African Journal of Biotechnology

Volume 14 Number 25, 23 June, 2015
ISSN 1684-5315

Academic Journals

ABOUT AJB

The **African Journal of Biotechnology (AJB)** (ISSN 1684-5315) is published weekly (one volume per year) by Academic Journals.

African Journal of Biotechnology (AJB), a new broad-based journal, is an open access journal that was founded on two key tenets: To publish the most exciting research in all areas of applied biochemistry, industrial microbiology, molecular biology, genomics and proteomics, food and agricultural technologies, and metabolic engineering. Secondly, to provide the most rapid turn-around time possible for reviewing and publishing, and to disseminate the articles freely for teaching and reference purposes. All articles published in AJB are peer-reviewed.

Submission of Manuscript

Please read the **Instructions for Authors** before submitting your manuscript. The manuscript files should be given the last name of the first author

Click here to Submit manuscripts online

If you have any difficulty using the online submission system, kindly submit via this email ajb@academicjournals.org.

With questions or concerns, please contact the Editorial Office at ajb@academicjournals.org.

Editorial Board

Instructions for Author

Electronic submission of manuscripts is strongly encouraged, provided that the text, tables, and figures are included in a single Microsoft Word file (preferably in Arial font).

The **cover letter** should include the corresponding author's full address and telephone/fax numbers and should be in an e-mail message sent to the Editor, with the file, whose name should begin with the first author's surname, as an attachment.

Article Types
Three types of manuscripts may be submitted:

Regular articles: These should describe new and carefully confirmed findings, and experimental procedures should be given in sufficient detail for others to verify the work. The length of a full paper should be the minimum required to describe and interpret the work clearly.
Short Communications: A Short Communication is suitable for recording the results of complete small investigations or giving details of new models or hypotheses, innovative methods, techniques or apparatus. The style of main sections need not conform to that of full-length papers. Short communications are 2 to 4 printed pages (about 6 to 12 manuscript pages) in length.

Reviews: Submissions of reviews and perspectives covering topics of current interest are welcome and encouraged. Reviews should be concise and no longer than 4-6 printed pages (about 12 to 18 manuscript pages). Reviews are also peer-reviewed.

Review Process

All manuscripts are reviewed by an editor and members of the Editorial Board or qualified outside reviewers. Authors cannot nominate reviewers. Only reviewers randomly selected from our database with specialization in the subject area will be contacted to evaluate the manuscripts. The process will be blind review.
Decisions will be made as rapidly as possible, and the journal strives to return reviewers' comments to authors as fast as possible. The editorial board will re-review manuscripts that are accepted pending revision. It is the goal of the AJFS to publish manuscripts within weeks after submission.

Regular articles

All portions of the manuscript must be typed double-spaced and all pages numbered starting from the title page.

The Title should be a brief phrase describing the contents of the paper. The Title Page should include the authors' full names and affiliations, the name of the corresponding author along with phone, fax and E-mail information. Present addresses of authors should appear as a footnote.

The Abstract should be informative and completely self-explanatory, briefly present the topic, state the scope of the experiments, indicate significant data, and point out major findings and conclusions. The Abstract should be 100 to 200 words in length.. Complete sentences, active verbs, and the third person should be used, and the abstract should be written in the past tense. Standard nomenclature should be used and abbreviations should be avoided. No literature should be cited.
Following the abstract, about 3 to 10 key words that will provide indexing references should be listed.

A list of non-standard **Abbreviations** should be added. In general, non-standard abbreviations should be used only when the full term is very long and used often. Each abbreviation should be spelled out and introduced in parentheses the first time it is used in the text. Only recommended SI units should be used. Authors should use the solidus presentation (mg/ml). Standard abbreviations (such as ATP and DNA) need not be defined.

The Introduction should provide a clear statement of the problem, the relevant literature on the subject, and the proposed approach or solution. It should be understandable to colleagues from a broad range of scientific disciplines.

Materials and methods should be complete enough to allow experiments to be reproduced. However, only truly new procedures should be described in detail; previously published procedures should be cited, and important modifications of published procedures should be mentioned briefly. Capitalize trade names and include the manufacturer's name and address. Subheadings should be used. Methods in general use need not be described in detail.

Results should be presented with clarity and precision. The results should be written in the past tense when describing findings in the authors' experiments. Previously published findings should be written in the present tense. Results should be explained, but largely without referring to the literature. Discussion, speculation and detailed interpretation of data should not be included in the Results but should be put into the Discussion section.

The Discussion should interpret the findings in view of the results obtained in this and in past studies on this topic. State the conclusions in a few sentences at the end of the paper. The Results and Discussion sections can include subheadings, and when appropriate, both sections can be combined.

The Acknowledgments of people, grants, funds, etc should be brief.

Tables should be kept to a minimum and be designed to be as simple as possible. Tables are to be typed double-spaced throughout, including headings and footnotes. Each table should be on a separate page, numbered consecutively in Arabic numerals and supplied with a heading and a legend. Tables should be self-explanatory without reference to the text. The details of the methods used in the experiments should preferably be described in the legend instead of in the text. The same data should not be presented in both table and graph form or repeated in the text.

Figure legends should be typed in numerical order on a separate sheet. Graphics should be prepared using applications capable of generating high resolution GIF, TIFF, JPEG or Powerpoint before pasting in the Microsoft Word manuscript file. Tables should be prepared in Microsoft Word. Use Arabic numerals to designate figures and upper case letters for their parts (Figure 1). Begin each legend with a title and include sufficient description so that the figure is understandable without reading the text of the manuscript. Information given in legends should not be repeated in the text.

References: In the text, a reference identified by means of an author‘s name should be followed by the date of the reference in parentheses. When there are more than two authors, only the first author‘s name should be mentioned, followed by ’et al‘. In the event that an author cited has had two or more works published during the same year, the reference, both in the text and in the reference list, should be identified by a lower case letter like ’a‘ and ’b‘ after the date to distinguish the works.

Examples:

Abayomi (2000), Agindotan et al. (2003), (Kelebeni, 1983), (Usman and Smith, 1992), (Chege, 1998; 1987a,b; Tijani, 1993,1995), (Kumasi et al., 2001)
References should be listed at the end of the paper in alphabetical order. Articles in preparation or articles submitted for publication, unpublished observations, personal communications, etc. should not be included in the reference list but should only be mentioned in the article text (e.g., A. Kingori, University of Nairobi, Kenya, personal communication). Journal names are abbreviated according to Chemical Abstracts. Authors are fully responsible for the accuracy of the references.

Examples:

Chikere CB, Omoni VT and Chikere BO (2008). Distribution of potential nosocomial pathogens in a hospital environment. Afr. J. Biotechnol. 7: 3535-3539.

Moran GJ, Amii RN, Abrahamian FM, Talan DA (2005). Methicillinresistant Staphylococcus aureus in community-acquired skin infections. Emerg. Infect. Dis. 11: 928-930.

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

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

Short Communications

Short Communications are limited to a maximum of two figures and one table. They should present a complete study that is more limited in scope than is found in full-length papers. The items of manuscript preparation listed above apply to Short Communications with the following differences: (1) Abstracts are limited to 100 words; (2) instead of a separate Materials and Methods section, experimental procedures may be incorporated into Figure Legends and Table footnotes; (3) Results and Discussion should be combined into a single section.
Proofs and Reprints: Electronic proofs will be sent (e-mail attachment) to the corresponding author as a PDF file. Page proofs are considered to be the final version of the manuscript. With the exception of typographical or minor clerical errors, no changes will be made in the manuscript at the proof stage.

Fees and Charges: Authors are required to pay a $650 handling fee. Publication of an article in the African Journal of Biotechnology is not contingent upon the author's ability to pay the charges. Neither is acceptance to pay the handling fee a guarantee that the paper will be accepted for publication. Authors may still request (in advance) that the editorial office waive some of the handling fee under special circumstances

African Journal of Biotechnology

Table of Contents: Volume 14 Number 25, 23 June, 2015

ARTICLES

Table of Contents: Volume 14 Number 25, 23 June, 2015

academicJournals

Vol. 14(25), pp. 2066-2079, 23 June, 2015
DOI: 10.5897/AJB2014.14368
Article Number: DCB3C0253881
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Genetic diversity in *Jatropha* species from different regions of Brazil based on morphological characters and inters-simple sequence repeat (ISSR) molecular markers

Mariana Silva Rosa Pazeto*, Sandra Helena Unêda-Trevisoli, Aretha Arcenio Pimentel Corrêa, Viviane Formice Vianna, Daniel Carvalho Leite and Antônio Orlando Di Mauro

Departamento de Fitotecnia, Faculdade de Ciências Agrárias e Veterinárias de Jaboticabal, Universidade Estadual Paulista "Júlio de Mesquita Filho", Jaboticabal, SP, Brazil.

Received 13 December, 2014; Accepted 17 June, 2015

Jatropha **belonging to Euphorbiaceae family has around 170 species distributed throughout tropical semi-arid regions of Africa and the Americas. Some of its species include *Jatropha curcas* L. (Physic Nut), *Jatropha pohliana* Müll.Arg. (Brazilian Purging Nut) and *Jatropha gossypiifolia* L. (Black Physic Nut). Phenotypic and genetic studies of a population are important for plant improvements, helping in the characterization of accesses, as well as facilitating selection of parental for directed crossings. Thus, the present study aimed to evaluate accesses of three *Jatropha* species from different regions of Brazil, through morphological characters and ISSR molecular markers, in order to group them according to the existing degree of divergence. A higher interspecific variability, rather than intraspecific, was observed among accesses, for morphological as well as molecular characters. Qualitative and quantitative characters showed variability between accesses and may serve as reference for future genetic studies. There was no relation between similarity patterns and geographical origin of accesses in the group analysis. Average percentage of polymorphism found for inter-simple sequence repeat (ISSR) markers between the studied accesses was 40.6%. A higher genetic variability interspecific was observed than intraspecific, suggesting the search for genetic variability among species *Jatropha* through interspecific crosses.**

Key words: Germplasm, *Jatropha curcas*, *Jatropha gossypiifolia*, *Jatropha pohliana*, variability.

INTRODUCTION

Jatropha (Euphorbiaceae) is represented by around 170 species, distributed throughout tropical semi-arid regions of Africa and the Americas. Dehgan and Webster (1979) recognized two subgenres (*Jatropha* and *Curcas*) and 10

*Corresponding author. E-mail: mariana_silvarosa@yahoo.com.br. Tel: 55 16 992299881.

sections and subsections based on their morphological and intergeneric relations. Some of the genre's species are *Jatropha curcas* L. (Barbados Nut), *Jatropha pohliana* Müll.Arg. (Brazilian Purging Nut), and *Jatropha gossypiifolia* L. (Black Physic Nut). These species, classified as autogamous, have been studied due to the high oil content of their seeds, which represent an important source for plant biofuel production. *J. curcas* L. and *J. pohliana* Müll.Arg., seeds may contain an average of 33 to 38% (Nunes et al., 2008), and 25% (Targino and Silva, 2013) oil, respectively. While, *J. gossypiifolia* L. contains about 23% (Oliveira et al., 2006). Biofuel is a generic name for fuels and additives derived from renewable sources. Compared to petroleum-derived diesel, biofuel may reduce up to 78% CO_2 emission, considering reabsorption by plants (Accarini, 2006). Furthermore, it decreases smoke emission by 90% and practically eliminates sulfur dioxide emission (Sousa, 2006). Nowadays, little is known about genetic divergence (or genetic dissimilarity) between these species genotypes, which would be of great use to the selection of parentals for the creation of segregant populations, as well as for the maintenance of genetic diversity in improvement programs and germplasm banks. These estimates reveal availability of alternative alleles for characters of interest, which are the base for long-term selection gain. Crossings between genetic divergent parentals provide a much higher genetic variability between progenies than that obtained from the crossing of plants more closely related and, thus, increase opportunities of breeders obtaining superior progenies (Messmer et al., 1993). To this end, the creation of a germplasm bank which includes promising accesses that are mutually divergent is capital, warranting the need of further knowledge about the genetic diversity found in natural populations (Cruz et al., 2012).

Molecular markers constitute an important technological instrument in processes for the selection and increase of genetic variability, chiefly when associated with the phenotypic analysis of populations. Among those available and widely used for characterization of genetic diversity in a population, we have inter simple sequence repeat (ISSR), which are dominant markers, meaning they do not allow differentiation between homozygotes and heterozygotes. However, they are excellent tools to be used in initial studies on the genetic diversity due to its nonspecificity. Studies of this nature with *Jatropha* species are lacking in literature. Divakara et al. (2010), in a revision of biological aspects and improvement of *J curcas*, presents nine references of studies that used molecular markers to characterize accesses of *J. curcas* L., among those, Basha and Sujatha (2007) evaluated 42 *J. curcas* L. accesses from different regions in India. These authors used random amplified polymorphic DNA (RAPD) and ISSR markers to determine genetic diversity and reported on the immediate need to increase the genetic base of *J. curcas* L. in India, due to the low genetic diversity found between accesses. Góis et al. (2006) evaluated eight accesses in Minas Gerais, four in Goiás, one in Espírito Santo, and one in Sergipe, through the isoenzyme technique, finding highly similar accesses and others quite divergent. Therefore, the aim of this study was to evaluate genetic diversity in three species of *Jatropha* genre from different regions of Brazil, using morphological characters and ISSR molecular markers.

MATERIALS AND METHODS

Plant material

A total of 84 accesses were used, 76 *J. curcas* (Barbados Nut), seven *J. pohliana* (Brazilian Purging Nut) and one *J. gossypiifolia* (Black Physic Nut), for the most part belonging to the Banco Ativo de Germoplasma (Active Germplasm Bank) from Embrapa Algodão (Embrapa Cotton), Campina Grande – PB, and also spontaneous plants collected in the Northeast and Southeast regions of Brazil (Table 1). Accesses were cultivated in an experimental area of the Department of Plant Production at UNESP, in the Jaboticabal Campus.

Field evaluation

Jatropha spp. accesses were organized according to the coordinates 21° 15' 29" S and 48° 16' 47" W at an average altitude of 614 m. The spacing used was 3 x 2 m, respectively, between lines and plants. Local weather is Cwa, humid temperate climate with dry and hot summer winter, according to the Köppen Climate Classification System; mean annual rainfall averages of 1425 mm, with mean temperature of 22.2°C and relative air humidity of 75% (annual). Seventeen morphological characters were analyzed, having as criteria descriptors used for castor bean (*Ricinus communis* L.), adapted for colorations in the studied species (Embrapa Cotton, 2008). The qualitative descriptors used were: 1) leaf pilosity: present and absent; 2) stem waxiness: present and absent; 3) color of young leaves, observing coloration between 80 to 120 days of planting: green, red and purple; 4) color of adult leaves, observing the second adult leaf below primary raceme, taking an average of ten random leaves: dark green and light green; 5) color of foliar veins, noting the adaxial side of three mature leaves selected randomly from ten plants: green, purple and red; 6) petiole leaf position: opposed, alternate and mixed; 7) type of plant ramification, during maturation of raceme: trifurcate, when there are two lateral branches, and from these, two more, giving the appearance of a trident; bifurcate, while from the stem there's one lateral branch, having the appearance of a bipartite stem; cup-like, when branches emerge from the stem forming a structure similar to a wine glass, and universal, when branches outweigh the primary raceme in height; 8) seed pattern: single color, painted, spotted and stripped; 9) shape of seeds: elongated, rounded, and flattened; 10) caruncle type: protuberant and non-protuberant; 11) fruit dehiscence: dehiscent, semi-dehiscent and indehiscent; and the quantitative descriptors were: 12) number of leaf lobes, taking an average of 10 adult leaves from the middle third of plants; 13) plant height in centimeters; 14) stalk diameter in centimeters; 15) maturation cycle, which corresponds to the number of days between germination and physiological maturity, considering: precocious (up to 275 days), average (from 276 to 306 days) and late (over 306 days); 16) length of seeds in centimeters, and 17) width of seeds in centimeters.

Table 1. Identification of the 76 *Jatropha curcas* accesses, seven *Jatropha pohliana* accesses and one *Jatropha gossypiifolia* access evaluated.

Identification	Code	Origin	Latitude	Longitude	Species
Acess 1	CNPA PM IV P1	Tocantinópolis-TO	06°19'S	47°24'W	*J. curcas*
Acess 2	CNPA PM IV P2	Tocantinópolis-TO	06°19'S	47°24'W	*J. curcas*
Acess 3	CNPA PM II P1	Garanhuns-PE	08°53'S	36°30'W	*J. curcas*
Acess 4	CNPA PM II P2	Garanhuns-PE	08°53'S	36°30'W	*J. curcas*
Acess 5	CNPA PM II P3	Garanhuns-PE	08°53'S	36°30'W	*J. curcas*
Acess 6	CNPA PM II P4	Garanhuns-PE	08°53'S	36°30'W	*J. curcas*
Acess 7	CNPA PM II P5	Garanhuns-PE	08°53'S	36°30'W	*J. curcas*
Acess 8	CNPA PM II P6	Garanhuns-PE	08°53'S	36°30'W	*J. curcas*
Acess 9	CNPA PM II P7	Garanhuns-PE	08°53'S	36°30'W	*J. curcas*
Acess 10	CNPA PM II P8	Garanhuns-PE	08°53'S	36°30'W	*J. curcas*
Acess 11	CNPA PM II P9	Garanhuns-PE	08°53'S	36°30'W	*J. curcas*
Acess 12	CNPA PM II P12	Garanhuns-PE	08°53'S	36°30'W	*J. curcas*
Acess 13	CNPA PM II P16	Garanhuns-PE	08°53'S	36°30'W	*J. curcas*
Acess 14	CNPA PM II P17	Garanhuns-PE	08°53'S	36°30'W	*J. curcas*
Acess 15	CNPA PM II P19	Garanhuns-PE	08°53'S	36°30'W	*J. curcas*
Acess 16	CNPA PM II P20	Garanhuns-PE	08°53'S	36°30'W	*J. curcas*
Acess 17	CNPA PM II P21	Garanhuns-PE	08°53'S	36°30'W	*J. curcas*
Acess 18	CNPA PM II P22	Garanhuns-PE	08°53'S	36°30'W	*J. curcas*
Acess 19	CNPA PM II P24	Garanhuns-PE	08°53'S	36°30'W	*J. curcas*
Acess 20	CNPA PM II P25	Garanhuns-PE	08°53'S	36°30'W	*J. curcas*
Acess 21	CNPA PM II P26	Garanhuns-PE	08°53'S	36°30'W	*J. curcas*
Acess 22	CNPA PM II P30	Garanhuns-PE	08°53'S	36°30'W	*J. curcas*
Acess 23	CNPA PM II P31	Garanhuns-PE	08°53'S	36°30'W	*J. curcas*
Acess 24	CNPA PM XI P1	Mundo Novo-PE	07°35'S	37°11'W	*J. curcas*
Acess 25	CNPA PM XI P2	Mundo Novo-PE	07°35'S	37°11'W	*J. curcas*
Acess 26	CNPA PM XI P3	Mundo Novo-PE	07°35'S	37°11'W	*J. curcas*
Acess 27	CNPA PM XI P4	Mundo Novo-PE	07°35'S	37°11'W	*J. curcas*
Acess 28	CNPA PM VII P1	Mundo Novo-PE	07°35'S	37°11'W	*J. curcas*
Acess 29	CNPA PM VII P2	Mundo Novo-PE	07°35'S	37°11'W	*J. curcas*
Acess 30	CNPA PM VII P6	Mundo Novo-PE	07°35'S	37°11'W	*J. curcas*
Acess 31	CNPA PM VII P7	Mundo Novo-PE	07°35'S	37°11'W	*J. curcas*
Acess 32	CNPA PM IX P1	Pugmil- TO	06°24'S	37°48'W	*J. curcas*
Acess 33	CNPA PM IX P2	Pugmil- TO	06°24'S	37°48'W	*J. curcas*
Acess 34	CNPA PM IX P3	Pugmil- TO	06°24'S	37°48'W	*J. curcas*
Acess 35	CNPA PM IX P4	Pugmil- TO	06°24'S	37°48'W	*J. curcas*
Acess 36	CNPA PM IX P5	Pugmil- TO	06°24'S	37°48'W	*J. curcas*
Acess 37	CNPA PM IX P6	Pugmil- TO	06°24'S	37°48'W	*J. curcas*
Acess 38	CNPA PM IX P7	Pugmil- TO	06°24'S	37°48'W	*J. curcas*
Acess 39	CNPA PM IX P8	Pugmil- TO	06°24'S	37°48'W	*J. curcas*
Acess 40	CNPA PM VIII P1	Alagoinha-PB	06°57'S	35°32'W	*J. curcas*
Acess 41	CNPA PM VIII P2	Alagoinha-PB	06°57'S	35°32'W	*J. curcas*
Acess 42	CNPA PM VIII P4	Alagoinha-PB	06°57'S	35°32'W	*J. curcas*
Acess 43	CNPA PM VIII P5	Alagoinha-PB	06°57'S	35°32'W	*J. curcas*
Acess 44	CNPA PM VI P1	Tocantinópolis-TO	10°42'S	48°54'W	*J. curcas*
Acess 45	CNPA PM VI P2	Tocantinópolis-TO	10°42'S	48°54'W	*J. curcas*
Acess 46	CNPA PM VI P3	Tocantinópolis-TO	10°42'S	48°54'W	*J. curcas*
Acess 47	CNPA PM VI P5	Tocantinópolis-TO	10°42'S	48°54'W	*J. curcas*
Acess 48	CNPA PM VI P6	Tocantinópolis-TO	10°42'S	48°54'W	*J. curcas*
Acess 49	CNPA PM VI P7	Tocantinópolis-TO	10°42'S	48°54'W	*J. curcas*
Acess 50	CNPA PM VI P8	Tocantinópolis-TO	10°42'S	48°54'W	*J. curcas*

Table 1. Contd

Acess 51	CNPA PM X P1	Marizópolis- PB	09°47'S	49°39'W	*J. curcas*
Acess 52	CNPA PM X P2	Marizópolis- PB	09°47'S	49°39'W	*J. curcas*
Acess 53	CNPA PM X P3	Marizópolis- PB	09°47'S	49°39'W	*J. curcas*
Acess 54	CNPA PM X P4	Marizópolis- PB	09°47'S	49°39'W	*J. curcas*
Acess 55	IT1	Ituverava- SP	20°20'S	47°46'W	*J. curcas*
Acess 56	IT2	Ituverava- SP	20°20'S	47°46'W	*J. curcas*
Acess 57	IT3	Ituverava- SP	20°20'S	47°46'W	*J. curcas*
Acess 58	IT4	Ituverava- SP	20°20'S	47°46'W	*J. curcas*
Acess 59	IT5	Ituverava- SP	20°20'S	47°46'W	*J. curcas*
Acess 60	IT6	Ituverava- SP	20°20'S	47°46'W	*J. curcas*
Acess 61	IT7	Ituverava- SP	20°20'S	47°46'W	*J. curcas*
Acess 62	IT8	Ituverava- SP	20°20'S	47°46'W	*J. curcas*
Acess 63	IT9	Ituverava- SP	20°20'S	47°46'W	*J. curcas*
Acess 64	IT10	Ituverava- SP	20°20'S	47°46'W	*J. curcas*
Acess 65	IT11	Ituverava- SP	20°20'S	47°46'W	*J. curcas*
Acess 66	IT12	Ituverava- SP	20°20'S	47°46'W	*J. curcas*
Acess 67	IT13	Ituverava- SP	20°20'S	47°46'W	*J. curcas*
Acess 68	IT14	Ituverava- SP	20°20'S	47°46'W	*J. curcas*
Acess 69	IT15	Ituverava- SP	20°20'S	47°46'W	*J. curcas*
Acess 70	IT16	Ituverava- SP	20°20'S	47°46'W	*J. curcas*
Acess 71	IT17	Ituverava- SP	20°20'S	47°46'W	*J. curcas*
Acess 72	JB1	Jaboticabal- SP	21°15'S	48°17'W	*J. curcas*
Acess 73	CG1	Campina Grande- PB	07°13'S	35°52'W	*J. curcas*
Acess 74	CG2	Campina Grande- PB	07°13'S	35°52'W	*J. curcas*
Acess 75	CG3	Campina Grande- PB	07°13'S	35°52'W	*J. curcas*
Acess 76	RB1	Riachão do Bacamarte- PB	07°14'S	35°39'W	*J. curcas*
Acess 77	GT1	Galante- PB	07°17'S	35°45'W	*J. pohliana*
Acess 78	GT3	Galante- PB	07°17'S	35°45'W	*J. pohliana*
Acess 79	GT4	Galante- PB	07°17'S	35°45'W	*J. pohliana*
Acess 80	GT5	Galante- PB	07°17'S	35°45'W	*J. pohliana*
Acess 81	GT6	Galante- PB	07°17'S	35°45'W	*J. pohliana*
Acess 82	GT7	Galante- PB	07°17'S	35°45'W	*J. pohliana*
Acess 83	RB2	Riachão do Bacamarte- PB	07°14'S	35°39'W	*J. pohliana*
Acess 84	GT2	Galante- PB	07°17'S	35°45'W	*J. gossypiifolia*

DNA extraction and amplification

The genomic DNA was extracted from leaves of each access and the methodology used varied according to the species. For *J. curcas* L. the protocol proposed by Doyle and Doyle (1990) was used with a few modifications described by Ferreira and Grattapaglia (1998). Due to singularities in *J. gossypiifolia* L. and *J. pohliana* Müll.Arg. DNA extractions for these species followed the methodology proposed by Elias et al. (2004) with modifications, based on CTAB extraction buffer, method (Dellaporta et al.,1983), in which leaves undergo a period of dehydration in a greenhouse at 60°C for 72 h, and are then macerated in a porcelain mortar, without the use of liquid nitrogen. The temperature and duration of the dehydration process proposed for cassava in the original method by Elias et al. (2004) were modified in order to expedite maceration, due to the fleshy character of *J. gossypiifolia* L. and *J. pohliana* Müll.Arg., leaves. One hundred ISSR primers from collection # 9 synthesized by the University of British Columbia (UBC) Biotechnology Laboratory in Canada, and described by Zietkiewicz et al. (1994) were used. Polymerase Chain Reaction (PCR) was carried out for all 84 *Jatropha* accesses. The reaction was carried out for a final volume of 25 µl containing 0.12 µl of Taq polymerase (5 units μl^{-1}), 2.5 µl 10 x amplification buffer (no Mg^{++}), 2.0 µl $MgCl_2$ (25 mM), 1.0 µl primer (10 µM), 0.5 µl dNTPs (2.5 mM each), 1.25 µl DNA (50 ng μl^{-1}), and 17.63 µl ultrapure water. The reaction was carried out in a Bio rad thermal cycler, according to the following parameters: denaturation step for 4 min at 94°C, followed by 35 denaturation cycles (92°C at 30 s), 1 min for the primer annealing at a specific temperature for each primer used, and extension for 2 min at 72°C. The final extension was extended to 7 min (Soares, 2010). Annealing temperatures used were determined through subjacent temperature gradient tests (Table 4). Subsequently, amplification products were separated by electrophoresis on 3% agarose gel treated with ethidium bromide, at 80 V and 400 mA, for approximately 3 h. Visualization was done on Bio Rad Gel Doc 2000 transilluminator and digitalized by Quantity One® software. The sizes of amplified fragments were estimated by comparison with the 100 pb DNA ladder molecular

Table 2. Description of qualitative characters adopted and frequency of occurrence of the categories in the 84 accesses of *Jatropha* spp. Jaboticabal, SP.

