase production by an alkalophilic marine Beauveria bassiana in solid state fermentation. Process Biochem. 34:257-267.
Trachuk LA, Revina LP, Shemyakina TM, Chestukhina GG, Stepanov VM (1996). Chitinases of Bacillus licheniformis B-6839: isolation and properties. Can. J. Microbiol. 42:307-315.
Thomas KR, Davis B, Mills J (1979). The effect of B-glucuronidase and chitinase on the cell wall of Aspergillus niger and Aspergillus fumigates. Microbios 25:111-123.
Ueno H, Miyashita K, Swada Y, Oba Y (1990). Purification and some properties of extracellular chitinase from Streptomyces sp. S-84. J. Gen. Appl. Microbiol. 36:377-392.
Wang SL, Chang WT (1997). Purification and characterization of two Bifunctional chitinases lysozyme extracellularly produced by Pseudomonas aeruginosa K-187 in a shrimp-and crab shell powder medium. Appl. Environ. Microbiol. 63:380-386.
Wang SL, Lin TY, Yen YH, Liao HF, Chen YJ (2006). Bioconversion of shellfish chitin wastes for the production of Bacillus W-118 chitinase. Carbohydrate Res. 341:2507-2515.
Zhao Z, Wang Q, Wang K, Brian K, Liu C, Gu Y (2010). Study of the antifungal activity of Bacillus vallismortis ZZ185 in vitro and identification of its antifungal components. Bioresour. Technol. 101:292-297.

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Full Length Research Paper

Adsorption of heavy metals by agroforestry waste derived activated carbons applied to aqueous solutions

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Activated carbons prepared from macadamia nut shells, baobab shells, pigeon pea husks, rice husks, *Moringa oleifera* husks, and marula stones were investigated for their abilities to adsorb Pb(II), Zn(II), Cu(II), Ni(II), Fe(II), Mn(II), Hg(II), Cr(III), As(III) and Cd(II) from aqueous solutions. Batch adsorption experiments were conducted at pH values of 4, 5 and 6. Metal ion adsorption generally increased as the pH was increased from 4 to 6. Percentage adsorption values were above 60% for adsorption of Hg(II) by all the activated carbons at pH 6. The adsorption of Pb(II) by carbons from Baobab shells, pigeon pea husks, *Moringa oleifera* husks and Marula stones was at least 22% higher than that of the commercial carbons used for comparison. Carbons derived from pigeon pea husks and baobab shells showed better metal ion adsorption compared to the other carbons and were used to determine the effects of initial metal concentration, contact time and adsorbent quantity on metal adsorption. The metal ion adsorption data fitted the Langmuir adsorption model.

Key words: Agroforestry, wastes, activated, carbon, adsorption, metals.

INTRODUCTION

The removal of heavy metals from industrial effluents is a major challenge of wastewater treatment. Heavy metals occur as contaminants of liquid waste discharged from various industries such as electroplating, tanneries, textiles, radiator manufacturing, chloralkali, oil refineries, mining and smelting (Kula et al., 2008; Garcia-Reyes et al., 2010; Kantali and Yanik, 2010). The most common toxic metals found in industrial wastewater are chromium, nickel, manganese, mercury, cadmium, lead, copper and zinc (Kazemipour et al., 2008). Potential exists for the

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accumulation and exposure of the metals to humans and other biological systems via water or food (Farooq et al., 2010; Ye and Du, 2010; Zakhama et al., 2011). When heavy metals exceed tolerance levels, they have a harmful effect on human physiology causing various diseases and disorders among which are nervous and renal breakdown, brain damage and convulsions (Kula et al., 2008; Kazemipour et al., 2008; Farooq et al., 2010).

Conventional techniques used for the removal of metals from wastewater include precipitation, membrane filtration, flocculation, ion exchange, reverse osmosis and adsorption (Chang et al., 2002; Kantarli et al., 2010). The methods tend to have relatively high capital and operating costs and are therefore not suitable for developing countries and small-scale industries in developed countries.

Worldwide, research has been focused on the indigenous production of water treatment chemicals using locally available raw materials (Farooq et al., 2010; Mussatto et al., 2010; Rivera-Utrilla et al., 2011). Among many water treatment technologies, utilizing plant residues as adsorbents for the removal of dyes and metal ions from wastewater is a prominent technology (Kadirvelu et al., 2001).

Activated carbon, due to its high surface area, micro porous character and surface chemistry, has been proven to be an effective adsorbent for the removal of heavy metals from industrial wastewater (Avelar et al., 2010; Mussato et al., 2010; Utrilla et al., 2011). Despite its frequent use in the water and waste industries, activated carbon remains an expensive material (Farooq et al., 2010; Mussatto et al., 2010). In view of the high cost of activated carbon, a lot of research has been directed to the development of low-cost activated carbon from cheap and readily available materials. Among the low-cost raw materials are the agroforestry and agro-industrial residues. A wide variety of carbons have been prepared from agricultural wastes such as olive seeds, rice husks, walnut shells, palm shells, hazelnut shells, almond shells, pistachio nut shells and apricot stone (Kula et al., 2008; Kazemipour et al., 2008; Ye and Du, 2010; Zabihi et al., 2010; Owlad et al., 2010; Ozcimen and Ersoy-Mericboyu, 2009). Each of the carbons has characteristic properties and variation exists in the efficiency of removal of a range of heavy metal ions from solution. There is a constant need to investigate the potential of various raw materials as activated carbon precursors, depending on their costs and availability.

We report in this study, the efficiencies of agroforestry waste-derived activated carbons in the removal of heavy metal ions from aqueous solutions. Common heavy metals found in industrial wastewater, Pb(II), Zn(II), Cu(II), Ni(II), Fe(II), Mn(II), Hg(II), Cr(III), As(II) and Cd(II) were used in the study.

MATERIALS AND METHODS

Activated carbon

Activated carbons prepared from macadamia nut shells (MNS), *Adansonia digitata* (Baobab) fruit shells (BBB), pigeon pea husks (PPH), rice husks (RH), *Moringa oleifera* husks (MH) and *Sclerocarya birrea* (Marula) fruit stones (AML) were used in this study. The carbons were prepared at the Centre for Water Sanitation and Hygiene, Malawi Polytechnic, University of Malawi. All the biomass samples were activated and carbonized in a one-step pyrolysis method in the presence of steam at 750^{o}C and final soak time of 30 min (Warhurst et al., 1997). Pyrolysis was carried out in a cube furnace (Podmore and Sons Ltd, Type J2HT, S/N 5655) at a temperature of 750°C, a soak time of 30 min and a water flow rate of 30 ml/min. A stainless steel box, 240 mm high, 210 mm wide and 400 mm deep, with three trays in which the raw materials were placed, was inserted into the furnace and water was injected at a rate of 30 ml/min using a pump (Watson Marlow Ltd. Type 501 S170, S/N 70256). After carbonization, the carbons were ground using a pestle and mortar and sieved to a size of <250 µm, dried in an oven at 110°C then stored in a desiccator until use. All the agroforestry derived carbons were compared against the commercially produced carbon typically used at municipal water treatment works in Harare, Zimbabwe, and with Eurocarbon PHO 14 x 45 from Eurocarbon Products Ltd., England.

Effect of pH on metal ion adsorption

Batch adsorption experiments were conducted in triplicate to investigate the effect of pH on the adsorption of metal ions by activated carbons. Solutions containing 10 mg/l of each heavy metal ion (Pb(II), Zn(II), Cu(II), Ni(II), Fe(II), Mn(II), Hg(II), Cr(III), As(III) and Cd(II) were prepared from the dilution of 1000 mg/L metal ion standard solutions (Fisher Scientific, Loughborough, UK). Metal ion solutions were prepared in acetate buffer (0.1 M) at pH values of 4, 5 and 6. The initial pH values of the solutions were measured using a Mettler Toledo 340 pH meter (Leicester, UK). Powdered activated carbon (100 mg) (<200 µm) from the various agroforestry wastes was added to solutions of the metal ions (20 ml) at different initial pH values. Mixtures were agitated on a flask shaker (Barlowworld Ltd., Staffordshire, UK) at 500 strokes/min at room temperature (25 $\pm$ 2°C) for 120 min. The mixtures were then centrifuged at 3000 rpm for 10 min (ALC Centrifuge, model PK130, Cologne, Germany) and the supernatants were filtered through 0.45 µm syringe filters (Millipore Corporation, USA). The concentration of residual metal ions in the supernatants were determined by ICP-OES using a Perkin Elmer Optima 5300 DV instrument (Perkin Elmer, UK) at an RF power of 1300 W and with plasma, auxiliary and nebuliser argon gas flows of 15, 0.2 and 0.75 L min^{-1} respectively, and a pump flow rate of 1.5 ml/min. Multi-element calibration standards in the concentration range 1 - 10 mg/L were used and the emission intensity measured at appropriate wavelengths. For all elements, analytical precision (RSD) was typically 1 to 5% for individual aliquots (n=3).

Effect of contact time on metal ion adsorption by BBB and PPH

Activated carbons pigeon pea husks (PPH) and baobab shells

(BBB) were used to determine the effect of contact time and quantity of adsorbent on metal ion adsorption. Batch adsorption experiments were carried out as described for the determination of initial pH, for contact times of 15, 30, 60, 90 and 120 min, with a fixed quantity of adsorbent of 5 g/L at pH 6 for solutions of mixtures of the metal ions at a concentration of 10 mg/L and for solutions containing Pb(II) ions only at a level of 10 mg/L.

Effect of quantity of carbon on metal ion adsorption

To investigate the effect of varying the quantity of carbon on metal ion adsorption, the quantity of carbon was varied from 0.5 to 10 g/L, whilst keeping all the other experimental variables constant, that is, pH 6.0, initial metal concentration of 10 mg/L and contact time 60 min.

Langmuir adsorption isotherm

The adsorption isotherm data produced from varying quantities of BBB in the adsorption of Pb(II) was analyzed using a Langmuir aqueous phase adsorption model. The Langmuir adsorption model assumes that maximum adsorption corresponds to a saturated monolayer of solute molecules on the adsorbent surface (Bansode et al., 2003). The Langmuir equation can be described by the linearized form:

$$C_e/q_e = 1/Q_o b + C_e/Q_o \quad (1)$$

Where, q_e is the amount of solute adsorbed on the carbon (mg/g), C_e is the equilibrium concentration of solute (mg/l). The adsorption capacity, Q_o; and the energy of adsorption capacity, b; can be derived from a linear plot of C_e/q_e against C_e.

The essential characteristics of the Langmuir isotherm can be expressed in terms of a dimensionless constant separation factor R_L that is given by the equation:

$$R_L = 1/(1+bC_o) \quad (2)$$

R_L values between 0 and 1 indicate favorable adsorption of Pb(II) onto BBB (Bansode et al., 2003).

RESULTS AND DISCUSSION

Effect of pH on adsorption of metal ions

The adsorption efficiency of the carbons was initially determined at pH values of 4, 5 and 6 and a contact time of 120 min, which was considered as adequate for an efficient adsorbent to reach equilibrium. The adsorption of all target metal ions by macadamia nut shells (MNS), rice husks (RH), *Moringa oleifera* husks (MH) and marula stones (AML) increased as initial pH increased from 4 to 6 (Tables 1, 2 and 3). The trend is consistent with the results obtained for the adsorption of Pb(II) using activated carbon prepared from various agricultural wastes (Ayyappan et al., 2005), for the adsorption of heavy metal ions using activated carbon prepared from apricot stone (Kobya et al., 2005) and for the adsorption of nickel by activated carbon prepared from almond husk (Hasar, 2003). For all the carbons used in this study, maximum metal ion adsorption was observed at pH 6 (Table 3) and on raising the pH above 6, precipitation occurred. The removal of metals from solution is dependent on the pH of the solution, which affects the surface charge of adsorbents, the degree of ionization and the species of adsorbate (Ayyappan et al., 2005; Karagoz et al., 2008; Dermibas et al., 2008). The increase in metal removal as pH increased can be attributed to the decrease in competition between H^+ ions and positively charged metal ions at the surface sites of the activated carbon (Hasar, 2003). Also, the binding of metal ions to the adsorbent should increase as the surface of the adsorbent changes from positive to negative, a transition which is influenced by the equilibrium pH. As the equilibration pH is varied, a point of zero charges (pH_{ZPC}) is attained, which is a pH value where the net charge on the surface of the adsorbent is zero (Noh and Schwarz, 1989). It is expected that as equilibrium pH is increased from 4 to 6, the transition towards negative charge on the carbons takes place as the pH moves towards or above pH_{ZPC} and therefore the carbon surfaces become more negatively charged resulting in increased binding of the metal ions.

Under all the pH conditions, RH had adsorption efficiencies below 42% for most of the metal ions. Adsorption efficiencies of Cd(II) and Zn(II) were less than 40% for all the adsorbents under all the pH conditions investigated, except PPH, which had 63% adsorption of Zn(II) at pH 6. On comparing the metal ion adsorption efficiencies of the various adsorbents at pH 6, it was observed that the percentage adsorption values were above 60% for adsorption of Hg(II) by all the activated carbons (Table 3). MNS had adsorption efficiencies of above 60% for Cu(II) and Hg(II) under all the pH conditions investigated, with a relatively high value of 97% for Hg(II) at pH 6. At the same pH, BBB was able to adsorb more than 84% of Pb(II), Cu(II), Fe(II) and Hg(II). Adsorption efficiencies higher than 68% were observed for adsorption of Pb(II), Cu(II), Fe(II), Hg(II) and As(III) using PPH. Heavy metal ion adsorption efficiencies of the experimental carbons were compared with commercially available carbons. In comparison with the two commercial carbons, the adsorption of Pb(II) by BBB, PPH, MH and AML was at least 22% higher than that of commercial carbons. The differences in adsorption efficiencies of the carbons can be attributed to possible differences in the pH_{ZPC} of the carbons. The pH_{ZPC} is in-turn influenced by the nature of the active groups on the carbon, an aspect that will vary depending on the precursor material (Al-Degs et al., 2000).

Table 1. Percentage adsorption of metal ions onto various adsorbents at pH 4.

Metal ion	Adsorbent							
	MNS	BBB	PPH	RH	MH	AML	CC 1	CC 2
Pb(II)	19.3	51.2 ± 0.1	49.2	12.8 ± 0.1	51.9 ± 0.1	28.2 ± 0.1	14.2±0.1	7.4 ± 0.5
Zn(II)	12.6 ± 0.2	19.1 ± 0.2	19.8 ± 0.3	1.5 ± 0.1	4.7 ± 0.1	1.7 ± 0.1	1.3	17.9 ± 0.3
Cu(II)	78.2	90.7	61.8 ± 0.1	16.1 ± 0.2	34.9 ± 0.2	35.7 ± 0.1	17.7	43.3 ± 0.6
Ni(II)	13.6	30.7 ± 0.1	16.9 ± 0.2	0.3	3.1	1.9	0	14.5 ± 0.2
Fe(II)	38.6 ± 0.2	50.1 ± 0.1	68.3 ± 0.1	8.2	28.4 ± 0.1	9.1	0	13 ± 0.1
Mn(II)	0	12.3 ± 0.1	16.7 ± 0.1	2 ± 0.1	6 ± 0.1	1.6	1.4	2.3 ± 0.2
Hg(II)	62.4 ± 0.1	43.7 ± 0.1	39.2 ± 0.1	22 ± 0.3	66.7	45.6 ± 0.1	100	50.8 ± 0.7
Cr(III)	22.8 ± 0.1	35.3 ± 0.1	31	9.4 ± 0.2	17	9.5 ± 0.6	6.2	13.6
As(III)	9.5 ± 0.1	18.6 ± 0.2	17.4 ± 0.1	5.2	5.4	7.5	5.8	13.8 ± 0.1
Cd(II)	10.7 ± 0.2	20.6 ± 0.2	22.9	2.3	7.3	3.2 ± 0.1	6.1	13 ± 0.4

MNS: Macadamia nut shells activated carbon, BBB: baobab shells activated carbon, PPH: pigeon pea husks activated carbon, RH: rice husks activated carbon, MH: *Moringaoleifera* husks activated carbon, AML: marula stones activated carbon, CC1: commercial carbon 1, CC2: commercial carbon 2

Table 2. Percentage adsorption of metal ions onto various adsorbents at pH 5.

Metal ion	Adsorbent							
	MNS	BBB	PPH	RH	MH	AML	CC 1	CC 2
Pb(II)	28.7±0.1	38.3±0.1	35.1±0.1	14.3	54	36.1±0.1	17.8	26.2±0.1
Zn(II)	6.9±0.1	14.2±0.1	6.6±0.2	1.6	7±0.1	4.1	2.5	8.6±0.2
Cu(II)	75.3±0.1	84.3	48	25.8±0.4	47.9±0.1	51.2±0.1	29.1	43.3±0.4
Ni(II)	11.4±0.1	24.1±0.1	8.9±0.1	2.8±0.1	6.6±0.1	8.2±0.1	2.3±0.1	7.1
Fe(II)	47.7±0.1	48.5±0.1	50.8	16±0.1	12.3±0.1	17.6±0.1	20.6±0.1	17.2±0.2
Mn(II)	0±0.1	5.7	8.6	2.8±0.1	5.4±0.1	2.8	0.7	0.7
Hg(II)	61±0.2	41.7±0.1	37.7±0.1	29.2±0.2	72.2±0.1	57.2	92.8±0.1	51.3±0.2
Cr(III)	30.2±0.1	34.5	27.4	7.7±0.5	18.3±0.1	18±0.1	21.9±0.1	17±0.1
As(III)	0.3±0.1	3.4±0.1	3.6±0.1	6.9	8.3±0.1	7±0.1	8.4	0.7
Cd(II)	0±0.1	6±0.1	10.9±0.1	0.4	6.5±0.1	2.7	3.6	1.2±0.1

MNS: Macadamia nut shells activated carbon, BBB: baobab shells activated carbon, PPH: pigeon pea husks activated carbon, RH: rice husks activated carbon, MH: *Moringaoleifera* husks activated carbon, AML: marula stones activated carbon, CC1: commercial carbon 1, CC2: commercial carbon 2

Effect of contact time on adsorption of metal ions

On monitoring the adsorption of the metal ions by the most efficient carbons, PPH and BBB, over time, percentage adsorption increased up to 60 min then reached a plateau for adsorption of Pb(II), Cu(II), Hg(II), Zn(II), Cr(III), Ni(II) and As(III) onto PPH (Figures 1 and 2). The adsorption of Fe(II) remained constant at the different contact times while percentage adsorption continued to increase slightly for the adsorption of Mn(II), As(III) and Cd(II). Apparently, there was competitive binding of the metal ions onto the carbon in the decreasing order of Fe(II)>Pb(II)>Hg(II)>Cu(II)>Zn(II)> Cr(III)>As(III)>Ni(II)>Mg(II)>Cd(II). When BBB was used as the adsorbent, there was no apparent effect of increasing contact time on the adsorption of all the metals except for Cr(III), where a slight increase in adsorption was adsorbed, reaching equilibrium at 90 min (Figure 2). Yu et al. (2001), reported that removal of Cr(VI) from aqueous solution increased with time and attained saturation in about 100 to 200 min. In adsorption of nickel by activated carbon prepared from almond husks, Hasar

Table 3. Percentage adsorption of metal ions onto various adsorbents at pH 6.

Metal ion	Adsorbent							
	MNS	BBB	PPH	RH	MH	AML	CC 1	CC 2
Pb(II)	66.3	84.3	94.2±0.1	40.2 ± 0.1	76.2	64.8	40.8 ± 0.1	61.8
Zn(II)	11.7	36.6	62.8	7.9 ± 0.2	28.4 ± 0.1	16.3 ± 0.1	0 ± 0.2	14.5 ± 0.1
Cu(II)	87.7 ± 0.2	98.3±0.1	87.5 ± 0.1	42.2 ± 0.2	62.7 ± 0.1	75.9 ± 0.1	59.9	79.5 ± 0.1
Ni(II)	17.6	61.4	38.4	12.7 ± 0.1	19.8 ± 0.1	19.5 ± 0.1	0 ± 0.2	19.9 ± 0.1
Fe(II)	80.9	88.6	98.5	58.8 ± 0.1	58.0	63.8	83.3	73.3
Mn(II)	6.9	26.1±0.1	34.7±0.1	11.5 ± 0.1	24.8 ± 0.1	12.9 ± 0.1	0	6.6
Hg(II)	97.3	89.3±0.1	95.5±0.1	64.5	95.1	90.9	100 ± 0.1	88.0 ± 0.1
Cr(III)	59	48.8	51.8	24.8 ± 0.1	28.4 ± 0.1	36.0 ± 0.1	61.3 ± 0.1	56.3 ± 0.1
As(III)	50.5	57.2	68.5	40.1 ± 0.2	34.5 ± 0.1	41.5 ± 0.1	57.8 ± 0.2	46.0 ± 0.1
Cd(II)	4.9	16.5	25.5±0.1	5.0 ± 0.2	18.1 ± 0.1	8.3 ± 0.1	0	4.4 ± 0.2

MNS: Macadamia nut shells activated carbon, BBB: baobab shells activated carbon, PPH: pigeon pea husks activated carbon, RH: rice husks activated carbon, MH: *Moringaoleifera* husks activated carbon, AML: marula stones activated carbon, CC1: commercial carbon 1, CC2: commercial carbon 2

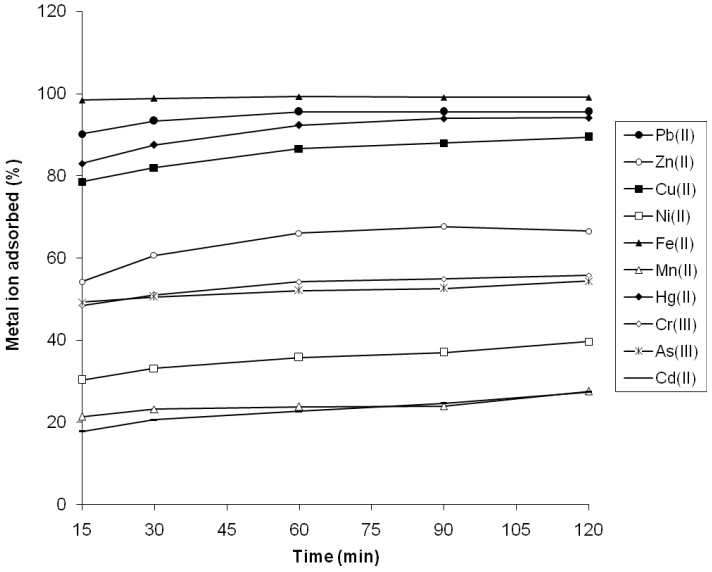

Figure 1. Effect of contact time on metal ion adsorption onto carbon prepared from pigeon pea husks. (Contact times: 15-20 min, carbon dose: 5 g/l, pH:6, metal ion concentration: 10 mg/l each in a mixture of the ions).