Descriptors	Categories	Relative frequency (%)	Descriptors	Categories	Relative frequency (%)
Leaf pilosity	Present	8	Type of plant ramification	Trifurcate	14
	Absent	92		Bifurcate	4
				Cup-like	79
Stem waxiness	Present	99		Universal	4
	Absent	1			
Color of young leaves	Green	7	Seed pattern	Single color	90
	Red	5		Painted	10
	Purple	88		Spotted	0
				Stripped	0
Color of adult leaves	Dark green	89	Shape of seeds	Elongated,	92
	Light green	11		Flattened	8
				Rounded	0
Color of foliar veins	Green	93	Caruncle type		
	Red	1		Protuberant	10
	Purple	6		Non-protuberant	90
Petiole leaf position	Opposed	0	Fruit dehiscence	Dehiscent	92
	Alternate	100		Semi-dehiscent	8
	Mixed	0		Indehiscent	0

weight marker.

Statistical analysis

Morphological and phenotypic analysis

Variables were initially standardized, preceding the multivariate statistical analysis for quantitative and qualitative characters. All analyzes were carried out using the Genes Software (Universidade Federal de Viçosa, Viçosa, MG, Brazil) (Cruz, 2013). Dissimilarity estimates were acquired by the Euclidean Distance function for quantitative characters, while morphological qualitative characters were obtained from Cole-Rodgers et al. (1997), where the traits, which cannot normally be ordered, can be analyzed as discrete quantitative traits (Cruz and Carneiro, 2006). With this index, a determined value expresses the percentage of coincidences considering the various characters being analyzed for each binary and multicategorical trait, contemplating concordance and discordance information from each class. Both clusters were done by the UPGMA (Unweighted Pair-Group Method Arithmetic Average) hierarchical clustering method.

Molecular evaluations

Polymorphism indicative binary matrices were formed from the results observed on the gel, based on the presence (1), or absence (0) of marks (bands) for each allele found. The matrix thus found was used for genetic distance calculations, based on dissimilarity measures the formation of clusters by the Unweighted Pair-Group Method Arithmetic Average (UPGMA) method, using the software Genes (Cruz, 2013). The obtained electrophoretic profiles will enable the similarity measures (Cii') to be converted to dissimilarity through the arithmetic complement (D), using the Jaccard Coefficient = a/a + b + c, where a is the number of concordances type 1-1, b is the number of discordances type 1-0 and c is the number of discordances type 0-1.(Cruz and Carneiro, 2006). The use of this coefficient was opted for not only due to its mathematical properties, but also because it enables intraspecific differences to be evinced and will not consider the absence of bands as synonym of genetic similarity.

RESULTS

Morphological and phenotypic data analysis

Observing frequency data on binary and multicategorical qualitative morphological characters evaluated for the 84 *Jatropha* accesses (Table 2), the existence of little phenotypic variability is noted, with many individuals concentrated in a single class. In order to carry out the multivariate analysis, the character for position of the leaves on the branches was removed, since all accesses presented the same pattern, that is, alternate leaves throughout the petiole. Plants presented glabrous leaves, with purple coloration when young and green in adulthood, green foliar veins, waxy stems; a character that possibly helps control excess loss of water under

long periods of drought. Other striking characteristics between accesses, also shown in Table 2, were the plant structure in a glass-like shape, alternate leaves throughout the branches, fruits containing three elongated seeds, black coloring, and non-protuberant caruncle. Most accesses presented dehiscent fruits, which indicate populations that are still wild. The *J. pohliana* access code GT1 (77), from Galante, Paraíba, presented glabrous leaves, differently than other *J. pohliana* accesses from this site, which have hairy adaxial and abaxial leaf surfaces. Similarly, GT1 presented young leaves with purple coloring, while other accesses from Galante had green young leaves. Access GT2, the single *J gossypiifolia* access, possesses morphological structures much different than *J. curcas* and *J. pohliana*, with small, delicate, glabrous, purplish leaves, rather exotic in appearance, with characteristics similar to ornamental plants. Accesses of all three species presented green adult leaves, with the exception of eight accesses from Garunhuns – PE and 1 access from Mundo Novo – PE, which had mostly discolored adult leaves. *J. curcas* accesses presented mostly leaves with green veins, whereas for *J. pohliana* accesses veins were purplish, with the exception of access RB2 (82), which had veins of red coloring. All plants from access GT2 (84), *J. gossypiifolia*, presented purplish veins, with darker, more intense purple veins in young, and lighter in adult leaves. Observing Figure 1 and considering a percentage of dissimilarity close to 34%, the formation of four distinct clusters is noted. Two of these groups were composed of *J. pohliana* accesses, where access 77 was isolated from the other accesses of the same species, 80, 81, 78, 79, 82 and 83, which in their turn composed a second group. The formation of a large group occurred, which encompassed all 76 *J. curcas* accesses. The *J. gossypiifolia* access constitutes the fourth group, isolated from the other *J. curcas* and *J. pohliana* accesses. Observing the group formed by *J. curcas* accesses it is possible to assume that accesses sharing the same origin have had greater proximity within the group. Analyzing maximum, minimum, average and standard deviation values for the five quantitative characters evaluated (Table 3), it is evident that the number of lobes character varied among the studied accesses, oscillating from 3 to 7 lobes. In regards to *J. curcas*, such variation was observed even in a single plant, in which case, the criteria adopted was ten adult leaves from the middle third of the plant. For *J. pohliana* accesses there was no such a variation because all the plants presented serrated, fleshy leaves with 5 lobes (Table 3). The GT2 (84) access, the only *J. gossypiifolia* sample, presented jagged leaves with 3 well defined lobes and it was the only one, among the 84 accesses studied, that did not present visible stem waxiness (Table 3). There was great variability in regards to plant height and diameter of the stem among the 84 accesses studied (Table 3), being that this difference are even greater among interspecific accesses. Plant height and stem diameter were, in general, quite regular between accesses of *J. curcas* with most accesses being over 100 cm in height, reaching up to 230 cm. The average diameter of the stem was around 5 cm (Table 3). Plants derived from Garanhuns – PE became taller than the other accesses of *J. curcas.* Accesses of *J. pohliana* presented an average height superior to all accesses of the other studied species, often reaching 2.00 m in height.

However, the diameter of the stem remained close to the average of the *J. curcas* accesses. *J. gossypiifolia* plants, access GT2 (84), are visibly smaller than the other accesses, yet their stem diameter is close to *J. curcas* and *J. pohliana*. In regards to the maturation cycle, it is possible to recognize a great variability among accesses, with a minimum of 240 and maximum of 342 days (Table 3). As for the seeds, values largely varied among accesses, ranging from 0.82 and 0.11 cm, maximum of 1.93 and 1.24 cm and average 1.76 and 1.08 cm, respectively for length and width (Table 3). Observing the dendrogram formed based on quantitative characters (Figure 2) and taking clustering criteria at the same percentage of genetic dissimilarity of 34%, the formation of 3 distinct groups is noted, where only access 84 (*J. gossypiifolia*) and 83 (*J. pohliana*) formed isolated groups. *J. pohliana,* access 83, originated from the state of Paraíba, in the region of Riachão do Bacamarte, was isolated from other accesses of this species mainly due to the reduced size of its seeds, both in length (average 1.12 cm) and width (average 0.11 cm), values quite similar to those observed for *J. gossypiifolia* seeds, 0.82 cm and 0.50, respectively, for length and width. All other accesses of *J. pohliana* (77, 78, 79, 80, 81, and 82) were allocated along with the 76 *J. curcas* accesses forming a single group. However, it is possible to see that *J. pohliana* accesses remained together in the lower extremity of the dendrogram and the same happened to *J. curcas* accesses which were allocated in the top extremity of the cluster, always mutual neighbors, meaning there was no mixture of accesses of different species within the group. Such results show the low variability found among accesses of the same species and that only accesses of different species had morphological differences.

Molecular data analysis

Of all 100 ISSR markers used, 43 were selected because they presented quantity, quality and reproducibility of amplified bands. All 43 primers presented polymorphism for the 84 studied accesses. A total of 451 bands were produced (Table 4), of which 185 (39.6%) were polymorphic, revealing an intermediate level of similarity among accesses. The average genetic dissimilarity among accesses of each species and between species was high (Table 5). The *J. gossypiifolia* species,

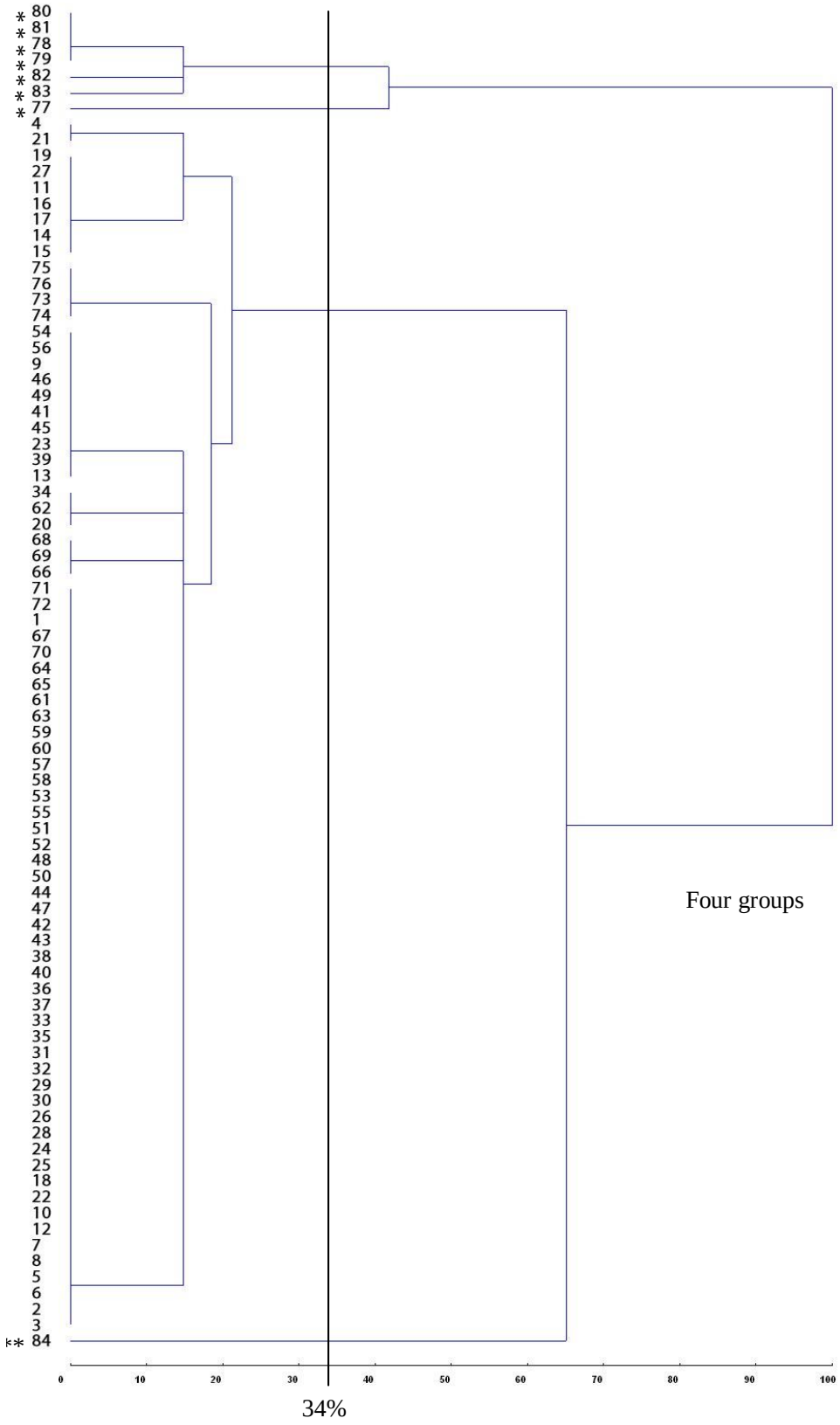

Figure 1. Dendrogram by UPGMA method, originated from the dissimilarities of the 84 accessions of *Jatropha* spp., based on qualitative morphological characters. The numbering of the access matches that specified in Table 1. Unmarked, *J. curcas; *J. pohliana; **J. gossypiifolia*

Table 3. Minimum values, mean, maximum and standard deviations for the quantitative characters used in the evaluation of the 84 accessions of *Jatropha* spp.

Quantitative descriptors	Values			Standard deviations
	Minimum	Mean	Maximum	
Number of leaf lobes	3.00	6.00	7.00	0.75
Plant height (cm)	74.80	117.05	230.00	28.91
Stalk diameter (cm)	4.18	5.31	6.18	0.47
Maturation cycle (days)	240.00	291.00	342.00	22.94
Length of seeds (cm)	0.82	1.76	1.93	0.19
Width of seeds (cm)	0.41	1.08	1.24	0.12

represented only by access 84, presented a null value for intraspecific variation, as expected. The *J. curcas* species, in its turn, despite being represented by more accesses than the others, presented low intraspecific variation. These results can be related to self-reproduction facilitated by the occurrence of individual plants and how this species was introduced in Brazil. The lowest estimated interspecific variation value (10.54) occurred between *J. pohliana* and *J. gossypiifolia* and the highest (12.96) between *J. pohliana* and *J. curcas*, very close to the value found between *J. curcas* and *J. gossypiifolia* (12.68). The number of bands produced by primer varied from five (ISSR 840) to 21 (ISSR 834), with an average of 10.5 bands per primer (Table 4). The level of polymorphism by starter was approximately 4.3 bands, with a variation from 1 (ISSR 840) to 15 polymorphic bands (ISSR 834). The size of the amplified fragments varied from 150 to 1900 pb, approximately. It is important to state that the differences between accesses were determined based on the more frequency of bands than on the presence or absence of specific bands. Nevertheless, it is important to point out the occurrence of specific bands in certain accesses by means of a determined primer, being that these bands are potentially useful for future studies on genetic improvement. The result of the hierarchization of all 84 *Jatropha* accesses is shown in Figure 3. There was a formation of ten distinct groups, generally, *J. curcas* accesses formed five distinct groups and *J. pohliana* and *J. gossypiifolia* accesses composed the five remaining groups. With the exception of the *J. gossypiifolia* access 84, which had been allocated along with a *J. pohliana* access (82), to the *J. pohliana* access (77), allocated to a group composed only of *J. curcas* accesses; as well as *J. curcas* accesses 70, 72, and 76, which had been allocated in distinct groups containing *J. pohliana* accesses (78, 79, and 81, respectively, a greater divergence between interspecific rather than intraspecific accesses was noted. The average genetic dissimilarities between accesses of each species were lower than dissimilarity among species (Table 5). In a study realized by Dhillon et al. (2009), interspecific hybrids of *J. curcas* and *Jatropha intergerrima* were developed to combine desirable agronomic traits and RAPD was used to distinguish the legacy of fragments in the hybrid progeny. The species *J. gossypiifolia*, being represented only by the access 84, presented a null value for intraspecific variation, as expected. The species *J. curcas*, however, despite being represented by more accesses than the others, presented low intraspecific variation. The lowest estimated interspecific variation value (10.54) occurred between *J. pohliana* and *J. gossypiifolia* and the highest (12.96) between *J. pohliana* and *J. curcas*, very close to the value found between *J. curcas* and *J. gossypiifolia* (12.68).

DISCUSSION

Very similar qualitative characters were observed in all the accesses, even among different species, for instance alternate and spiral leaves along the petiole (Table 2). Vasconcelos et al. (2010) while studying phytoalexin of *J. curcas* accesses from the states of Bahia and Paraíba found similar results to those observed in the present study. The same authors characterized *J. curcas* leaves as being greenish and glabrous when fully formed; however, the leaves when young had a vinaceous color, indicating photosynthetic inactivity, which was also observed in *J. curcas* accesses in this study (Table 2). Melo et al. (2007), on the other hand, described leaves with pilosity on the adaxial surface, indicating intraspecific variability in *J. curcas.* Regarding the ramification structure, a predominance of the goblet-shape was observed, 79% of the *Jatropha* spp accesses, which corresponds to the monopodial stem type, present in the 57 *J. curcas* accesses studied by Vasconcelos et al. (2010). Previous works corroborate for the classification found for coloration of leaf veins. Melo et al. (2007) when morphologically characterizing accesses of *J. curcas* from the active germplasm bank of the Federal University of Sergipe, verified that of the 15 accesses studied, all had greenish veins. Nonetheless, both Vasconcelos et al. (2010) and Avelar et al. (2008) observed leaf nerves with whitish protrusions on the abaxial surface of *J. curcas* accesses. Many of the characteristics presented by *J.*

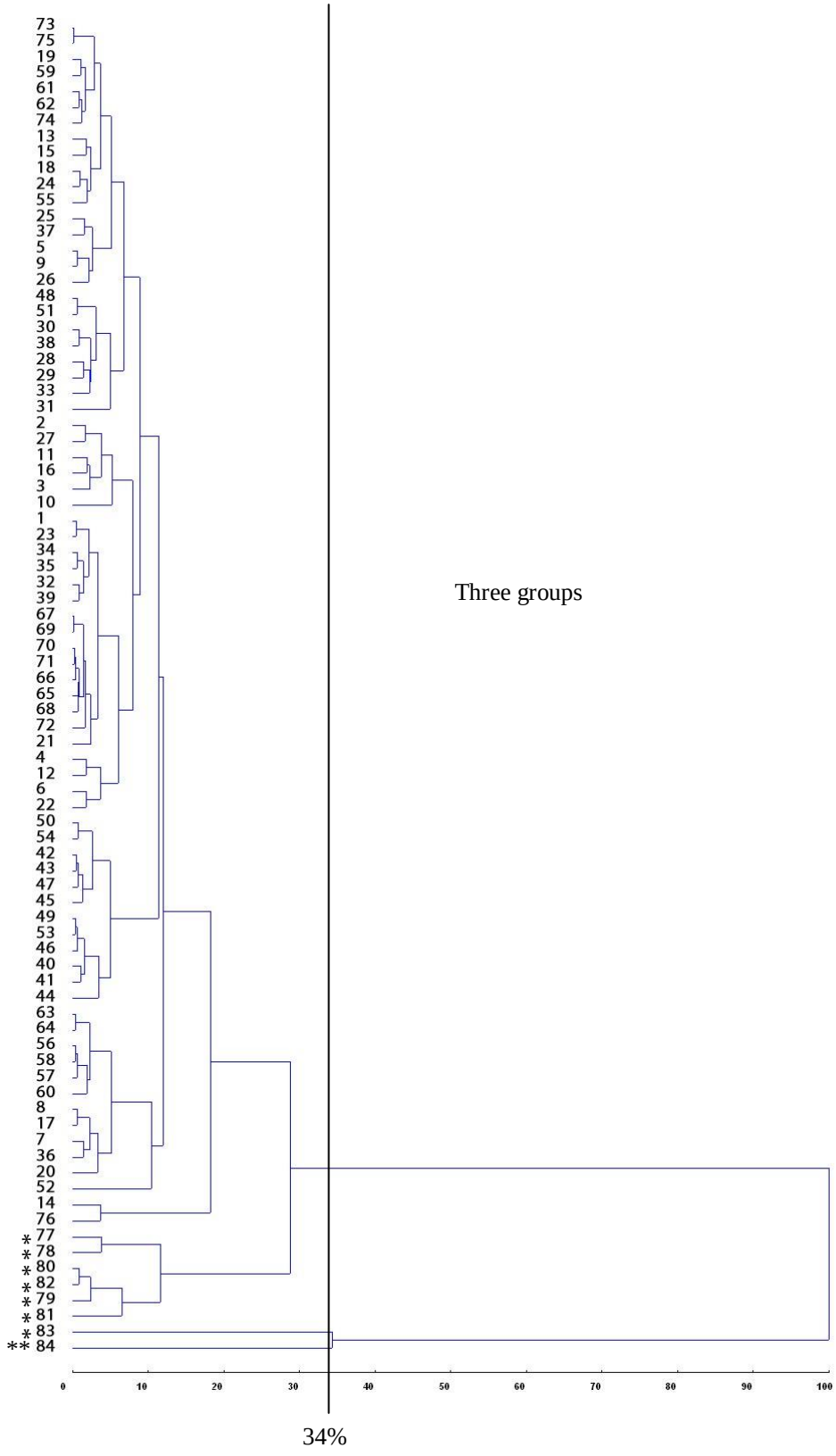

Figure 2 Dendrogram by UPGMA method that originated from the dissimilarities of the 84 accessions of *Jatropha* spp., based on quantitative morphological characters. The numbering of the access matches that specified in Table 1. Unmarked, *J. curcas; *J. pohliana; **J. gossypiifolia*

Table 4. Sequence of nitrogenous bases, annealing temperature (Ta), total number of fragments (TNF), number of polymorphic fragments (NPF) and percentage of polymorphism (P%) obtained in the analyzes of the 43 ISSR markers in 84 accessions of *Jatropha* spp.

Primer	Sequence (5' 3') (1)	Ta (°C)	TNF	NPP	P%
807	AGA GAG AGA GAG AGA GT	49.8	9	4	44.4
808	AGA GAG AGA GAG AGA GC	56.0	9	4	44.4
809	AGA GAG AGA GAG AGA GG	51.5	11	5	45.5
810	GAG AGA GAG AGA GAG AT	55.0	17	9	52.9
811	GAG AGA GAG AGA GAG AC	55.0	17	8	47.1
812	GAG AGA GAG AGA GAG AA	50.0	13	7	53.8
813	CTC TCT CTC TCT CTC TT	55.0	7	4	57.1
815	CTC TCT CTC TCT CTC TG	55.0	9	3	33.3
817	CAC ACA CAC ACA CAC AA	55.0	15	5	33.3
818	CAC ACA CAC ACA CAC AG	55.0	14	2	14.3
819	GTG TGT GTG TGT GTG TA	55.0	10	6	60.0
821	GTG TGT GTG TGT GTG TT	55.0	11	7	63.6
822	TCT CTC TCT CTC TCT CA	55.0	8	4	50.0
823	TCT CTC TCT CTC TCT CC	55.0	14	8	57.1
824	TCT CTC TCT CTC TCT CG	55.0	12	9	75.0
825	ACA CAC ACA CAC ACA CT	55.0	12	4	33.3
826	ACA CAC ACA CAC ACA CC	55.0	15	7	46.7
827	ACA CAC ACA CAC ACA CG	52.0	17	7	41.2
828	TGT GTG TGT GTG TGT GA	55.0	8	4	50.0
829	TGT GTG TGT GTG TGT GC	50.0	10	6	60.0
830	TGT GTG TGT GTG TGT GG	52.0	9	6	66.7
834	AGA GAG AGA GAG AGA GYT	52.5	21	15	71.4
835	AGA GAG AGA GAG AGA GYC	53.9	9	4	44.4
836	AGA GAG AGA GAG AGA GYA	53.7	9	3	33.3
840	GAG AGA GAG AGA GAG AYT	54.2	5	1	20.0
841	GAG AGA GAG AGA GAG AYC	55.0	8	4	50.0
842	GAG AGA GAG AGA GAG AYG	55.0	8	1	12.5
847	CAC ACA CAC ACA CAC ARC	53.3	10	4	40.0
850	GTG TGT GTG TGT GTG TYC	55.0	6	2	33.3
853	TCT CTC TCT CTC TCT CRT	55.0	7	1	14.3
858	TGT GTG TGT GTG TGT GRT	55.0	9	4	44.4
860	TGT GTG TGT GTG TGT GRA	55.0	10	4	40,0
863	AGT AGT AGT AGT AGT AGT	55.0	8	1	12.5
864	ATG ATG ATG ATG ATG ATG	55.0	10	2	20.0
866	CTC CTC CTC CTC CTC CTC	55.0	13	1	7.7
867	GGC GGC GGC GGC GGC GGC	50.0	13	2	15.4
876	GAT AGA TAG ACA GAC A	55.0	9	2	22.2
880	GGA GAG GAG AGG AGA	55.0	9	1	11.1
881	GGG TGG GGT GGG GTG	55.0	7	3	42.9
884	HBH AGA GAG AGA GAG AG	55.0	7	3	42.9
887	DVD TCT CTC TCT CTC TC	55.0	7	2	28.6
889	DBD ACA CAC ACA CAC AC	53.6	11	3	27.3
891	HVH TGT GTG TGT GTG TG	50.4	8	3	37.5
Total			451	185	
Averages		54.0	10.5	4.3	39.6

(1)B, D, H, R, V and Y mean degenerate oligonucleotide: B = (T, C, G); D = (A, G, T); H = (A, T, C); R = (A, G); V = (A, C, G) and Y = (C, T).

Table 5. Averages of genetic dissimilarities (%) estimated by molecular ISSR primers, adopting the arithmetic complement of Jaccard coefficient for three species of *Jatropha*. Jaboticabal, SP.

	J. curcas	***J. pohliana***	***J. gossypiifolia***
J. curcas	2.09	12.96	12.68
J. pohliana	-	2.56	10.54
J. gossypiifolia	-	-	0.00

curcas, *J. pohliana* and *J. gossypiifolia* fruits in this study were also verified in *Jatropha elliptica* fruits (Añez et al., 2005), another species of the genre, with the same explosive propulsion mechanism of seeds. The dehiscence observed in *J. curcas*, *J. pohliana* and *J. gossypiifolia* fruits (Table 2) is also present in fruits of other genres of Euphorbiaceae. Barroso et al. (1999) pointed out that all of these species are related to one another and must have originated from a common ancestor. In the present study, the height of *J. curcas* and *J. pohliana* plants, in general among accesses, was very high (Table 2). In this case, the selection of smaller genotypes in order to facilitate harvest operations is optimal (Dias et al., 2007). A study conducted with 57 *J. curcas* accesses from Petrolina - BA and Columbia-PB showed an average height of 164.00 cm in accesses from the first group and 190.00 cm in the second group (Vasconcelos et al., 2010), only confirming the results observed here. Studies on the contribution of characters for diversity are important in order to select those characters which best differentiate accesses and exclude those that do not effectively contribute to genotype discrimination (Cruz et al., 2012). There was great variability among accesses regarding trait for number of days for fruit maturation (Table 4), which may be justified by the fact that those are allogamous species, dependant on pollinator insect, which in their turn can be insufficiently or irregularly distributed in the environment. Such differences may be still explained by genetic divergences that naturally occur among accesses of species of a determined kind and origin.

Also, heterogeneous fructification was observed within a plant, which probably occurred for the reasons previously cited. Table 2 also presents seed length and width data for the 84 accesses studied. Lower values than those found in the present study have been observed by Añez et al. (2005) for *J. elliptica* seeds, having an average of 0.82 cm length and 0.43 cm width. However, *J. gossypiifolia* seeds were the smallest among the studied species, with average values of 0.82 and 0.50 cm, respectively, for length and width, much closed values to those found by Añez et al. (2005) for *J. elliptica*. The high variability observed between accesses for the number of lobes, height of plants, stem diameter, maturation cycle, and length and width of seeds traits can, and certainly will serve as base for future studies. As for the other characters studied, namely, the qualitative characters, there was also variability, which enabled the separation of accesses into a number of different groups, superior even to the quantitative characters. However, by observing Table 2, it is possible to observe many accesses placed within the same classification category, for instance, 92% of accesses presented glabrous leaves, and only 8% hairy leaves, however when using a larger number of qualitative characters, as in the case in this study, the chances of obtaining variability between accesses were increased. By observing the results for each qualitative character individually (Table 2), low variation between accesses is noted, which may be explained by the narrow genetic base of the studied plants from the germplasm bank, or by the monogenic nature of these characters. There was congruency between some of the groups formed in the clustering analysis for qualitative and quantitative characters (Figures 1 and 2). In both cases, the totality of *J. curcas* accesses was arranged in one large group. *J. pohliana* accesses, in general, were allocated in the same group and access 84, the only *J. pohliana* sample, formed and isolated group in the two analysis carried out. Such observations show low intraspecific variation of these *Jatropha* species, as opposed to the interspecific diversity found.

The molecular analysis indicated that the investigated species shared 260 alleles (57.6%), with a polymorphism rate of 42.3%, which considered a relatively high number. Cai et al. (2010) evaluated a set of 224 *J. curcas* accessions including 219 from all the adaptation areas in China and five from Myanmar, 15 UBC primers provided among the 169 amplified bands, 127 (75.15%) were polymorphic which meant that Chinese *Jatropha* had high genetic diversity. Souza et al. (2009), however, analyzing 11 ISSR primers, obtained a low polymorphism rate (23.09%). Few accesses were distinguished by their diversity, the interspecific variability (between accesses of different species) higher than the intraspecific variability (occurring within a species) (Table 5), which is justified by the higher degree of relationship existing between accesses of the same species as well as by the narrow genetic base of the studied species. Ram et al. (2008) found 80.2% polymorphism when studying genetic diversity of eight species of *Jatropha* genre, including *J. curcas* and *J. gossypiifolia*, and indicative of the existence of high interspecific variability within *Jatropha* genre. The authors also observed, through UPGMA, the

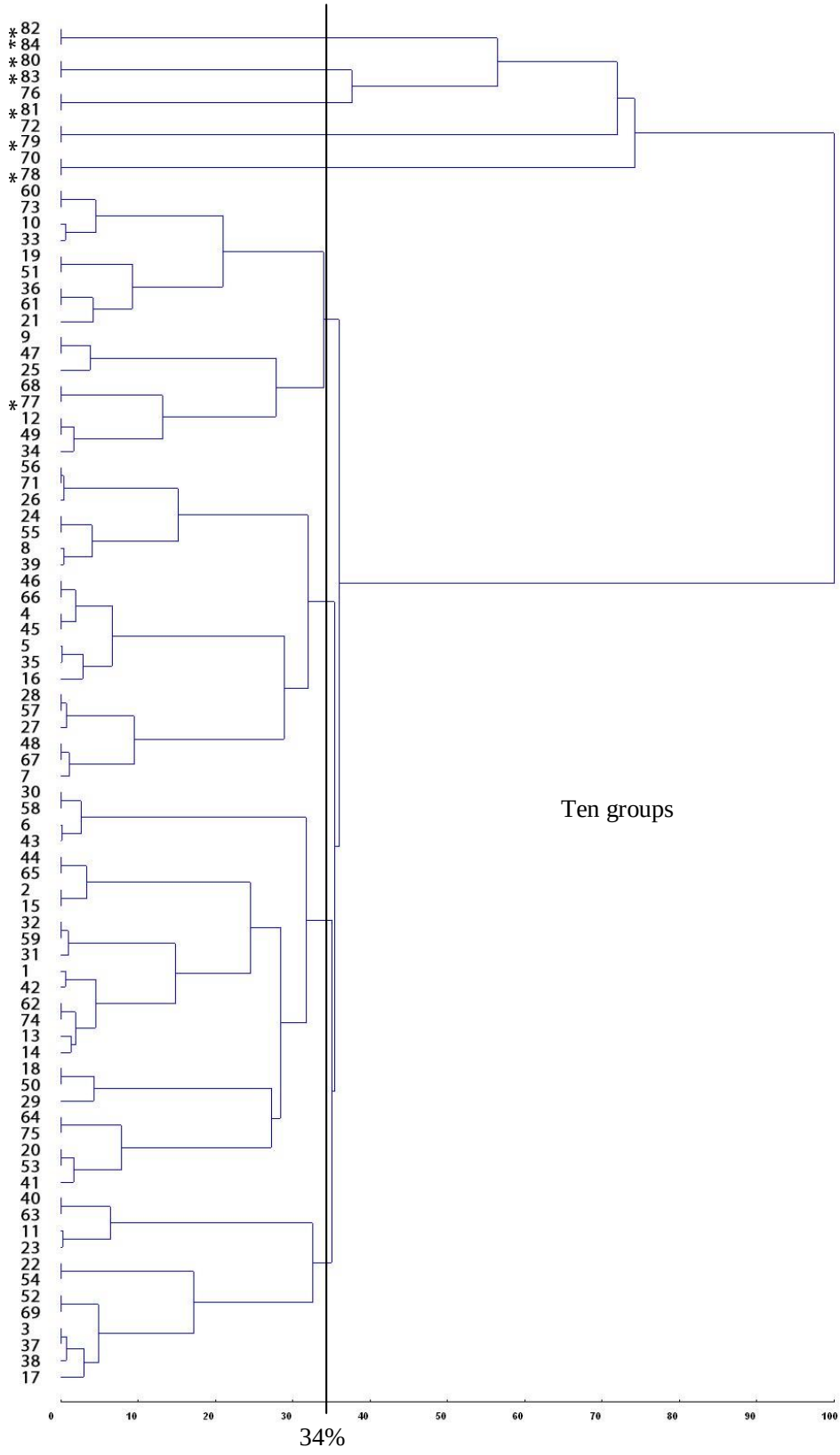

Figure 3. Dendrogram by UPGMA method, originated from arithmetic complement of Jaccard Coefficient between 84 accessions of *Jatropha* spp., based on ISSR molecular markers. The numbering of the access matches that specified in Table 1 Unmarked, *J. curcas; *J. pohliana; **J. gossypiifolia*

formation of three distinct groups, one of them formed exclusively of *J. curcas* accesses. In this study, the dendrogram formed by the UPGMA method based on molecular data (Figure 3), created ten distinct groups, where it was possible to observe *J. curcas* accesses in distinct, but very close groups. It is still possible to observe that *J. curcas* accesses 70, 72, and 76 were allocated into groups with *J. pohliana* accesses in the top extremity of the dendrogram. *J. pohliana* access 77 was allocated in a group composed only by *J. curcas* accesses, and the *J. gossypiifolia* access 84 was in a group with *J. pohliana* access 82, also from Galante – PB. Therefore, there was interspecific mixing, when 34% dissimilarity was used, however it is possible to observe that this mixing kept intraspecific accesses in close groups, in other words, with a high degree of genetic similarity.

Dehgan and Webster (1979), in their study on the classic taxonomy of *Jatropha* genre, concluded that *J. curcas* is a primitive ancestor species of this genre, due to its morphological differentiation, and that species from other sections evolved from it and other ancestor forms. By observing Figure 3, where *J. curcas* accesses distinguish themselves molecularly from the other two species, this hypothesis is once again reinforced. Past studies with *Jatropha*-related species using RAPD and ISSR markers reported the *J. curcas* accessions in a distinct group (Ganesh Ram et al., 2008; Senthil Kumar et al., 2009, respectively). Sujatha et al. (2005), studying the genetic diversity between toxic and non-toxic *J. curcas* using RAPD markers, found a percentage of 96.3% genetic similarity. In another study, Sudheer et al. (2009) reported 84.9 and 83.6% between toxic and nontoxic *J. curcas* plants analyzed by RAPD and AFLP, respectively, and identified specific RAPD and AFLP markers for both varieties. A inter and intrapopulation study conducted with RAPD and ISSR markers on 42 *J. curcas* accesses from different regions in India, and one non-toxic genotype from Mexico presented 42.0 and 37.4% polymorphism through RAPD and ISSR, respectively (Basha and Sujatha, 2007). The same authors in further studies, working with 200 RAPD, 100 ISSR and 50 organelle specific microsatellite primers for *Jatropha* species from India, observed 98.5% polymorphism, with high interspecific genetic variation (Basha and Sujatha, 2009). Furthermore, ISSR primers have also been successfully used to estimate the degree of intra and interspecific genetic diversity in other species, including rice (Joshi et al., 2000), wheat (Nagaoka and Ogihara, 1997), Eleusine coracana (Salimath et al., 1995), Vigna (Ajibade et al., 2000), sweet potato (Huang and Sun, 2000), and Plantago (Wolff and Morgan-Richards, 1998). The formation of main groups containing the majority of *J. curcas* accesses are a clear indication of the low genetic diversity between individuals of different origins and reinforces the idea that *J. curcas* genotypes from different origins share a common ancestry, excluding the genetic concept of diversity by origin.

Oliveira (2007), working with RAPD markers, verified the formation of four distinct groups independent of the origin of the genotypes. The lack of relationship between the similarity pattern found and the geographical origin on the accesses has been reported in other studies (Basha and Sujatha, 2007). Additional efforts to cover the lack of knowledge on the genetic diversity of the *Jatropha* genre using molecular markers and morphological characters are obviously needed. Finally, the ISSR markers and morphological characters used allowed us to distinguish the species and accesses of the *Jatropha* genre, detecting low variation between intraspecific, and high variation between interspecific accesses, and may be very useful in monitoring genetic variability in germplasm banks when nuclear collection are eventually established.

Conflict of interests

The author(s) did not declare any conflict of interest.

ACKNOWLEDGEMENTS

The authors would like to thank Conselho Nacional de Desenvolvimento Cientifico e Tecnológico (CNPq) and Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) for their funding of this research.

REFERENCES

Accarini JH (2006). Biodiesel no Brasil: estágio atual e perspectivas. Revista Bahia, Análise & Dados 16(1):51-63.

Ajibade SR, Weeden NF, Chite SM (2000). Inter-simple sequence repeat analysis of genetic relationships in the genus *Vigna*. Euphytica 111(1):47–55.

Añez, LMM, Coelho MFB, Albuquerque MCF, Dombroski JLD (2005). Caracterização morfológica dos frutos, das sementes e do desenvolvimento das plântulas de *Jatropha elliptica* Müll.Arg.. Arg. (Euphorbiaceae). Rev. Bras. Bot. 28(3):563-568.

Avelar RC, Silva FM, Castro Neto P, Fraga AC (2008). Avaliação do desenvolvimento de pinhão- manso (*Jatropha curcas* L.) do banco de germoplasma da UFLA. In: Anais do Congresso Brasileiro de Plantas Oleaginosas, Óleos, Gorduras e Biodiesel, Lavras (CD Room).

Barroso MB, Morin NP, Peixoto AL, Ichaso CLF (1999). Frutos e sementes. Morfologia Aplicada à Sistemática de Dicotiledôneas. 2ª edição. Editora UFV, Viçosa, 444 pp.

Basha SD, Sujatha M (2007). Inter and intra-population variability of *Jatropha curcas* (L.) characterized by RAPD and ISSR markers and development of population-specific SCAR markers. Euphytica 156(3):375-386.

Basha SD, Sujatha M (2009). Genetic analysis of *Jatropha* species and interspecific hybrids of *Jatropha curcas* using nuclear and organelle specific markers. Euphytica 168(2):197–214.

Cai Y, Suna D, Wud G (2010). ISSR-based genetic diversity of *Jatropha curcas* germplasm in China. Biomass Bioenergy 34(12):1739-1750.

Cole-Rodgers P, Smith DW, Bosland PW (1997). A novel statistical approach to analyze genetic resource evaluations using Capsicum as an example. Crop Sci. 37(3):1000-1002.

Cruz CD (2013). GENES - a software package for analysis in experimental statistics and quantitative genetics. Acta Sci. 35(3):271-

276.
Cruz CD, Carneiro PCS (2006). Modelos Biométricos Aplicados ao Melhoramento Genético – Volume 2. 2ª edição. Editora UFV, Viçosa, 585 pp.
Cruz CD, Regazzi AJ, Carneiro PCS (2012). Modelos Biométricos Aplicados ao Melhoramento Genético- Volume 1. 4ª edição. EditoraUFV, Viçosa, 514 pp.
Dehgan B, Webster GL (1979). Morphology and intergeneric relationship of the genus *Jatropha*. Univ. Calif. Publ. Bot. 74:1–73.
Dellaporta SL, Wood J, Hick JB (1983). A plant DNA minipreparation. Plant. Mol. Biol. Rep. 1(4):19-21.
Dhillon RS, Hooda MS, Jattan M, Chawla V, Bharadwaj M, Goyal SC (2009). Development and molecular characterization of interspecific hybrids of Jatropha curcas X Jatropha integerrima. Indian J. Biotechnol. 8:384-390.
Dias LAS, Leme LP, Laviola BG, Pallini Filho A, Pereira OL, Carvalho M, Manfio CE, Santos AS, Sousa LCA, Oliveira TS, Dias DCFS (2007). Cultivo de Pinhão-manso (*Jatropha curcas* L) para Produção de Óleo Combustível. 1ª edição. Editora UFV, Viçosa, 40 pp.
Divakara BN, Upadhyaya HD, Wani SP, Gowda CLL (2010). Biology and genetic improvement of *Jatropha curcas* L.: a review. Appl. Energy 87(3):732-742.
Doyle JJ, Doyle JL (1990). Isolation of plant DNA from fresh tissue. Focus 12:13-15.
Elias M, Mühlen GS, McKey D, Roa AC, Tohme J (2004). Genetic diversity of traditional South American landraces of cassava (*Manihot esculenta* Crantz): an analysis using microsatellites. Econ. Bot. 58(2):242-256.
Embrapa Algodão (2008). Descritores de Mamona Utilizados pela Embrapa Algodão. Campina Grande, Embrapa Algodão Campina Grande (ed), documento 192, Campina Grande, 39pp.
Ferreira ME, Grattapaglia D (1998). Introdução ao Uso de Marcadores Moleculares em Análise Genética. 3ª edição. Embrapa Recurso Genéticos Vegetais Brasília (ed), Brasília, 220 pp.
Ganesh Ram S, Parthiban KT, Senthil Kumar R, Thiruvengadam V, Paramathma M (2008). Genetic diversity among Jatropha species as revealed by RAPD markers. Genet. Resour. Crop Evol. 55(6):803–809.
Góis IB, Silva R, Boari A de J, Oliveira A dos S, Fraga AC (2006). Caracterização isoenzimática de acessos de pinhão- manso (*Jatropha curcas* L.). In: Embrapa Algodão Campina Grande (ed). Proceedings of the 2º Congresso Brasileiro de Mamona, Aracaju, http://www.cnpa.embrapa.br/produtos/mamona/publicacoes/trabalhos_cbm2/2006.pdf (September 2, 2011)
Huang J, Sun SM (2000). Genetic diversity and relationships of sweet potato and its wild relatives in *Ipomoea* series Batatas (Convolvulaceae) as revealed by inter-simple sequence repeat (ISSR) and restriction analysis of chloroplast DNA. Theor. Appl. Genet. 100(7):1050-1060.
Joshi SP, Grupta VS, Aggarwal RK, Ranjekar PK, Brar DS (2000). Genetic diversity and phylogenetic relationship as revealed by inter-simple sequence repeat (ISSR) polymorphism in the genus *Oryza*. Theor. Appl. Genet. 100(8):1311-1320.
Melo MFV, Silva-Mann R, Santos HO, Souza EM (2007). Caracterização morfológica de acessos de *Jatropha curcas* L. do banco de germoplasma da Universidade Federal de Sergipe. In: Anais do Congresso da Rede Brasileira de Tecnologia de Biodiesel, Brasília (CD Room).
Messmer MM, Melchinger AE, Herrmann RG, Jurgen B (1993). Relationships among early European maize inbreds: II. Comparison of pedigree and RFLP data. Crop Sci. 33(5):944-950.
Nagaoka T, Ogihara Y (1997). Applicability of inter-simple sequence repeat polymorphisms in wheat for use as DNA markers in comparison to RFLP and RAPD markers. Theor. Apppl. Genet. 94(5):597–602.
Nunes CF, Pasqual M, Santos DN dos, Custódio TN, Araújo AG de (2008). Diferentes suplementos no cultivo in vitro de embriões de pinhão manso. Pesq. Agropec. Bras. 43(1):9-14.
Oliveira AS (2007). Diversidade genética entre acessos de *Jatropha* sp. In: Anais do 2º Congresso da Rede Brasileira de Tecnologia de Biodiesel. MCT/ABIPTI, Brasília, 451 pp.
Oliveira JS, Leite PM, Souza LB de, Mello VM, Silva EC, Rubim JC, Meneghetti SMP, Suarez PAZ (2006). Avaliação Preliminar de parâmetros físico-químicos do óleo e biodiesel de Pinhão Roxo (*Jatropha gossypiifolia* L.). In: Anais da 29ª Reunião Anual da Sociedade Brasileira de Química, Águas de Lindóia, https://sec.sbq.org.br/cd29ra/resumos/T0777-1.pdf (November 19, 2011).
Ram GS, Parthiban KT, Senthil KR, Thiruvengadam V, Paramathma M (2008). Genetic diversity among *Jatropha* species as reveled by RAPD markers. Genet. Resour. Crop Evol. 55(6):803–809.
Salimath SS, Oliveira AC, Godwin ID, Bennetzen JL (1995). Assessment of genome origins and genetic diversity in the genus *Eleusine* with DNA markers. Genome 38(4):757–763.
Senthil Kumar R, Parthiban KT, Govinda Rao M (2009). Molecular characterization of *Jatropha* genetic resources through intersimple sequence repeat (ISSR) markers. Mol. Biol. Rep. 36(7):1951–1956.
Soares BO (2010). Diversidade genética de genótipos de pinhão-manso por meio de RAPD e ISSR. Dissertação (Mestrado)– Universidade Estadual de Montes Claros, Jarnaúba, 54 pp.
Sousa MTB (2006). Análise da utilização do biodiesel como alternativa para o desenvolvimento sustentável. In: Anais do 1º Congresso de Pesquisa e Inovação da Rede Norte Nordeste de Educação Tecnológica, Natal. Natal: CONNEPI.
Souza DA, Moreira GBR, Guimarães JFR, Librelon SS, Fernandes TP, Nietsche S, Costa MR (2009). Análise da diversidade genética intrapopulacional de pinhão-manso (*Jatropha curcas* L.) com marcadores ISSR. In: Anais do Congresso Brasileiro de Plantas Oleaginosas, Óleos, Gorduras e Biodiesel. Montes Claros, pp.2158-2164.
Sudheer PDVN, Singh S, Mastan SG, Patel J, Reddy MP (2009). Molecular characterization and identification of markers for toxic and non-toxic varieties of *Jatropha curcas* L. using RAPD, AFLP and SSR markers. Mol. Bio. Rep. 36(6):1357-1364.
Sujatha M, Makkar HPS, Becker K (2005). Shoot bud proliferation from axillary nodes and leaf sections of non-toxic *Jatropha curcas* L. Plant Growth Regul. 47(1):83–90.
Targino KC de F, Silva FFM da (2013). Rendimento e análise físico-química do óleo extraído das sementes do pinhão-bravo (*Jatropha mollissima* Müell.Arg.). In: Anais do IX Congresso de Iniciação Científica do IFRN, Natal, pp.1326-1334.
Vasconcelos GCL, Arriel NHC, Medeiros KAAL, Medeiros OS, Melo JIM, Lucena AMA, Freire MAO (2010). Caracterização botânica de acessos de pinhão manso do banco de germoplasma da Embrapa Algodão. In: Embrapa Algodão Campina Grande (ed), Anais do Congresso Brasileiro de Mamona & Simpósio Internacional de Oleaginosas Energéticas, João Pessoa, pp.1606-1611.
Wolff K, Morgan-Richards M (1998). PCR markers distinguish *Plantago major* subspecies. Theor. Appl. Genet. 96(2):282-286.
Zietkiewicz E, Rafalki A, Labuda D (1994). Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. Genomics 20(2):176-183.

academicJournals

Vol. 14(25), pp. 2080-2087, 23 June, 2015
DOI: 10.5897/AJB2015.14427
Article Number: 10A3D2B53882
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

In vitro microtuberization of Black Zira (*Bunium persicum* Boiss.)