(2003) established that at 50 min, equilibrium had been attained. Apparently, the adsorption of most metal ions by activated carbon generally reaches equilibrium within 120 min (Ayyappan et al., 2005; Yu et al., 2001; Hasar, 2003). When BBB was used to adsorb Pb(II) from a solution containing Pb(II) only, percentage adsorption of Pb(II) was higher than the values obtained when Pb(II) was adsorbed from a mixture of metal ions (Figure 3).

Effect of quantity of carbon on adsorption of Pb(II)

The effect of increasing the quantity of adsorbent on the adsorption of Pb(II) by PPH and BBB is shown in Figure

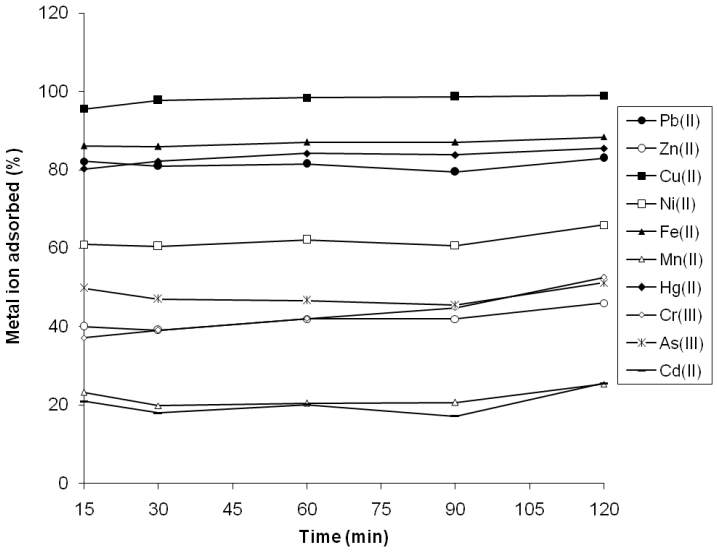

Figure 2. Effect of contact time on metal ion adsorption onto carbon prepared from baobab shells. (Contact times: 15-20 min, carbon dose: 5g/l, pH:6, metal ion concentration: 10 mg/l each in a mixture of the ions).

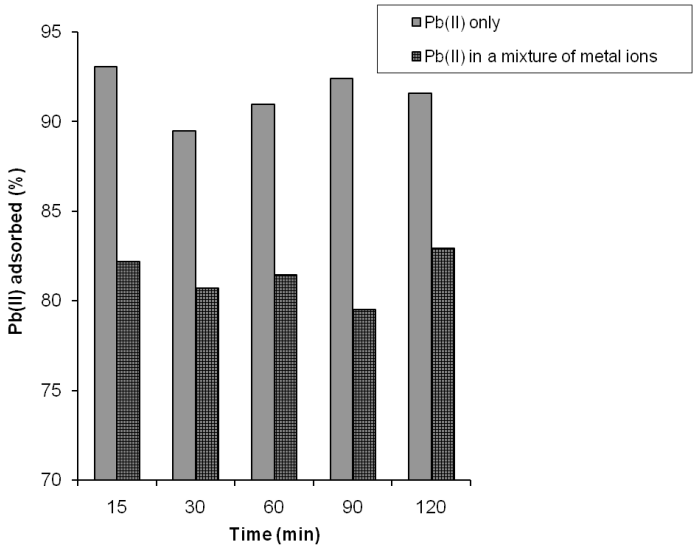

Figure 3. Comparison of the adsorption of Pb(II) onto carbon prepared from baobab shells while alone in solution and in a mixture with other metal ions. (Contact times: 15-20 min, carbon dose: 5g/l, pH:6, metal ion concentration: 10 mg/l).

4. On increasing the quantity of carbon from 0.5 g/l, the percentage removal of Pb(II) increased up to carbon quantities of 4 g/l and 6 g/l for PPH and BBB respectively then remained constant as the adsorbent quantities increased. The trend is consistent with results obtained by Hasar (2003), who observed an increase in adsorption

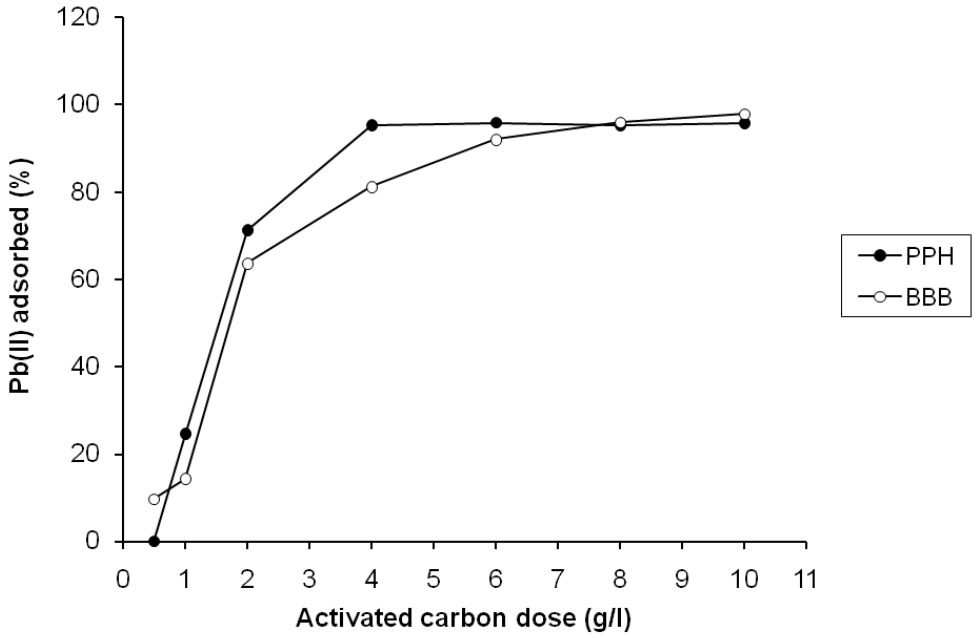

Figure 4. Effect of adsorbent dose on the adsorption of Pb(II) onto carbon prepared from pigeon pea husks and baobab shells. (Contact times: 15-20 min, carbon dose: 5g/l, pH:6, Pb(II) concentration: 10 mg/l).

of Ni(II) onto activated carbon prepared from almond husks as quantity of carbon was increased from 0.5 to 10 g/L then the adsorption remained constant upon further increase in carbon quantity. Ayyappan et al. (2005), recorded that percent sorption of Pb(II) onto activated carbon prepared from various agroforestry wastes increased with the increase in quantity of carbon from 0.5 to 2.0 g and remained constant on further increase in the quantity of the carbon.

Adsorption isotherms

The linear plot of C_e/q_e versus C_e for the adsorption of Pb(II) onto BBB shows that the adsorption obeys the Langmuir adsorption isotherm and indicates the formation of monolayer coverage of the adsorbate at the outer surface of the adsorbent (Figure 5). Q_o and b values of 1.52 and 1.96 respectively were determined from the linear plot. The R_L value was 0.05, indicating favorable adsorption of Pb(II) onto BBB (Bansode, 2003). The implications of this trend are that the adsorption of Pb(II) is due to specific interactions of the metal ion with groups on the surface of the carbon. Thus, when the monolayer covers these groups, there is no further adsorption of the metal ions.

Conclusion

The results of this study demonstrate the efficacy of carbon derived from agroforestry waste products in the removal of heavy metal ions from aqueous solutions. The optimum pH for removal of most of the ions by adsorption on all the carbons studied was 6. Equilibrium adsorption data for Pb(II) adsorption onto BBB followed the Langmuir isotherm. The results of this study show great potential for the removal of toxic metals from water bodies using low cost, domestic and environmentally safe technology. It is possible to desorb the metal ions so as to regenerate the carbons by equilibrating the metal ion-carbon complexes in acid solutions. Since the raw materials obtained from agroforestry wastes are freely available and abundant, the cost of preparation of the carbons is expected to be low and the method should be adopted with ease.

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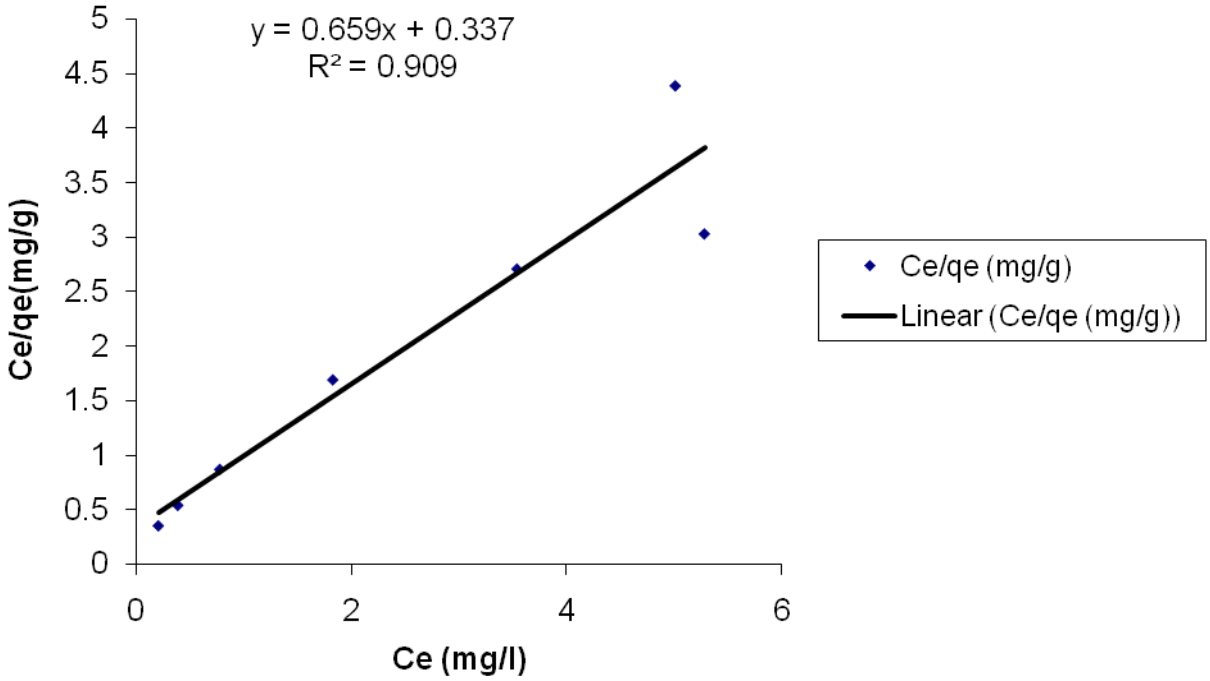

Figure 5. Langmuir isotherm for Pb(II) adsorption onto carbon prepared from baobab shells.

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Conflict of Interests

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

REFERENCES

Al-Degs Y, Khraisheh MAM, Allen SJ, Ahmad MN (2000). Effect of carbon surface chemistry on the removal of reactive dyes from textile effluent. Water Res. 927-935.

Avelar FF, Bianchi ML, Goncalves M, Gaspa da Mota E (2010). The use of piassava fibres (*Attaleafunifera*) in the preparation of carbon. Bioresour. Technol. 101: 4639-4656.

Ayyappan R, Sophia AC, Swaminathan K,Sandhya S (2005). Removal of Pb(II) from aqueous solution using carbon derived from agricultural wastes. Proc. Biochem. 40: 1293-1299.

Bansode RR, Loss JN, Marshall WE, Rao RM, Portier JR (2003). Adsorption of metal ions by pecan shell based granular activated carbons. Bioresour Technol. 89:115-119.

Chang YK, Chang JE, Lin TT, Hsu, YM (2002). Integrated copper containing wastewater treatment using xanthate process. J. Hazard. Mater. 94:89-99.

Dermibas E, Kobya M, Sulak MT (2008). Adsorption kinetics of a basic dye from aqueous solutions onto apricot stone activated. Bioresour Technol. 99:5368-5373.

Farooq U, Kozinski JA, Khan, MA, Athar M (2010). Biosorption of heavy metals using wheat based biosorbents- A review of recent literature. Bioresour. Technol. 101:5043-5053.

Garcia-Reyes RB, Rangel-Mendez JR (2010).Adsorption kinetics of Chromium (III) ions onto agro-waste materials.Bioresour. Technol. 101:8099-8108.

Hasar H (2003).Adsorption of nickel from aqueous solution onto activated carbon prepared from almond husk. J. Hazard. Mater. B97:49-57.

Kadirvelu K, Thamaraiselvi K, Namasivayam C (2001). Removal of heavy metals from industrial wastewaters by adsorption onto activated carbon prepared from an agricultural solid waste. Bioresour. Technol. 76:63-65.

Kantarli IC, Yanik J (2010). Activated carbon from leather shaving wastes and its application in removal of toxic materials. J Hazard Mater. 179:348-356.

Karagoz S, Tay T, Ucar S, Erdem M (2008). Activated carbons from waste biomass by sulfuric acid activation and their use on methylene blue adsorption. Bioresour. Technol. 99:6214-6222.

Kazemipour M, Ansari M, Tajrobehkar S, Majdzdeh M, Kermani HR (2008). Removal of lead, cadmium, zinc and copper from industrial wastewater by carbon developed from walnut, hazelnut, almond, pistachio shell and apricot stone. J. Hazard. Mater. 150:322-327.

Kobya M, Dermirbas E, Senturk E, Ince M (2005). Adsorption of heavy metal ions from aqueous solutions by activated carbon prepared from apricot stone. Bioresour Technol. 96:1518-1521.

Kula I,Ugurlu M, KaraoguluH,Celik A (2008). Adsorption of Cd(II) ions from aqueous solutions using activated carbon prepared from olive stone by $ZnCl_2$ activation. Bioresour. Technol. 99:492-501.

Mussatto SI, Fernandes M, Rocha GJM, Órfão JJM, Teixeira JA, Roberto IC (2010). Production, characterization and application of activated carbon from brewer's spent grain lignin. Bioresour. Technol.

101:2450-2457.
Noh JS, Schwarz JA (1989). Estimation of the point of zero charge of simple oxides by mass titration. J. Colloid Interface Sci. 130:157-164.
Owlad M, Aroua MK, Wan Daud WMA (2010). Hexavalent chromium adsorption on impregnated palm shell activated carbon with polyethyleneimine. Bioresour. Technol. 101:5098-5103.
Ozcimen D, Ersoy-Mericboyu A (2009). Removal of copper from aqueous solutions by adsorption onto chestnut shell and grapeseed activated carbons. J. Hazard Mater. 168:1118-1125.
Rivera-Utrilla J, Sanchez-Polo M, Gomez-Serrano V, Alvarez PM, Alvim-Ferraz MCM, Dias JM (2011). Activated carbon modifications to enhance its water treatment applications. An overview. J Hazard Mater. 187:1-23.
Warhurst AM, McConnachie GL, Pollard SJT (1997). Characterisation and applications of activated carbon produced from *Moringaoleifera* seed husks by single step steam pyrolysis. Water Res. 31:759-766.
Ye H, Zhu Q, Du D (2010). Adsorptive removal of Cd(II) from aqueous solution using natural and modified rice husk. Bioresour. Technol. 2010:101:5175-5179.
Yu B, Zhang y, Shukla A, Shukla SS, Dorris KL (2001).The removal of heavy metals from aqueous solutions by sawdust adsorption-removal of lead and comparison of its adsorption with copper. J Hazard Mater. 84:83-94.
Zabihi M, HaghighiAsl A, Ahmadpour A (2010). Studies on adsorption of mercury from aqueous solution on activated carbons prepared from walnut shell. J. Hazard Mater. 174:251-256.
Zakhama S, Daouadi H, Henni FM (2011). Nonlinear modelisation of heavy metal removal from aqueous solution using *Ulvalactuca* algae. Bioresour. Technol. 102:786-796.

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Full Length Research Paper

Expression, production and renaturation of a functional single-chain variable antibody fragment (scFv) against human intercellular adhesion molecule-1 (ICAM-1)

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The single-chain variable antibody fragment (scFv) against human intercellular adhesion molecule-1 (ICAM-1) was expressed at a high level in *Escherichia coli* as inclusion bodies. We attempted to refold the scFv by ion-exchange chromatography (IEC), dialysis and dilution. The results show that the column chromatography refolding by Q Sepharose high performance (Q HP) had remarkable advantages over the conventional dilution and dialysis methods. Furthermore, the anti-ICAM-1 scFv yield was higher by this method, which is about 60 mg/l. The purity of the final product was greater than 90%, as shown by denaturing gel electrophoresis. Enzyme-linked immunosorbent assay (ELISA), cell culture and animal experiments were used to assess the immunologic properties and biologic activities of the renatured scFv.

Key words: Intercellular adhesion molecule-1, single-chain variable antibody fragment, expression, purification, renaturation, biological activity.

INTRODUCTION

Intercellular adhesion molecule-1 (ICAM-1) is a member of the immunoglobulin supergene family and is a cell surface ligand for lymphocyte function-associated antigen-1 (LFA-1). ICAM-1 is mainly expressed on the surface of endothelial cells. It is also expressed on activated lymphocytes in inflamed regions; however, the expression in peripheral blood lymphocytes is normally very low. The level of ICAM-1 is upregulated in the presence of various stimuli (for example, inflammatory mediators, oxidative stress and viral infection) (Dustin et

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Abbreviations: scFv, Single-chain variable antibody fragment; **IEC,** ion-exchange chromatography; **Q HP,** Q Sepharose high performance; **ELISA,** enzyme-linked immunosorbent assay; **ICAM-1,** intercellular adhesion molecule-1; **SDS-PAGE,** sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

al., 1986). The interaction of ICAM-1 with the LFA-1 plays an important role in leukocyte adhesion and in the execution of immunological and inflammatory functions mediated by leukocyte adhesion (Dustin et al., 1988). Increased ICAM-1 levels result in the transmigration of neutrophils during the initial phase of inflammation. Inhibition of this process could decrease inflammatory response and tissue damage (Sadowska et al., 2004). Many diseases are associated with the over-expressed ICAM-1, such as acute pancreatitis (Kaufmann et al., 1996), inflammatory bowel disease and colonic neoplasms (Sadowska et al., 2004), organ transplantation (Vainer et al., 2006; Zhao et al., 2007; Aronni et al., 2006), angiocardiopathy (Sadowska et al., 2004; Bowes et al., 1993), ischemia-reperfusion injury (Souza-Moraes et al., 2003) and cancer (Alexiou et al., 2001; Coskun et al., 2006; Thomas and Speight, 2001). These reports suggested that the anti-ICAM-1 strategy has a potential application in the treatment of ICAM-1-mediated immunological and inflammatory diseases.

Today, the single-chain variable antibody fragment (scFv) strategy has become one of the most popular methods in antibody engineering because of its lower level of immunogenicity, and its small molecular size endows scFv with better tissue penetration (Yokota et al., 1992). Thus, scFv has a wide range of applications in diagnosis and therapy. It is possible for anti-ICAM-1 scFv to block the biological activity of ICAM-1, and it may be effective in preventing the progression of the above mentioned diseases. Therefore, it is necessary to prepare large-scale amounts of the anti-ICAM-1 scFv protein for further research and application. In this study, we expressed the anti-ICAM-1 scFv at a high level in the form of the inclusion bodies in *Escherichia coli*. We successfully refolded the denatured scFv by ion exchange chromatography (IEC). The study paves the way for preparing a large amount of anti-ICAM-1 scFv to be used for the application against diseases correlated with inflammation.

MATERIALS AND METHODS

The following reagents were used in this study: isopropyl-β-D-thiogalactoside (IPTG) (Merck), horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody (KeyGen Biotech), reduced glutathione hormone (GSH), glutathione (GSSG), lipopolysaccharide (LPS) and bovine serum albumin (BSA) (Sigma), Ficoll-Paque (Amersham Biosciences), rat ICAM-1 (R and D); Q Sepharose high performance (Pharmacia Biotech), ÄKTA (Prime protein purification system) (Amersham Biosciences), fermenter (10 L, East China University Science and Technology), anti-ICAM-1 mAb (prepared in our laboratory) (Sun et al., 2008a). A cell line (ECV-304) and Kunming mice (weight 18 to 22 g) were purchased from Maisha Biotechnology Limited Company (Shanghai, China) and the Center of Experimental Animal Changchun Institute of Biological Products (Changchun, China), respectively. All the other chemicals used were of analytical grade. Anti-scFv rabbit polyclonal (the antiserum against scFv) was obtained by hypodermic injecting of the rabbit with the purified inclusion bodies protein.

Plasmids and strains

E. coli BL-21(DE3) was obtained from Novagen. The expression plasmid of pET22b-(ICAM-1) scFv was constructed by our laboratory (Sun et al., 2008b).

Expression of anti- ICAM-1 scFv

Bacterial cells with the expression plasmid pET22b-(ICAM-1) scFv were grown overnight in 5 ml of LB with 50 mg/l ampicillin (amp) at 37°C. The overnight culture was used at 1:100 dilutions. Expression was induced by adding IPTG to a final concentration of 0.8 mM when the culture was grown to OD_{600} of 0.6 to 0.8. The mixture was further incubated for another 4 h. Cells were harvested by centrifugation at 5000 rpm for 20 min at 4°C.

Fermentation procedure

Large-scale scFv were produced in the bioreactor as follows; the prepared strain was added to the fermentation medium (pH 7.0 to 7.3). The rate of rotation was 400 rpm and aeration rate was 20. Meanwhile, the dissolved oxygen was controlled at the level of 20 to 30%. Then scFv production was induced by the addition of 0.8 mM IPTG, and the culture was allowed to incubate at 37°C for 4 h.

Preparation of the samples

The scFv was expressed as described above. After extraction from *E. coli* cells by the combination of lysozyme and sonication, the inclusion bodies were washed three times with 100 ml of 0.5% TritonX-100 (v/v) and 2 M urea for 30 min each time. Two grams of the pellet was suspended in 10 ml of denaturing buffer (50 mM Tris-HCl, 50 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 8 M urea, and 10 mM dithiothreitol (DTT), pH 8.0) and kept at room temperature for 2 to 4 h to dissolve the inclusion bodies. Residual insoluble matter was removed by centrifuging at 12000 rpm for 30 min. The supernatant was filtered on 0.22 μm filter (Durapore Membrane Filter, Millipore, USA) before chromatography.

Refolding of scFv

Refolding by dilution

The above solubilized inclusion bodies (7 ml) with a concentration of 7 mg/ml were slowly dropped into the refolding buffer A (30 mM Tris-HCl, 1 mM EDTA, 1 mM GSH, 0.2 mM GSSG, pH 8.0) and adjusted to a protein concentration of 100 μg/ml. The solution was stirred for 2 h at room temperature, followed by incubation at 4°C for more than 48 h (Takai et al., 2005).

Purification by ion-exchange chromatography (IEC): The 500 ml diluted supernatant was applied to the 10 ml IEC (Q HP) which was pre-equilibrated with buffer B (30 mM Tris-Cl, 1 mM EDTA, pH 8.0). The ÄKTA Prime protein purification system was used. The column was eluted with a 50 ml linear gradient of buffer B to buffer B containing 1 M NaCl. Finally, protein concentration was determined with the Bradford assay.

Refolding by urea gradient dialysis

The 100 ml solubilized denatured scFv (0.5 mg/ml) was loaded into a dialysis bag with a membrane molecular weight cutoff of 10000

Daltons, which was dialyzed against 50 columns of buffer A at 4°C for 24 h. Denaturant was slowly removed by a series of equilibrations with buffers of decreasing urea. The urea concentration was reduced as follows: 6 M → 4 M → 2 M → 1 M → 0.5 M → 0 M (Tan et al., 1998). After centrifugation, the supernatant was applied to Q HP for further purification, as described above.

Refolding by IEC

An IEC system was used with the XK16/20 column containing 10 ml of Q HP of the ÄKTA Prime protein purification system. The column was equilibrated with denaturing buffer A (30 mM Tris-Cl, pH 8.0, with 6 M urea, 1 mM EDTA, 1 mM GSH and 0.2 mM GSSG). Following equilibration, urea concentration of the solubilized inclusion bodies (7 ml; 7 mg/ml) was adjusted to 6 M, and the samples were loaded. After sample was loaded, the refolding procedure was performed with a linear gradient of 25 column volumes by decreasing urea concentration from 6 to 0 M, maintaining the flow rate at 0.5 ml/min. Protein was gradually being refolded within the column. Following refolding, another linear gradient of buffer (30 mM Tris-Cl, pH 8.0, 1 mM EDTA) without GSH-GSSG from 0 to 1 M NaCl was performed with the gradient length of six column volumes. The eluate fractions were collected and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The procedure was done at 4°C.

Protein determination

The relative protein concentration of the denatured and purified scFv was determined by Bradford assay using BSA as a standard protein (Bradford, 1976). The refolding yield was calculated as a percentage of the soluble protein after refolding against the total protein of inclusion bodies before refolding.

Indirect cellular ELISA for antigen-binding activity of anti-ICAM-1 scFv

The antigen-binding activity of the refolded scFv was detected and identified routinely by noncompetitive ELISA. Cultured ECV-304 cells were seeded overnight in the 96-well culture plate at 10^5 cells/well. Cells were fixed in 10% formalin-phosphate buffered saline (PBS) (pH 7.4) for 15 min at room temperature, washed three times with 1% BSA-PBS and blocked by 3% BSA-PBS for 2 h at 37°C. After washing, the refolded scFv concentration was diluted serially and added to the plate. The control well was prepared without scFv, and the plate was incubated for 1 h at 37°C. The second antibody (anti-rabbit IgG antibody) and HRP-conjugated goat anti-rabbit IgG were added in turn and incubated for 1 h at 37°C. Finally, tetramethylbenzidine (TMB) was used as the color developer, and absorbance was measured at 450 nm (Chen et al., 2006). The binding of purified scFv to rat ICAM-1 was also determined by indirect ELISA (the 96-well culture plate was coated with 1 μg/ml rat ICAM-1), as described above, which paved the way for further active research.

Cell adhesion assay for peripheral mononuclear cells (MCs) to ECV-304

ECV-304 cells grown in monolayers in a 96-well plate were treated with LPS (100 ng/ml) for 24 h at 37°C (Beck-Schimmer et al., 2002), followed by adding the anti-ICAM-1 mAb (5 μg/well), purified scFv (5 μg/well), or left untreated (PBS control). The 96-well plate was cultured for 0.5 h. Human MCs were isolated from healthy human peripheral blood using Ficoll-Paque according to the manufacturer's instructions. MCs were adjusted to a concentration of 10^6/ml with RPMI 1640, and added to the monolayers of MC in a final volume of 100 μl. After a 0.5 h co-culture in a CO_2 incubator, the populations of non-adherent cells was removed from the plate, and were manually counted under the microscope at ×20 magnification (Takai et al., 2005). The inhibition (%) was calculated by: 100 × [1 - (the cell population of non-adhesion) / (total cellular score)].

Inhibitory effect on the swelling of mouse auricle that was induced by dimethyl benzene

The Kunming mice weighing 18 to 22 g (females and males) were randomly divided into five groups, namely: experiment group 1 (intraperitoneal injection scFv, 2 mg/kg), experiment group 2 (intravenous injection scFv, 2 mg/kg), experiment group 3 (intravenous injection mAb, 2 mg/kg), hexadecadrol group (intraperitoneal injection hexadecadrol, 2 mg/kg), and the control group (0.9% saline). Thereafter, dimethyl benzene was dropped onto the left ear conch uniformly 1 h after injection. After another 2 h, the mice were sacrificed, and both ears were cut by scissors. The ear pieces were punched with a puncher at the same place and weighed. The inhibition (%) of the engorgement was calculated by: 100 × [(the average tumescent degree of control group - the average tumescent degree of experimental group) / the average tumescent degree of control group]. The animal experiment was conducted under applicable laws and guidelines and after approval by the Animal Care and Use Committee of Jilin University.

RESULTS

The expression of scFv

The constructed expression vector was transferred into BL21 (DE3) and induced with IPTG at 37°C. As shown in Figure 1, a protein of 30 kDa was strongly expressed after 4 h incubation with 0.8 mM IPTG. It was found that the scFv was expressed as insoluble inclusion bodies. The wet weight cell was about 5 g in 1 l of *E. coli* flask culture. After large scale production of anti-ICAM-1 scFv in the fermentor, the overall yield of the harvested cells (wet weight) was about 10 g/l.

Preparation of the samples

Isolation is the first step for the recovery of active protein from the *E. coli* which involves breaking the cells to release the cell contents, including the inclusion bodies. Lysis is more efficient if the cells are pretreated with lysozyme, which weakens the cell walls. Therefore, in our experiments, the cells were treated by lysozyme before sonication. Washing can remove non-target protein and may also remove proteases that could degrade the expressed product. In this study, we washed the inclusion bodies three times with buffer containing 0.5% TritonX-100 buffer and 2 M urea. Finally, 350 mg of inclusion body protein per liter of culture were obtained by fermentor, and the purity of inclusion bodies was up to 70%, as determined by SDS-PAGE (Figure 2A).

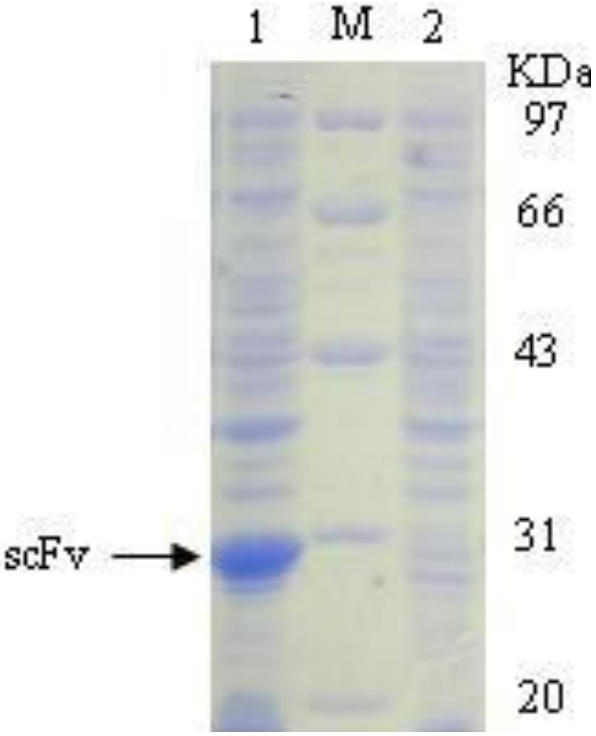

Figure 1. Analysis of scFv expressed in *E. coli.* The amount of inclusion bodies was about 30% of the total cell proteins of *E. coli*. Lane 1, induced total cellular protein; lane 2, non-induced pET22b-anti ICAM-1 scFv/BL21 (λDE3); M, protein molecular weight markers.

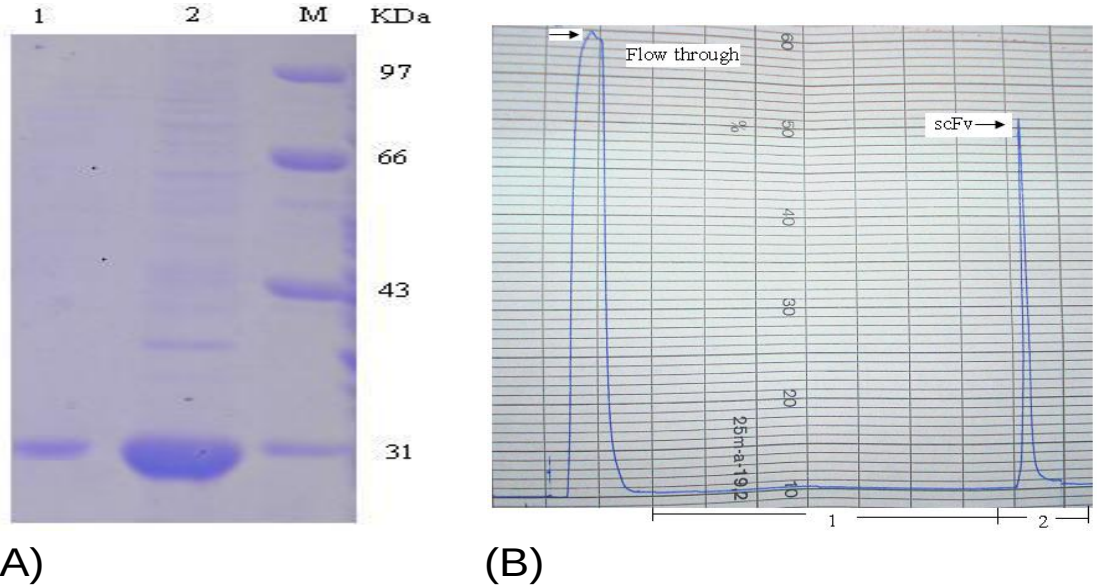

Figure 2. (A) Analysis of purified and refolded scFv on SDS–PAGE. Lane 1, scFv refolding by Q HP; lane 2, solubilized inclusion bodies; M, protein molecular weight markers. (B) Chromatographic elution profile of refolding scFv by Q Sepharose HP column. Protein peaks were observed at 280 nm. Process 1, refolding procedure; process 2, gradient elution. The arrow indicates flow through protein and interest protein.

Table 1. The comparison of three refolding methods.

Refolding method	Dilution	Dialysis	IEC
The protein yield (%)	22	28	17
Protein concentration (mg/ml)	0.03	0.15	0.4-0.5
Purity (%)	>70	>70	>90
Activity (OD)	1.86	2.05	2.53
Time required (h)	>48	>72	<10
Reagent consumption (relative amount)	2	100	1

Activity is the OD value of refolded and purified scFv analyzed by indirect cell-ELISA at the same protein concentration (2.5 μg/mL). (The OD value of negative control was 0.080); reagent consumption is relative amount that is calculated on the basis of 1 g inclusion bodies.

Table 2. Purification summary of scFv.

Purification step	Total protein (mg)	scFv (mg)	Purity (%)	Recovery yield (%)
Total cell lysate[a]	1200	372	31	100
Inclusion bodies after washing	494	346	70	93
Refolded scFv after IEC column	67	60	90	17

[a]Approximately, 10 g of wet-weight cells and a total cell lysate containing 1200 mg protein were obtained from 1000 ml cell culture.

Refolding of scFv

For comparison of the methods, the dilution and dialysis were both carried out. During the two processes, a slight protein aggregation was found. Perhaps due to the prolonged experimental time, the activities of the refolded scFv proteins by dilution and dialysis were lower than that refolded by IEC. Comparing the renatured scFv by three refolding methods (Table 1), and considering purity, activity, time required and the consumption of reagents, the column chromatography method for refolding was the most suitable for large scale production. After refolding by IEC, we obtained a single protein peak during the elution procedure and collected approximately 20 ml of the protein (Figure 2B). The overall purification is summarized in Table 2. The purity of product was about 90%, as shown on the 12% SDS-PAGE (Figure 2A).

Effect of pH on the refolding by IEC

The protein yield was affected by the pH value significantly. To study the influence of the pH on the refolding recovery, denatured scFv was loaded and eluted by a buffer with different pH (7.0, 7.5, 8.0 and 8.5) each time. As shown in Figure 3, the pH 8.0 elution buffer led to a significantly increased yield of the functional anti-ICAM-1 scFv (17%). It is suggested that the condition is suitable for the native disulfide bond formation. So the condition of pH 8.0 was used throughout the experiment.

The activity of refolded scFv

The non-competitive ELISA results demonstrated that refolded anti-ICAM-1 scFv was able to bind specifically to human ICAM-1-expressing cells in a dose dependent manner. Specifically, when gradually increasing the concentrations of refolded scFv, the extent of ICAM-1 and scFv binding increased (Figure 4). Meanwhile, the indirect ELISA showed the specific antigen-binding activity of the refolded scFv to rat ICAM-1 (Figure 5).

Cell adhesion assay for peripheral MCs to ECV-304

Statistical analysis was carried out using SPSS 13.0, and the statistical significance was set at $P < 0.05$. It can be seen from the data that the MCs adhesion to LPS-stimulated ECV-304 monolayers was largely inhibited in the presence of a neutralizing ICAM-1 mAb and scFv, both at 30 min, compared with the untreated condition (with PBS). As a result, the ratio of adhesion in the presence of mAb and scFv were 31 and 36% (data not shown), respectively, which was a statistically significant difference compared with the control (42%) ($P < 0.05$). Although both mAb and scFv could inhibit the MCs adhesion to ECV-304, the effectiveness of scFv was lower than that of the mAb ($p > 0.05$).

The analysis of the inflammation depressant effect

Statistical analysis was carried out as described above. We found that injection of anti-ICAM-1 scFv or mAb reduced the severity of swelling of auricle to a different degree, but the depressant effect of scFv and mAb was a little weaker than that of hexadecadrol. As shown in Table 3, there was a statistically significant difference in

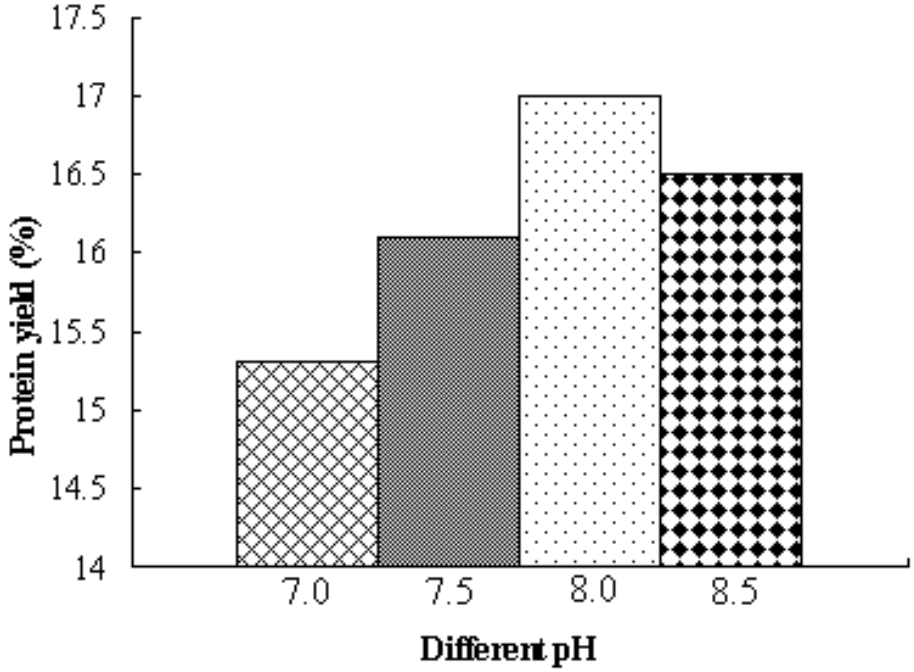

Figure 3. Effect of pH on refolding yield. Refolding was performed at different pH, 7.0, 7.5, 8.0 and 8.5, to measure pH dependence of refolding. The data indicates that the optimal pH for refolding of scFv may be 8.0.

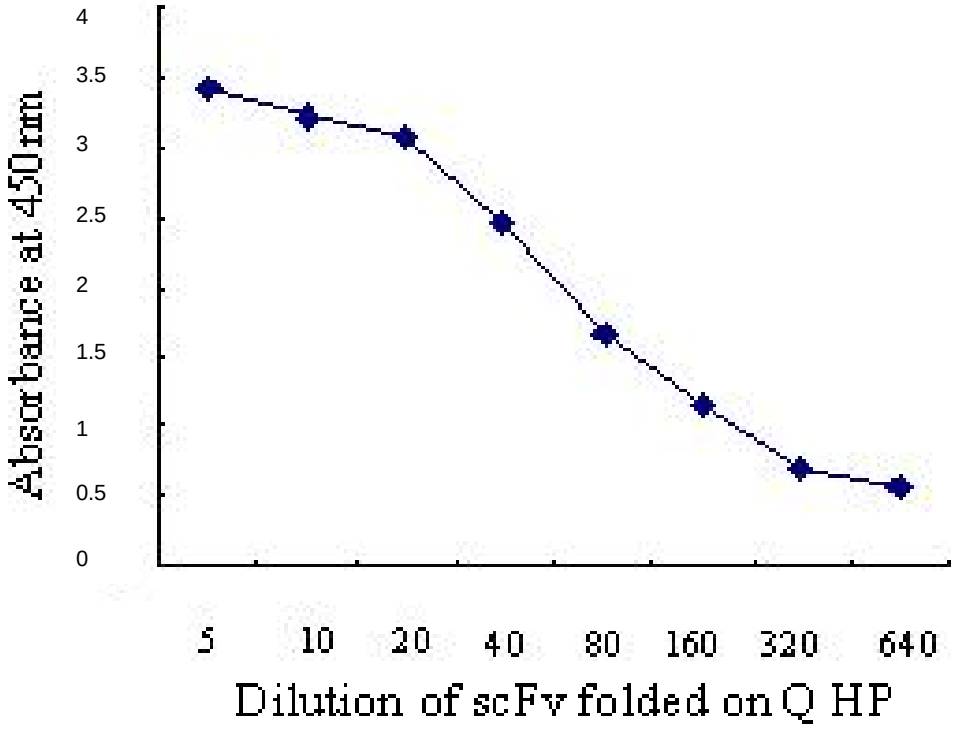

Figure 4. Antigen-binding activity of refolded anti-ICAM-1 scFv. Specific antigen-binding activity was determined by indirect cellular ELISA. The 96-well culture plate was coated by EC-304 cells and 2-fold serially diluted, purified scFv was added to the wells. As can be seen, when gradually increasing the concentrations of refolded scFv, absorbance values increased.