Hossein Mardani[1]*, Seyed Mahdi Ziaratnia[2], Majid Azizi[3], Han Phyo Aung[1], Kwame Sarpong Appiah[5] and Yoshiharu Fujii[4]

[1]Laboratory of International Agro-Biological Resources and Allelopathy, Tokyo University of Agriculture and Technology, Japan.
[2]Department of Food Technology, Research Institute of Food Science and Technology, Mashhad, Mashhad, Iran.
[3]Department of Horticulture, Faculty of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran.
[4]Tokyo University of Agriculture and Technology, Division of International Environmental Agriculture, Laboratory of International Agro-Biological Resources and Allelopathy, Japan.
[5]Department of International Environmental and Agricultural Science in Tokyo University of Agriculture and Technology, Japan.

Received 15 January, 2015; Accepted 17 June, 2015

Bunium persicum or **Black Zira is one of the endangered species in the land of Persia. The main purpose of this study was to investigate microtuberization of *B persicum*in in order to use in germplasm storage and commercial production. Seeds of *B. persicum* were used as explant. Different culture media (MS, ½MS and B_5) along with different concentrations of jasmonic acid (JA) (0, 2 and 5) were used individually as basal media and also in combination with two different temperatures (15 and 20°C) to develop appropriate media for microtuberization. Moreover, propagated microtubers were then vernalized and acclimatized in order to transfer to greenhouse. The results revealed that by increasing in concentration of JA, weight and length of microtubers increased significantly. MS medium seemed to be the most effective basal medium for this plant. In contrary, this study indicated that MS medium and 5 mM JA were the most suitable combination for *in vitro* culture establishment and short-term maintenance of tested *B. persicum.* Also, 15°C showed significant effect on increasing the weight of microtubers.**

Key words: Microtuberization, *Bunium persicum*, jasmonic acid, temperature, medium.

INTRODUCTION

Bunium persicum (Boiss.) or Black Zira is a species from the Apiaceae family, especially grown in the northeast areas of Iran. *B. persicum* seeds are called ‘‘zireh kuhi’’, meaning ‘‘wild cumin’’, and are used as a culinary spice (Mortazavi et al., 2010). Apiaceae family has a short growth period (75 days) (Omidbigi, 2012). It is found wildly growing in Iran, Pakistan, India and central parts of Asia (Omidbigi, 2012). *B. Persicum* seeds have been widely used for medicinal purposes especially as anti-fungus (Sharififar et al., 2010). Several remedial impacts

*Corresponding author. E-mail: 50014951904@st.tuat.ac.jp.

including digestive disorders, urinary tract disorders, and diuretic, gynaecologic, anticonvulsion, anti-helmetic have been defined for *B. persicum* seeds (Sekine et al., 2007). It has also been reported that this species has strong anti-inflammatory activities and reduces the pain (Hajhashemi et al., 2012). The demand for this crop is rapidly increasing while its habitat has been shrinking across the country because of overharvesting. Despite all properties of this valuable plant, there are lots of unknown and unstudied cases about this amazing species. Appropriate germination and crop establishment is most critical stage of growth period of this plant (Sharifi and Pouresmael, 2003). It is well documented that the main reason of low germination percentage of Apiaceae species are because of its minute embryo in this family and oxygen deficit in germination process (Carol et al., 1992). Seeds of *B. persicum* need cold temperature for stratification process and cold demands of this plant eliminate across December to January in northeast of Iran. In nature, seeds germinate in 3 to 4 month after passing through the winter and consequently produce only a few leaves and a small tuber (Garval and Rani, 1999). Plant growth rate in first year of cultivation is significantly slow and maximum diameter of *B. persicum* tuber is approximately 4 mm (Omidbigi, 2012). Reproductive phase (seed production) of *B. persicum* starts after 4 years and may maintain up to 12 years as tuber continues growing (Garval and Rani, 1999; Omidbigi, 2012). Generally, economic production of this plant set up after almost 4 years which is the main reason for low tendency of local farmers to cultivation of this valuable plant. Khosravi (1994) reported that the production of *B. persicum* is limited because of seed dormancy and several biotic stresses of which wilt diseases are the most serious. Also, potential genetic variability for conventional production is limited in *B persicum* (Hunault et al., 1989). Chizzola et al. (2014) reported the genetic variability of fruits from different Iranian wild populations.

The use of *in vitro* tuberization has some advantage such as higher control of the different environmental factors in microtuberization and can boost the crop production. Rapid production of microtubers could be useful for the production of pathogen-free seed tubers (Omokolo et al., 2003). Moreover, it has been proved that tissue culture is one of the main methods for gene conservation (Turner et al., 2001). Also, microtubers are important in fundamental researches in plant science and plant germplasm storage. Components of the culture medium vary according to the type of plant and the propagation stage. These components include inorganic salts, organic compound, natural ingredients and inert support. There are few reports about micro-propagation and somatic embryo development of *B. persicum*. Wakhlu et al. (1990) and Valizadeh et al. (2007) obtained the callus of *B. persicum* Boiss from mericarps. In this regard, the present study was built on determination of more suitable medium for enhancing of *B. persicum* tuber size in order to promote its commercial production in future and save this plant from extension.

Numerous investigators engaged in other systems have shown that some growth regulators, such as Jasmonic acid, can act as growth induced stimulator in tuber development processes (Zel et al., 1997; Cenzanoa et al., 2007; Pelacho et al., 1999). It has shown that microtuberization is generally induced directly on the explants culture and is influenced by factors including growth regulators such as Jasmonic acid (Jasik and Mantell, 2000). Hence, the main purpose of this study was to find out the appropriate condition for microtuber development of *B. persicum* under different controlled conditions such as different medium and temperature, and their interactions with Jasmonic acid as a growth regulator. To achieve this, an *in vitro* experiment under different temperatures, mediums and growth regulators was established.

MATERIALS AND METHODS

Plant material

This study was conducted at Ferdowsi University of Mashhad and Khorasan Research Institute for Food Science and Technology in Iran from 2010 to 2012. *B. persicum* seeds were collected from the experimental field of Ferdowsi University of Mashhad. Seeds were washed with tap water for 10 min and surface sterilized with 70% ethanol for 1 min and 1.5% (w/v) sodium hypochlorite solution for 15 min. Seed were then washed with distilled water 3 times. A simple seed medium containing agar (Sigma) (5 g/l) and sucrose (Sigma) (5 g/l) were used in each micro-tube. Each seed were sown individually in 1.5 ml microtubes containing 1 ml prepared seed medium to prevent contamination. All microtubes were kept in 4°C for stratification. Following a period of time (8 weeks), seeds started to germinate. Those seeds which had developed 4 mm of root were used for tuber induction stage. Figure 1 shows the germinated seeds after developing a full radicle.

Preparation of the plant growth regulators and media

For tuber induction, different basal media MS (Murashige and Skoog, 1962) (3% sucrose), B_5 (Gamborg et al., 1968) and MS basal media of half strength of macro- and micro-salts (1/2 MS) with 825 mg/l NH_4NO_3 and 950 mg/l KNO_3, 185 mg/l $MgSO_4.7H_2O$, 85 mg/l KH_2PO_4, 13.9 mg/l $FeSO_4 \cdot 7H_2O$, 110 mg/l $CaCl_2.2H_2O$, 3.1 mg/l H_3BO_3, 0.12 mg/l $NaMoO_4.2H_2O$, 0.012 mg/l $CoCl_2.5H_2O$, 4.3 mg/l $ZnSO_4.4H_2O$, 11.1 mg/l $MnSO_4.4H_2O$, 0.41 mg/l KI, 18.6 mg/l Na_2 EDTA.$2H_2O$ supplemented with 3% sucrose were used. Vitamins were added to all media in same concentration. The pH of the media was adjusted to 5.7 and autoclaved at 121°C for 15 min. Stock solution of Jasmonic acid (JA) was prepared by dissolving in a small amount of 70% ethanol and then adding distilled water to volume for the next steps of experiment (Gao et al., 2003). Also, to assess the effect of JA on morphological characteristics of microtubers, JA was used in concentration of 0 (control), 2 and 5 mg/l. Each germinated seed was planted in a glass tube containing 5 ml of each medium. Temperature treatment was applied after seeds had been germinated at 4°C under darkness conditions. *B. persicum* germinated seeds were transferred to growth chambers controlled to temperature of 15 and 20°C (18 h light/6 h dark

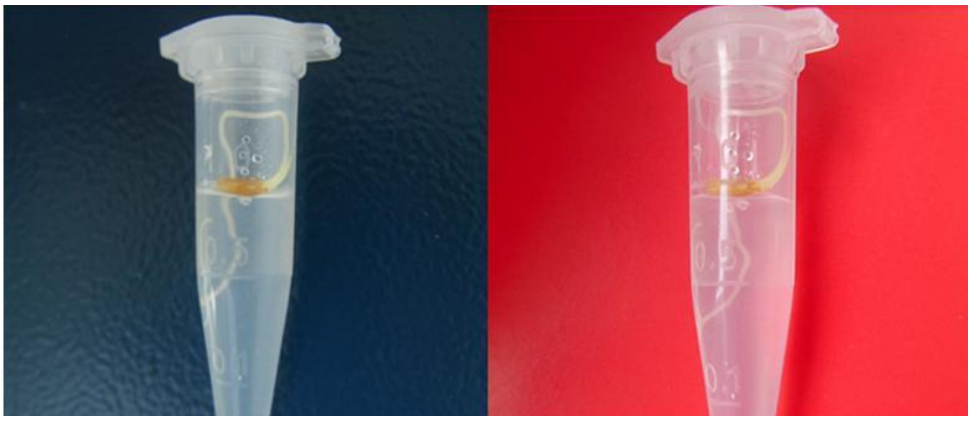

Figure 1. Germinated seeds of *B. persicum* after chilling period at 4°C.

photoperiod) conditions. Light was supplied by cool white fluorescent lamps (40 mMol/m/s).

Data collection

Following a period of growth (usually after 8 weeks), fresh weight, length, width of each single microtuber from each culture tube were recorded. The average final dry weight for the replicates of each treatment was recorded after 8 weeks.

Microtuber vernalization

Some visual morphological changes in the plantlets and micro tubers after vernalization stage *in vitro* and in greenhouse conditions were observed and recorded. To access this purpose, 20 well developed healthy microtubers obtained from all treatments were acclimatized and subcultured in the same medium which had been applied during the incubation stage. At this time, all the microtubers had been passed 8 weeks after the seed culture. Furthermore, to induce the vernalization, microtubers were transplanted to the refrigerator (4°C). After detecting the first true leaves on microtubers, all germinated microtubers were transferred to the greenhouse controlled to temperatures of 25±3°C. MS medium was removed, and washed from the microtubers using distilled water to prevent subsequent infection. All the microtubers were planted in medium composed of peat (20%) and coco peat (20%) and soil (60%). Plants were then maintained in the greenhouse (25°C, 85% humidity). Vernalisation steps of microtubers are provided in Figure 2.

Data analysis

Statistical analysis was performed using the SAS software program (Version 9.0). Data were subjected to the analysis of variance (ANOVA) to detect the significant differences between level of Jasmonic acid, level of temperature and level of medium. The general linear model (GLM) was constructed to generate three-way ANOVA. The results of three-way ANOVA for dry weight and length of microtuber are summarized in Table 1. Mean separation was conducted using Least Significant Difference (LSD) test at 0.01 probability level. All samples, with the exception of occasional contaminated tubes, were computed for tuberization. Among all calculated factor (length, width, weight, weight ratio) impacts of medium and temperature and jasmonic acid had shown a significant influence on length and weight of *B. persicum* microtubers. The effective results were obtained as shown in the present report.

RESULTS AND DISCUSSION

Effect of jasmonic acid

Jasmonic acid significantly increased the growth of *B. persicum* microtubers (2 and 5 mM) on the weight and length of microtubers in comparison with the control ($p \leq 0.01$) (Table 1). This effect of JA on tuber induction and tuber growth ratio has been previously reported. Pelacho and Mingo-Castel (1999) showed that final JA promoted tuberization percentage (100%) after 30 days in culture and it induced potato tuber fresh weight 6.4 times the kinetin induced tuber weight. Similar to those effects, in *B. persicum,* JA might act as a chemical signal to trigger senescence related processes such as tuber induction, which took place after a sufficient vegetative development (8 weeks).

Effect of jasmonic acid and temperature

There was a significant effect in both concentration of Jasmonic acid (2 mM and 5 mM) on the weight and length of microtubers in comparison with the control ($p \leq 0.01$) (Figures 3 and 4). Regardless of culture medium effect, JA promoted the weight of *B. persicum* microtubers under both 15 and 20°C temperature when used at concentrations of 2 and 5 mM (Figure 3). This effect of JA was prominent in microtubers length (Figure 4). A more complete effect of JA occurred when samples were exposed to 5 mM and JA. The response of microtubers to 2 mM JA and 20°C were significantly lower than the other two levels of JA ($p \leq 0.01$). This might be due to pleiotropic effects associated with the result of external addition of Jasmonates, with induction or inhibition of physiological and biochemical processes in specific

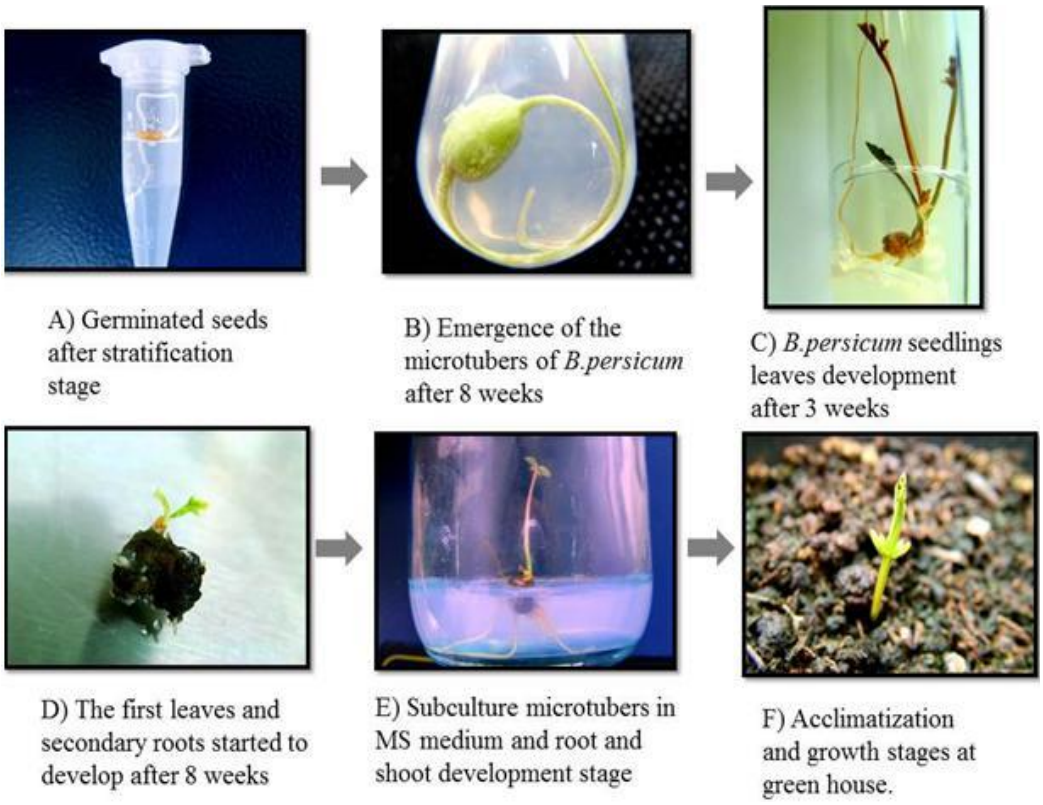

Figure 2. *In vitro* microtuberization, vernalization and acclimatization stages of *B. persicum* (A-f).

Table 1. Summary of the three-way analysis of variance for dry weight and length of *B. persicum* microtubers under three levels of Jasmonic acid (JA), three levels of medium (Med) and two levels of temperature (Temp).

Variable source	Degree of freedom (DF)	Dry weight of microtuber (mg)		Length of microtuber (mm)	
		Means of square	*p*-level	Means of square	*p*-level
Replicate	2	1986.71	<0.0001**	45.45	<0.0001**
JA	2	32368.12	<0.0001**	117.85	<0.0001**
Med	2	23618.74	<0.0001**	34.04	<0.0001**
Temp	1	7302.94	<0.0001**	19.03	<0.0001**
JA*Med	4	2119.99	<0.0001**	1.44	0.0013**
JA*Temp	2	880.19	<0.0001**	0.68	0.0828^{ns}
Med*Temp	2	266.81	<0.0001**	0.47	0.1725^{ns}
JA*Med*Temp	4	215.15	<0.0001**	1.12	0.0056**
Model	19	7099.19	<0.0001**	22.44	
Error	34	10.41		0.25	
Corrected total	53				
Coefficient variance (CV)			1.84		5.67
R^2			0.99		0.98

**- significant at 0.01 level of probability, ^{ns}- non-significant.

organs of a plant or in the whole plant. Previous studies demonstrated that JA induced tuber formation (Zel et al., 1997; Pelacho et al., 1999; Jasik and Mantell, 2000; Cenzanoa et al., 2007). Result of this study shows that JA supported microtubers development in all medium combination tests. However, JA could not influence the microtubers weight ratio as much as weight (Data not shown). It is also believed that this particular manner of

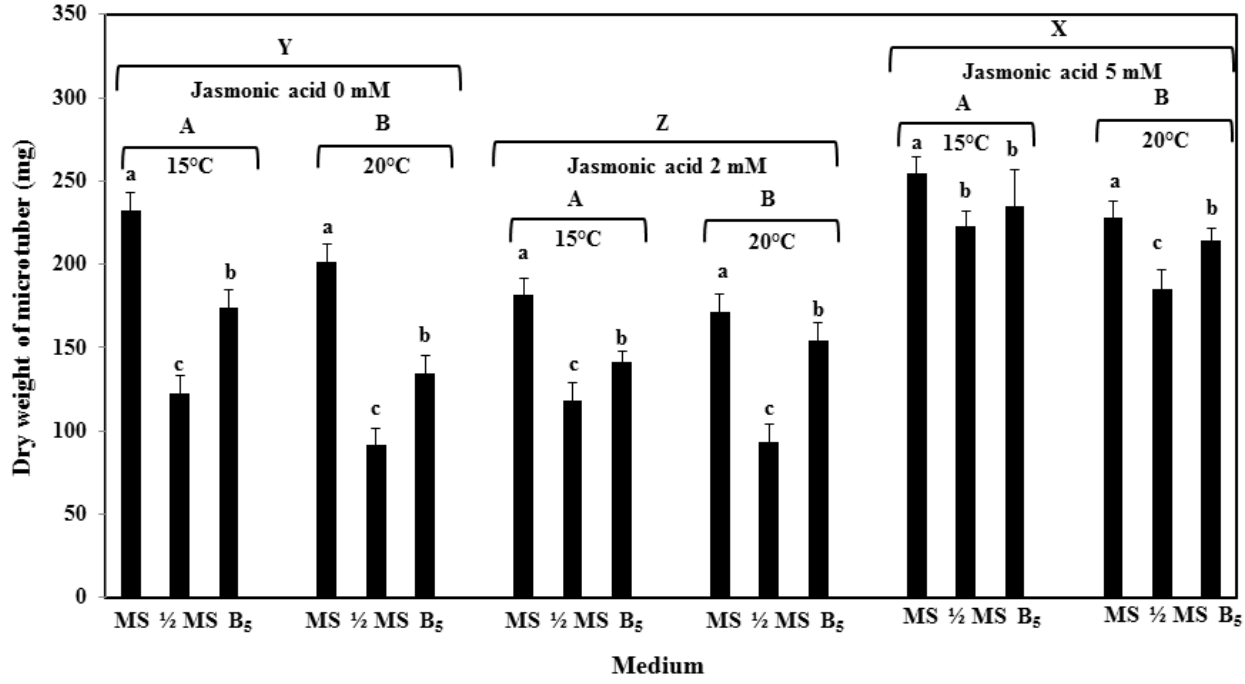

Figure 3. The effect of different mediums (MS, ½MS and B_5), different temperatures (15°C and 20°C) and Jasmonic acid on *B. persicum* microtuber dry weight.. Error bars indicate standard deviation. Small letters (a, b and c) represent significant differences among different mediums. Capital letters (A, B and C) represent significant differences between different temperatures. Capital letters (X, Y and Z) represents significant differences among different concentrations of Jasmonic acid.

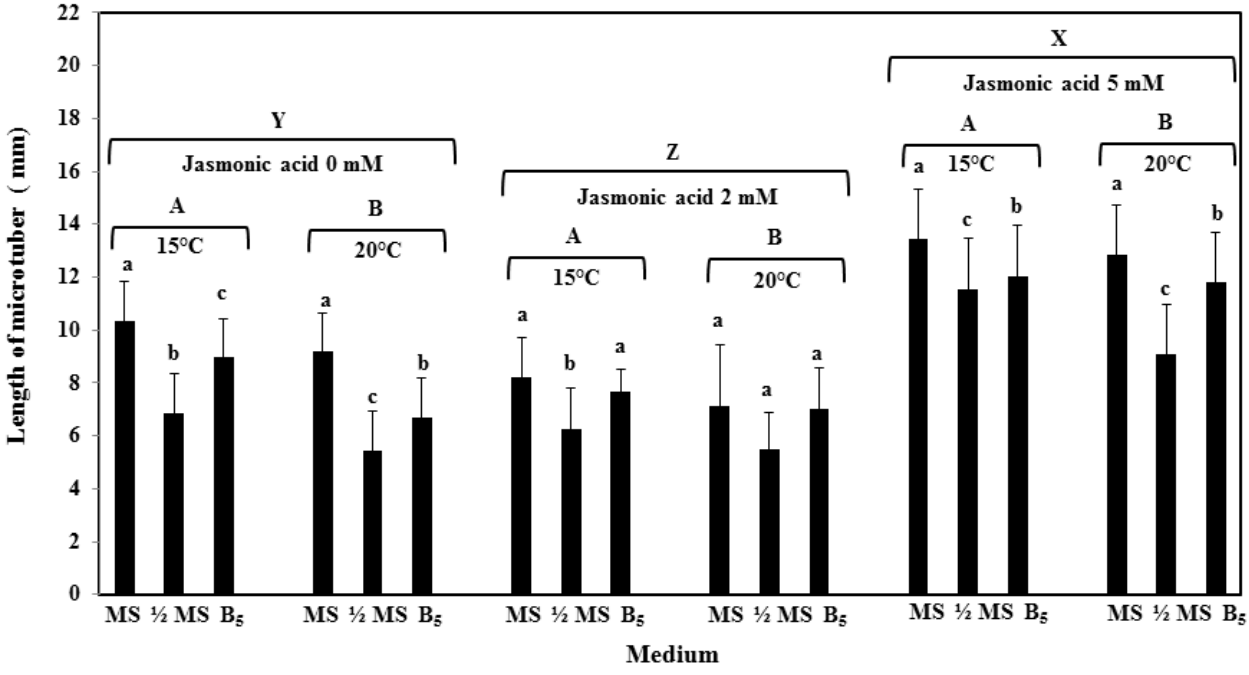

Figure 4. The effect of different mediums (MS, ½MS and B_5), different temperatures (15°C and 20°C) and Jasmonic acid on *B. persicum* microtuber length. Error bars indicate standard deviation. Small letters (a, b and c) represent significant differences among different mediums. Capital letters (A, B and C) represent significant differences between different temperatures. Capital letters (X, Y and Z) represents significant differences among different concentrations of Jasmonic acid.

the JA was greater when *B. persicum* microtubers were grown in 15°C. JA and related compounds play an important role in many morphogenetic events in plants, such as tuberization, flowering, bulb and tuberous root formation and others. All these areas have been extensively reviewed (Castro et al., 1999; Jasik and Mantell, 2000). Moreover, some other reports have suggested that exogenously applied JA affected the formation of storage organs like bulbs in garlic (Ravnikar et al., 1993). In some cases, Jasmonic acid and its related compounds act in an inhibitory manner (Sembdner and Parthier, 1993). Methyl Jasmonates inhibited tubrization when added as vapour, but stimulated this process if added to the medium (Jasik and Mantell, 2000). It seems that JA could stimulate microtuber induction in *B. persicum* specifically when added in lower concentration. It might be because JA increases the young cell expansion in potato buds. Increasing the microtuber weight can be the result of cell expansion in *B. persicum* microtubers. JA increases the tuberonic acid and its glucoside (TAG) in plant which are signals for tuberization (Castro et al., 1999).