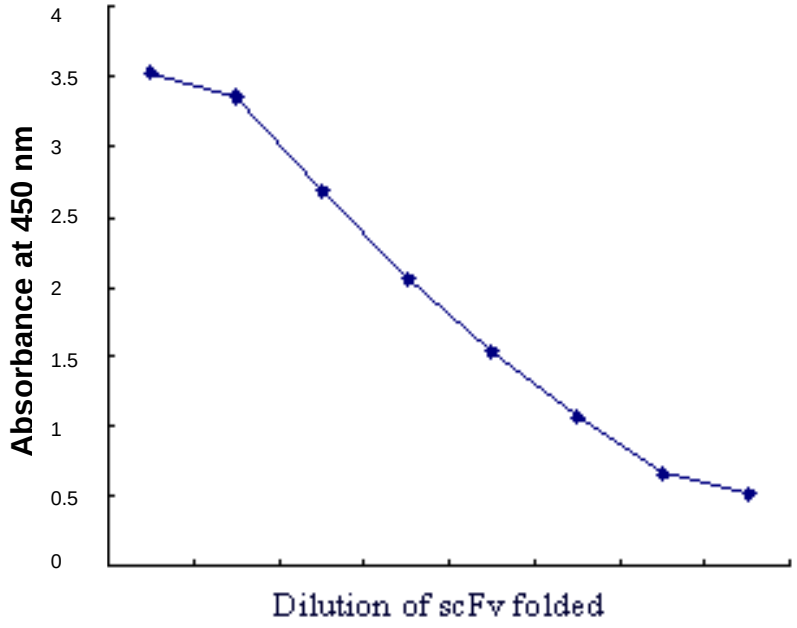

Figure 5. Indirect ELISA for antigen-binding to rat ICAM-1. The 96-well culture plate was coated with 1 μg/ml rat ICAM-1, the refolded scFv concentration was diluted serially and added to the plate.

Table 3. The effect of anti-ICAM-1 scFv and mAb on dimethyl benzene-induced swelling of auricle ($\overline{X} \pm S$).

Group	n	Dosage (mg/kg)	Tumescent degree (mg)	Inhibition (%)
Control group	10	-	12.6±1.05	-
Hexadecadrol group	10	2	5.15±1.23*	59
Experiment group 1	10	2	6.95±1.52*	45
Experiment group 2	10	2	7.61±2.02*	39
Experiment group 3	10	2	5.94±1.69*	53

Statistically significant reduction by *t*-test compared with control group, *P < 0.01.

the inhibition (%) between the experimental group and the control group.

DISCUSSION

In previous studies, it has been shown that increasing ICAM-1 *in vivo* contributed to the pathogenesis of the inflammation-related diseases. Many reports had suggested the protective effects of anti-ICAM-1 mAb, which could block the inflammation response *in vitro* or *in vivo*. The results in our laboratory also indicate that ICAM-1 and its receptors exhibit a high expression level in highly pathogenic avian influenza (H5N1) and viral pneumonia (HPAIV), and may play an important role in the pathogenesis (Coskun et al., 2006). Moreover, we gained excellent results in curing mice of avian influenza with the anti-ICAM-1 antibody we applied for a patent in 2007, in which we named the anti-ICAM-1 antibody as a treatment for avian influenza. However, mAb molecules are large and have more immunogenicity, so it may be advisable to use the anti-ICAM-1 scFv in diagnostic and therapeutic applications. In our present work, the active scFv against human ICAM-1, successfully prepared from the inclusion bodies by chromatography renaturation, had a significant effect on the aseptic inflammation.

To obtain the active protein, refolding the expressed products from the inclusion bodies is the most important and fundamental procedure. If we can develop a refolding strategy *in vitro* with lower cost, higher yield and activity, then less expensive and easier prokaryotic expression, namely, bacterial fermentation systems, may become

feasible for the inclusion body protein production. In the past, dilution and dialysis were convenient and traditional refolding strategies. Now, there are many reports about the two methods, which had been used for refolding many proteins. Most of them were prepared at the laboratory scale but not industrial scale production. These refolding techniques had some disadvantages, such as that the dialysis procedure needed large amounts of reagents, long treatment times (Tan et al., 1998), and also can cause the adhesion of protein on the membrane (Verma et al., 1998). Sometimes, it is easy to form protein aggregates (West et al., 1998). The dis-advantages of the dilution method are the large processing volumes involved, and the increasing costs and the “step-change” in denaturant concentration to native conditions may result in aggregation (Chaudhuri, 1994). Moreover, the concentration of denatured protein during the two refolding process has to be controlled at a low level to prevent aggregate formation, which restricted their application on a large scale production.

In recent years, chromatographic methods have been developed for the refolding of the inclusion body proteins from *E. coli*. In many cases, they appeared to be more effective than traditional refolding strategies (Lanckriet and Meddelberg, 2004; Fahey et al., 2000; Gu et al., 2002). IEC has the characteristics of simple operation, good biological compatibility and high capacity. Furthermore, the medium could be reused, which decreases the cost of materials. In addition, the IEC method is the concomitant purification of the target protein during the refolding process (Müller and Rinas, 1999), so considered from all perspectives, the IEC process is the more valuable method for refolding of the engineered proteins. In the case of proteins containing cysteine, the isolated inclusion bodies usually contain a certain amount of interchain disulfide bonds (Schoemaker et al., 1985). There are two disulfide bonds in anti-ICAM-1 scFv. For disulfide-containing proteins, the refolding yields are strongly dependent upon the redox environment, which helps to form the proper disulfide bond and to associate different domains (Wetlaufer et al., 1987).

In our study, 1 mM GSH and 0.2 mM GSSG were included in the first gradient buffers during the refolding procedure. In the second gradient, we investigated two conditions and found that introducing redox conditions to the refolding buffer did not lead to a significantly increased yield of functional anti-ICAM-1 scFv (data not shown). So GSH-GSSG was not added in the second gradient. It may be that the first gradient helps to form the disulfide bonds. The stage is a critical refolding period for native disulfide bond formation, not the post refolding stage. Due to the lack of Fc domain in the structure of scFv, the refolded scFv did not bind with common HRP-conjugated second antibody. In the noncompetitive ELISA experiment, we prepared second antibody (rabbit anti-mouse IgG antibody) which can bind to scFv.

Conclusion

A high-level production of scFv in the *E. coli* expression system has been successfully established, and an inexpensive convenient refolding strategy for scFv recovery has also been developed. We could obtain 60 mg of active scFvs from 1 liter cultivation of *E. coli* cells by fermentation. The established on-column refolding procedure for the efficient recovery of anti-ICAM-1 scFv from the inclusion bodies had a practical significance for further research on other scFv or recombinant protein. The advantages of this method include the biophysical and biochemical characteristics, and the fact that the purified scFv can markedly suppress the MCs adhesion to LPS-stimulated ECV-304 monolayers. Moreover, we have established the mouse model of aseptic inflammation and showed that the scFv significantly inhibited the inflammatory swelling of auricle in mice induced by dimethyl benzene. The renaturation process was significant; it is possible that the IEC could be very useful in refolding inclusion body protein on a large scale but refolding efficiency still needs to improve so as to magnify its commercial application on other recombinant proteins.

Conflict of Interests

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

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REFERENCES

Alexiou D, Karayiannakis AJ, Syrigos KN, Zbar A, Kremmyda A, Bramis I, Tsigris C (2001). Serum levels of E-selectin, ICAM-1 and VCAM-1 in colorectal cancer patients:correlations with clinicopathological features, patient survival and tumour surgery. Eur. J. Cancer 37(18):2392-2397.

Aronni S, Cortes M, Sacchetti M, Lambiase A, Micera A, Sgrulletta R, Bonini S (2006). Upregulation of ICAM-1 expression in the conjunctiva of patients with chronic graft-versus-host disease. Eur. J. Ophthalmol. 16(1):17-23.

Beck-Schimmer B, Madjdpour C, Kneller S, Ziegler U, Pasch T, Wüthrich RP, Ward PA, Schimmer RC (2002). Role of alveolar epithelial ICAM-1 in lipopolysaccharide-induced lung inflammation. Eur. Respir. J. 19:1142-1150.

Bowes MP, Zivin JA, Rothlein R (1993). Monoclonal antibody to the ICAM-1 adhesion site reduces neurological damage in a rabbit cerebral embolism stroke model. Exp. Neurol. 119(2):215-219.

Bradford MM (1976). A rapid method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. 7:248-254.
Chaudhuri JB (1994). Refolding recombinant proteins: process strategies and novel approaches. Ann. N.Y. Acad. Sci. 721:374-385.
Chen LH, Huang Q, Wan L (2006). Expression, purification, and *in vitro* refolding of a humanized single-chain Fv antibody against human CTLA4 (CD152). Protein Expr. Purif. 46:495-502.
Coskun U, Sancak B, Sen I, Bukan N, Tufan MA, Gulbahar O, Sozen S (2006). Serum P-selection soluble vascular cell adhesion molecule-1(sVCAM-1) and soluble intercellular adhesion molecule-1 (sICAM-1) levels in bladder carcinoma patients with different stages. Immunopharmacology 6(4):672-677.
Dustin ML, Rothlein R, Bhan AK, Dinarello CA, Springer TA (1986). Induction by IL-1 and interferon-gamma: tissue distribution, biochemistry, and function of a natural adherence molecule (ICAM-1). J. Immunol. 137:245-254.
Dustin ML, Staunton DE, Springer TA (1988). Supergene families meet in the immune system. Immunology 9(7-8):213-215.
Fahey EM, Chaudhuri JB, Binding P (2000). Refolding and purification of a urokinase plasminogen activator fragment by chromatography. J. Chromatogr. B. 737(1-2):225-235.
Gu ZY, Weidenhaupt M, Ivanova N, Pavlov M, Xu B, Su ZG, Janson JC (2002). Chromatographic methods for the isolation of, and refolding of proteins from. *Escherichia coli* inclusion bodies. Protein Expr. Purif. 25:174-179.
Kaufmann P, Tilz GP, Smolle KH, Demel U, Krejs GJ (1996). Increased plasma concentrations of circulating intercellular adhesion molecule-1(ICAM-1) in patients with necrotizing pancreatitis. Immunobiology 95(2):209-219.
Lanckriet H, Meddelberg AP (2004). Continuous chromatographic protein refolding. J. Chromatogr. A 1022(1-2):103-113.
Müller C, Rinas U (1999). Renaturation of heterodimeric platelet-derived growth factor from inclusion bodies of recombinant *Escherichia coli* using size-exclusion chromatography. J. Chromatogr. A 855(1):203-213.
Sadowska AM, van Overveld FJ, Luyten C, Germonpré P, De Backer WA (2004). Use of ICAM-1 antibodies and antisense oligonucleotides to inhibit transmigration of neutrophils. Inflamm. Res. 53(4):143-149.
Schoemaker JM, Brasnett AH, Marstion FAO (1985). Examination of calf prochymotrypsin accumulation in *Escherichia coli*: disulfide linkages are a structural component of prochymotrypsin-containing inclusion bodies. EMBOJ 4:775-780.
Souza-Moraes MR, David-Filho R, Baptista-Silva JC, Ullian M, Franco MF, Gabriel Jr A, Smith B, Burihan E (2003). Effect of antibodies to intercellular adhesion molecule type 1 on the protection of distant organs during reperfusion syndrome in rats. J. Med. Biol. Res. 36(5):605-612.
Sun H, Wan ZH, Zhang GL, Wu GM, Zhu P, Yue YH (2008). Construction and expression of anti-human ICAM-1 scFv in *E.coli*. Chin. J. Biologicals 21(5):82-86.
Sun H, Wan ZH, Zhang GL, Wu GM, Zhu P, Yue YH (2008). Preparation of monoclonal antibody against human ICAM-1 and identification of its activity. Chin. J. Biologicals 21(4):308-311.
Takai T, Takaoka M, Yasueda H, Okumura K, Ogawa H (2005). Dilution method to refold bacterially expressed recombinant Der f 2 and Der p 2 to exhibit the secondary structure and histamine-releasing activity of natural allergens. Int. Arch. Allergy Immunol. 137(1):1-8.
Tan PH, Sandmaier BM, Stayton PS (1998). Contributions of ahighly conserved VH/VL hydrogen bonding interaction to scFv folding stability and refolding efficiency. Biophys. J. 75:1473-14821.
Thomas GJ, Speight PM (2001). Cell adhesionmolecules and oral cancer [J]. Crit. Rev. Oral. Biol. Med. 12:479-498.
Vainer B, Horn T, Nielsen OH (2006). Colonic epithelial cell expression of ICAM-1 relates to loss of surface continuity: a comparative study of inflammatory bowel disease and colonic neoplasms. Scand. J. Gastroenterol. 41(3):318-325.
Verma R, Boleti E, George AJ (1998). Antibody engineering: comparison of bacterial yeast insect and mammalian expression systems. J. Immunol. Methods 216(1-2):165-181.
West SM, Chaudhuri JB, Howell JA (1998). Improved protein refolding using hollow-fibre membrane dialysis. Biotechnol. Bioeng. 57(5):590-599.
Wetlaufer DB, Branca PA, Chen G (1987). The oxidative folding of proteins by disulfide plus thiol does not correlate with redox potential. Protein Eng. 1:141-146.
Yokota T, Milenic DE, Whitlow M, Schlom J (1992). Rapid tumor penetration of a single-chain Fv and comparison with other immunoglobulin forms. Cancer Res. 52:3402-3408.
Zhao X, Song GM, Xu JL, Wang XJ, Song HM, Qu X (2007). Expression of intercellular adhesion molecule-1 and vascular cell adhesion molecule in cardiac allografts and significance thereof: experiment with rats. Zhonghua Yi Xue Za Zhi 87(37):2657-2659.

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Full Length Research Paper

Production of *Spirulina platensis* by adding sodium bicarbonate and urea into chicken manure medium

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In this study, dry chicken manure (DCM) was used as the source of nitrogen in the production of *Spirulina platensis*. Experiment was conducted in two different types of nutrition. In the type I nutrition, the highest average cell increase was recorded as 289.70 filaments mL^{-1} in value in the type I group (DCM+urea). In the type II nutrition, the highest average cell increase was recorded as 296.50 filament mL^{-1} in value in the type II group (DCM+urea+sodium bicarbonate). In the type I nutrition (DCM+Urea), the highest specific growth rate was detected as 0.21 division day^{-1} on the sixth day and k value as being 0.30 division day^{-1}. In the type II nutrition, the highest specific growth rate was recorded as 0.08 division day^{-1} in the group II (DCM+Urea+NaHCO$_3$) and k value as being 0.12 division day^{-1}. In both types of nutrition, the protein rates of the type I group (DCM+urea) and the group III (DCM+sodium bicarbonate) are found to be higher than that in the other groups. The lipid levels in both types of nutrition, in DCM (Control) and in the type I group (DCM+Urea) were found to be higher than the other groups.

Key words: *Spirulina platensis,* dry chicken manure, sodium bicarbonate, urea.

INTRODUCTION

Spirulina platensis (Geitler, 1932) is a species of cyanobacteria with a commercial value. It is a species of microalgae with thread-like structure constituting intensive populations in tropic and sub-tropic waters characterized with high carbonate, bicarbonate and the highest pH value (Binaghi et al*.,* 2003). *S. platensis* production has been launched in Turkey and has find a place in the region of nutraceuticals and pharmaceuticals fields' market (Blunden et al*.*, 1992). *Spirulina* sp. is being used in combined feed of poultry, being an active biological additive due to high protein concentration, essential amino acids, vitamins, β-carotene, minerals, polysaccharides and essential fatty acids (Babadzhanov et al*.,* 2004; Venkataraman et al., 1994). *Spirulina* is also stated to be a natural colorant. Hence, it is stated that it is being used in chicken eggs and fish feed; in aquaculture as natural colorant (Challem, 1981). It is also revealed that the cost can vary for the various nutrition compositions and nutrient environment for the biomass productivity of *Spirulina* sp. (Kendirli, 2010).

Microalgae are in the need of basic elements such as carbon (C), nitrogen (N) and phosphorus (P) in order to conduct the organic substance synthesis (Davis, 1977). In cultures, the most important inorganic N sources that

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Table 1. Nutrient media used in the experiment.

Nutrition type I groups of nutrient media	Nutrition type II groups of nutrient media
DCM (Control): tap water was added every 24 h	DCM (Control): tap water was added every 24 h
(DCM+Urea): urea (2.0 mg/L) was added every 24 h	(DCM+Urea): urea (1.0 mg/L) was added every 24 h
(DCM+Urea+$NaHCO_3$): urea (2.0 mg/L) and sodium bicarbonate (40 mg/L) were added every 24 h	(DCM+Urea+$NaHCO_3$): urea (1.0 mg/L) and sodium bicarbonate (20 mg/L) were added every 24 h
(DCM+$NaHCO_3$): sodium bicarbonate (40 mg/L) was added every 24 h	(DCM+NaHCO3): sodium bicarbonate (20 mg/L) was added every 24 h

are able to be used by the cells are as follows; NO_3 nitrogen (NO_3-NN), NH_4 nitrogen (NH_4-N) and urea nitrogen ($(NH_2)_2CO$-N) (Gökpınar, 1991). *S. platensis*, as opposed to the other photosynthetic microorganisms is able to use ammonia in high pH values. The penetration of ammonia into cell for an average inner cell pH (pH_{av}) and the outer environment pH (pH_{out}) is depended on the difference (delta pH) and limits the high pH average of inner cell. This high pH value is found to be pressured by inner thylakoid pH (Boussiba, 1989). Urea is converted to ammonia by hydrolysis in the alkaline culture media. This emerging ammonia in high concentration is able to create toxic impact for microalgae. However, addition of urea in discontinuous nutrition period enables the use of KNO_3 as the source of nitrogen in culture medium. On the other hand, it is stated that working in the industrial scale might be simpler with constant urea addition (Danesi et al*.,* 2004).

Another way of reasonable utilization of farm manure is to use it in the production of microalgae. Nitrogen source for *S. platensis* is known to be nitrate. Furthermore, the farmers managed to produce *Spirulina* sp. using the manure disposals of pig and chicken farms as the source of nitrogen (Ungsethaphand et al., 2007). By doing so, they cut back on purchasing expensive feed additive, at the same time utilizing from the waste water, they have provided as an economic contribution (Cheunbarn et al*.,* 2010). Algae use solar energy whilst absorbing nutrients using the carbonized substances from the waste waters in order to produce biomass. Biomass which is obtained from the useful part of the waste water ingredient is able to be used in animal feed as being a valuable nutrient source (Ratana et al*.,* 2009). Later, researches showed that dry chicken manure provides the necessary nutrient source for *S. platensis* culture (Ungsethaphand et al., 2007).

In Turkey, the research on organic manure usage for *S. platensis* production is not yet sufficient. The aim of this study is to achieve the low cost production of *S. platensis* by adding urea and sodium bicarbonate into dry chicken manure and to determine the protein and lipid levels of the yield.

MATERIALS AND METHODS

S. platensis was produced in the Plankton Laboratory of Mersin University of Fisheries Faculty. As the culture environment *Spirulina* liquid medium was used by having been modified. $NaNO_3$ was not used in the modified *Spirulina* medium (Schlosser, 1982). Part I: 180.6 g $NaHCO_3$; 80.6 g Na_2CO_3; 10.0 g K_2HPO_4; Part II: 20.0 g K_2SO_4; 20.0 g NaCl; 4.0 g $MgSO_4.7H_2O$; 2.0 g $CaCl_2.2H_2O$; 20.0 g $FeSO_4.7H_2O$; 1.6 g $EDTANa_2$ and 100 m, micro nutrient solution (0.001 g $ZnSO_4.7H_2O$; 0.002 g $MnSO_4.7H_2O$; 0.01 g H_3BO_3; 0.001 g $Na_2MoO_4.2H_2O$; 0.001 g $Co(NO_3)_2.6H_2O$; 0.00005 g $CuSO_4.5H_2O$; 0.7 g $FeSO_4.7H_2O$; 0.8 g $EDTANa_2$). Part I and II were prepared by being added into 10 L of pure water. To constitute a inoculation culture; firstly, the cells of *Spirulina* are filtered through a 30 µm mesh net and to purify from sodium nitrate washed three times with 0.8% NaCl solution and harvested. Stabilizing the initial pH value at 10 and the temperature at 34±1°C, cells of *Spirulina* was inoculated into modified *Spirulina* medium. Initial culture was carried out by providing moderate aeration in 1 L flasks. 24 h of lighting was applied with daylight fluorescent lamps and when the culture reached to maximum intensity, the cell was inoculated into the prepared manure syrup.

In the experiment, 200 g of dry chicken manure which has been provided from a chicken farm was used for the culture medium. Dry chicken manure was powdered with a feed grinding machine and the powder sieved through 30 µm mesh net. Then, it was waited in 10 L tap water filtered through 0.45 µm membrane filter and aerated for seven days. Furthermore, sodium metabisulfide (5 mg L^{-1}) was added into the medium in order to prevent the microbial contamination. In addition, the manure syrup was waited in 121°C in an autoclave for 15 min sterilizing. 24 h after the commencement of the experiment, 8.5 g L^{-1} of sodium bicarbonate was also added. The experiment was conducted in 10 L of glass tanks in discontinuous mode for 10 days. Each of them was aerated by a central aeration system.

The nutrient media and quantities used in the experiment are shown in Table 1. The addition of urea and sodium bicarbonate was conducted at the time of feeding. The experiments were conducted in two different types of nutrition on 4 x 3 experimental designs, in 8 groups each, three times repeatedly.