Effect of medium and temperature

Basically, it is necessary to optimize the best combination of medium and temperature to develop a beneficial protocol for a particular species. In the present experiment, MS medium could significantly increase the length and weight of microtubers in comparison to the other mediums (½MS and B_5) ($p \leq 0.01$) (Figures 3 and 4). In contrast, ½MS implied the lowest effect on microtubers final dry weight and length of *B. persicum*. Moreover, combination of MS medium and lower temperature seemed to be more effective on growth of microtubers. However, it was found under the conditions employed that MS medium and 15°C is suitable for *in vitro* tuberization and maintenance of *B. persicum*. Several reports show that MS medium can be a good option for microtuberization. Valizadeh et al. (2007) revealed that MS medium was suitable for callus induction of *B. persicum.* Karam and Al-Majathoub (2000) found that MS medium was a satisfactorybasic medium for *invitro* tuber cultures of *Cyclamen persicum*. MS medium contains more nutrients and salts in comparison with the ½MS and possibly it can influence the weight ratio and weight of microtubers. Yamaner and Erdag (2008) also showed that combination of MS and ½MS with 24°C is the best condition for tuber development of *Cyclamen persicum*. It has been reported that the optimum temperature for developing the microtubers of *B. persicum* in the natural habitat is 15°C (Omidbaigi, 2012). Temperature can influence rooting by interfering with nutrient uptake, metabolism and its control, mainly in temperate climates. This environmental factor can influence the plant life cycle in a particular year. Regarding the result of the present study, it looks that higher nutrients (MS) and lower temperatures stimulate (15°C) the development of *B. persicum* microtubers. The effect of the lower temperature might be due to the different origin of the seeds. Apparently, MS medium contains main nutrients necessary for predevelopment of the microtubers of *B. persicum*. The performance of MS medium and 15°C showed the best result on microtubers of *B. persicum*.

Effect of medium, temperature and jasmonic acid

The ½MS induced the lower weight and length in combination with 2 mM JA. This effect of the ½MS basal medium was significant when the microtubers were kept on 20°C (Figures 3 and 4). MS medium combined with 5 mM JA and 15°C demonstrated the greatest effect on stimulating the weight increase on the microtubers. This result is in agreement with the result of Jasik and Mantell (2000) who showed that JA and its compound were able to improve microtuber weight but when *Dioscorea alata* were planted in the medium supplemented with low concentrations of salt and sucrose. This result might be due to the role of JA in uptake of sucrose and nutrients in the medium (Jasik and Mantell, 2000). However, the main storage compounds of the *B. persicum* tubers are unknown. Moreover, this manner of the JA can be greater when added in the lower temperature. In order to improve microtuberization in *B. persicum* in different solid medium, the effect of temperature and JA were tested. According to the present result, JA, temperature and medium combination increased the weight and length of microtubers significantly ($p \leq 0.05$) (Figures 3 and 4). Results show that when microtubers were grown to MS solid medium containing 5 mM JA and kept in 15°C the average weight of microtubers increased up to 250 mg. As the Figure 3 depicts, in general MS medium influenced microtubers weights more than other medium (½MS and B_5) when used in combination with other treatments (JA and temperature). Having analyzed the data, it was found that combination of MS medium, 15°C and 5 mM JA seems to be beneficial for the *B. persicum* microtuber development. In addition, the lowest length of microtubers was obtained with B_5, 20°C and in the absence of JA (Figure 2).

Microtuber acclimatization and vernalization

Microtubers started to grow and the first true leaf emerged 8 weeks after chilling periods. At first, a small green leaf started to emerge followed by very small with secondary roots. In this stage, germinated microtubers were transported to MS basal medium. Results show that 16 (80%) transported microtubers germinated 8 weeks after chilling periods. Based on the result of this study, it seemed that a minimum period of 8 weeks (1344 h) was necessary for vernalization of *B. persicum* microtubers.

Vernalization is a substantial stage for many plant species including many species in Apiaceae family (Alessandro and Galmarini, 2007). However, there is no report about the cold period necessary for verbalization of *B. persicum* tubers. The stage of growth when seedlings are not responsive to low-temperature vernalization is known as juvenility (Alessandro and Galmarini, 2007). In the case of carrot, this condition usually ended when carrot plants had initiated 8 to 12 leaves, and storage roots were larger than 4 to 8 mm in diameter (Atherton et al., 1990). The juvenility stage of *B. persicum* has not been well known and needs further investigation. Acclimatization is an important stage for plant establishment of plant *in vitro* propagation. The development of optimization process in one or more stages of plant micro propagation is of fundamental importance to increase the competitiveness of micro propagated plantlets produced in commercial laboratories (Cardosoa et al., 2013). In this study, it was found that regardless of the effect of the different medium on microtubrisation of *B. persicum* during laboratory stages, 20% of microtubers adapted to the greenhouse condition and started to develop new leaves after 1 week.

To sum it up, *in vitro* microtuber production is very beneficial to propagate and store valuable plants stock and may be adaptable for automated commercial propagation and large scale mechanized field planting. However, there is no particular report on optimization, a medium for development of *B. persicum* microtubers. In the present study three different culture media MS, ½MS and B5 along with different concentrations of JA were used individually as basal media and also in combination with two different temperatures (15 and 20°C) to develop appropriate media for microtubrization of *B. persicum*. The use of JA in a concentration of 5 mM could enhance the *B. persicum* microtubers development. MS medium and JA level of 5 mM seem to be satisfactory for *in vitro* culture establishment and short-term maintenance of tested *B. persicum.* However, more researches are needed to develop a protocol suitable for commercial micro propagation, specifically in plant establishment and acclimatization of *B. persicum.*

ACKNOWLEDGEMENTS

Authors would like to thank Department of Food Technology, Research Institute of Food Science and Technology, Mashhad, Iran, providing the budget and facilities for this work. This work was supported by the grant-in aid for research on Agriculture and Food Science (25029AB) from the Ministry of Agriculture, Forestry and Fisheries of Japan and also by JSPS KAKENHI Grant Number 26304024.

Conflict of interests

The author(s) did not declare any conflict of interest.

REFERENCES

Alessandro MS, Galmarini CR (2007). Inheritance of Vernalization Requirement in Carrot. J. Am. Soc. Hortic. Sci. 123: 525-529.

Atherton JG, Craigon J, Basher EA (1990). Flowering and bolting in carrot. I. Juvenility, cardinal temperatures and thermal times for vernalization. J. Hort. Sri. 65:423-429.

Cardosoa JC, Rossi ML, Rosalemc IB, Da Silva JAT (2013). Pre-acclimatization in the greenhouse: An alternative to optimizing the micropropagation of gerbera. Sci. Hortic. 164: 616-624.

Carol CB, Chester EW, Baskin JM (1992). Deep Complex Morphophysiological Dormancy in Seeds of *Thaspium pinnatifidum* (Apiaceae). Int. J. Plant Sci. 153: 565-571.

Castro G, Kraus T, Abdala G (1999). Endogenous jasmonic acid and radial cell expansion in buds of potato tubers. J Plant Physiol. 155:706-10.

Cenzanoa A, Abdalaa G, Hauseb B (2007). Cytochemical immuno-localization of allene oxide cyclase, a jasmonic acid biosynthetic enzyme, in developing potato stolons. J. Plant Physiol. 164:1449-1456.

Chizzola R, Saeidnejad AH, Azizi M, Oroojalian F, Mardani H (2014). *Bunium persicum*: variability in essential oil and antioxidants activity of fruits from different Iranian wild populations. Genet. Resour. Crop. Evol. 61:1621-163.

Gamborg OL, Miller RA, Ojima K (1968). Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. 50:151-158.

Gao X, yang Q, Minami C, Matsuura H, Kimura A, Yoshihara T (2003). Inhibitory effect of salicylhydroxamic acid on theobroxide-induced potato tuber formation. Plant Sci. 165:993-999.

Garval S, Rani M (1999). Repetitive somatic embryogenesis in aggregated liquid culture of *Bunium persicum* Boiss. Indian J. Exp. Biol. 37:70-74.

Hajhashemi V, Sajjadi SE, Zomorodkia M (2012). Antinociceptive and anti-inflammatory activities of *Bunium persicum* essential oil, hydroalcoholic and polyphenolic extracts in animal models. Pharm. Biol. 49:146-51.

Hunault G, Desmarest P, Manoir JD (1989). Medicinal and Aromatic Plants II, Biotechnology in Agriculture and Forestry, In: *Foeniculum vulgare* Miller: Cell Culture, Regeneration and the Production of Anethole., Springer Berlin Heidelberg, 7: pp 185-212.

Jasik J, Mantell SH (2000). Effect of Jasmonic acid and its methylster on in vitro microtubrization of three food yam (*Dioscorea*) species. Plant. Cell. Rep. 19: 863-867.

Karam NS, Al-Majathoub M (2000). Direct shoot regeneration and microtuberization in wild *Cyclamen persicum* Mill. using seedling tissue. Sci. Hortic. 86:235-246.

Khosravi M (1994). *Bunium persicum*, botany, ecology and investigation the possibility of crop production. [M.Sc. Thesis.] Agricultural College, Ferdowsi University of Iran (In Persian).

Mortazavi SV, Eikani MH, Mirzaei H, Jafari M, Golmohammad F (2010). Extraction of essential oils from *Bunium persicum* Boiss. using superheated water. Food Bioprod. Process. 88:222-226.

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

Omidbaigi R, (2012). Production and Processing of Medicinal Plants.Vol. 1.3rd edition. Astan Quds Publication, Tehran, pp. 75-80 (in Persian).

Omokolo ND, Boudjeko T, Takadong TJJ (2003). *In vitro*tuberization of *Xanthosoma sagittifolium* L. Schott: effects of phytohormones, sucrose, nitrogen and photoperiod. Sci. Hortic. 98: 337-345.

Pelacho AM, Martin-Closas L, Sanfeliu JLI, (1999). *In vitro* induction of potato tuberization by organic acids. Potato Res. 42: 585-591.

Ravnikar M, zel J, Plaper I, Spacapan A (1993). Jasmonic acid stimulates shoot and bulbs formation in garlic in vitro. J. Plant. Growth Regul. 12:73-77.

Sekine T, Sugano M, Azizi M, Fujii Y (2007). Antifungal effects of volatile compounds from black zira (*Bunium persicum*) and other spices and herbs. J. Chem. Ecol. 33: 2123-2132.

Sembdner G, Parthier B, (1993). The biochemistry and physiological and molecular actions of Jasmonats. Annu. Rev. Plant Physiol. Plant Mol. Biol. 44:569-589.

Sharifi M, Pouresmael M, (2003). Effect of some chemical substances

on dormancy broking and in induction of seed germination on *Bunium persicum* Boiss. J. of Agri. Sci. Reco. 10:33-41(In Persian).
Sharififar F, Yassa N, Mozaffarian V (2010). Bioactivity of major components from the seeds of *Bunium persicum* (Boiss.) Fedtch. Pak. J. Pharm. Sci. 23:300-304.
Turner S, Krauss LS, Bunn E, Senaratna T, Dixon K, Tan B, Touchelld (2001). Genetic fidelity and viability of *Anigozanthos viridis* following tissue culture, cold storage and cryopreservation. Plant Sci. 161: 1099-1106.
Valizadeh M, Safarnejad A, Nematzadeh GH, Kazemitabar SK (2007). Regeneration of Plantlets from Embryo Explants of *Bunium persicum* (Boiss.) Fedtsch. J. Sci. Technol. Agric. Nat. Resour. Water Soil Sci. 11:33-38 (In Persian).
Wakhlu AK, Nagari S, Barna S (1990). Somatic embryogenensis and plant regeneration from callus culture of *Bunium persicum* Boiss. Plant Cell Rep. 9:137-138.
Yamaner Ö, Erdag B (2008). Direct shoot formation and microtuberization from aseptic seedlings of *Cyclamen mirabile* Hildebr. Biotechnology 7: 328-332.
Zel J, Debeljak N, Ucman R, Ravnikar M (1997). The Effect of Jasmonic Acid, Sucrose and Darkness on Garlic (*Allium Sativum* L. cv. Ptujski Jesenski) Bulb Formation *in Vitro. In Vitro* Cell. Dev. Plant 33:231-235.

academicJournals

Vol. 14(25), pp. 2088-2094, 23 June, 2015
DOI: 10.5897/AJB2013.13113
Article Number: 91278C653883
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Effects of aqueous extracts of *Mucuna sloanei* (Fabaceae) seed on haematological parameters of albino rats (*Rattus novergicus*)

Ejere, Vincent C.*, Ugwu, Godwin C., Chukwuka, Christian O. and Nnamonu, Emmanuel I.

Department of Zoology and Environmental Biology, University of Nigeria Nsukka, Enugu State, Nigeria.

Received 2 August, 2013; Accepted 15 June, 2015

***Mucuna sloanei* is a legume used as a soup thickener by communities in some parts of Africa countries. The effect of aqueous seed extract of *M. sloanei* on the haematological profile of normal albino rats was investigated for 28 days using standard methods. The results show no overall dose dependent significant difference ($p > 0.05$) in the serum levels of the white blood cell count, red blood cell count and its indices (haemoglobin content, pack cell volume, mean corpuscular volume, mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration) of the rats throughout the duration of the experiment when compared with the control. However, a dose independent significant reduction ($p < 0.05$) in the white blood cell level was observed in week 3, while the decrease observed in the remaining week was not significant. The effects of the interaction between the doses and duration of treatment were not statistically significant ($p > 0.05$) in all the haematological parameters tested. These findings suggest that consumption of the crude seed extracts of *M. sloanei* may not constitute any adverse effect on the haematological indices of the consumers. However, the significant reduction in white blood cell levels observed in week 3 could compromise the body's immunity and may predispose consumers to opportunistic and supra-infections in the long run.**

Key word: *Mucuna sloanei* seed, aqueous extract, haematological parameters, albino rats.

INTRODUCTION

Several forest resources have been domesticated and today they are contributing in feeding the world's teeming population especially in the developing world. The growing demands in plant-based proteins for humans and livestock, have elicited several researches on the possibilities of employing underutilized legumes as inexpensive and elegant source of protein than the conventional sources such as, soybean (*Glycine max*), groundnut (*Arachis*

*Corresponding author. E-mail: vincent.ejere@unn.edu.ng. Tel: +234 806 707 7741

Abbreviations: RBC, Total red blood cells; **Hb,** haemoglobin; **WBC,** white blood cells; **PCV,** packed cell volume; **MCV,** mean cell volume; **MCH,** mean cell haemoglobin; **MCHC,** mean cell haemoglobin concentration; **F-LSD,** Fisher's least significant difference; **SEM,** standard error of mean.

hypogea) and animal based proteins (Siddhuraju et al., 1995; Krause et al., 1996; Chel-Guerrero et al., 2002). *Mucuna sloanei*, an annual leguminous climber commonly called the “horse eye” or “hamburger” bean is one of such important legumes and is widely used among the various ethnic groups resident in Nigeria. It is variously called ‘ukpo’ by the Ibos; ‘karasuu’ by the Hausas; ‘yerepe’ by the Yorubas (Nwosu, 2011) and ‘ibabat’ by the Efiks of Nigeria (Obochi et al., 2007). *M. sloanei* seeds contain high protein, carbohydrate, crude fat and fiber contents (Akpata and Miachi, 2001) as well as a very rich amino acid profile (Ojiako et al., 2012). These constituents potentiate the seeds as a nutritious source of meal with high water binding capacity arising from the formation of hydrogen bonds between water and polar residues of the protein molecules (Obochi et al., 2007). The seeds are used as thickener in soups as well as a source of edible vegetable oil in many Igbo communities of Southeastern Nigeria (Afolabi et al., 1985; Ukachukwu et al., 2002). According to Ukachukwu and Obioha (1997), several rural populations of Nigeria, fall back on seeds of *M. sloanei* as soup thickeners during famine and scarcity of alternative soup thickeners such as melon.

M. sloanei seeds like all *Mucuna* species are very rich in many important bioactive substances such as L-3, 4-dihydroxyphenylalanine (L-DOPA) (Adebowale et al*.,* 2005), which has been reported to be a potent precursor of the brain neurotransmitter, dopamine (Hornykiewicz, 2002; Kostrzewa et al., 2005; Nagatsua and Sawadab, 2009). Similarly, lectin from *M. sloanei* has been reported as an effective and suitable cell receptor signal inducer as a result of its agglutinating ability in various blood cells of humans, goat, cow and chicken (Obochi et al., 2007). The Efiks of South south Nigeria claim that consumption of seeds of *M. sloanei* lowers libido in men as well as possessing sedative properties (Obochi et al., 2007). Despite all these varied perceptions, there is paucity of information regarding the effects of this important food condiment on blood parameters in experimental animal *vis-à-vis* the possible risks associated with its consumption by humans. The present study was therefore initiated to ascertain the haematological problems associated with oral consumption of aqueous extracts of shade dried dehulled seeds of *M. sloanei*.

MATERIALS AND METHODS

Collection and preparation of *M. sloanei* crude seed extract

Dried and mature nuts of *M. sloanei* were purchased from local markets around Nsukka metropolis. The seeds were identified using the identification key of Anyanwu and Okoli (2004). They were dehulled, dried at room temperature and pulverized into fine powder using a milling machine (Honda: model 622, China). The method of extraction followed that of Akintayo et al. (2000). A total of 100 g of the powdered sample was introduced into 2000 ml flat bottom flask and 1500 ml of distilled water was added. The content was mixed thoroughly and left for about 24 h with an occasional shaking to increase the extraction capacity. Thereafter, the soaked substance was filtered with a muslin clothe (number 60 mesh size) and concentrated to dryness. The solid extract was weighed and re-dissolved in normal saline according to the body weights of the animals for oral administration.

Procurement and management of experimental animals

Forty-eight (48) adult male albino rats weighing between 150 to 250 g were obtained from the Genetics and Animal breeding Laboratory of the Department of Zoology and Environmental Biology, University of Nigeria, Nsukka. The rats had no history of drug consumption (that is, they have not been used for any investigation). They were kept in stainless wire rat cages with dimensions (12 × 40 × 15 cm) equipped with drinkers and fecal collecting trays, in a clean and fly proof experimental animal house. The rats were fed with commercial growers chick mash (18% crude protein) made by Vital Feeds, Nigeria Limited and clean drinking water. They were allowed to acclimatize 14 days before the start of the experiment. All the animals were maintained under standard laboratory conditions for temperature, humidity and light throughout the experiment and were allowed unhindered access to food and water. The fecal droppings in the tray were removed daily. All the experiment was carried out under the approval of the Faculty Ethical Committee on Animal Research, Faculty of Biological Sciences, University of Nigeria Nsukka, Enugu State, Nigeria.

Toxicity test and experimental design

The LD_{50} of the extract in mice was determined orally using Lorke (1983) methodology with slight modification. The forty eight (48) male albino rats were then assigned into four groups (A, B, C, and D) of 12 rats per group. Each group was further replicated three times comprising of 4 rats per replicate. The rats in group A (control) were fed with normal rat feed and 1 ml/kg body weight of normal saline *ad libitum*. The treatment groups, B, C and D were administered 100, 200 and 400 mg/kg body weight of the seed extract, respectively. All the doses were administered once daily orally for 28 days (four weeks) for all the groups using 1 ml syringe.

Collection of blood sample

About 5 ml of the blood samples was collected from each of the anaesthetized rats using the ocular puncture method described by Hoff (2000). This was done before the start of the experiment (Week 0) and at weekly intervals during treatment (weeks 1 to 4) for the various haematological profile tests.

Determination of haematological parameters

Total red blood cells (RBC) count, haemoglobin (Hb) content and white blood cells (WBC) count were determined according to the methods described by Sood (2006). The packed cell volume (PCV) was ascertained using the methodology of Coles (1986). A heparinized capillary tube was filled to approximately three fourth (¾) of its length with well-mixed anticoagulated blood. The coloured end of the capillary tube was sealed with plasticin. The capillary tube was then placed into a microhaematocrit centrifuge set at 7826 g for 5 min. The height of the packed cell as well as the total height in millimeter was measured using the haematocrit reader. The packed cell volume was then calculated using the formula:

$$\text{PCV (\%)} = \frac{Height\ of\ red\ cell\ (mm)}{Total\ height\ (mm)} \times 100$$

Table 1. Effects of the aqueous seed extract of *M. sloanei* on red cell indices of albino rats.

Parameter	Concentrations (mg/kg)	Duration (week)				
		0	1	2	3	4
MCV (μ^3)	Control	54.20±0.06^{a1}	54.30±0.10^{b1}	54.20±0.10^{a1}	54.33±0.07^{a1}	54.20±0.12^{a1}
	100	54.23±0.07^{a1}	54.03±0.03^{a1}	54.10±0.12^{a1}	54.17±0.15^{a1}	54.30±0.06^{a1}
	200	54.13±0.07^{a1}	54.07±0.07^{a1}	54.00±0.10^{a1}	54.10±0.15^{a1}	54.23±0.09^{a1}
	400	54.23±0.03^{a1}	54.33±0.03^{b1}	54.03±0.09^{a1}	54.17±0.03^{a1}	54.10±0.00^{a1}
MCH (Pg)	Control	18.10±0.00^{a1}	18.10±0.00^{ab1}	18.03±0.03^{a1}	18.10±0.00^{a1}	18.07±0.03^{ab1}
	100	18.07±0.03^{a1}	18.00±0.00^{c1}	18.03±0.03^{a1}	17.33±0.82^{a2}	18.10±0.00^{b1}
	200	18.07±0.03^{a1}	18.03±0.03^{ac1}	18.03±0.03^{a1}	18.03±0.03^{a1}	18.07±0.00^{ab1}
	400	18.10±0.00^{a1}	18.17±0.03^{b2}	18.03±0.03^{a1}	18.07±0.03^{a1}	18.00±0.00^{a1}
MCHC (g/dl)	Control	33.33±0.03^{a1}	33.33±0.03^{a1}	33.30±0.00^{a1}	33.33±0.03^{a1}	33.30±0.00^{a1}
	100	33.57±0.22^{a1}	33.37±0.03^{a2}	33.33±0.03^{a2}	33.33±0.03^{a2}	33.30±0.00^{a2}
	200	33.37±0.03^{a1}	33.37±0.3^{a1}	33.30±0.00^{a1}	33.37±0.03^{a1}	33.30±0.00^{a1}
	400	33.30±0.00^{a1}	33.37±0.03^{a1}	33.33±0.03^{a1}	33.30±0.00^{a1}	33.30±0.00^{a1}

Values with different alphabetic superscripts differ significantly ($p < 0.05$) between different concentrations within the same exposure duration. Similarly, values with different numeric superscripts differ significantly ($p < 0.05$) between different exposure periods within the same concentration. MCV, mean cell volume; MCH, mean cell haemoglobin; MCHC, mean cell haemoglobin concentration.

Similarly the red blood cell indices namely, mean cell volume (MCV), mean cell haemoglobin (MCH) and the mean cell haemoglobin concentration (MCHC) were determined according to the methods described by Baker et al. (2001). The reagents used for the analyses were all analytical grades.

Statistical analysis

Data accumulated was analyzed using the GENSTAT (VSN International, Hemel Hempstead, Herts, UK). Whereas, a one-way analysis of variance (ANOVA) was used to test the effect of treatment, a Two-way ANOVA was used to determine the interactive effects of treatment and duration. Fisher's least significant difference (F-LSD) was used in the separation of means of the different treatment groups. All results were expressed as mean ± standard error of Mean (SEM), while values were considered significant at $p < 0.05$.

RESULTS

The oral LD_{50} of the aqueous seed extract in the rats showed no mortality at the different doses of 1000, 3000 and 5000 mg/kg. However, such cage side characteristics as shivering, bulging eyes and dullness were observed in the rats administered the 5000 mg/kg dose. Table 1 shows the results of the weekly effects of the seed extracts of *M. sloanei* on the haematological parameters assayed, namely, red blood cell (RBC) count, white blood cell (WBC) count, packed cell volume (PCV) level, haemoglobin (Hb) content and the red blood cell indices (MCV, MCH and MCHC) in the albino rats. There was no overall dose dependent significant difference ($p > 0.05$) observed in the levels of all the parameters in the weeks when compared with the control. However, minimal variations were observed in the weekly levels of some of the parameters; whereas, a significant decrease ($p < 0.05$) was observed in the RBC levels of the rats administered 100 and 200 mg/kg in week 1, the WBC levels decreased significantly ($p < 0.05$) in all the dose levels in week 3. Similarly, there was a sharp increase in the mean WBC levels observed in week 2 of the animals given 200 and 400 mg/kg, followed by a somewhat marked sharp decreases in values from week 2 to 4, respectively (Table 1).

The PCV and Hb levels also decreased significantly ($p < 0.05$) in rats administered the dose levels of 100 mg/kg (weeks 1 and 3) and 200 mg/kg (week 1), respectively. In the same vein, minor variations were observed in the levels of the red cell indices. A significant decrease ($p < 0.05$) in the MCV level was observed in rats administered the doses of 100 and 200 mg/kg in week 1. Thereafter, a very slight increase in the mean values of MCV was observed in the rats administered 100 mg/kg from week 2 to 4. Similarly, the MCV levels of the rats that received 200 mg/kg slightly increased from week 3 to 4, respectively. The MCH level of those animals given 100 mg/kg body weight was also observed to show a significant decrease ($p < 0.05$) in week 1, followed by a further decline in week 3.

DISCUSSION

The possible toxic effect of aqueous extracts of *M. sloanei* seed on haematological profile of normal albino rats was investigated for a total of 28 days. Toxicity test using raw unprocessed seed extract of *M. sloanei*

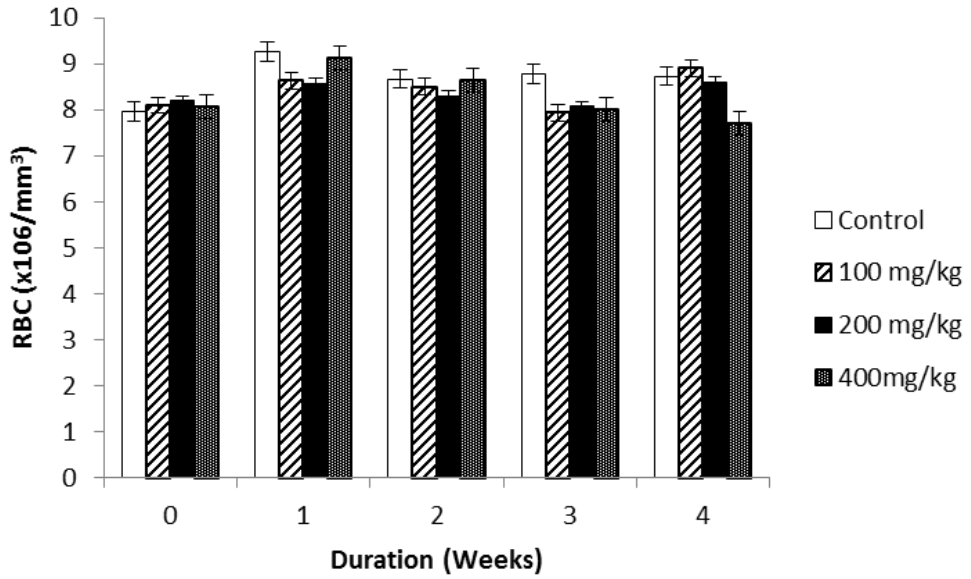

Figure 1. Effects of the aqueous seed extract of *M. sloanei* on red blood cell of albino rats.

showed that although at a dose level of 5,000 mg/kg, no death occurred, there was shivering, bulging of eyes as well as dullness in the rats. This observation contradicts the report of Egwurugwu et al. (2012) who recorded lethal effects of the *M. sloanei* seed extract at a dose level of 3,872.98 mg/kg in experimental rats. This discrepancy aptly calls for more studies to fully unravel the confusion surrounding the identity of the *Mucuna* species involved. Blood parameters are good indicators of physiological and nutritional status of animals, and changes in haematological parameters have the potential of being used to elucidate the impact of nutritional factor and additives supplied in diets of any living creature (Majid et al., 2010). Assessment of haematological parameters can be used to determine the extent of deleterious effect of foreign compounds including plant extracts on the blood constituents of an animal (Ashafa et al., 2009). It can also be used to explain blood relating functions of chemical compounds including plant extracts (Yakubu et al., 2007). The various haematological parameters investigated in this study are useful indices that can be employed to assess the toxic potentials of plant extracts/botanicals in living systems (Sunmonu and Oloyede, 2010). Such toxicity testing is relevant to risk evaluation as changes in the haematological system have higher predictive value for human toxicity, when data are translated from animal studies (Olson et al., 2000). The packed cell volume (PCV), haemoglobin (Hb) and red blood cells (RBC) are associated with the total population of red blood cells (Ashafa et al., 2011).

However, except in week 1, there were no significant effects of the extracts at various dose levels (100, 200 and 400 mg/kg body weight) on the RBC and its indices (Hb, PCV, MCV, MCH and MCHC) from the first to the last week of treatment when compared with the control (Table 1; Figures 1 to 4). This clearly indicates that there was no change in the rate of production of RBCs (erythropoiesis) as well as destruction of matured RBCs within the study period. It further showed that the extract did not have the potential to stimulate erythropoietin release in the kidney, which is the humoral regulator of RBC production (Polenakovic and Sikole, 1996; Sanchez-Elsner et al., 2004). The little or non-significant effect on the RBC and Hb also implies that there was no change in the oxygen-carrying capacity of the blood and amount of oxygen delivered to the tissues following the extract administration, since RBC and Hb are very important in transferring respiratory gases (De Gruchy, 1976). The significant reduction in the levels of RBC, PCV, HB and MCV (Table 1) when compared with the control at dose levels of 100 and 200 mg/kg in week 1 could be as a result of the animal's initial reaction to the assault by the extracts. The non-significant effects on these RBC indices further suggest that the average size of RBC (microcytes) as well as the haemoglobin weight per RBC were not affected. This implies that the aqueous seed extract does not seem to possess the potential of inducing anaemia following a prolonged period of administration. This is more so because MCV, MCH and MCHC levels have been found to be important indicators of anaemia diagnosis in most animals (Coles, 1986).

Similarly, the absence of observable significant effects of the extracts on the MCH, MCHC and MCV levels between the treatment groups within the duration of the present experiment may be an indication that neither the incorporation of haemoglobin into the red blood cells nor

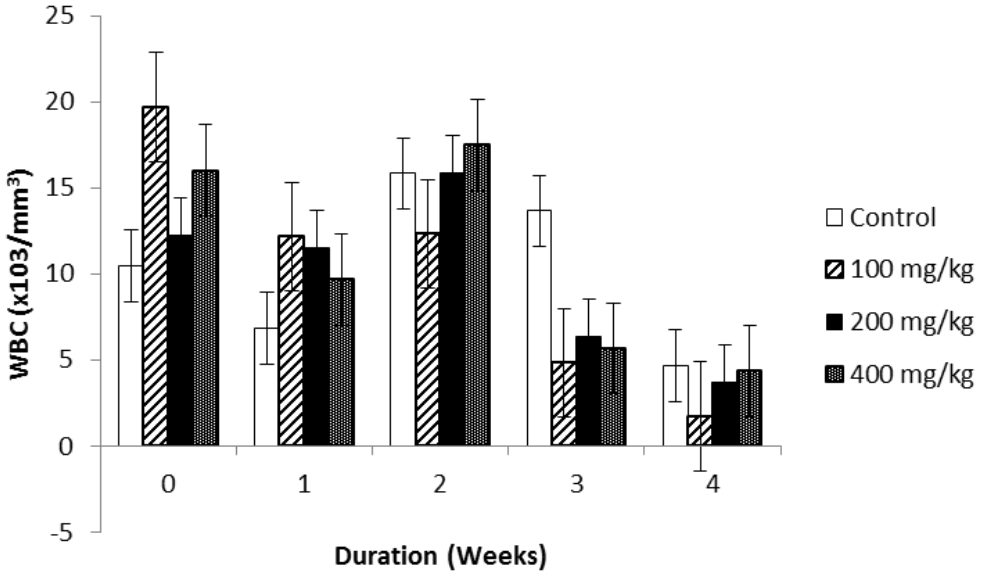

Figure 2. Effects of the aqueous seed extract of *M. sloanei* on white blood cell of albino rats.

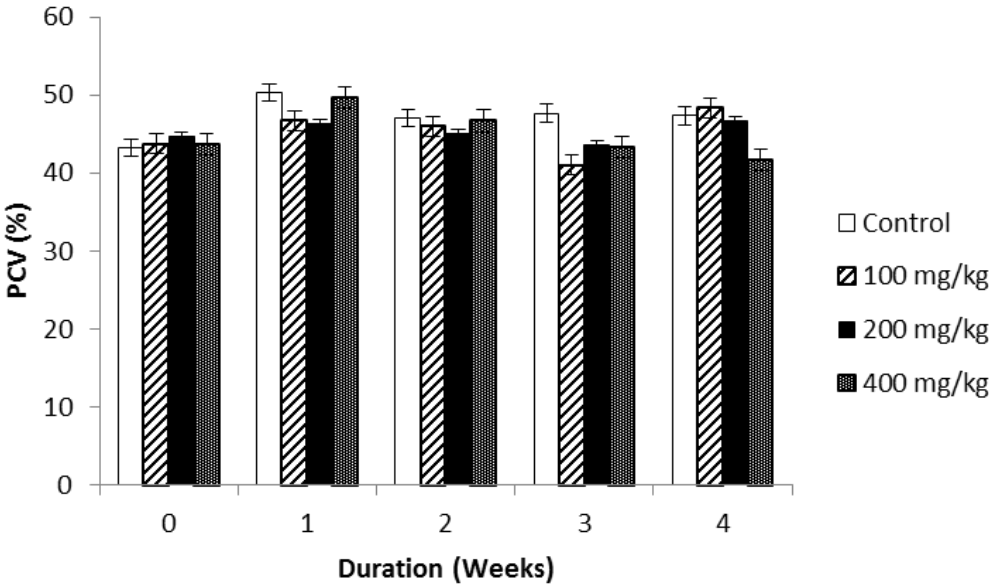

Figure 3. Effects of the aqueous seed extract of *M. sloanei* on pack cell volume (PCV) of albino rats.

the morphology and osmotic fragility of the red blood cells was altered (Adebayo et al., 2005). Nevertheless, the intermittently observed significant decreases in the values of these haematological parameters among treatment groups and the control, still falls within established rat haematological reference ranges (Johnson-Delaney, 1996).

The results obtained in the present research however, largely corroborate that obtained earlier for other *Mucuna* species in Nigeria. Odoh and Osadebe (2010) observed that aqueous extracts of *Mucuna flagellipes* produced no significant changes in the values of haemoglobin (HB), red blood cell (RBC), white blood cell (WBC) and packed cell volume (PCV) of normal albino rats. Similarly, Adepoju and Odubena (2009) posited that extracts of *Mucuna pruriens* did not produce any significant difference in the packed cell volume (PCV) of albino rats. Contrarily, the data obtained in the present study is at variance with the findings of Esonu et al. (2001) who stated that feeding of raw *Mucuna* (Velvet bean) seed

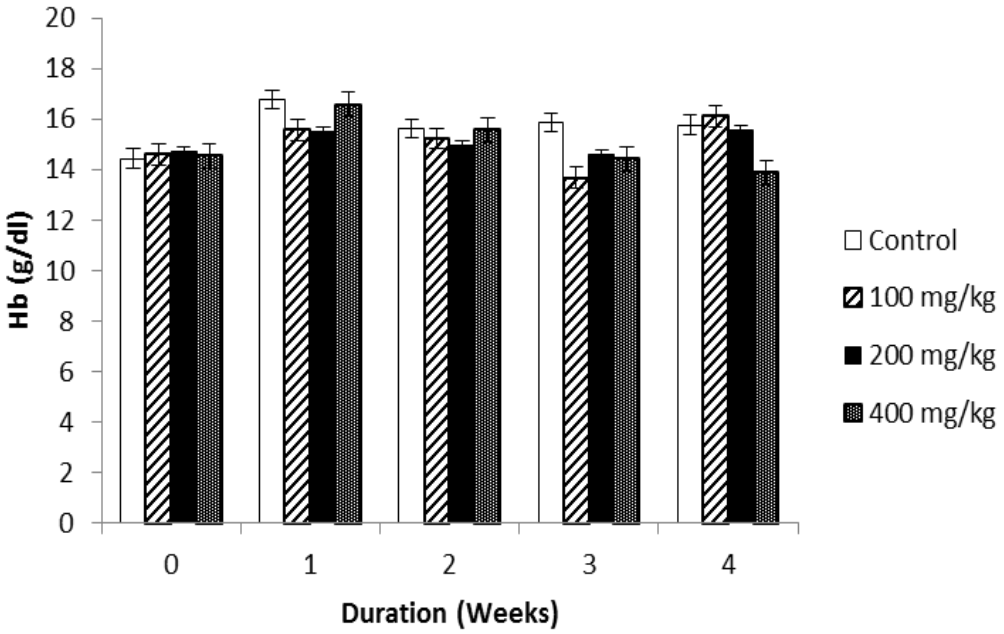

Figure 4. Effects of the aqueous seed extract of *M. sloanei* on haemoglobin level of albino rats.

meal resulted in deleterious effects on the performance and blood constituents of weaned pigs.

Nevertheless, the observed significant reduction in white blood cells in week 3 (Table 1) could compromise the body's immunity and may predispose consumers to opportunistic and supra-infections despite the acclaimed nutritional benefits of the *M. sloanei* seed. This is because the total WBC content contributes to the defense mechanism of the body (Lioyd and Mary, 1999). Any explanation for this observed discrepancy in the WBC level at present would at best be subjective. Hence, the need for more studies to actually unravel the active ingredient in *M. sloanei* implicated. However, it is instructive that the lack of significant differences between doses and duration of treatment portends that consumption of seeds of *M. sloanei* is beneficial even in the short term.

Conclusively, the study has demonstrated that the aqueous seed extract is safe for consumption since it does not cause any adverse effect on the haematological profile of rats. We therefore posit that, there is the need for further researches on the seed extracts of *M. sloanei* in order to fully understand the actual effects on the prolonged administration *vis-à-vis* its stability and suitability in clinical trials. Also, to fully explore the exact roles being played by the individual phytochemical constituent of *M. sloanei* seed in the general wellbeing of the body.

Conflict of interests

The author(s) did not declare any conflict of interest.

ACKNOWLEDGEMENTS

We express our profound gratitude to Prof. Okonta, M. I of the Department of Clinical Pharmacy and Dr. Nwani, C. D., of the Department of Zoology and Environmental Biology, both of University of Nigeria, Nsukka, Nigeria for their critique of the earlier manuscript.

REFERENCES

Adebayo JO, Adesokan AA, Olatunji LA, Buoro DO, Soladoye AO (2005). Effect of ethanolic extract of Bougainvillea spectabilis leaves on haematological and serum lipid variables in rats. Biokemistri 17:45-50.

Adebowale YA, Adeyemi A, Oshodi AA (2005). Variability in the physicochemical, nutritional and anti-nutritional attributes of six Mucuna species. Food Chem. 89(1):37-48.

Adepoju GKA, Odubena OO (2009). Effects of Mucuna pruriens on some haematological and biochemical parameters. J. Med. Plant Res. 3(2):73-76.

Afolabi OA, Oshuntogun BA, Adewusi SR, Fapojuwo OO, Ayorinde FO, Grisson FE, Oke OL (1985). Preliminary nutritional and chemical evaluation of raw seeds from Mucuna solanei: An underutilized food source. J. Agric. Food Chem. 33:122-124.

Akintayo ET, Oshodi AA, Adebowale KO (2000). Physicochemical Properties of Lima bean (Phaseolus lunatus) unmodified and modified starches. Nig. J. Sci. 34(2)187-193.

Akpata AO, Miachi EU (2001). Chemical composition and selected functional properties of sweet oranges and legumes flours. Plant Foods Hum. Nutr. 54: 353-362.

Anyanwu PC, Okoli BE (2004). Comparative Studies of the Morphology and Anatomy of Mucuna Prurienes (L.) D C and Mucuna sloanei (Fawc and Rendle). Nig. J. Bot. 17:21-27.

Ashafa AOT, Sunmonu TO, Afolayan AJ (2011). Effects of leaf and berry extracts of phytolacca dioca L. on haematological and weight parameters of wistar rats. Afr. J. Pharm. Pharmacol. 5(2):150-154.

Ashafa AOT, Yakubu MT, Grierson DS, Afolayan AJ (2009). Effects of aqueous leaf extract from the leaves of Chrysocoma ciliate L. on

some biochemical parameters of Wistar rats. Afr. J. Biotechnol. 8: 1425-1430.
Baker FJ, Silverton RE, Pallister CJ (2001). Introduction to Medical Laboratory Technology. Bounty Press Limited, Nigeria.
Chel-Guerrero L, Perez-Flores V, Bentacur-Ancona D, Davila-Ortiz G (2002). Functional properties of flours and protein isolates from Phaseolus lunatus and Canavalia ensiformis seeds. J. Agric. Food Chem. 50:584-591.
Coles EH (1986). Veterinary Clinical Pathology. 4th Edition. W.B. Souders Co., Philadelphia, USA.
De Gruchy GC (1976). Clinical Haematology in Medical Practice. Blackwell Scientific Publication. Oxford, London.
Egwurugwu JN, Nwafor A, Olorunfemi OJ, Anaduaka SC (2012). Effects of methanolic seed extracts of Mucuna sloanei on male sex hormones and sperm quality in rats. J. Med. Plants Res. 6(22): 3919 – 3922.
Esonu BO, Emenalom OO, Udedibie ABI, Okoloi IC, Herbert U, Ekpor CF (2001). Performance and blood chemistry of weaner pigs fed with raw Mucuna bean (velvet bean) meal. Trop. Anim. Prod. Investig. 4: 49-54.
Hoff J (2000). Methods of blood collection in the mouse. Lab. Anim. 29(10):50-51
Hornykiewicz O (2002). L-DOPA: from a biologically inactive amino acid to a successful therapeutic agent. Amino Acids 23(1-3):65-70.
Johnson-Delaney C (1996). Exotic Animal Companion Medicine Handbook for Veterinarians. Zoological Education Network, Chico, California, USA.
Kostrzewa RM, Nowak P, Kostrzewa JP, Kostrzewa RA, Brus R (2005). Peculiarities of L-DOPA treatment of Parkinson's disease. Amino Acids 28(2): 157 – 164.
Krause JP, Mothes R, Schwenke KD (1996). Some physicochemical and interfacial properties of native and acetylated legume from faba bean (Vicia faba L.). J. Agric. Food Chem. 44:429-437.
Lioyd YY, Mary AKK (1999). The clinical use of drugs. 6th Edition. Applied Therapeutic Incoporation, Vancouver. In: Mario AT, Venancio AF, Cesar AN, Helena SF, Edna S (2004). Clinical significance of elevated alanine aminotransferase in blood donors: a follow-up study. Pakistan J. Nutr. 2(2):112-117.
Lorke D (1983). A new approach to practical acute toxicity testing. Arch. Toxicol. 54: 275-287.
Majid T, Mohsen T, Abas AG, Sayed AT (2010). Performance, immunity, serum biochemical and haematological parameters in broiler chicks fed dietary thyme as alternative for an antibiotic growth promoter. Afr. J. Biotechnol. 9(40): 6819-6825.
Nagatsua T, Sawadab M (2009). L-dopa therapy for Parkinson's disease: past, present, and future. Parkinsonism Relat. Disord. 15 (Suppl. 1): S3 – S8.
Nwosu J (2011). The Effect of Storage Condition on the Rheological/Functional Properties of Soup Thickner Mucuna sloanei (Ukpo). Researcher 3:6-10.
Obochi GO, Malu SP, Effiom EO, Bassey GA (2007). Efficacy and suitability of lectin from Mucuna sloanei seeds extracts as a cell receptor signal inducer. Res. J. Biol. Sci. 2(6):667-669.
Odoh UE, Osadebe PO (2010). Effects of Mucuna flagellipes (Leguminosae) seeds on haematology and serum chemistry of rats. J. Trop. Med. Plant 11(1): 35-38.
Ojiako OA, Anugweje K, Igwe CU, Alisi CS (2012). Evaluation of the amino acid profile and haemoglobin polymerization inhibition potential of some Nigerian legumes. Br. J. Pharm. Res. 2(2): 80-88.
Olson H, Betton G, Robinson D, Thomas K, Monro A, Kolaja G, Lilly P, Sanders J, Sipes G, Bracken W, Dorato M, Deun KV, Smith, Berger B, Heller A (2000). Concordance of toxicity of pharmaceuticals in humans and in animals. Regul. Toxicol. Pharmacol. 32:56-67
Polenakovic M, Sikole A (1996). Is erythropoietin a survival factor for red blood cells? J. Am. Soc. Nephrol. 7(8):1178-1182.
Sanchez-Elsner T, Ramirez JR, Rodriguez- Sanz F, Varela E, Bernabew C, Botella LM (2004). A cross talk between hypoxia and TGF-beta orchestrates erythropoietin gene regulation through SPI and smads. J. Mol. Biol. 36(1):9-24.
Siddhuraju P, Vijayakumari K, Janardhanan K (1995). Studies on the under exploited legume, Indigofera linifolia and Sesbania bispinosa: Nutrient composition and antinutritional factors. Int. J. Food Sci. Nutr. 46:195-203.
Sood R (2006). Medical Laboratory Technology. Jaypee Brothers medical Publishers Limited. New Delhi
Sunmonu TO, Oloyede OB (2010). Performance and haematological indices in rats exposed to monocrotophos contamination. Exp. Toxicol. 2:7-12
Ukachukwu SN, Obioha FC (1997). Chemical evaluation of Mucuna cochinchinensis as alternative protein feedstuff. J. Appl. Chem. Agric. Res 4:33-38.
Ukachukwu SN, Ezeagu IE, Tarawali G, Ikeogu JEG (2002). Utilization of Mucuna as a Food and feed in West Africa. In: Flores BM, Eilittä M, Myhrman R, Carew LB, Carsky RJ (Editors). Food and Feed from Mucuna: Current Uses and the Way Forward Workshop. CIDICCO, CIEPCA and World Hunger Research Center, Tegucigalpa, Honduras.
Yakubu MT, Akanji MA, Oladiji AT (2007). Haematological evaluation in male albino rats following chronic administration of aqueous extract of Fadogia agrestis stem. Pharmacog. Mag. 3:34-43.

academicJournals

Vol. 14(25), pp. 2095-2102, 23 June, 2015
DOI: 10.5897/AJB2015.14505
Article Number: C03CD9153884
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Investigation of stress tolerance of endoglucanases of the cellulosomes of *Clostridium cellulolyticum* to ethanol

Ugochukwu Anieto

Biological Sciences Department, University of North Texas, Denton Texas 76203, USA.

Received 16 February, 2015; Accepted 17 June, 2015

Current energy and environmental challenges are driving the use of cellulosic materials for biofuel production. A major obstacle in this pursuit is poor ethanol tolerance among cellulolytic *Clostridium* species. The objective of this work was to establish a potential upper boundary of ethanol tolerance for the cellulosome itself. The hydrolytic function of crude cellulosome extracts from *Clostridium cellulolyticum* on carboxymethyl cellulose (CMC) with 0, 5, 10, 15, 20 and 25% ethanol was determined. Results indicate that the endoglucanase activity of the cellulosome incubated in 5 and 10% ethanol was significantly different from a control without ethanol addition. Furthermore the endoglucanase activity for the cellulosomes incubated in 5, 10, 15, 20 and 25% ethanol in a standalone experiment was significantly different from the control without ethanol. Endoglucanase activity continued to be observed for up to 25% ethanol, indicating that cellulosome function in ethanol will not be an impediment to future efforts towards engineering increasing production titers to levels at least as high as the current physiological limits of the most tolerant ethanologenic microbes.

Key words: Ethanol, *Clostridium cellulolyticum* carboxymethyl cellulose, endoglucanase activity, cellulosome.

INTRODUCTION

The continual depletion of fossil fuel reserves, increase in the world's population, environmental catastrophes associated with crude oil drilling, price fluctuations, political unrest in major oil producing countries, concern over greenhouse gases (GHG) emissions, and the need to preserve the environment through sustainable practices require an aggressive search for alternative fuels. In spite of the development of numerous alternatives, each with technical merits, bioethanol continues to lead the pack in adoption and commercial production. Ethanol is readily produced from a variety of agriculture-based renewable materials like sugarcane juice, molasses, potatoes, corn and barley (Panesar et al., 2006) and its potential for use as transportation fuel was conceived as early as 1890 (Esser and Karsch, 1984). The yeast *Saccharomyces cerevisiae* is in use all over the world as the major ethanol-producing microorganism, but despite its extensive use, it has a number of disadvantages

E-mail: ugochukwuanieto@my.unt.edu. Tel: +19404659176.

Abbreviations: CMC, Carboxymethyl cellulose; **GHG,** greenhouse gases; **PFO,** pyruvate ferridoxin

including high aeration cost, high biomass production, low temperature requirements, substrate range limitations, and finite ethanol tolerance (Saigal, 1993).

Many efforts to produce biofuel from biomass have focused on members of the genus *Clostridium*, known for their efficient degradation of lignocelluloses. *Clostridium cellulolyticum* is one such member of this group of Gram-positive anaerobic microorganisms. Its ability to utilize cellulose as a carbon and energy source is dependent upon the cell surface expression of multiple enzymes organized into a structure known as the cellulosome. Although, *C. cellulolyticum* is an efficient cellulose degrader, it has a low ethanol production yield, possibly due to pyruvate overflow arising from carbon flux surpassing the level of synthesis of pyruvate ferridoxin (PFO) and lactate dehydrogenase in its sugar utilization pathway (Senthilkumar, 2005). *C. cellulolyticum* ATCC 35319 (formerly identified as strain H_{10} was isolated from decayed grass compost at the Université de Nancy, France. It is a Gram-positive bacillus that forms spores in cultures of cellulose media three or more days old (Petitdemange et al., 1984). Growth of *C. cellulolyticum* on cellulose occurs via several cellulases, which are re-grouped into an extracellular enzymatic complex, called the cellulosome. Lignocellulosic fermentation results in mixed products including carbon dioxide, hydrogen, acetate, ethanol, lactate and formate (Desvaux, 2005). The term cellulosome was first introduced with the thermophilic cellulolytic anaerobic bacterium *Clostridium thermocellum* (Lamed et al., 1983). The cellulosome is of particular interest since it permits a highly efficient degradation of crystalline cellulose and offers exceptional biotechnological potential (Bayer et al., 1994). Advantages of the cellulosome include (i) a direct and specific adhesion to the substrate of interest permitting efficient competition with other microorganisms and (ii) ensuring efficient cellular uptake of the soluble cellodextrins by limiting their diffusion in the extracellular milieu (Shoham et al., 1999). The final products of cellulose digestion are oligodextrans, whose subsequent fermentation results in water, CO_2, and a number of mixed organic acids under anaerobic conditions (Ljungdahl and Eriksson, 1985). Since, the first step in cellulose degradation involves the action of cellulases, many researchers have focused on these enzymes (Bhat, 2000).