Inoculation

The *Spirulina* cells (500 filaments mL^{-1} density) purified from nitrate were inoculated into the water with manure. Culture tanks were prepared with 5.5 L water with manure and by the inoculation of 4.5 L *Spirulina* cells.

Obtaining of the *S. platensis* yields

S. platensis yields harvested from the type I and II nutrition were filtered through a 30 µm mesh net. Yields were kept waiting in the laboratory environment for one day and then the drying process was applied at 50°C temperature for three days in the incubator. The dried yields were preserved in the refrigerator and a second

drying process was applied at 65°C for 45 min before analyzing process.

The calculation of filament number and specific growth rate of *S. platensis*

Single drop method was used in the calculation of filament number (Semina, 1978). After each sample was counted at least 3 repeats, the filament number at 1 ml was calculated. In the experiment, the specific growth rate of *Spirulina* was calculated according to the formula below (Guillard, 1973);

$$\mu = \frac{\ln Nt - \ln N0}{t1 - t0}$$

μ: Specific growth rate, Nt: Number of cells at the end of the experiment, N_0: Initial cell number, t: Time (day).

$$k = \mu / 0.6931$$

Divisions per day were calculated according to (k) equation (Guillard, 1973; Mubeen et al., 2011).

Chemical analysis

Kjeldahl method (AOAC, 1984) was applied in the protein analysis and Blig and Dyer (1959) method was applied in lipid analysis.

Statistical analysis

The statistical analysis were made using SPSS 16.0 software package and the comparisons were carried out with one-way ANOVA-Duncan method.

RESULTS AND DISCUSSION

In the type I nutrition, the increase of cells in the type I group of the sixth day are detected respectively as follows: 326.67 filament ml^{-1}; 336.33 filament ml^{-1}; 357.67 filament ml^{-1}; 317.67 filament ml^{-1}; 260.33 filament ml^{-1} and are found to be different statistically in comparison to other groups ($p<0.05$). In the type I nutrition, average cell increase (289.70 filament ml^{-1}) was found to be in the highest level (Figure 1). In the experiment; in the type I nutrition, the ideal cell increase was detected in the type I group in the urea added nutrient medium. Costa et al. (2004) reported that the highest cell increase is obtained from the type I group with the addition of urea in which no sodium bicarbonate is added. The result which the researchers reported matches the results we had obtained. In various studies that have been conducted, it is stated that in *S. platensis* cultures, as the source of nitrogen increases, the use of urea causes an increase of cell (Stanca and Popovici, 1996). The result that they had obtained matches the findings we obtained as the result of our studies. Xu et al. (2001) reported that nitrogen sources and concentrations are one of the important factors in effecting the growth and biochemical composition in algae cultures. In the chicken manure medium with rich nitrogen, 2.0 mg L^{-1} of urea urged the increase of cell of *S. platensis* and affected its growth and biochemical structure positively in our study. Vonshak et al. (1982) reported that only when sodium bicarbonate is added, the increase of cell was affected negatively. They reported that in dark cycle at high temperatures, due to the increase in respiratory activity, cells apply the consumption of carbohydrates for respiration and this resulted in a decrease of cell growth.

According to the results of this study, the increase of cell that was found in the group with the sodium bicarbonate added is consistent with the Vonshak et al. (1982) result which they had reported. In that sense, the decrease in the cell increase can be connected to high CO_2 and high bicarbonate rates depended on the acceleration of photosynthesis and fast carbohydrate consumption. In the type II nutrition, the second day, the increase of cell in the type II group were defined respectively; 324.00 filament ml^{-1}; 311.33 filament ml^{-1}; 320.33 filament ml^{-1}; 318.67 filament ml^{-1}; 345.00 filament ml^{-1}; 325.00 filament ml^{-1}; 259.33 filament ml^{-1}; 233.00 filament ml^{-1}; 220.00 filament ml^{-1}. However, in the type II group nutrition, the average increase of cell (296.50 ml^{-1} filament) was found to be the ideal ($p<0.05$) (Figure 1). In the type II nutrition, from the second day, the increase of cell in the type I group were defined respectively; 340.00 filament ml^{-1}; 321.33 filament ml^{-1}; 329.00 filament ml^{-1}; 302.67 filament ml^{-1}; 270.00 filament ml^{-1}; 231.33 filament ml^{-1}; 165.33 filament ml^{-1}. From sixth day, any considerable difference was not found between type II group with type I group and other groups($p>0.05$). But, significant differences were found between type II group with other groups after sixth day ($p<0.05$). The highest increase of cell took place in the type II nutrition due to the rates of urea and sodium bicarbonate which was halved compared to the type I nutrition. However, as opposed to the increase of cell, decrease in the rates of protein was observed. The reason for this is the nitrogen source and levels being one of the most important factors affecting the growth and biochemical contents in algae cultures. The growth is affected by concentrations as well as by nourishing elements that are used in the nutrition environments (Xu et al., 2001). It is concluded that the decrease in the protein ratios is the result of sodium bicarbonate pressuring the nitrogen levels in the environment, slowing down the protein metabolism and augmenting carbohydrate metabolism.

In the type I nutrition, the specific growth rates in the second, fourth, sixth, seventh and eighth days were recorded as being respectively; 0.07 division day^{-1}; 0.01 division day^{-1}; 0.21 division day^{-1}; 0.03 division day^{-1}; 0.06 division day^{-1}. The most ideal specific growth rate in the type I group was found to be 0.21 division day^{-1} and k value as being 0.30 division day^{-1}. The specific growth rate in the type II group, in the second day was found to be 0.06 division day^{-1} and the k value as 0.09 division

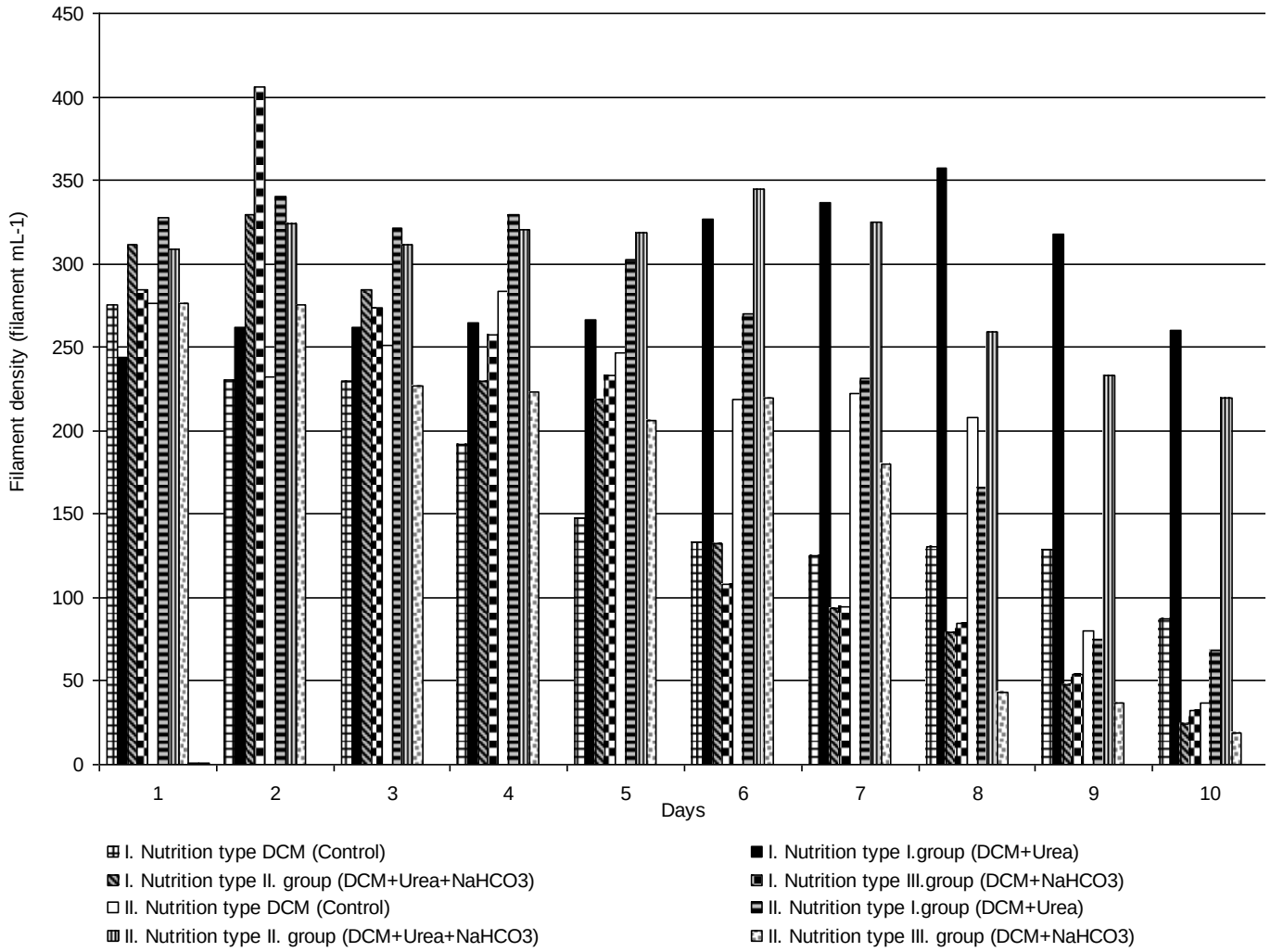

Figure 1. Daily increases in filament densities of *S. platensis* were cultivated in I and II type of nutrition with DCM.

day^{-1}. The specific growth rate in the type III group, in the second day was found to be 0.36 division day^{-1} and the k value as 0.52 division day^{-1}. The specific growth rate in the control group, in the eighth day was found to be 0.04 division day^{-1} and the k value as 0.06 division day^{-1} (Figures 2 and 3). In the type II nutrition, the specific growth rates in the second, fourth and the fifth day were recorded respectively; 0.04 division day^{-1}; 0.03 division day^{-1}; 0.05 division day^{-1}. The specific growth rates in the second day were recorded to be 0.04 division day^{-1} and the k value as being 0.06 division day^{-1}; in the fourth day was 0.03 division day-$^{-1}$ and the k value as being 0.04 division day^{-1}; in the fifth day was 0.05 division day^{-1} and the k value as 0.07 division day^{-1}. The specific growth rates in the second, fourth and the sixth day were recorded respectively; 0.05 division day^{-1}; 0.03 division day^{-1}; 0.08 division day^{-1}.

The highest specific growth rate in the type II group was found to be 0.08 division day^{-1} and the k value is recorded as 0.12 division day^{-1}. The specific growth rate in the type III group is recorded as 0.06 division day^{-1} and the k value as 0.09 division day^{1}. The specific growth rates in the control group in the third, fourth and the seventh day were recorded respectively; 0.08 division day^{-1}; 0.12 division day^{-1}; 0.02 division day^{-1}. The specific growth rate in the control group in the fourth day is recorded to be 0.12 division day^{-1} and the k value as 0.17 division day^{-1} (Figures 2 and 3). Vonshak et al. (1982) reported that in the group where low cell increase had taken place, high specific growth was observed. Similarly, in our study in the groups where low cell increase had taken place, a maximum specific growth rate (µmax) was obtained. Vonshak et al. (1982) detected the µmax rates as 0.048 to 0.054 division day^{-1} and this is consistent with the specific growth rates (0.04 to 0.08 division day^{-1}) as we had obtained in our study.

There was no statistical discrepancies observed in the two different nutrition media in terms of lipid levels ($p>0.05$). Between I to II groups and III to IV groups of the type I nutrition media statistical discrepancies were found

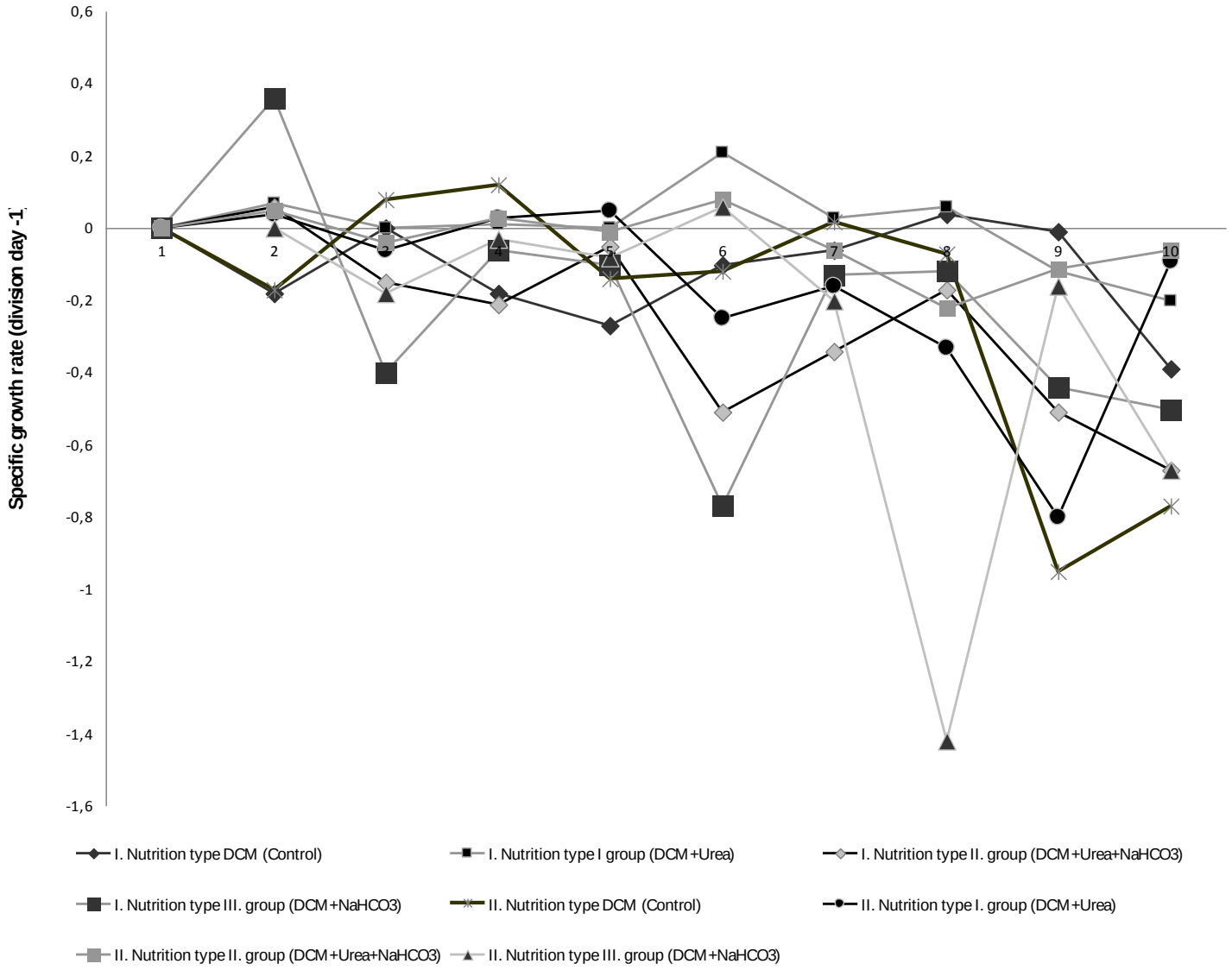

Figure 2. Daily increases in specific growth rates of *S. platensis* were cultivated in I and II type of nutrition with DCM.

($p<0.05$). In the type II nutrition medium, there was a discrepancy found between I, II, III and IV groups ($p<0.05$). The lipid levels of *S. platensis* which was produced in the types I and II groups are found to be higher than that found in the types III and IV groups of both nutrition media (Figure 4). It can be concluded that the addition of sodium bicarbonate into the III and IV groups could be responsible. Vonshak et al. (1982) reported that this took place due to the partial CO_2 pressure of biomass values being high at high temperatures and the acceleration of photosynthesis. The results that the researchers reported support our findings. Danesi et al. (2002) reported that the lipid contents in the biomass of *S. platensis* are not under the influence of nitrogen source that was used. In our study, there was no discrepancy found in the lipid rates of the various concentrations of urea and sodium bicarbonate in both nutrition media. Our results are consistent with the results that Danesi et al. (2002) reported. Ungsethaphand (2009) reported that the highest protein content is obtained from the media in which urea and urea + sodium bicarbonate is added into the chicken manure. The increase in the protein content takes place due to the nitrogen levels increase in the environment (Ungsethaphand, 2009). In our study, the highest protein ratio is obtained from the groups in which urea and urea + sodium bicarbonate were added into the chicken manure. Our findings are consisted with the results that were obtained from the studies conducted by Ungsethaphand (2009).

In the study conducted by Koru and Cirik (2003), the protein ratio of *S. platensis* was reported to be 57.5% and the lipid ratio as 8%. In our study, the ratios of lipid and protein are found respectively, between 2.59 to 4.96, 35.51 to 44.98%. Based on the results that the researchers

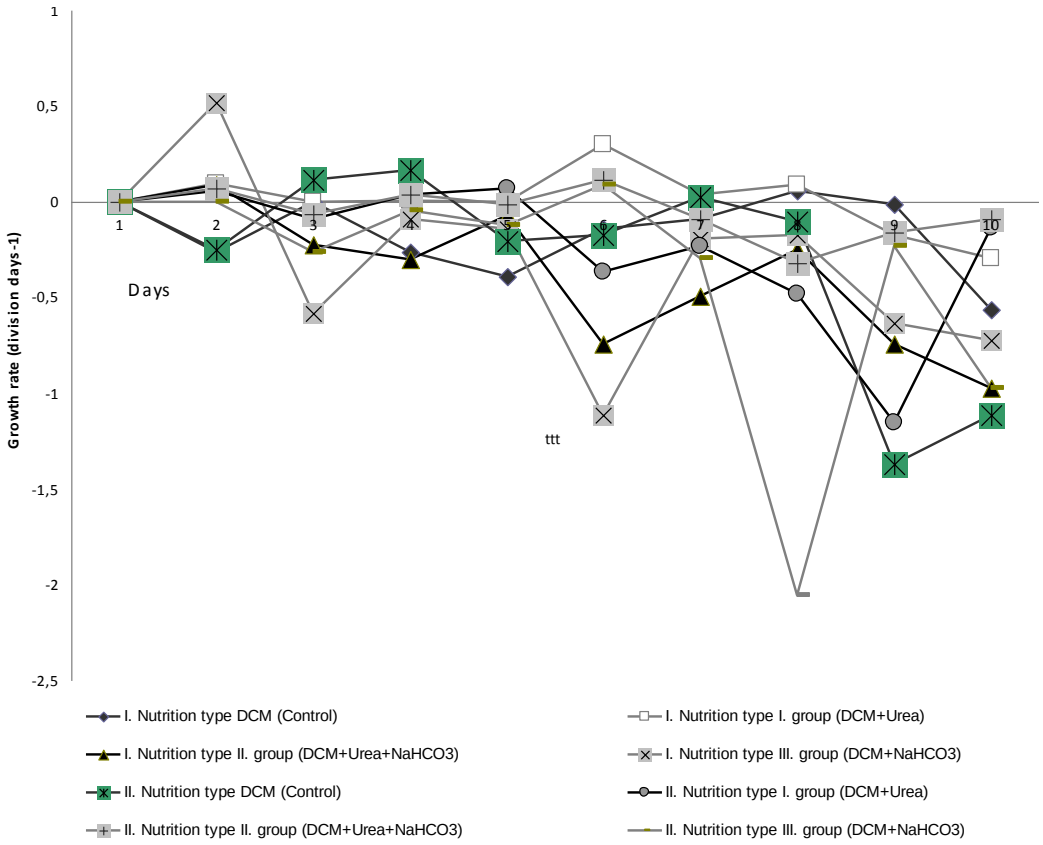

Figure 3. Daily growth rates (division day^{-1}) of *S. platensis* were cultivated in I and II type of nutrition with DCM.

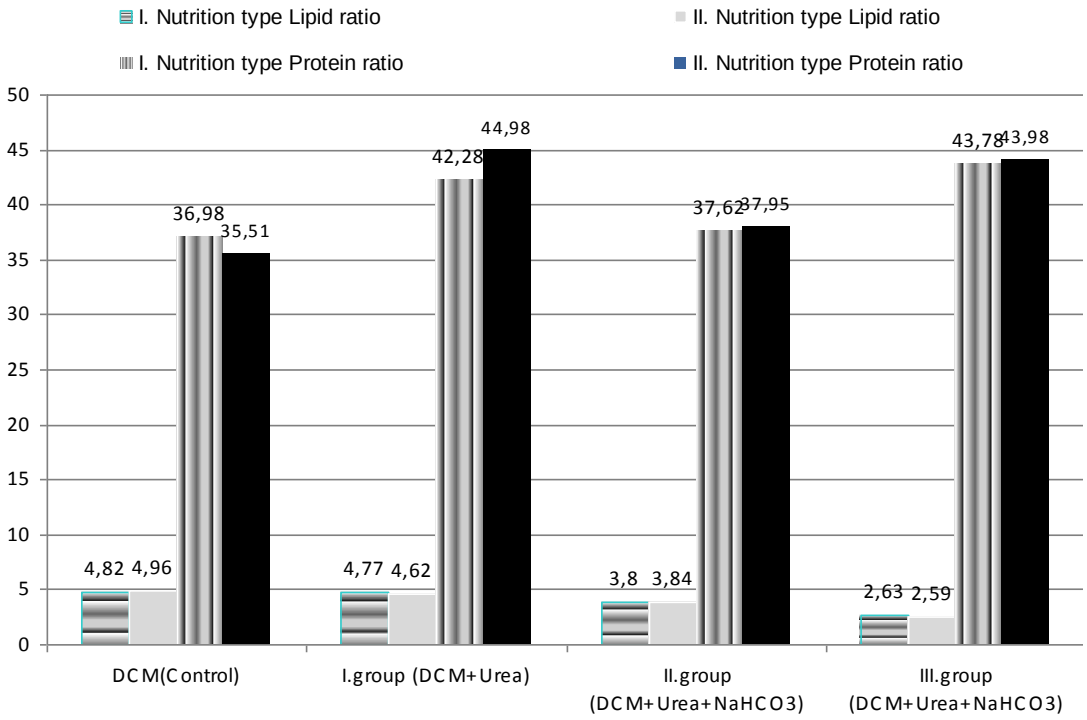

Figure 4. Lipid and protein ratio of *S. platensis* were cultivated in I and II type of nutrition with DCM.

reported, it was concluded that the low protein and lipid ratios that was obtained in our study is due to the differences in the nutrition media.