The cellulosome from an enzymatic viewpoint (i) allows optimum concerted activity and synergism of the cellulases, (ii) avoids non-productive adsorption of the cellulases, (iii) limits competition between cellulases for the sites of adsorption and (iv) allows optimal processivity of the cellulases all along the cellulose fiber (Schwarz, 2001). Given that *C. cellulolyticum* fermentation results in poor ethanol yield, efforts have been made to improve overall yield, including one attempt to improve productivity by introducing heterologous genes from *Zymomonas mobilis*, with mixed results (Senthilkumar, 2005). The cellulosome of various cellulolytic bacteria have been subjects of intense studies in recent times with the genus *Clostridia* receiving the most attention partly due to the advantages listed above and due to its readily available substantial genomic information. For this reason, various attempts are geared towards the cell surface expression of the cellulosome in more efficient ethanol producers and these attempts have necessitated studies describing bioengineered *Clostridia,* stress tolerance of *Clostridia* under various environmental conditions, cellulosome activity etc. In a study by Brown et al. (2011) on ethanol adapted *C. thermocellum,* it was discovered that a mutant alcohol dehydrogenase (*adhE*) confers increased ethanol tolerance. The results show that strain of *C. thermocellum* DSM 1313 WT carrying the mutant allele showed marked improvement in growth in the presence of 20 and 24 g/L added ethanol and was also the only strain able to grow in the presence of 40 g/L added ethanol.

Xu et al. (2010) in studies of factors influencing cellulosome activity in Consolidated Bioprocessing of cellulosic ethanol concluded that formate, acetate and lactate with concentrations below 100, 200 and 50 mM, respectively, could increase cellulosome activities for cellulosome degradation. They further added that cellulosome exhibited higher ethanol tolerance and thermostability than cellulase and was tolerant up to 5 mM furfural, 50 mM *p*-hydroxybenzoic acid and 1 mM catechol and finally 491 mM ethanol was generated. In another study by Yang et al. (2012), the transcriptomic, metabolic and proteomic profiles of *C. thermocellum* ATCC27405 after ethanol stress, revealed several previously unknown information which include (i) medium supplementation with ethanol negatively influenced *C. thermocellum* growth and cellobiose consumption, with cellobiose consumption rate reduced from 0.46 g/L/h in the absence of ethanol to 0.24 g/L/h after ethanol treatment; (ii) Ethanol treated cells indicated a decline in glutamic acid, a 2.8- fold increase in phenylalanine and a doubling of sugar phosphates that were significant at 60 and 120 min post treatment; (iii) 326 genes showed high levels of expression whereas 361 genes had relatively low expression intensity; (iv) 77 proteins exhibited a ≥ 1.5-fold and significant change ($p \leq 0.05$) in abundance. Of the 77 proteins, 42 were more-abundant within ethanol-treated cells while 35 were down regulated following ethanol treatment;(v) Out of the 158 ethanol-responsive genes, six cellulosomal genes were defined as ethanol-responsive with only one Cthe_3078 (*olpB)* down-regulated while the rest which were up-regulated at earlier stages, had no or few peptides detected following ethanol treatment. Several researchers have studied the surface assembly and expression of cellulosome primarily on the yeast *S. cerevisiae* and a few bacteria. In the study of the heterologous expression of a *Clostridium* minicellulosome in *S. cerevisiae,* Lilly et al. (2009) reported the establishment of the phenotypic evidence for

cohesion-dockerin interaction with a detection of a twofold increase in tethered endoglucanase activity in *S. cerevisiae* expressing the scalffoldin protein Scaf3 compared with the parent strain. In another study, Fan et al. (2012) reported the self-surface assembly of the minicellulosome of *C. cellulolyticum* on *S. cerevisiae*. The engineered *S. cerevisiae* was applied in the fermentation of carboxymethyl cellulose (CMC), phosphoric acid-swollen cellulose (PASC), or Avicel. It showed a significant hydrolytic activity towards microcrystalline cellulose, with an ethanol titer of 1412 mg/L. They concluded that the simultaneous saccharification and fermentation of crystalline cellulose to ethanol can be accomplished by the yeast, engineered with minicellulosome. Other researchers have similarly studied surface assembly varying degrees of success for example Anderson et al (2011) successfully attached a three-enzyme-containing minicellulosome on the cell surface of *Bacillus subtilis.* Other studies include the works of Tsai et al 2009 and Wen et al 2010.

Most studies on environmental stress have concentrated on cultures of *Clostridia* species with little or none addressing stress response of the cellulosome itself *in vitro.* There is lack of sufficient literature detailing ethanol stress on the cellulosome of the genus *Clostridia* and other genera that possesses the cellulosome, particularly at the time researchers have found novel ways to express the genes coding for the cellulosome proteins in microorganisms that are better suited to produce industrially useful titers of ethanol. The aim of this study was to determine the upper boundary of *C. cellulolyticum* cellulosome ethanol tolerance. The data will serve as a guide to a better understanding of the cellulosome of this and other cellulose degraders, as well as identify potential limitations to increasing ethanol yield in biofuel systems.

MATERIALS AND METHODS

Bacterial strain and media

C. cellulolyticum (ATCC 35319) was purchased from the American Type Culture Collection (Manassas, Virginia USA). The culture was first grown in *C. cellulolyticum* medium (ATCC, 1368) using glucose as the carbon source. Medium preparation involved the addition of resazurin to indicate for the presence of oxygen. The medium was boiled under pressure, with the removal of oxygen using a vacuum pump. At the point at which the resazurin changed color from blue to pink, the boiling was stopped and the medium allowed to cool down to room temperature. The medium was transferred into the Coy Anaerobic chamber (Great Lakes, Michigan USA) where 10 ml each were dispensed into sterile serum bottles. The bottles were sealed to prevent any introduction of oxygen and the medium sterilized under standard autoclave conditions. Thereafter, the *C. cellulolyticum* culture was aseptically transferred into the 10 ml medium, gassed for 1 minute using 90% hydrogen and 10% carbondioxide gas mix. The culture was incubated for 24 h in 37°C shaking incubator at 225 revolutions per minute. After 24 h, they were routinely transferred into *C. cellulolyticum* medium (ATCC 1368) containing microcrystalline cellulose as carbon source and incubated for 72 h under the same conditions.

Cellulosome purification

Cultures were harvested according to the methods as described by Gal et al. (1997). Cells were cultivated anaerobically in two 500-ml flasks at 37ºC and 160 revolutions per minute (rpm) in *C. cellulolyticum* medium. After 120 h of growth, cells and residual cellulose were harvested by centrifugation at 13000 *g* for 20 min. The pellets were washed five times in 50 ml of 25 mM Tris-HCl (pH 8.0), re-suspended in the same medium and filtered through a 3-µm pore size glass filter (glass microfiber filter GF/D; WHATMAN; GE Healthcare Pittsburgh Pennsylvania USA) to remove cellulose fibers, washed on the filter, first with 100 ml of 25 mM Tris-HCl (pH 8.0) and second with 100 ml of 12.5 mM Tris-HCl (pH 8.0) and the cellulosome eluted with 150 ml of water on the filter. The eluted fraction was then filtered on a 0.2-µm pore size nylon membrane filter and concentrated using Amicon Ultra centrifugal filters (30 KDa molecular weight cutoff filter, Billerica Massachusetts USA). The final 1-ml sample of filtrate (eluate) extract containing the crude cellulosome preparation was subjected to vacuum drying for 20 min to concentrate the dilute cellulosome extracts to approximately 0.5 ml.

Protein quantification of cellulosome preparations

Total protein concentrations of crude cellulosome extracts were determined by the Bradford assay using Bovine serum albumin (BSA) as a standard. Bovine serum albumin standards at concentrations of 0, 20, 40, 80 and 100 µg/ml were prepared in 0.1 M sodium acetate buffer pH 5.5, the standard and cellulosome extracts incubated at 37ºC for 30 min, and absorbance readings were taken at 595 nm on a Synergy 2 Multi-Detection Microplate Reader (BioTek Instruments Inc., Winooski VT USA).

Substrates

Medium-viscosity carboxymethyl cellulose (CMC) originally purchased from Nutritional Biochemicals Corp. (Cleveland, Ohio USA) was used.

Hydrolysis with crude cellulosome preparation

Reactions were carried out according to the method of King et al., (2009) for the quantification of free reducing ends of released mono and oligosaccharides as described below. Each tube contained 90 µL of 0.25% CMC in 0.1 M sodium acetate buffer pH 5.5. Five tubes were set up as follows: (a) 90 µL crude cellulosome with 90 µL of water (negative control); (b) 90 µl CMC substrate with 90 µL of water (negative control); (c) 90 µL CMC substrate in 0% ethanol with 90 µL of crude cellulosome (d) 90 µL CMC substrate in 10% ethanol with 90 µL of crude cellulosome (5% final ethanol concentration), and (e) 90 µL CMC substrate in 20% ethanol with 90 µL of crude cellulosome (10% final ethanol concentration), these were incubated at 37ºC first for 24 h and then for another 24 h. These time points were chosen to allow for adequate hydrolysis of the substrate and release of the reducing sugars. This exact procedure was replicated two additional times under the same conditions. A standalone fourth sample was analyzed and it included 15, 20 and 25% final ethanol concentrations in the set to gauge the upper ethanol concentration limit of activity.

Reducing sugar quantification

The DNS reagent was prepared according to method of Ghose

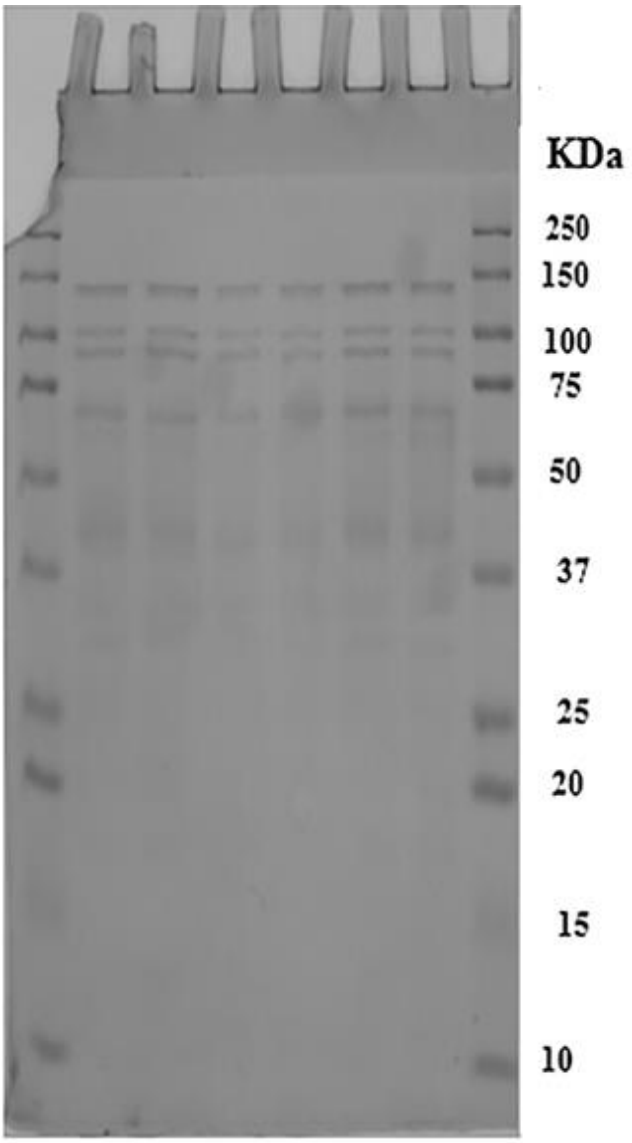

Figure 1. Cellulosomes Extract on SDS-PAGE. Wells 1 and 8 contain 250 KDa protein ladder. Wells 2 to 7 are replicates of the cellulosome proteins.

(1987). The DNS reagent is non-specific and reacts with both five and six carbon reducing sugars (King et al., 2009). Glucose standards curve of 0, 0.25, 0.50, 0.75, and 1.0 mg ml^{-1} were prepared in 0.1 M sodium acetate buffer pH 5.5 with additional sets of the same standards containing 10% and 20% ethanol, respectively. The glucose standards prepared under different conditions were used to determine if the presence of ethanol would interfere with quantification of reducing sugars. 60 µL of each standard or reaction hydrolysis product was added to 120 µL DNS reagent in a 2.0 ml PCR microtube for a total volume of 180 µL. The DNS reactions were carried out in thermocyclers (Bio-Rad, Hercules California USA) by heating at 95ºC for 5 min followed by cooling to 4ºC for 1 minute and holding at 20ºC. A 36 µL aliquot of each completed DNS reaction was added to 160 µL of deionized water in a flat bottom, 96-well microplate and mixed thoroughly using the micropipette. Absorbance was immediately determined at 540 nm.

Statistical analyses

The data obtained from the glucose standard assay and the endoglucanase activities were analyzed using ANOVA and the t-test of means from the statistical software package SigmaPlot (San Jose California USA, www.sigmaplot.com).

SDS-PAGE

SDS-PAGE was performed by the procedure of Laemmli (1970) by preparing 10% polyacrylamide gels. The samples used for the SDS-PAGE analyses were boiled in sample buffer before use. The SDS-PAGE was run under a constant flow of cold water using the Hoefer slab apparatus (Holliston, Massachusetts USA) for approximately 3 h.

RESULTS

SDS-PAGE

The cellulosome extracts were run on SDS-PAGE containing 0.25% CMC to determine the likely fractions of the cellulosome retained in the extract. The largest fraction at approximately 150 KDa suggests the presence of the scaffoldin protein CipC. The individual enzymes of the cellulosome range between 40 and 90 KDa in size. Figure 1 shows the SDS-PAGE with the inclusion of 0.25% CMC.

Glucose Standard Curve

Glucose standard curve without ethanol, with 10% ethanol, and with 20% ethanol was determined and plotted. The regression values under the three conditions were determined using the R programme to be 0.992, 0.993 and 0.992 respectively. This experiment was repeated two more times and designated as batch 1, 2 and 3 with each batch consisting of glucose standard 0%, 10% and 20% ethanol. To further determine whether the addition of ethanol had any significant effect on the measurement of Absorbance in the reducing sugar reaction for the standard curves, ANOVA was applied to each batch ($\alpha = 0.05$) and the values obtained were 0.9994, 0.9962 and 0.9916, respectively. From the values obtained, it was concluded that ethanol up to 20% did not interfere with the reducing sugar quantification reaction and would therefore not affect the determination of the endoglucanase activity of the crude cellulosomes.

Measurement of endoglucanase activity

To determine endoglucanase activity of the cellulosome from the Absorbance values obtained at 540 nm, the standard endoglucanase determination formula for carboxymethyl cellulose was calculated using the method of (Xiao et al., 2005).

Table 1. Endoglucanase activities measured from CMC per batch determined at 24 and 48 hours of incubation. The numbers in parentheses represent the standard deviations of triplicate values

Parameter	Endoglucanase activity (10^{-6}, IU/ml) after 24 h	Endoglucanase activity (10^{-6}, IU/ml) after 48 h
Samples (Batch 1)		
Cellulosome without substrate	3.000	3.000
CMC without cellulosome	3.000	3.000
CMC in 0% ethanol with cellulosome	11.22 (1.025)	10.80 (0.177)
CMC in 5% ethanol with cellulosome	8.72 (0.537)	10.76 (0.042)
CMC in 10% ethanol with cellulosome	8.28 (0.170)	8.90 (0.113)
Samples (Batch 2)		
CMC in 0% ethanol with cellulosome	8.19 (0.244)	9.24 (0.100)
CMC in 5% ethanol with cellulosome	8.66 (0.330)	9.83 (0.127)
CMC in 10% ethanol with cellulosome	8.17 (0.140)	9.25 (0.159)
Samples (Batch 3)		
CMC in 0% ethanolwith cellulosome	7.55 (0.138)	8.85 (0.052)
CMC in 5% ethanol with cellulosome	7.10 (0.216)	8.46 (0.132)
CMC in 10% ethanol with cellulosome.	6.97 (0.261)	8.15 (0.360)
Samples (Batch 4)		
CMC in 5% ethanol with cellulosome	4.0775 (0.03)	4.210 (0.04)
CMC in 10% ethanol with cellulosome	3.960 (0.08)	4.09 (0.111)
CMC in 15% ethanol with cellulosome	3.820 (0.09)	3.905 (0.03)
CMC in 20% ethanol with cellulosome	3.7175 (0.02)	3.845 (0.02)
CMC in 25% ethanol with cellulosome	3.68 (0.04)	3.63 (0.05)

IU/ml = (A_{540} sample/A_{540}/µg standard) (1/180 µg/µmol glucose) (1/30 min) (1/x ml) Where one international unit (IU) is defined as an average of 1 µmol of glucose equivalents released per min in the assay reaction. A_{540} sample is the absorbance obtained from the reducing sugar assay for CMC at λ = 540 nm; A_{540}/µg standard is the absorbance for 1 µg of glucose as derived from the glucose standard curve. 180 µg/µmol glucose is the amount of glucose in 1 µmol; 30 min is the assay incubation time; and *x* ml is the volume of the enzyme used in the assay (25), (in this case 0.09 ml). Table 1 shows the calculated values for the endoglucanase activities under the different ethanol concentrations after 24 and 48 h, the standard deviations of triplicate samples are shown in parenthesis.

Figure 2 shows the bar plot of endoglucanase activity for batch 1, 2, and 3 determined after 24 and 48 h. The time points were arbitrarily chosen. The bar plots clearly indicate an improvement in reducing sugars released after 48 h (5 and 10%, respectively) and the extracts incubated in substrates containing 10% ethanol showed slightly lower values of reducing sugars released overall when compared with those without ethanol and those with 5% ethanol concentration. The values obtained from the three independent experiments for 0, 5 and 10% conditions were normalized before plotting and subjected to statistical analyses using ONE-WAY ANOVA. There is a statistical significant difference in the amount of reducing sugar released under the three conditions, p = 0.033 (α = 0.05). Additionally, the t-test of means (using the Mann-Whitney Ranked Sum Test) was applied to the values to determine whether any difference existed between the 24 h samples and the 48 h sample. The difference in the median values between the two groups is greater than would be expected by chance; there is a statistically significant difference (P = <0.001) (Figure 3). The data indicate that the reaction was highly affected by the addition of ethanol up to 10% and that endoglucanase activity could have continued beyond 48 h. Figure 4 shows the bar plot of a standalone batch 4 with 15, 20 and 25% ethanol concentrations included for 24 and 48 h incubation time. Replicates of the sample were statistically analyzed separately from other batches using the Kruskal-Wallis One Way Analysis of Variance on Ranks. The differences in the median values among the ethanol concentrations are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

DISCUSSION

The result of the statistical analysis obtained for the batch 4 supports the result earlier seen in batch 1, 2 and 3 even

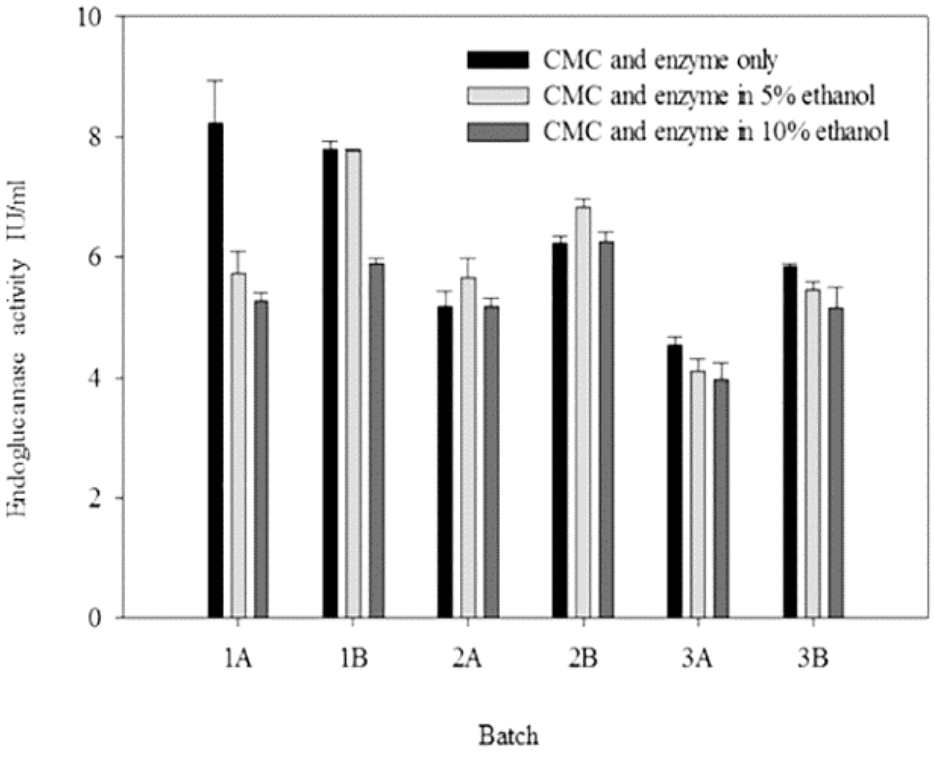

Figure 2. Effects of 0%, 5% and 10% ethanol on endoglucanase activities. A, 24-hour incubation; and B, 48-hour incubation. Error bars represent standard deviations among three replicates (One-way ANOVA).

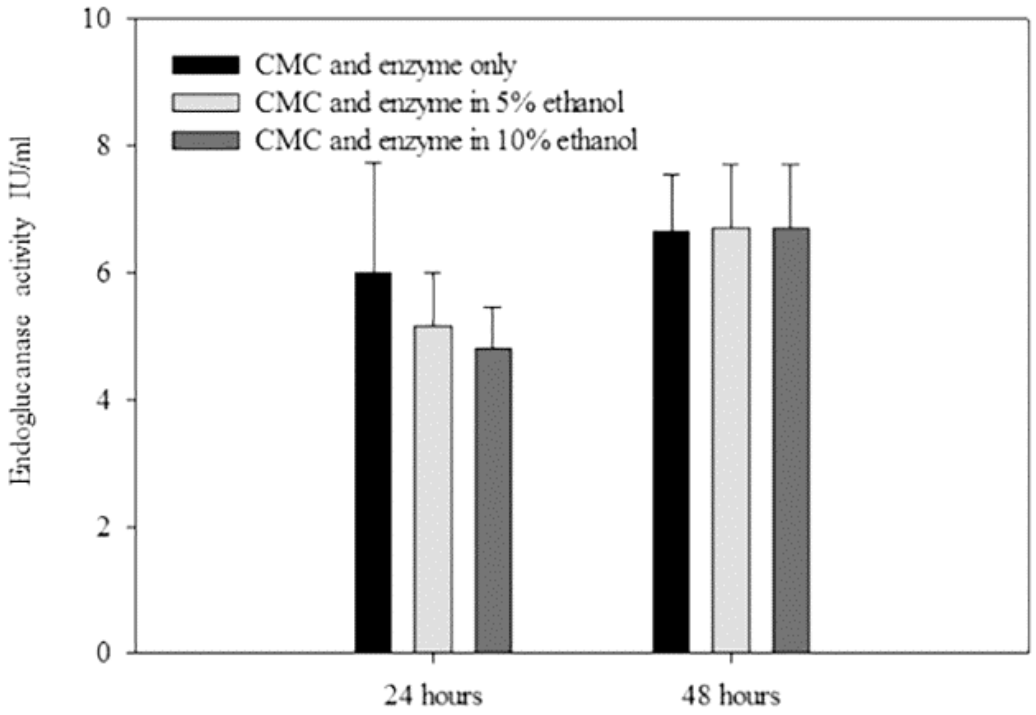

Figure 3. Effects of 0%, 5% and 10% ethanol on endoglucanase activities after 24 and 48 hours respectively. Error bars represent standard deviations among three replicates. (Mann-Whitney T-test).