It was determined that usage of 1.0 mg/L urea+20 mg/L $NaCO_3$ added 200 mg/10 L of DCM (in type II nutrition) gave the best cellular increase and usage of 2.0 mg/L urea added 200 mg/10 L of DCM (in type I nutrition) gave the best cellular increase and the best protein ratio. In this case, we can say that suppressive effect of $NaCO_3$ decreased when the amount of urea and $NaCO_3$ had been halved.

Conflict of interests

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

REFERENCES

AOAC (1984). Official Methods of Analysis 14th. Ed. Association of Official Analytical chemists, Washington, DC, USA.

Babadzhanov AS, Abdusamatova N, Yusupova FM, Faizullaeva N, Mezhlumyan LG, Malikova MK (2004). Chemical composition of *Spirulina platensis* cultivated in Uzbekistan. Chem. Nat. Compounds. 40(3): 276-279.

Binaghi L, Del Borghi A, Lodi A, Converti A, Del Borghi M (2003). Batch and fed-batch uptake of carbon dioxide by *Spirulina platensis*. Proc. Biochem. 38:1341-1346.

Blig EG, Dyer WJ (1959). A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37: 911-917.

Blunden G, Whapham C, Jenkins T (1992). Seaweed Extracts in Agriculture and Horticulture: Their Origins, Uses and Modes of Action. School of Pharmacy and Biomedical Science and School of Biological Sciences, University of Portsmouth, King Henry John Street, Portsmouth, Hampshire P01 U.K. p. 202.

Boussiba S (1989). Ammonia uptake in the alkalophilic cyanobacteria *Spirulina platensis*. Plant Cell Physiol. 30:303-308.

Challem JJ (1981). Spirulina:What It Is. The Health Benefits It Can Give You. Keats Publishing Inc., New Canaan, CT.

Cheunbarn S (2010). Yuwadee Peerapornpisal, "Cultivation of *Spirulina platensis* using Anaerobically Swine Wastewater Treatment Effluent". Faculty of Science, Maejo University, Chiang Mai 50290, Thailand.

Costa JAV, Colla LM, Filho PFD (2004). Improving *Spirulina platensis* biomass yield using a fed-batch process. Bioresour. Technol. 92: 237-241.

Danesi EDG, Rangel-Yagui CO, Carvalho JCM, Sato S (2004). Effect of reducing the light intensity on the growth and production of chlorophyll by *Spirulina platensis.* Biomass and Bioenergy. 26: 329-335.

Davis AR (1977). Principles of Oceanography. University of South Florida Aquafarms, Inc., Florida. p. 126.

Geitler L (1932). Cyanophyceae. In: Rabenhorst L. (ed). Rabenhorst's Kryptogamen-Flora von Deutchland. O¨ sterreich und der Schweiz, Akademische Verlagsgesellschaft, Leipzig. 14: 1-1196.

Gökpınar Ş (1991). Akuakültürde önemli beş deniz flagellatlatının inorganik N alınımları üzerine sıcaklık değişimlerinin etkisi. The Institute of Marine Sciences and Technolohgy of Dokuz Eylül University. PhD Thesis, İzmir, p. 88.

Guillard RRL (1973). Division rates. In, Stein (ed), Handbook of Phycological Methods,V. 1, Cambridge University Press, Cambridge. pp. 289-312.

Kendirli K (2010). *Spirulina* Kültürlerinde Besin Elementlerinin Farklı Oranlarda Kullanımının Kuru Madde, Protein ve Klorofil-*a* Düzeyine Etkisi, Çukurova University Institute of Natural and Applied Sciences, MSc Thesis. p. 5.

Koru E, Cirik S (2003). *Spirulina platensis* (Cyanophyceae) Mikroalg'inin Büyümesine ve Bazı Biyokimyasal Özelliklerine Sıcaklığın Etkisi. Ege University. J. Fish. Aquat. Sci. 20(3-4): 419-422.

Mubeen, U., Hussain, W., Ikram-ul-Haq and Kauser Abdullah Malik 2011. Study of Native Algal Species for Growth Potential and Lipid Yield. Current Res. J. Biol. Sci. 3(3): 240-245, ISSN: 2041-0778.

Ratana C, Chirasuwan N, Siangdung W (2009). Kalyanee Paithoonrangsarid and Boosya Bunnag, "Cultivation of *Spirulina platensis* Using Pig Wastewater in a Semi-Continuous Process". J. Microbiol. Biotechnol. 20(3): 609-614.

Schlosser UG (1982). Sammlung von Algenkulturen. Berichte der Deutschen Botanischen Gesellschaft. 95: 181-276.

Semina HJ (1978). Treatment of an Aliquot Sample. (Editor; In Sournia, A., Phytoplankton Manual,), UNESCO, Paris. p. 181.

Stanca D, Popovici E (1996). Urea as nitrogen source in modified Zarrouk medium. Review Roum. Biology 41: 25-31.

Ungsethaphand T, Peerapornpisal Y, Whangchai N (2009). Production of *Spirulina platensis* using dry chicken manure supplemented with urea and sodium bicarbonate. Maejo Int. J. Sci.Technol. 3: 379-387.

Ungsethaphand T, Peerapornpisal Y, Whangchai N, Sardsud U (2007). Productivity and chemical composition of *Spirulina platensis* using dry chicken manure as nitrogen sources. Proceedings of the 19th Annual Meeting of the Thai Society for Biotechnology, Bangkok, Thailand, pp. 43-48.

Vonshak A, Abeliovich A, Boussiba S, Arad S, Richmond A (1982). Production of *Spirulina* biomass: effects of environmental factors and population density. Biomass 2: 175-185.

Xu N, Zhang X, Fan X, Han L, Zeng C (2001). Effect of nitrogen source and concentration on growth rate and fatty acid composition of Ellipsoidion sp. (*Eustigmatophyta*). J. Appl. Phycol. 13: 463-469.

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Full Length Research Paper

Efficiency of fatty acid accumulation into breast muscles of chickens fed diets with lycopene, fish oil and different chemical selenium forms

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The purpose of the investigation was to determine the effect of the addition of 12 ppm lycopene (Lyc), 2% fish oil (FO) or 0.25 ppm Se as selenate (SeVI) or selenized yeast (SeY) to an isoenergetic and isonitrogenous basal diet containing sunflower oil (SO) as the source of energy on the concentrations of fatty acids (FA), especially saturated- (SFA), mono- (MUFA) and polyunsaturated (PUFA) acids, in breast muscles of female and male chickens for six weeks. The influence of these additives on the capacity of Δ9-, Δ4- and Δ5-desaturations, the elongation of FA, and the yield of PUFA peroxidation (an oxidative stress) in breast muscles of female and male chickens were also studied. Dietary SeY most efficiently decreased the concentrations of SFA, MUFA and PUFA as well as malondialdehyde (the marker of the oxidative stress) in muscles of female and male chickens. The addition of FO most efficiently increased the concentration of n-3 long-chain PUFA (n-3LPUFA) and most effectively increased the concentration ratio of n-3LPUFA to SFA (n-3LPUFA/SFA), while most effectively decreased the concentration ratio of n-6PUFA to n-3PUFA (n-6PUFA/n-3PUFA) in muscles of chickens that are beneficial to human health. We conclude that further studies are necessary to determine if diets containing other chemical form of selenium compounds and other vegetable oils induce changes in the profiles of fatty acids in muscles of chickens that are beneficial to human health.

Key words: Chicken, lycopene, selenium, fish oil, sunflower oil, breast muscles, fatty acids, malondialdehyde.

INTRODUCTION

There is interest in meat containing higher levels of polyunsaturated fatty acids (PUFA), especially long-chain PUFA (LPUFA), because of their beneficial effects on human health, mainly in the prevention of cardiovascular

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disease (Harris et al., 2007, 2009). For this reason, there are numerous investigations concerning the enrichment of chicken meat with PUFA by the addition of fish or plant oils to diets (Betti et al., 2009a; b; Zuidhof et al., 2009; Rozbicka-Wieczorek et al., 2012).

However, chicken meat enriched with PUFA contains especially LPUFA with a high number of double bonds, which increases the susceptibility of meat to oxidation (Betti et al., 2009b; Cortinas et al., 2005; Rozbicka-Wieczorek et al., 2012). PUFA oxidation causes loss of sensory and nutritional values as well as the formation of potentially toxic species that compromise meat and adipose tissue quality and reduce its shelf life. One such product is malondialdehyde (MDA), which has long been considered as the index of oxidative rancidity. Indeed, MDA as well as other carbonyl compounds are naturally occurring byproducts of PUFA peroxidation and prostaglandin biosynthesis (Cortinas et al., 2005; Czauderna et al., 2011; Urso and Clarkson, 2003). Therefore, the oxidative stability of stored edible carcass parts of farm animals (e.g. pigs, ruminants or poultry) decreases when animals are fed a ration enriched in plant or/and fish oils (Perez et al., 2010; Rahimi et al., 2011; Rozbicka-Wieczorek et al., 2012; Urso and Clarkson, 2003).

Fortunately, the oxidative stability of edible carcass parts of animal can be manipulated by supplements added to a diet. The balance of unsaturated fatty acids (UFA), especially LPUFA, and antioxidants, like lycopenes, seleno-compounds or tocopherols in diets is a fundamental factor affecting the quality of edible carcass parts of farm animals (Betti et al., 2009b; Czauderna et al., 2009, Navarro-Alarcon and Cabrera-Vique, 2008; Rozbicka-Wieczorek et al., 2012; Tapiero et al., 2003; Yu et al., 2008). Indeed, numerous studies documented that the concentration of PUFA, especially phospholipids and cholesterol esters, in living organisms, was positively correlated with the selenium (Se) level in diets (Pappas et al., 2008; Schweizer et al., 2005; Yu et al., 2008). Really, Se is an important part of at least 25 Se-proteins possessing antioxidant, anti-inflammatory and chemoprotective properties; the most important are glutathione peroxidases, iodothyronine deiodinases, thioredoxin reductases, selenoprotein P, selenophosphate synthetase and selenoprotein (Navarro-Alarcon and Cabrera-Vique, 2008; Rayman, 2004; Tapiero et al., 2003).

Another approach to obtain animal products with low levels of lipid oxidation could be the addition of lycopene (Lyc) to diets (Boileau et al., 2013; Heber and Lu, 2002; Rao and Agarwal, 1998). Indeed, Lyc, an open-chain hydrocarbon carotenoid (C40), is the most potent antioxidant among common carotenoids (Boileau et al., 2013); Lyc can trap singlet oxygen and reduce mutagenesis and the risk of chronic diseases such as cardiovascular diseases (Rao and Agarwal, 1998). Moreover, Lyc has been found to inhibit the proliferation of several types of cancer cells, including those of the breast, lung and endometrium (Heber and Lu, 2002). Recent investigations have found that the influence of dietary Se (as high-selenized yeast (SeY) or and selenate (SeVI) affected the concentration of MDA, Lyc and/or fatty acids, especially PUFA, in tissues of experimental animals (Cortinas et al., 2005; Czauderna et al., 2009; Perez et al., 2010; Rao and Agarwal, 1998; Rozbicka-Wieczorek et al., 2012). Therefore, we hypothesized that dietary Lyc or Se as SeY and SeVI stimulate the accumulation of UFA, especially LPUFA, while decrease the concentration of MDA in breast muscles of chickens. Moreover, we intended to compare the impact of dietary anti-oxidants (that is, Lyc, SeY and SeVI) on the accumulation of UFA in breast muscles with dietary FO.

Therefore, the aim of the present study was to explore the effect of dietary Lyc, SeY, SeVI and FO on the concentration of UFA and the size of the oxidative stress in breast muscles of female and male chickens. The use of chickens significantly lowered the cost of our preliminary investigations.

MATERIALS AND METHODS

Birds, housing, nutrition and experimental design

One hundred and eighty two (182) one-day-old non-sexed hybrid Ross-308 chicks were obtained from a commercial hatchery and raised in five pens (groups) with 35 to 38 chicks per pen at the Poultry Research Station of the National Research Institute of Animal Production in Kraków-Balice (Poland). The experiment was carried out in accordance with established standards for use of birds. The chick room temperature, air exchange and humidity were maintained according to the recommendations for zoo-hygiene for young chickens. The protocol was approved by the local ethics and scientific authorities. Chicks were kept in metal cages (18 birds/m^2), on litter from deciduous trees. After 21 days of the experiment, the number of birds in pens was controlled, so, as to be in line with the Council Directive 2007/43/EC. Thus, at the end of the experiment, there were approximately 33 kg of live weight of chickens per m^2. Throughout the study, feed and water were provided for consumption *ad libitum*. Starter (1 to 21 days) and grower (22 to 42 days) rations were formulated based on maize-wheat-soyabean meal as was presented in our previous publication (Rozbicka-Wieczorek et al., 2012).

Basal and experimental diets were formulated to be isoenergetic and isonitrogenous. All rations contain sunflower oil (SO) as the source of energy (Table 1). The mean fatty acid composition (%) of dietary SO was as follows: C12:0 ~0.05, C14:0 ~1.9, C16:0 5.4, C16:1 0.3, C17:0 0.2, C17:1 ~0.03, C18:0 2.7, cis9C18:1 (*c9*C18:1) 39.1, *c9c12*C18:2 48.3, *c9c12c15*C18:3 0.3, C20:0 0.25, C20:1 0.14, C22:0 0.9, C22:1 0.15, C22:2 ~0.05, C24:0 0.25, C24:1 ~0.27. Basal and experimental diets were administered as a powder. During the experiment, feed consumption was monitored and feed intake was calculated per kg body weight gain of chickens; mortality was monitored. On the fifth day of breeding, the chicks were vaccinated against Goomboro disease and on the twelfth day, against the Newcastle disease (NCD). The vaccines against Goomboro and NCD were supplied by CEVAC® IBD L and Intervet

Table 1. The experimental design and the composition of the control and experimental diets.

Group	Additives added to the basal diet
I Control (negative)	4% SO[1]
II	4% SO and 12 ppm Lyc[2]
III	4% SO and 0.25 ppm Se as SeVI[3]
IV	4% SO and 0.25 ppm Se as SeY[4]
V	2% SO and 2% FO[5]

[1]SO, Sunflower oil; the iodine value of SO: 119 to 135; the acid value of SO: 1.3 mg KOH/g SO; gross energy of SO: 23.1 MJ Kg^{-1}; [2]Lyc, lycopene; [3]SeVI, sodium selenate; [4]SeY selenized yeast; [5]FO, fish oil; the iodine value of FO: 50 to 65 g/100 g FO; the acid value of FO: 20 mg KOH/g FO; the fatty acid profile of FO: C14:0 3.3%; C14:1 0.3%; C15:0 0.2%; C16:0 10.5%; C16:1 3,9%; C16:2 0.3%; C16:3 0.2%; C17:0 0.2%; C17:1 0.1%; C18:0 2.5%; *trans*C18:1 0.5%; *cis9*C18:1 31.8%; *cis11*C18:1 3.0%; other *cis*C18:1 0.4%; C18:2n-6 10.4%; C18:3n-3 3.9%; C20:0 0.3%; C18:4n-3 1.2%; *cis11*C20:1 5.9%; *cis11cis14*C20:2 0.9%; *cis8cis11cis14*C20:3 0.2%; C20:4n-6 0.3%; C20:5n-3 4.0%; C22:0 0.1%; *cis13*C22:1 6.6%; C22:4n-6 0.3%; C22:5n-3 1.6%; C22:6n-3 5.6%; C24:0 0.1%; C24:1 0.5% (the total PUFA abundance in FO: 42%); poultry digestible energy: 31.46 MJ Kg^{-1}; poultry gross energy: 34.2 MJ Kg^{-1}.

Sp. z o.o. (Poland), respectively. For a total of eight times in intervals of a few days during the entire experimental period, the chickens were fed a vitamin mixture, "Vitasol" (as a lyophilizate in drinking water). 'Vitasol' (BIOWET DRWALEW S.A., Poland) stimulated survival and the weight gain of birds. The body weight of chickens was determined after 21 and 42 days of the experiments; before each weighing, chickens were fasted for 12 h. Prior to feeding chickens, all experimental diets (that is, starter and grower) were supplemented with 12 ppm Lyc, 0.25 ppm Se (as SeVI or SeY) or 2% FO; the experimental design is shown in Table 1. The ingredients in the rations were determined by chemical analysis (AOAC, 2005).

On day 43 of the experimental period, 12 birds from each group, six male chickens (♂) and six female chickens (♀), were randomly selected. These birds were slaughtered by decapitation after stunning. The right breast muscles were quickly removed, weighed, homogenized and frozen at -32°C until chemical analyses. Each right breast muscle was analysed individually.

Reagents

All organic solvents were of high performance liquid chromatography (HPLC) grade and chemicals were of analytical grade. KOH, NaOH, dichloromethane (DCM), Na_2SO_4 and conc. HCl were purchased from POCH (Gliwice, Poland). Methanol, acetonitrile, n-hexane and n-heptane (99%, GC) were supplied by Lab-Scan (Ireland), whereas the CLA isomer mixture (2.1% *tt*CLA, 7.1% *c11t13*CLA, 40.8% *c9t11*CLA, 41.3% *t10c12*CLA, 6.7% *c8t10*CLA and 2.0% *cc*CLA) by the Industrial Chemistry Research Institute (Warsaw, Poland). Fatty acid methyl ester standards, sodium selenate (SeVI), and 25% BF_3 in methanol were purchased from Supelco and Sigma-Aldrich Co. (St. Louis, MO, USA). SeY (Se-*Saccharomyces cerevisiae*) was donated by Sel-Plex (non-commercial yeast sample; Alltech Inc., USA). About 83% of the total selenium content of SeY represents Se in the form of seleno-methionine (Se-Met) and 5% in the form of seleno-cysteine (Se-Cys) incorporated into the proteins of *S. cerevisiae* (Rayman, 2004); the chemical composition of the selenized yeast was presented in our previous publication (Czauderna et al., 2009). Trichloroacetic acid, 2,6-di-tert-butyl-p-cresol, 25% aqueous 1,5-pentanedi-aldehyde (PDA) solution, 2,4-dinitrophenylhydrazine (DNPH), containing about 30% water and 1,1,3,3-tetra-methoxy-propane (99%) were supplied by Sigma-Aldrich (St. Louis, MO, USA). Lycopene (Lyc), 10% in sunflower oil (LycoVit Dispersion 10%; Product-No. 30264388) was donated by BASF, the Chemical Company (Germany). SO was donated by Company AGROSOL (Pacanów, Poland), while FO, by Meals Manufacturing Company ZPM (Bokiny-Łapy, Poland). Water used for the preparation of mobile phases and chemical reagents was prepared using an Elix™ water purification system (Millipore). The mobile phases were filtered through a 0.45 µm membrane filter (Millipore).

Saponification and gentle base- and acid-catalyzed methylation of fatty acids

Homogenized chicken breast muscle samples (45 to 55 mg) were placed in vials and treated with a mixture of 2 ml of 2 M KOH in water and 2 ml of 1 M KOH in methanol. Next, 50 µl of the internal standard (IS) solution (17 mg ml^{-1} nonadecanoic acid in chloroform) were added to the obtained mixture. The resulting mixture was flushed with argon (Ar) for ~4 min. The vial was then sealed and the mixture vortexed were heated under Ar at 95°C for 10 min, cooled for 10 min at room temperature, and sonicated for 10 min. The resulting mixture was protected from the light and stored in the sealed vial under Ar at ~22°C overnight. Next, 3 ml of water were added to the hydrolysate and the solution was again vortexed. The obtained solution was acidified with 4 M HCl to ~pH 2 and free fatty acids were extracted four times with 3 ml of DCM. Extraction was repeated four times using 3 ml of n-hexane. The upper n-hexane layer was combined with the DCM layer, and next the resulting organic phase was dried with ~0.1 g of Na_2SO_4. The organic solvents were removed under a stream of Ar at room temperature. The obtained residue (I) was stored at -20°C until gentle base- and acid-catalyzed methylation of free fatty acids. 2 ml of 2 M NaOH in methanol were added to the residue (I) while mixing, then flushed with Ar, and reacted for 1 h at 40°C. After cooling the reaction mixture to ~4°C, 2 ml of 25% BF_3 in methanol were added, flushed with Ar, and heated for 1 h at 40°C. To the cooled reaction mixture 5 ml of water were added and then methylated fatty acids (FAME) were extracted with 5 ml of n-hexane. The supernatant was transferred to a GC vial.

Gas chromatographic equipments and analyses of FAME

The analyses of all FAME were performed on a SHIMADZU GC-MS-QP2010 Plus EI equipped with a BPX70 fused silica capillary column (120 m × 0.25 mm i.d. × 0.25 µm film thickness; SHIM-POL), quadrupole mass selective (MS) detector (Model 5973N) and injection port. Helium as the carrier gas operated at a constant pressure (223.4 kPa) and flow rate of 1 ml/min. Injector and MS detector temperatures were maintained at 200 and 240°C, respectively. The FAME profile in a 1 µl sample at a split ratio of 10:1 was determined using the column temperature gradient programme. The oven temperature was programmed as follows: initially 70°C for 4 min, then increasing by 12°C/min to 150°C, held for 6 min, programmed at 8°C/min to 168°C, held for 27 min, programmed at 0.75°C/min to 190°C, held for 10 min, programmed at 1.8°C/min to 210°C, held for 15 min, programmed at 6°C/min to 234°C, held for 4 min, programmed at 6°C/min to 236°C, held for 20 min. FAME identification was validated based on electron impact ionization spectra of FAME and compared with authentic FAME

standards and NIST 2007 reference mass spectra library.

Derivatisation of MDA in breast muscles, liquid chromatographic equipments and analyses

The MDA concentrations in muscle were determined after saponification followed by derivatization with DNPH to MDA-DNPH according to Czauderna et al. (2011). The chromatographic separation of MDA-DNPH from muscles was conducted using an ultra-fast liquid chromatography system (SHIMADZU, Kyoto, Japan), incorporating two LC-20AD$_{XR}$ liquid chromatographic pumps (UFLC$_{XR}$), a SIL-20AC$_{XR}$ autosampler (LFLC$_{XR}$), a CBM-20A communications bus module (UFLC), a CTO-20A column oven, a DGU-20A5 degasser, and a SPD diode array detector. The column was a Phenomenex C18-column (Synergi 2.5 µm, Hydro-RP, 100 Å, 100 mm in length) with an inner diameter of 2 mm. MDA in samples was analysed using a linear gradient of acetonitrile in water and the photodiode detector was set to 307 nm for UV detection (Czauderna et al., 2011). The concentration of MDA was calculated based on the fresh weight of breast muscle samples.

Statistical analyses

Results are presented as means of individually analysed samples of breast muscles. Mean values in columns having the same superscripts are significantly different at $^{a,b}P<0.05$ and $^{A,B}P<0.01$, while differences at $^{\alpha,\beta}P<0.1$ are indicated as tendencies. These one-factorial statistical analyses of the effects of additives (Lyc, SeVI, SeY or OF) in the ration were conducted using the non-parametric Mann-Whitney U test for comparing independent experimental groups. Statistical analyses were performed separately between the female chicken groups and between male chicken groups. Statistical analyses were performed using the Statistica v. 10 software package (Statistica, 2010).

RESULTS AND DISCUSSION

In the current study, neither macroscopic lesions nor pathological changes were found in the internal organs and muscles and adipose tissues of chickens fed the experimental diets enriched with Lyc, SeVI, SeY or FO (Table 1). Indeed, only long-term consumption of Se compounds, particularly selenite or selenide, at rates of more than 5 ppm Se can be teratogenic and hepatotoxic in animals and humans (Navarro-Alarcon and Cabrera-Vique, 2008; Tapiero et al., 2003). On the other hand, SeVI is less reactive and toxic in living organisms. Moreover, seleno-methionine (Se-Me), the predominant chemical form of Se in dietary SeY, is least reactive, as $tRNA_{Met}$ does not discriminate between Se-Met and methionine. Therefore, dietary Se-Met (derived from SeY) is incorporated into body protein in place of methionine (Rayman, 2004; Tapiero et al., 2003). Se-Met as Se-Met residue in proteins is a stable and safe-storage mode for Se in the body of animals and humans. Moreover, our recent studies with chickens documented that feeding chickens the experimental diets containing extra Se (like SeY) increased the average live body weight and breast muscle masses of chickens compared with the control chickens (Rozbicka-Wieczorek et al., 2012). In line with the above, we found also that SeY added to a diet improved feed conversion efficiency, stimulated the body mass gain and protein synthesis (the repartition) in body of experimental animals (Czauderna et al., 2009; Rozbicka-Wieczorek et al., 2012). These observations are consistent with the effect of the diet enriched in SeY (group IV) on the decrease in the concentration sum of all assayed fatty acids (ΣFA) in breast muscles of chickens (Table 2). Similarly, our newest studies documented that the diet with SeY resulted in the lower concentration of ΣFA in thigh muscles of female and male chickens compared with the control birds (Rozbicka-Wieczorek et al., 2014).

Effect of the experimental diets on the saturated fatty acid profile of breast muscles

The effects of the experimental diets on the concentration of saturated fatty acids (SFA) in chicken breast muscles are summarized in Table 2. The addition of SeY to the diet (Groups IV) resulted in the decrease in the concentrations of C14:0, C16:0 and C18:0 as well as the concentration sum of SFA and atherogenic (A-SFA) and thrombogenic (T-SFA) saturated fatty acids in muscles of female and male chickens in comparison with the control birds and usually other experimental female and male chickens (Groups II, III and V). Similarly, the diet with SeY most efficiently decreased the concentration sum of assayed FA (ΣFA) in muscles of female and male chickens. In line with the above, our recent studies revealed also that dietary extra SeY decreased the concentrations of SFA, A-SFA and T-SFA in thigh muscles of female and male chickens compared with the control birds (Rozbicka-Wieczorek et al., 2014). The values of the A-SFA and T-SFA indexes ($_{index}A^{SFA}$ and $_{index}T^{SFA}$) (Ulbricht and Southgate, 1991) in muscles of chickens fed the diets with SeIV, SeY or FO were greater, however, than those in the control birds and the chickens fed with the diet Lyc (Group II). These findings are due to the concentrations of PUFA, including n-6PUFA, n-3PUFA, being usually more significantly decreased in muscles of SeVI-, SeY- or FO-fed chickens (Groups III, IV and V) compared with those in the control birds or Lyc-fed chickens (Group II). As can be seen from the data summarized in Table 2, the addition of SeY or FO to the diets (Groups IV and V) also increased the concentration ratios of A-SFA or T-SFA to ΣFA (that is, A-SFA/ΣFA and T-SFA/ΣFA) in muscles of chickens in comparison with the control group and other experimental groups. These results are due to the concentration of ΣFA being usually more significantly decreased in muscles compared with the muscle concentrations of A-SFA and T-SFA in SeY- or FO-fed chicken.

Our results show more profound effects of dietary SeY

Table 2. The concentration of C14:0, C16:0, C18:0, the sum of saturated fatty acids (SFA), atherogenic (A-SFA) and thrombogenic (T-SFA) saturated fatty acids, indexes of A-SFA ($_{index}A^{SFA}$)[1] and T-SFA ($_{index}T^{SFA}$)[2], and ratios of A-SFA to ΣFA[3] and T-SFA to ΣFA in breast muscles[4].

Group		C14:0 µg/g	C16:0 mg/g	C18:0 µg/g	SFA[5] mg/g	A-SFA[6] mg/g	A-SFA / ΣFA[2]	$_{index}A^{SFA}$	T-SFA[7] mg/g	T-SFA / ΣFA	$_{index}T^{SFA}$	ΣFA[3] mg/g
I Negative Control	♂	28^{β}	$1.34^{B\alpha d}$	598^{α}	2.06	1.39	0.202^{cd}	0.291^{IJKL}	1.97	0.287	0.632^{GHIc}	6.86^{df}
	♀	47^{B}	1.67^{A}	689^{Bgh}	2.51^{Bde}	$1.72^{Ad\alpha}$	$0.197^{EF\beta\alpha}$	$0.288^{\beta OP}$	2.41^{Ade}	$0.276^{\sigma cd}$	$0.607^{KL\alpha}$	8.73^{Be}
	Σ[8]	38^{A}	1.51^{a}	644^{Aab}	$2.29^{A\alpha}$	1.55^{a}	0.199^{ABa}	0.289^{ABCa}	2.20^{a}	0.281^{AB}	0.618^{ABCD}	7.79^{Aa}
II	♂	37^{σ}	$1.71^{\sigma B}$	675^{ef}	2.53^{c}	1.78^{α}	0.205^{ef}	0.295^{IMN}	2.42^{α}	$0.279^{\beta\Delta b}$	0.648^{cdeJ}	8.68^{f}
	♀	34^{Δ}	0.97^{AC}	466^{B}	1.54^{BC}	1.01^{AB}	$0.209^{GH\beta}$	$0.316^{\Delta R}$	1.47^{AB}	$0.305^{\sigma\gamma}$	$0.680^{\alpha\beta f}$	$4.83^{B\alpha}$
	Σ	$35^{a\alpha}$	1.34^{b}	570^{c}	2.04^{a}	1.39^{b}	0.206^{CD}	0.303^{DEFGa}	1.95^{b}	0.288^{CD}	0.660^{AEab}	6.76^{b}
III	♂	$21^{\sigma c}$	1.24	523	1.87	1.27	0.213	$0.310^{J\alpha}$	1.79	0.301^{β}	0.737^{Gd}	5.95
	♀	42	$1.41^{C\mu}$	$563^{\beta\delta}$	$2.10^{CD\beta}$	$1.45^{B\sigma}$	0.211^{α}	$0.318^{\beta c}$	$2.01^{B\beta}$	0.294^{e}	0.687	$6.85^{\alpha\beta}$
	Σ	31^{α}	1.33^{c}	543^{ad}	1.98^{b}	1.36^{c}	0.212^{ab}	0.315^{AHb}	1.90^{c}	0.297^{a}	0.710^{Ba}	6.40^{c}
IV	♂	20^{d}	$1.00^{\sigma\alpha d}$	$399^{\alpha e}$	1.48^{c}	1.02^{α}	0.225^{ce}	0.338^{KM}	1.42^{α}	0.312^{Δ}	0.757^{HJ}	4.55^{d}
	♀	$17^{B\Delta}$	0.99	$408^{g\beta}$	1.46^{dD}	1.00^{α}	0.229^{EG}	$0.341^{O\Delta}$	1.41^{d}	0.322^{c}	0.763^{Kfg}	4.39^{e}
	Σ	19^{Aab}	0.99^{abc}	403^{Acd}	1.47^{Aab}	1.01^{abc}	0.227^{ACb}	0.339^{BDFb}	1.42^{abc}	$0.317^{ACa\alpha}$	0.760^{CEF}	4.47^{Abc}
V	♂	$42^{\beta cd}$	1.41	536^{f}	2.04	1.46	0.228^{df}	$0.356^{LN\alpha}$	1.99	0.311^{b}	0.707^{le}	6.40
	♀	28	0.99^{μ}	$449^{h\delta}$	$1.51^{e\beta}$	$1.02^{d\sigma}$	0.224^{FH}	0.353^{PRc}	$1.46^{e\beta}$	$0.323^{d\gamma e}$	$0.711^{L\beta g}$	$4.54^{d\beta}$
	Σ	35^{b}	1.20	492^{b}	1.72^{α}	1.12	0.226^{BD}	0.360^{CEGH}	1.73	$0.317^{BD\alpha}$	0.710^{DFb}	5.47^{a}

[1]The atherogenic index = (C12:0 + 4*C14:0 + C16:0)/(MUFA +PUFAn-6 + PUFAn-3); [2]the thrombogenic index = (C14:0 +C16:0+ C18:0)/0.5*MUFA + 0.5*PUFAn-6 + 3*PUFAn-3 + PUFAn-3/PUFAn-6); [3]the sum of all assayed fatty acids; [4]mean values in columns having the same superscripts are significantly different at $^{a,b}P<0.05$ and $^{A,B}P<0.01$; [5]SFA, the concentration sum of C8:0, C10:0, C12:0, C14:0, C16:0, C18:0, C20:0, C22:0 and C24:0; [6]A-SFA: C12:0+C14:0+C16:0; [7]T-SFA: C14:0+C16:0+C18:0; [8]the concentration of FA in muscles of broilers of both sexes (Σ).

On the concentration of ΣFA in muscles (Table 2). Indeed, it has been shown that dietary SO, rich in *c9c12*C18:2, act synergistically with SeY towards the activation of the carnitine palmitoyltransferase 1 (CPT1) gene through the peroxisomes proliferator-activated receptor a (PPARa) (Stahle et al., 2009). Thus, we argue that the substantial decrease in the concentrations of SFA, MUFA and PUFA in thigh muscle of SeY supplemented chickens may be the upregulation of CPT1, with increased activity of PPARa (Goto et al., 2011). In addition, a study on laboratory animal lymphocytes showed that dietary Se addition was effective for improving β-oxidation (Kuryl et al., 2008). In line with the above, we found that dietary extra SeY also significantly reduced the concentrations of SFA, MUFA and PUFA in breast muscles of female and male chickens compared with the control birds (Rozbicka-Wieczorek et al., 2012). Moreover, our results show more profound dietary effects of SeY than SeVI on the abundance of SFA, MUFA and PUFA in breast muscles (Tables 2 to 5). This may reflect the lover yield of accumulation of Se in the body of

Table 3. The concentration of *c9*C18:1, the sum of monounsaturated fatty acids (MUFA), *c9t11*CLA (*c9t11*), linoleic acid (LA), α-linolenic acid (αLNA), *c5c8c11c14*C20:4 (AA), *c7c10c13c16c19*C22:5 (DPA), *c4c7c10c13c16c19*C22:6 (DHA), n-6PUFA (n-6) and n-3PUFA (n-3) in breast muscles.

Group		*c9*C18:1 mg/g	MUFA mg/g	Desaturase index $_{C18:0}\Delta9$ [3]	Desaturase index $_{C16:0}\Delta9$ [4]	*c9t11* µg/g	LA mg/g	AA µg/g	αLNA µg/g	DPA µg/g	DHA µg/g	n-6 mg/g	n-3 µg/g
I	♂	2.11^{g}	2.63^{bcd}	0.779	0.021^{a}	-	1.63^{α}	259^{Ja}	97^{abc}	33^{GHc}	22^{Fc}	2.18^{π}	238^{bc}
Negative	♀	2.90^{Bef}	3.63^{Eef}	0.808^{bc}	0.039^{HIJ}	-	2.02^{Bfg}	237^{Oc}	140^{FGef}	$36^{Ke\Delta}$	$30^{K\Delta\pi c}$	$2.54^{gh\Delta}$	$294^{KLd\alpha}$
Control	Σ[2]	$2.51^{A\alpha\alpha ab}$	3.13^{ABCD}	$0.796^{A\alpha\Delta a}$	$0.030^{ABa\alpha}$	-	1.82^{Aab}	248^{AB}	119^{ABCa}	35^{ABC}	$26^{ABa\sigma}$	2.36^{ab}	266^{ABa}
II	♂	2.42^{β}	2.68^{α}	0.782	0.034^{FGab}	-	2.50^{ef}	296^{KLM}	$82^{\Delta Ed}$	$24^{\alpha d}$	18^{G}	$3.14^{\pi fg}$	214^{GH}
	♀	1.17^{Δ}	1.58^{E}	0.715^{b}	0.039^{KL}	-	1.23^{B}	250^{RS}	38^{e}	28^{β}	$13^{\Delta L}$	$1.78^{g\delta\beta}$	164^{de}
	Σ	$1.79^{\alpha c}$	2.13^{A}	0.759^{α}	$0.036^{CDE\alpha}$	-	1.86^{c}	273^{CD}	$60^{Da\alpha}$	26^{Dab}	$15^{C\sigma}$	2.46^{cd}	189^{CDa}
III	♂	1.50	$1\ 87^{b}$	0.742	0.016^{F}	1.75^{b}	1.82	221^{KNb}	$40^{a\Delta}$	$10^{GI\alpha}$	10^{Hc}	2.30	115^{Glb}
	♀	1.83^{Δ}	2.32^{Δ}	0.764	$0.016^{HK\beta}$	1.83	$1.94^{\Delta\beta}$	227^{T}	$59^{fg\delta}$	14^{eL}	24^{π}	$2.44^{\delta\mu}$	183^{α}
	Σ	1.67^{ad}	2.09^{Ba}	0.754^{Δ}	0.016^{AC}	1.79^{a}	1.88^{ad}	224^{CFH}	49^{Ab}	12^{AEa}	17^{ADb}	$2.37^{e\sigma}$	149^{ACE}
IV	♂	1.21^{β}	$1.55^{c\alpha}$	0.753	0.019^{b}	2.68	$1.20^{\alpha e}$	160^{Lab}	25^{Eb}	13^{HJ}	14^{I}	1.55^{f}	111^{HJc}
	♀	1.16^{e}	$1.47^{e\Delta}$	0.741^{c}	0.014^{IL}	3.11	$1.11^{f\Delta}$	182^{RZc}	$23^{F\delta}$	$11^{KN\beta}$	$18^{Nc\chi}$	1.50^{Δ}	120^{KNe}
	Σ	1.19^{Acd}	1.51^{Ca}	0.747^{A}	0.017^{Da}	2.89	1.15^{Acd}	171^{ADFI}	$24^{BDb\beta}$	12^{BFb}	16^{Eab}	1.52^{ace}	115^{BDF}
V	♂	1.44^{g}	1.90^{d}	0.777	0.017^{G}	5.84^{b}	1.55^{f}	124^{JMN}	46^{cd}	52^{IJcd}	85^{FGI}	1.85^{g}	236^{IJ}
	♀	1.24^{f}	1.56^{f}	0.735^{d}	$0.014^{J\beta}$	3.89	$1.10^{g\beta}$	109^{OSTZ}	29^{Gg}	$43^{\Delta LN}$	$72^{KLN\chi}$	$1.36^{h\beta\mu}$	200^{LN}
	Σ	1.34^{b}	1.73^{D}	0.748^{a}	0.015^{BE}	4.85^{a}	1.32^{b}	113^{BEHJ}	$37^{C\alpha\beta}$	47^{CDEF}	78^{BCDE}	$1.60^{bd\alpha}$	217^{EF}

[1]Mean values in columns having the same superscripts are significantly different at $^{a,b}P<0.05$ and $^{A,B}P<0.01$; [2]the concentration of FA in muscles of broilers of both sexes; [3] Δ9-desaturase index ($_{C18:0}\Delta9$ index) = *c9*C18:1/(*c9*C18:1+C18:0); [4]Δ9-desaturase index ($_{C16:0}\Delta9$ index) = *c9*C16:1/(*c9*C16:1+C16:0); [5]below the quantification limit (L_Q).

chickens fed with the diet enriched in SeVI than SeY (Rayman, 2004).

Influence of the experimental diets on the monounsaturated fatty acid profile of breast muscles

As can be seen from the results summarized in Table 3, the addition of SeVI, SeY or RO to the diet resulted in the decrease in the concentration of MUFA, including *c9*C18:1, compared with the control birds and Lyc-fed chickens. Interestingly, SeY added to the diet most efficiently decreased the concentration of MUFA, including *c9*C18:1, in breast muscles. The presented experiments document the significant negative influence of dietary SeVI and SeY on the capacity of Δ 9-desaturation of C18:0 and C16:0 compared with the control birds and Lyc-fed chickens. Thus, the current studies support our earlier investigations in which dietary SeVI and SeY also decreased the capacity of Δ9-desaturase (that is, stearoyl-CoA desaturase-1) in thigh muscles of broiler chickens and rats (Czauderna et al., 2009; Rozbicka-Wieczorek et al., 2014). Our recent studies (Rozbicka-Wieczorek et al., 2012) and the current investigation also reinforce the finding that the yield of Δ9-desaturation depends on the length of the carbon atom chain of desaturated fatty acids (Table 3). Indeed, the values of the Δ9-desaturase index for C18:0 (substrate of Δ9-desaturase) are higher than the values of this

Table 4. The concentration of long-chain PUFA (LPUFA), n-6LPUFA (n-6_{LPUFA}), n-3LPUFA (n-3_{LPUFA}) and values of the ratio of n-6PUFA/n-3PUFA (n-6/n-3), the elongase index, Δ4- and Δ5-desaturase indexes ($\Delta4_{index}$, $\Delta5_{index}$), the ratios of PUFA, LPUFA, n-6_{LPUFA} and n-3_{LPUFA} to SFA in breast muscles[1].

Group		LPUFA µg/g	LPUFA, µg/g		n-6 n-3	elongase index[3]	$\Delta4_{index}$[4]	$\Delta5_{index}$[5]	PUFA SFA	LPUFA SFA	n-6_{LPUFA} SFA	n-3_{LPUFA} SFA
			n-6_{LPUFA}	n-3_{LPUFA}								
I	♂	$438^{d\alpha}$	297^{cd}	$141^{I\beta a}$	9.2^{Fe}	0.588^{Ec}	$0.393^{B\alpha}$	0.882^{de}	1.047^{b}	0.212	0.144^{FGHI}	$0.068^{\Delta Eb}$
Negative	♀	434^{Hf}	280^{NO}	$154^{\Delta R}$	$8.6^{\beta f}$	0.480^{c}	0.457	0.846^{h}	1.035^{β}	$0.173^{\delta\Delta}$	0.122^{J}	0.061^{c}
Control	Σ[2]	436^{ABa}	288^{ABa}	$147^{ABC\alpha}$	8.9^{ABab}	0.539^{Aa}	0.428^{Aa}	0.865^{a}	1.040^{Aa}	0.191^{α}	$0.126^{ABC\alpha}$	0.064^{Aa}
	♂	$475^{FG\alpha e}$	343^{Ie}	132^{JKb}	14.7^{Ge}	0.646^{F}	0.422^{β}	$0.863^{\alpha f}$	1.221^{bcF}	0.187	0.136^{Fc}	0.052^{Δ}
II	♀	424^{Jg}	298^{RS}	$126^{\Delta\delta e}$	$10.8^{\beta\Delta g}$	0.603^{I}	0.317	0.838^{δ}	1.117^{de}	$0.275^{A\delta}$	$0.193^{JLK\Delta}$	$0.082^{\mu\gamma}$
	Σ	449^{CDE}	321^{CDE}	$129^{DEF\alpha}$	13.0^{Acc}	$0.624^{B\alpha}$	0.371^{b}	0.851^{b}	1.181^{ABC}	$0.221^{\alpha a}$	0.157^{ADa}	$0.063^{B\alpha}$
	♂	328^{Fid}	253^{Je}	76^{IJL}	20.0^{FH}	$0.750^{c\Delta G}$	0.489^{δ}	0.875^{g}	1.185^{α}	0.176	0.135^{Gd}	0.040^{EF}
III	♀	393^{i}	269^{fU}	124^{λ}	$13.3^{Ff\Delta}$	0.711^{J}	0.644	0.846^{β}	1.156^{fg}	0.187^{A}	0.128^{L}	$0.059^{d\mu}$
	Σ	361^{Cab}	261^{CGHa}	100^{ADG}	15.9^{BDcd}	$0.729^{C\ a\alpha b}$	0.592^{c}	0.860^{c}	1.169^{DE}	$0.182^{a\beta}$	0.131^{Bab}	$0.050^{Ca\alpha\beta}$
	♂	$276^{G\beta}$	190^{Ic}	$86^{N\beta b}$	14.0^{α}	$0.621^{\Delta H}$	$0.509^{\alpha d}$	$0.841^{Bdg\alpha}$	1.032^{c}	0.187	0.129^{He}	0.058
IV	♀	304^{HJi}	207^{NRfh}	$97^{RO\delta}$	12.5^{μ}	0.646^{K}	0.634	$0.882^{h\delta\beta\lambda}$	0.999^{df}	0.209^{Δ}	$0.142^{\Delta\lambda}$	0.066^{σ}
	Σ	290^{ADbc}	199^{ADGb}	91^{BEH}	13.2^{Ead}	0.633^{Db}	0.574^{Aa}	0.862^{A}	1.016^{BD}	0.198^{β}	0.135^{CE}	0.062^{BD}
	♂	$363^{e\beta}$	173^{Jd}	190^{KLNa}	$7.8^{GH\alpha}$	0.241^{EFH}	$0.619^{B\beta\delta d}$	0.816^{ef}	$0.972^{F\alpha}$	0.178	0.085^{Icde}	0.092^{Fb}
V	♀	324^{fg}	153^{OSUh}	$171^{Oe\lambda}$	$6.8^{g\mu}$	0.259^{IJKc}	0.625	0.834^{λ}	$0.979^{\beta eg}$	0.215	$0.102^{K\lambda}$	$0.113^{cd\gamma\sigma}$
	Σ	339^{BEc}	160^{BEHb}	180^{CFGH}	7.3^{CDEFb}	0.253^{ABCD}	0.621^{bc}	0.820^{ABabc}	0.975^{CEa}	0.209	$0.098^{DEb\alpha}$	0.103^{ABCD}

[1]Mean values in columns having the same superscripts are significantly different at $^{a,b}P<0.05$ and $^{A,B}P<0.01$; [2]the concentration of FA in muscles of broilers of both sexes; [3]the elongase index = the concentration ratio: C24:5n-3/(C24:5n-3+C22:5n-3); [4]Δ4-desaturase index ($\Delta4_{index}$) = C22:6n-3/(C22:6n-3+C22:5n-3); [5]Δ5-desaturase index ($\Delta5_{index}$) = C20:4n-6/(C20:4n-6+C20:3n-6).

index for C16:0 and C14:0 (that is, $_{C18:0}$ Δ9 > $_{C16:0}$ Δ9 > $_{C14:0}$ Δ9; Table 3). Consequently, the values of the Δ9-desaturase index for C14:0 are close to zero (that is, 0.0011 to 0.0007; data not shown). Our results indicate that the diet enriched in FO, reach in LPUFA, also decreased the Δ9-desaturase index, especially for C16:0 ($_{C16:0}$ Δ9) compared with the control female and male chickens.

Considering the above results, we suggest that dietary FO-long-chain fatty acids (e.g.: *c11*C20:1, *c11c14*C20:2, C20:4n-6, C20:5n-3, *c13*C22:1, C22:4n-6, C22:5n-3, C22:6n-3; Table 1) decreased the capacity of Δ9-desaturase, elongation and Δ5-desaturase in muscles of female and male chickens compared with the control birds (Tables 3 and 4). On the other hand, it was found that the addition of Lyc to the diet decreased the Δ9-desaturase index for C18:0 in muscles of female chickens, whereas it increased the Δ9-desaturase indexes for C18:0 and C16:0 in muscles of male chickens (Group II) compared with the control chickens. As consequence, dietary Lyc resulted in the decrease in the concentration of MUFA, including *c9*C18:1 in muscles of female chickens, while increased in muscles of male chickens compared with the control birds.

Influence of the experimental diets on the polyunsaturated fatty acid profile of breast muscles

As can be seen from the data summarized in Tables 3 to 5, the addition of SeY to the diet decreased the concentrations of n-6PUFA, n-3PUFA, including n-6LPUFA and n-3LPUFA, as well as the concentration sum of PUFA and LPUFA in muscles in comparison with the control birds. Fortunately, the diet enriched in SeY revealed negligible influence on the concentration ratios of PUFA/SFA, LPUFA/SFA, n-6LPUFA/SFA and n-3PUFA/SFA in muscles of chickens

Table 5. The concentration of malondialdehyde (MDA), and the concentration sum of PUFA, and the MDA indexes in breast muscles[1].

Group		MDA ng/g	MDA/(FA-PUFA) (ng/g)/(mg/g) ($^{simple}MDA_{index}$)[2]	Peroxidation index (MDA_{index})[3]	PUFA mg/g
I	♂	$1.34^{E\alpha ef}$	0.285^{EFG}	1.620^{FGef}	2.16^{μ}
Negative	♀	1.92^{IKL}	$0.313^{de\beta}$	1.741	$2.59^{ef\sigma}$
control	Σ	1.63^{Aab}	$0.300^{Aab\alpha}$	1.685^{ABa}	$2.38^{a\alpha}$
	♂	$1.50^{\alpha gF}$	0.268^{Hic}	1.485^{HIJeg}	$3.09^{\mu cd}$
II	♀	1.85^{NMO}	0.504^{LNdf}	1.899	$1.72^{e\lambda}$
	Σ	1.68^{Bcd}	0.386^{Ba}	1.697^{Cbc}	$2.41^{A\beta}$
	♂	1.71^{Eg}	0.428^{EHJ}	1.763^{HKfhi}	2.21
III	♀	1.14^{INR}	$0.258^{Lgh\beta\gamma}$	1.469	$2.43^{\lambda\delta}$
	Σ	1.41^{Cac}	$0.343^{C\alpha}$	1.608^{Dabd}	$2.32^{b\Delta}$
	♂	0.52^{EFGH}	0.172^{FJKc}	1.340^{FIKLgi}	1.53^{c}
IV	♀	0.40^{KMRS}	$0.137^{NReg\gamma}$	1.274	1.46^{σ}
	Σ	0.46^{ABCD}	0.154^{ABCD}	1.309^{ACEd}	1.49^{Aab}
	♂	1.69^{fgH}	0.406^{GIK}	1.900^{GJLh}	1.99^{d}
V	♀	1.19^{LOS}	0.365^{Rfh}	1.762	$1.47^{f\delta}$
	Σ	1.44^{Dbd}	0.387^{Db}	1.823^{BDEc}	$1.75^{\alpha\beta\Delta}$

[1]Mean values in columns having the same superscripts are significantly different at $^{a,b}P<0.05$ and $^{A,B}P<0.01$, while differences at $^{\alpha,\beta}P<0.1$ are indicated as tendencies; [2]the concentration ratio (r) of MDA (ng/g) to the difference between the sum of all assayed fatty acids (mg/g) and polyunsaturated fatty acids (mg/g): r = MDA/(FA-PUFA);[3]peroxidation index: MDA_{index} = [MDA (ng/g) + PUFA (mg/g)] / PUFA (mg/g) (Rozbicka-Wieczorek et al., 2012)

compared with the control animals. Interestingly, dietary Lyc or SeVI most efficiently increased the ratio of PUFA/SFA in breast muscles of chickens compared with the control group and SeY- or FO-fed chickens. Data from the current investigation confirm our recent study in which the concentration ratio of PUFA/SFA was highest in thigh muscles of female and male chickens fed with the diets containing Lyc or SeVI (Rozbicka-Wieczorek et al., 2014). Considering the above, we argue that Lyc- or SeVI-dietary manipulations are linked with improved nutritional properties and quality of breast and thigh muscles obtained from chickens. Considering the current results and our other studies (Czauderna et al., 2009; Rozbicka-Wieczorek et al., 2012; 2014) we suggest that the PUFA/SFA ratio was significantly higher in muscles of Lyc- or SeVI-fed chickens, which possibly reflects the effect of oxidation protection of Lyc and SeVI (the antioxidants) on the unsaturated fatty acids in living organisms (Navarro-Alarcon and Cabrera-Vique, 2008; Rayman, 2004; Rao and Agarwal, 1998).

Interestingly, the concentration ratio of n-6PUFA/n-3PUFA (n-6/n-3; Table 4) was significantly greater in muscles of female chickens and especially male chickens fed with the diet enriched in Lyc, SeVI or SeY. The distinct difference in n-6PUFA versus n-3PUFA concentrations may reflect up-regulated control of enzyme transcription by circulating n-3PUFA over n-6PUFA. In addition, dietary Lyc most efficiently stimulated the accumulation of n-6LPUFA (e.g. AA) in breast muscles and thigh muscles (Rozbicka-Wieczorek et al., 2014), while decreased the concentration of n-3LPUFA (e.g. DPA or DHA) in these muscles compared with the control female and male chickens. Thus, our current studies carried out on chickens also reinforce the finding that *c9c12*C18:2 (LA) originating from dietary SO could be metabolized *in vivo* into n-6LPUFA (via Δ6-desaturation, elongation and Δ5-desaturation of LA) using the same pathway as *c9c12c15*C18:3 (that is, n-3PUFA).

As can be seen from the results summarized in Table 4, SeVI or SeY added the diet decreased the concentration of LPUFA, including n-6LPUFA and n-3LPUFA, in muscles compared with the control birds and Lyc-fed chickens. So, our results (Table 4) support our earlier observations that dietary SeY or SeVI activate the

carnitine palmitoyltransferase 1 (CPT1) gene and increased the yield of β-oxidation (Kuryl et al., 2008). On the other hand, dietary FO increased the accumulation of n-3LPUFA, especially DHA, in muscles compared with the control birds and other experimental groups. In line with the above, we found that the diet with FO most effectively increased the concentration ratio of n-3LPUFA to SFA and Δ4-desaturase indexes ($\Delta 4_{index}$), whereas most efficiently decreased the elongase index (Table 4). Indeed, dietary FO was reach in n-3LPUFA (like C20:5n-3, C22:5n-3 or C22:6n-3; Table 1), consequently, the diet enriched in FO stimulated the accumulation of n-3LPUFA, especially C22:6n-3 in breast muscles (Table 4) as well as in thigh muscles of female and male chickens (Rozbicka-Wieczorek et al., 2014).

Influence of the experimental rations on the yield of PUFA peroxidation in breast muscles

The influence of the experimental diets on the concentration of MDA and the MDA indexes of breast muscles are summarized in Table 5. The addition of SeY to the diet most effectively decreased the concentration of MDA as well as values of the peroxidation index (MDA_{index}) and our proposed original MDA index ($^{simple}MDA_{index}$) in breast muscles of male chickens and especially female chickens. Above, we found that dietary extra SeY also most efficiently decreased the concentrations of PUFA (Table 5) and MUFA (Table 3).

Considering the above, we argued that dietary SeY significantly decreased the oxidative stress in breast muscles as dosed SeY stimulated the β-oxidation of fatty acids (including PUFA and MUFA) as well as stimulated the biosynthesis of Se-proteins with antioxidant, chemopro-tective and anti-inflammatory properties (Rayman, 2004; Tapiero et al., 2003; Yu et al., 2008). Moreover, the results summarized in Table 5 documented also that dietary SeVI or SeY more effectively reduced the oxidative stress in breast muscles of female chickens than in muscles of male chickens. We suggest that this effect of dietary SeVI or SeY, may be due to the higher concentration of anti-inflammatory n-3PUFA, including n-3LPUFA, in breast muscles of female chickens compared with muscles of male chickens (Tables 3 and 4).

On the other hand, FO added to the diet increased values of $^{simple}MDA_{index}$ and MDA_{index} indexes in breast muscles in comparison with the control chickens; indeed, dietary FO revealed a negligible effect on the concentration of MDA, while decreased the concentration of PUFA in muscles compared with the control birds. In contrast, SeVI added to the diet significantly decreased the concentration of MDA and values of $^{simple}MDA_{index}$ and MDA_{index} indexes in breast muscles of female chickens, whereas increased those in muscles of male chickens compared with the control birds. Interestingly, the effect of dietary Lyc on the concentration of MDA and indexes also depend upon the gender of chickens. Indeed, the diet containing Lyc more effectively reduced the oxidative stress in breast muscles of male chickens than in muscles of female chickens. Hence, we suggest that this effect of dietary Lyc may be due to the higher concentration of anti-inflammatory n-3PUFA, including n-3LPUFA, in breast muscles of male chickens compared with muscles of female chickens (Tables 3 and 4). Moreover, the diet enriched in Lyc increased values of $^{simple}MDA_{index}$ and MDA_{index} indexes in breast muscles of female chickens, whereas decreased in muscles of male chickens in comparison with the control birds.

According to the above and all of the results summarized in Table 5, we documented that our proposed original $^{simple}MDA_{index}$ (calculated as: $^{simple}MDA_{index}$ = MDA/(FA-PUFA); Table 5) is the best indicator of the yield of PUFA peroxidation in tissues of living organisms; $^{simple}MDA_{index}$ takes into consideration the MDA concentrations as well as the concentration of PUFA and the presence of antioxidants in the examined samples. Indeed, the value of this index more significantly depend on the concentrations of MDA and PUFA than the values of MDA_{index} (calculated as: MDA_{index} = (MDA + PUFA)/PUFA).

Conclusion

Diets enriched in Se (as SeVI or SeY) or Lyc can be regularly used to increase the concentration of Lyc or Se (as the antioxidants) in the body of chickens without adversely influencing the performance of male and female chickens. Moreover, dietary SeY most effectively increased the oxidative stability of breast muscles, whereas decreased the concentration of FA in muscles of birds (that is, dietary SeY increases the leanness of breast muscles). Thus, we concluded that dietary SeY stimulated the repartition in breast muscles (that is, increased the level of proteins, while decreased the content of fat in breast muscles of chickens). The addition of FO to the diet most efficiently increased the concentration of n-3LPUFA and the concentration ratio of n-3LPUFA to SFA (n-3LPUFA/SFA), whereas most effectively decreased the concentration ratio of n-6PUFA to n-3PUFA (n-6PUFA/n-3PUFA) in muscles. Considering the above, we argue that dietary FO changes in the profiles of fatty acids in muscles of chickens that are beneficial to human health. Further investigations are necessary to determine if diets containing other chemical form of selenium compounds (like selenite or Se-cysteine), other vegetable oils or the higher concentrations of Lyc induce changes in the profiles of fatty acids in breast and thigh muscles and adipose tissues of chickens that are beneficial to human health.

Conflict of Interests

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

REFERENCES

AOAC (2005). Association of Official Analytical Chemists, Official Methods of Analysis. 18th Edition. Arlington, VA.

Betti M, Perez TI, Zuidhof MJ, Renema RA (2009a). Omega-3-enriched broiler meat: 3. Fatty acid distribution between triacylglycerol and phospholipid classes. Poult. Sci. 88:1740-1754.

Betti M, Schneider BL, Wismer WV, Carney VL, Zuidhof MJ, Renema RA (2009b). Omega-3-enriched broiler meat: 2. Functional properties, oxidative stability, and consumer acceptance. Poult. Sci. 88: 1085-1095.

Boileau TWM, Boileau AC, John W, Erdman JW (2013). Bioavailability of all-*trans* and *cis*-isomers of lycopene. Exp. Biol. Med. 227:914-919.

Cortinas L, Barroeta A, Villaverde C, Galobart J, Guardiola F, Baucells MD (2005). Influence of the dietary polyunsaturation level on chicken meat quality: lipid oxidation. Poult. Sci. 84:48-55.

Council Directive 2007/43/EC. (2007). Laying down minimum rules for the protection of chickens kept for meat production. Official J. Eur. Union, 12.7.2007: L 182/19 - L 182/28.

Czauderna M, Kowalczyk J, Marounek M (2011). The simple and sensitive measurement of malondialdehyde in selected specimens of biological origin and some feed by reversed phase high performance liquid chromatography. J. Chromatogr. B 879:2251-2258.

Czauderna M, Kowalczyk J, Niedźwiedzka KM, Leng L, Cobanova K (2009). Dietary selenized yeast and CLA isomer mixture affect fatty- and amino acid concentrations in the femoral muscles and liver of rats. J. Anim. Feed Sci. 18: 348-361.

Goto T, Lee J-Y, Teraminami A, Kim Y-I (2011). Activation of peroxisome proliferator-activated receptor-alpha stimulates both differentiation and fatty acid oxidation in adipocytes. J. Lipid Res. 52: 873-884.

Harris WS, Mozaffarian D, Rimm E, Kris-Etherton P, Rudel LL, Appel LJ, Engler MM, Engler MB, Sacks F (2009). Omega-6 fatty acids and risk for cardiovascular disease. Circulation (the American Heart Association) 119: 902-907.

Harris WS, Poston WC, Haddock CK (2007). Tissue n-3 and n-6 fatty acids and risk for coronary heart disease events. Atherosclerosis 193: 1-10.

Heber D, Lu Q-Y (2002). Overview of mechanisms of action of lycopene. Exp. Biol. Med. 227: 920-923.

Kuryl T, Dębski B, Martinik K (2008). The effect of microelements supplementation on b-oxidation activity in healthy and type 1 diabetic rats. Cent. Eur. J. Public Health 16: 205-208.

Navarro-Alarcon M, Cabrera-Vique C (2008). Selenium in food and the human body: A review. Sci. Total Envir. 400:115-141.

Pappas AC, Zoidis E, Surai PF, Zervas G (2008). Selenoproteins and maternal nutrition. Comp. Biochem. Physiol. Pt B 151: 361-372.

Perez TI, Zuidhof MJ, Renema RA, Curtis JM, Ren Y, Betti M (2010). Effects of vitamin E and organic selenium on oxidative stability of omega-3 enriched dark chicken meat during cooking. J. Food Sci. T25-T34.

Rahimi S, Kamaran Azad S, Karimi Torshizi MA (2011). Omega-3 enrichment of broiler meat by Rusing two oil seeds. J. Agric. Sci. Technol. 13: 353-365.

Rao AV, Agarwal S (1998). Bioavailability and *in vivo* antioxidant properties of lycopene from tomato products and their possible role in the prevention of cancer. Nutr. Cancer 31: 199-203.

Rayman PM (2004). Review article. The use of high-selenium yeast to raise selenium status: how does it measure up? Brit. J. Nutr. 92:557-573.

Rozbicka-Wieczorek AJ, Szarpak E, Brzóska F, Śliwiński B, Kowalczyk J, Czauderna M (2012). Dietary lycopenes, selenium compounds and fish oil affect the profile of fatty acids and oxidative stress in chicken breast muscle. J. Anim. Feed Sci. 21:705-724.

Rozbicka-Wieczorek AJ, Więsyk E, Brzóska F, Śliwiński B, Czauderna M, Kowalczyk J (2014). Lycopene, fish oil and selenium compounds added to the diet influence the profile of fatty acids and oxidative stress in chicken thigh muscles. Ann. Anim. Sci. *in press.*

Schweizer U, Streckfub F, Pelt P, Carlson BA, Hatfield DL, Kohrle J, Schomburg L (2005). Hepatically derived selenoprotein P is a key factor to kidney but not for brain selenium supply. Biochem. J. 386: 221-226.

Stahle JA, Vunta H, Reddy CC, Prabhu KS (2009). Regulation of expression of apolipoprotein A-I by selenium status in human liver hepatoblastoma cells. Eur. J. Nutr. 48: 283-290.

StatSoft (2010): STATISTICA (Data analysis software system), version 10.0. StatSoft® Inc., Tulsa, USA.

Tapiero H, Townsend DM, Tew KD (2003). The antioxidant role of elenium and seleno-compounds. Biomed. Pharmacother. 57:134-144.

Ulbricht TLV, Southgate DAT (1991). Coronary heart disease: seven dietary factors. Lancet 338:985-992.

Urso ML, Clarkson PM (2003). Oxidative stress, exercise, and antioxidant supplementation. Toxicology 189:41-54.

Yu LL, Wang RL, Zhang YZ, Kleemann DO, Zhu XP, Jia ZH (2008). Effects of selenium supplementation on polyunsaturated fatty acid concentrations and antioxidant status in plasma and liver of lambs fed linseed oil or sunflower oil diets. Anim. Feed Sci. Tech. 140:39-51.

Zuidhof MJ, Betti M, Korver DR, Hernandez FIL, Schneider BL, Carney VL, Renema RA (2009). Omega-3-enriched broiler meat: 1. Optimization of a production system. Poult. Sci. 88:1108-1120.

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