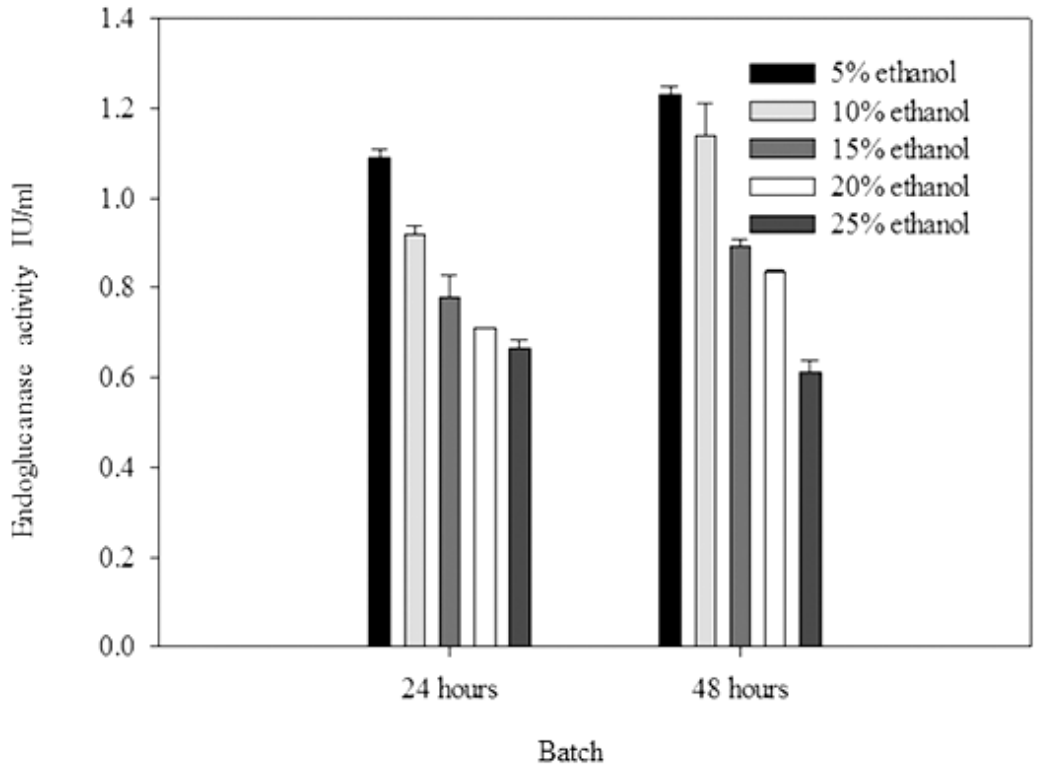

Figure 4. Effects of 0%, 5% 10%, 15%, 20% and 25% ethanol on endoglucanase activities on the standalone batch 4. Error bars represent standard deviations among three replicates. (Kruskal-Wallis One Way Analysis of Variance on Ranks).

though the standalone batch 4 did not produce the same level of endoglucanase activity as previously observed with the first 3 batches that were analyzed. Cellulosome activity could be highly dependent upon other extraneous factors, such as pH, temperature, presence of chemical elements in the aqueous medium etc. For batch 4, samples from 5 to 25% followed a similar pattern with the previously analyzed batch 1, 2 and 3 and a decline was observed in endoglucanase activity with increasing ethanol concentration. An attempt was made to analyze higher ethanol concentration above 30% and the results (not shown) were highly inconsistent with varying figures and therefore were deemed not reproducible and dropped. The Mann-Whitney Ranked Sum Test was performed for the 24 and 48 h reading of batch 3. The difference in the median values between the two groups is not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.228). This is not consistent with the previously observed result for the first three batches and could be as a result that of low endoglucanase activities obtained. The actual values for the endoglucanase activities determined could however be a function of the concentration of the cellulosomes, the composition of the cellulosomes, the physiological state of the cells in the culture medium, the culturing conditions used, the purification, handling and processing conditions etc. The cellulosome of *C. cellulolyticum* retained endoglucanase activity in the presence of ethanol in some cases up to 25%. Cellulosomes activity seemed improved after 48 h of incubation with CMC for 5, 10, 15, 20 and 25% samples, even though activity decreased as the ethanol concentration increased. However, at no point was activity totally eliminated. In a similar study by Skovgaard and Jorgensen (2013), a mixture of mesophilic and thermostable lignocelluloytic enzymes were exposed to a temperature of 55 to 65°C and up to 5% ethanol (w/v), the thermostable and mesophilic mixture remained active at up to 65°C. When the enzyme mixtures reached their maximum temperature limit, ethanol had a remarkable influence on enzyme activity, e.g., the more ethanol, the faster the inactivation. During hydrolysis, it has been found that ethanol is a non-competitive inhibitor binding to the allosteric site of the enzyme, which results in reversible denaturation because of the solvent properties of ethanol (Holtzapple et al., 1990).

Furthermore, ethanol destroys the tertiary hydrophobic interactions in the enzyme, breaking or loosening the compact structure of the enzyme complex (Shao et al., 2012; Yoshikawa et al., 2012). From these preliminary results, the various bioengineering attempts to increase

ethanol production in microbes will not likely be limited by cellulosome activity but further investigation will need to be carried out to determine whether a “ceiling” exists for ethanol stress that could be tolerated by the cellulosome. Additionally, the actual activity of cellulosome *ex vivo* could be different from that observed under the *in vitro* conditions tested here. This could occur if, for example, other yet-to-be annotated genes and their protein products may contribute to the resilience of the cellulosome *ex vivo*. Cellulosome, in spite of their size and complexity, are remarkably robust complexes. It is likely that these structures can withstand various degrees of other environmental stresses in addition to those discussed here. The use of carboxymethyl cellulose as a substrate for this experiment is a test bed for further investigation. In reality, the cellulosome would degrade other forms of cellulose in its various forms, these would be utilized in further testing of cellulosome behavior under ethanol stress. Additionally, this work provides the grounds for further research into other stressors that could impact on the activities of the cellulosome, taking into consideration that they could be used in different unrelated microorganisms that could be making other products besides ethanol. Future efforts would explore tolerances to alternative biofuel products, such as biodiesel, biobutanol, etc.

Conflict of interest

The author has not declared any conflict of interest.

ACKNOWLEDGEMENT

The author wishes to thank Dr. Michael Allen of the Department of Biological Sciences, University of North Texas, Denton TX for his support, Dr. Nathaniel Mills of Texas Woman’s University, Denton TX and the Department of Biological Sciences, University of North Texas for the funding.

REFERENCES

Anderson T, Robson S, Jiang X, Malmirchegini G, Fierobe H, Lazazzera B, Clubb R (2011). Assembly of Minicellulosomes on the Surface of *Bacillus subtilis*. Appl. Environ. Microbiol. 77(14):4849-4858.

Bayer E, Morag E, Lamed R (1994). The cellulosome- a treasure-trove for Biotechnology. Trends Biotechnol. 12(9):379-386.

Bhat M (2000). Cellulases and related enzymes in biotechnology. Biotechnol. Adv. 18(5): 355-383.

Brown S, Guss A, Karpinets T, Parks J, Smolin N, Yang S, Land M, Klingeman D, Bhandiwad A, Rodriguez M, Raman B, Shao X, Mielenz JR, Smith JC, Keller M, Lynd LR (2011). Mutant alcohol dehydrogenase leads to improved ethanol tolerance in *Clostridium thermocellum*. P. Natl. Acad. Sci. USA 108(33):13752-13757.

Desvaux M (2005). The cellulosome of *Clostridium cellulolyticum*. Enzyme Microb. Tech. 37(4): 373-385.

Esser K, Karsch T (1984). Bacterial ethanol production: advantages and disadvantages. Process Biochem. 19:116-121.

Fan L, Zhang Z, Yu X, Xue Y, Tan T (2012). Self-surface assembly of cellulosomes with two miniscaffoldins on Saccharomyces cerevisiae for cellulosic ethanol production. P. Natl. Acad. Sci. USA 109(33):13260-13265.

Gal L, Pages S, Gaudin C, Belaich A, Reverbel-Leroy C, Tardiff C, Belaich J (1997). Characterization of the cellulolytic complex (cellulosome) produced by *Clostridium cellulolyticum*. Appl. Environ. Microbiol. 63(3): 903-909.

Ghose T (1987). Measurement of cellulase activities. Pure Appl. Chem. 59(2): 257-268.

Holtzapple M, Cognata M, Shu Y, Hendrickson C (1990). Inhibition of *Trichoderma reesei* cellulase by sugars and solvents. Biotechnol. Bioeng. 36(3):275-287.

King B, Donnelly M, Bergstrom G, Walker L, Gibson D (2009). An optimized microplate assay system for quantitative evaluation of plant cell wall–degrading enzyme activity of fungal culture extracts. Biotechnol. Bioeng. 102(4):1033-1044.

Laemmli U (1970). Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4, Nature 227: 680-685.

Lamed R, Setter E, Bayer E (1983). Characterization of a cellulose-binding, cellulase-containing complex in *Clostridium thermocellum*. J. Biotechnol. 156(2): 828-836.

Lilly M, Fierobe H, Van ZW, Volschenk H (2009). Heterologous expression of a Clostridium minicellulosome in *Saccharomyces cerevisiae*. FEMS Yeast Res. 9(8): 1236-1249.

Ljungdahl L, Eriksson K (1985). Ecology of microbial cellulose degradation. Adv. Microb. Ecol. 8: 237-299.

Panesar P, Marwaha S, Kennedy J (2006). *Zymomonas mobilis*: an alternative ethanol producer. J. Chem. Tech. Biotech. 81(4): 623-635.

Petitdemange E, Caillet F, Giallo J, Gaudin C (1984). *Clostridium cellulolyticum* sp. nov., a Cellulolytic, Mesophilic: Species from Decayed Grass. Int. J. Sys. Bacteriol. 34(2): 155-159.

Saigal D (1993). Yeast strain development for ethanol production. Indian J. Microbiol. 33:159-168.

Schwarz W (2000). The cellulosome and cellulose degradation by anaerobic bacteria. App. Microbiol. Biotechnol. 56(5-6): 634-649.

Senthilkumar V, Gunasekaran P (2005). Bioethanol production from cellulosic substrates: Engineered bacteria and process integration challenges. J. Sci. Ind. Res. 64:845-853.

Shao Q, Fan Y, Yang L, Qin Y (2012). From protein denaturant to protectant: Comparative molecular dynamics study of alcohol/protein interactions. J. Chem. Phys. 136:115101.

Shoham Y, Lamed R, Bayer E (1999). The cellulosome concept as an efficient microbial strategy for the degradation of insoluble polysaccharides. Trends Microbiol. 7(7): 275-281.

Skovgaard P, Jorgensen H (2013). Influence of high temperature and ethanol on thermostable lignocellulolytic enzymes. J. Ind. Microbiol. Biotechnol. 40(5):447-456.

Tsai S, Oh J, Singh S, Chen R, Chen W (2009). Functional Assembly of Minicellulosomes on the *Saccharomyces cerevisiae* Cell Surface for Cellulose Hydrolysis and Ethanol Production. Appl. Environ. Microbiol. 75 (19):6087-6093.

Wen F, Sun J, Zhao H (2010). Yeast Surface Display of Trifunctional Minicellulosomes for Simultaneous Saccharification and Fermentation of Cellulose to Ethanol. Appl. Environ. Microbiol. 76(4):1251-1260.

Xiao Z, Storms R, Tsang A (2005). Microplate-based carboxymethyl cellulose assay for endoglucanase activity. Anal. Biochem. 342(1):176-178.

Xu C, Qin Y, Li Y, Ji Y, Huang J, Song H, Xu J (2010). Factors influencing cellulosome activity in Consolidated Bioprocessing of cellulosic ethanol. Bioresour. Technol. 101(24): 9560-9569.

Yang S, Giannone R, Dice L, Yang Z, Engle N, Tschaplinski T, Hettich R, Brown S (2012). *Clostridium thermocellum* ATCC27405 transcriptomic, metabolomic and proteomic profiles after ethanol stress. BMC Genomics 13:336.

Yoshikawa H, Hirano A, Arakawa T, Shiraki K (2012). Mechanistic insights into protein precipitation by alcohol. International J. Biol. Macromol. 50(3):865-871.

academicJournals

Vol. 14(25), pp. 2103-2113, 23 June, 2015
DOI: 10.5897/AJB2015.14696
Article Number: A2EDAB153885
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Fenthion induced toxicity and histopathological changes in gill tissue of freshwater African catfish, *Clarias gariepinus* (Burchell, 1822)

Peace Onas Somdare[1], Christopher Didigwu Nwani[2]*, Alfreda O. Nwadinigwe[3], Jacinta Chinedu Nwani[4], Gregory Ejike Odo[2], Ogechi Nnabuchi Ugbor[2], Juliana A Ukonze[5] and Ada Bridget Chidi A Ezeibe[6]

[1]Department of Biological Sciences, Federal University Lokoja, Kogi State, Nigeria.
[2]Department of Zoology and Environmental Biology, University of Nigeria Nsukka, Enugu State, Nigeria.
[3]Department of Plant Science and Biotechnology, University of Nigeria Nsukka, Enugu State, Nigeria.
[4]Department of Crop Science and Land Scape Management, Ebonyi State University Abakaliki, Ebonyi State, Nigeria.
[5]Department of Vocational Teacher Education, University of Nigeria Nsukka, Enugu State, Nigeria.
[6]Department of Agricultural Economics/CDR, University of Nigeria Nsukka, Enugu State, Nigeria.

Received 4 May, 2015; Accepted 15 June, 2015

Fenthion is an organophosphate pesticide commonly used in agriculture and public health for the control of insect pests. The present study investigated the toxic effect of fenthion and the histopathological alterations in the gill tissue of African catfish *Clarias gariepinus.* The 96 h LC_{50} value of fenthion in *C. gariepinus* determined by probit analysis was found to be 39.97 mg/L. Fish exposed to different fenthion concentrations showed clinical signs such as erratic swimming, attempt to jump out of water, increased opercula frequencies, air gasping, mucus secretion followed by exhaustion and death. To evaluate the histopathological changes in the gill tissue, fish were exposed to 2.0, 4.0 and 8.0 mg/L corresponding to 1/20, 1/10 and 1/5th, respectively of 96 h LC_{50} of fenthion for 21 days and 7 days recovery. Gill disorder and fusion of the secondary lamellar were pronounced in all treatments. Alterations in gill structure exposed to the highest concentration were oedema, lifting of lamellar epithelia, destruction of gill architecture and lamellar fusion. From the findings, it can be deduced that fenthion-induced alterations are irreversible and therefore should be applied with caution in the environment so as to reduce its damage to aquatic organisms.

Key words: *Clarias gariepinus*, fenthion, lethal concentration, histopathology.

INTRODUCTION

Organic insecticide poisoning remains one of the major health issues in both developing and developed communities (Peter and Cherian, 2000). A great proportion of acute poisoning cases are caused by exposure to pesticides, especially organophosphate (OP) compounds. The primary mechanism of action of OP pesticides is

*Corresponding author. E-mail: didigwunwani@yahoo.com, chris.nwani@unn.edu.ng. Tel: +2348037509910.

based on inhibition of the acetylcholinesterase (AChE) enzyme (Eto, 1979). Once AChE has been inactivated, acetylcholine (ACh) accumulates throughout the nervous system, resulting in overstimulation of muscarinic and nicotinic receptors. Fenthion (*O,O*-Dimethyl *O*-[3-methyl-4-(methylsulfanyl) phenyl] phosphorothioate) is a systemic OP insecticide widely used in both agricultural and urban areas for controlling many sucking and biting pests (Cong et al., 2009; Sevgiler and Uner, 2010). Like all organo-phosphate insecticides, fenthion acts on the nervous system as inhibitor of acetylcholinesterase (AchE), an enzyme that hydrolyzes acetylcholine (Ach). Acetylcholine is a molecule that is involved in the transmission of nervous signals from nerves to muscles and between neurons in the brain (Jungera, 2014). Due to fenthion toxicity, the Environmental Protection Agency (EPA) has classified it as restricted use pesticide (RUP) that warrants special handling (ATSDR, 2005). Large amounts of pesticides find their ways into water bodies due to repeated application for the control of pest. The indiscri-minate use of pesticides, careless handling, accidental spillage or discharge into natural water ways have harmful effects on fish population and other aquatic organisms and may contribute to long term effects (Nwani et al., 2013b).

Histopathological studies help to establish causal relations between contaminant exposure and various biological responses (Boran et al., 2012) and have proven to be a sensitive tool used in detecting direct effects of chemical compounds within target organs of fish in laboratory experiments (Altinok and Capkin, 2007; Capkin et al., 2009). A study on histopathology provides very important and useful data concerning changes in cellular or sub cellular structure of an organ much earlier than external notification. One of the advantages of using histopathological biomarkers in environmental studies is that it allows the examination of target organs (Gernhofer et al., 2001) and the alterations found in these organs are easier to identify than functional ones (Fanta et al., 2003). These alterations serve as warning signs of damage to the well-being of an organism.

Research has demonstrated that fenthion induced DNA damage and should be considered potentially hazardous to humans (Wu et al., 2011). Recent research by Kanter and Celik (2012) revealed that exposure of frogs to fenthion induced an increase in melondialdehyde (MDA) and antioxidant defense systems. The effect of fenthion on biochemical changes in *Cirrhina mrigala* (Israel and Sam, 2012), liver function and pigmentation (Muralidharan and Pillai, 2012) in *Cyprinus carpio* have been reported. Studies have demonstrated that exposure of fish to different pesticides resulted to histopathological changes in the gill, liver and kidney tissues (Auta, 2001; Maduenho and Martinez, 2008; Capkin and Altinok, 2013). The fish species *Clarias gariepinus* is an attractive model for ecotoxicological studies because of its hardiness in culture, omnivorous feeding habits and availability throughout the year. In spite of the wide use of fenthion in Nigeria, there is however paucity of information on its effects on indigenous fish species. The present study was thus aimed at determination of the lethal concentration, behavioral effects and histopathological changes in gill tissues of freshwater African catfish, *C. gariepinus* exposed to fenthion.

MATERIALS AND METHODS

Experimental fish specimen and chemical

Freshwater African catfish *C. gariepinus* (Burchell 1822) (Family: Clariidae, Order: Siluriformes), mean standard length of 27.36 ± 0.23 cm and weight of 197.39 ± 2.34 g were procured from Freedom Fisheries Ltd Nsukka, Enugu, Nigeria and transported to our wet laboratory where they were treated with 0.05% potassium permanganate ($KMnO_4$) for 2 min to avoid any dermal infections. The fish were acclimatized for three weeks in plastic tanks of 300 L capacity and fed *ad libitum* daily with commercial available food (Coppens International Helmond Netherlands) containing 35% crude protein. To maintain hygienic condition and prevent pollution caused by food and feces, fecal matter and other waste materials were siphoned off daily. Dead fish were removed with plastic forceps to avoid possible deterioration of water quality. During the period of acclimation, the water in the tanks was renewed daily with well aerated tap water. The feeding was terminated 24 h prior to the range finding and acute toxicity test to avoid interference of feces. The mean water quality of the test water analyzed daily during the experimental period following the standard methods (APHA, AWWA, WPCE, 2005) had the following physicochemical characteristics: temperature (24.40°C; SE 0.56), dissolved oxygen (6.77 mg/L; SE 0.80), conductivity (285 Mm/cm; SE 6.50), pH (7.22; SE 0.60), and total hardness (as $CaCO_3$ - 240 mg/L; SE 6.60). For the present study, commercial formulation of fenthion manufactured by Yufull Industry Co, Ltd., China containing 600 g/L fenthion was purchased from the local market and used.

Acute toxicity bioassay and behavioral responses

Acute toxicity bioassay to determine the 96 h LC_{50} values of fenthion was conducted using standard methods (APHA, AWWA, WPCF, 2005). The range finding test was carried out before determining the concentrations of the test chemical for the definitive test. For the definitive test, a set of 10 fish were randomly exposed to fenthion concentrations of 12, 24, 36, 48, 60 and 72 mg/L derived from a range finding test using plastic aquaria (60 × 30 × 30 cm size) containing 25 L aerated tap water. Another set of 10 fish was simultaneously maintained in equal amount of tap water but without the test insecticide and considered as the control. The experiments were set in triplicates to obtain the LC_{50} values of the test chemical. Fish were not fed throughout the experiment and lethality was the toxicity end point. Fish were visually examined daily and considered dead when no sudden swimming in response to gentle touch was observed. Dead fish were removed with plastic forceps and the mortality was recorded at intervals of 24, 48, 72 and 96 h. The LC_{10-90} values of the test insecticide for the fish at 24, 48, 72 and 96 h was determined by probit analysis (Finney, 1971). During the acute toxicity test, the no observed effect concentration (NOEC) was determined as the highest concentration in which no mortality was observed while lowest observed effect concentration **(**LOEC) was also determined using acute toxicity results. The safe levels of the test pesticide was estimated by multiplying the 96 h LC_{50} with

Table 1. Cumulative mortality of *Clarias gariepinus* exposed to various concentrations of fenthion.

Concentration (mg l^{-1})	Number of fish exposed	Number of deaths				Mortality (%)	Survival (%)
		24 h	48 h	72 h	96 h		
Control	30	0	0	0	0	0	100
12	30	0	0	0	0	0	100
24	30	2	2	2	4	13	87
36	30	2	6	10	12	40	60
48	30	4	10	12	18	53	47
60	30	12	16	22	24	80	20
72	30	24	30	30	30	100	0

Table 2. Lethal concentration of fenthion (mg/L) (95% confidence interval) depending on exposure time for *Clarias gariepinus* (n=10 in three replicates).

Lethal concentration (mg/L)	Exposure time (h)			
	24 h	48 h	72 h	96 h
LC_{10}	36.14^{a} (0.59-48.16)	30.90^{b} (8.03-40.36)	28.04^{b} (14.08-35.39)	23.97^{c} (18.84-27.85)
LC_{30}	49.70^{a} (17.79-79.51)	41.22^{b} (21.94-52.06)	37.11^{b} (25.37-44.43)	32.42^{c} (27.92-36.04)
LC_{50}	61.99^{a} (45.76-81.74)	50.32^{b} (37.15-73.60)	45.06^{c} (35.87-55.32)	39.97^{d} (35.95-43.93)
LC_{70}	77.30^{a} (57.83-458.20)	61.43^{b} (48.88-133.89)	54.71^{c} (45.75-76.34)	49.27^{d} (44.80-55.33)
LC_{90}	106.33^{a} (71.56-778.35)	81.94^{b} (61.55-374.99)	72.41^{c} (58.10-136.00)	66.64^{d} (58.74-80.92)

Values in rows with different superscript letters differ significantly ($p < 0.05$).

different application factors (AF) and were based on the methods of Hart et al., (1948), Sprague (1971), CWQC (1972), NAS/NAE (1973), IJC (1977), and CCREM (1991). The behavioral responses of *Clarias gariepinus* at different concentrations of fenthion were observed from 24 to 96 h of the exposure.

Sublethal concentrations and tissue preparation

The 96 h LC_{50} value of fenthion in the present study was determined to be 39.97 mg/L. Based on this value, three sublethal concentrations of 2.0, 4.0 and 8.0 mg/L corresponding to 1/20, 1/10 and 1/5th of fenthion, respectively were prepared by serial dilution of the stock solution. A total of 30 acclimatized fish were exposed to each of the aforementioned sublethal test concentrations in triplicates of 10 fish per replicate. Control fish specimens were maintained in dechlorinated tap water without fenthion. The exposure lasted for 21 days after which the fish were withdrawn from the insecticide and kept for seven days in dechlorinated tap water for possible recovery. To avoid catabolism due to starvation during the chronic exposure, the fish were fed daily small quantity of food (approximately 1% of total body weight) an hour before renewal of the test solution. On day 1, 7, 14, 21 and 7 days recovery, one fish from each replicate treatment group and control was sacrificed after anesthetizing with tricaine methanesulfonate (MS 222) to minimize stress. The fish were dissected and gill tissues were removed, preserved in 10% phosphate buffered formalin for 24 h, dehydrated by a series of upgraded ethanol solution, embedded in paraffin, and sectioned at 5 µm thick. A total of three tissue sections per fish for each replicate concentration were routinely processed and stained with Hematoxylin and Eosin (H & E) and examined by light microscopy according to Roberts (2001). Photomicrographs were then taken at (x100) objective and the fish of the control groups were compared with that of exposed groups under the guidance of a pathologist.

Statistical analysis

The data obtained were statistically analyzed using statistical package for social sciences (SPSS) version 21.0 software (SPSS, Inc., Chicago, Illinois, USA) and Duncan's multiple range test was used to determine the significant difference at 5% probability level.

RESULTS

Median lethal concentration (LC_{50}) and safe level

The mortality of *C. gariepinus* exposed to different concentrations of fenthion is presented in Table 1. A dose-dependent increase and time dependent decrease was observed in mortality rate such that as the exposure time increases from 24 to 96 h, the median lethal concentration required to kill the fish was reduced. The LC_{50} values (with 95% confidence limits) of different concentrations of fenthion were found to be 61.99 (45.76 to 81.74), 50.32 (37.15 to 73.60), 45.06 (35.87 to 55.32) and 39.97 (35.95 to 43.89) mg/L for 24, 48, 72 and 96 h exposure, respectively (Table 2). The statistical end points of acute toxicity test are shown in Figure 1. NOEC was not significantly different during acute toxicity test (p

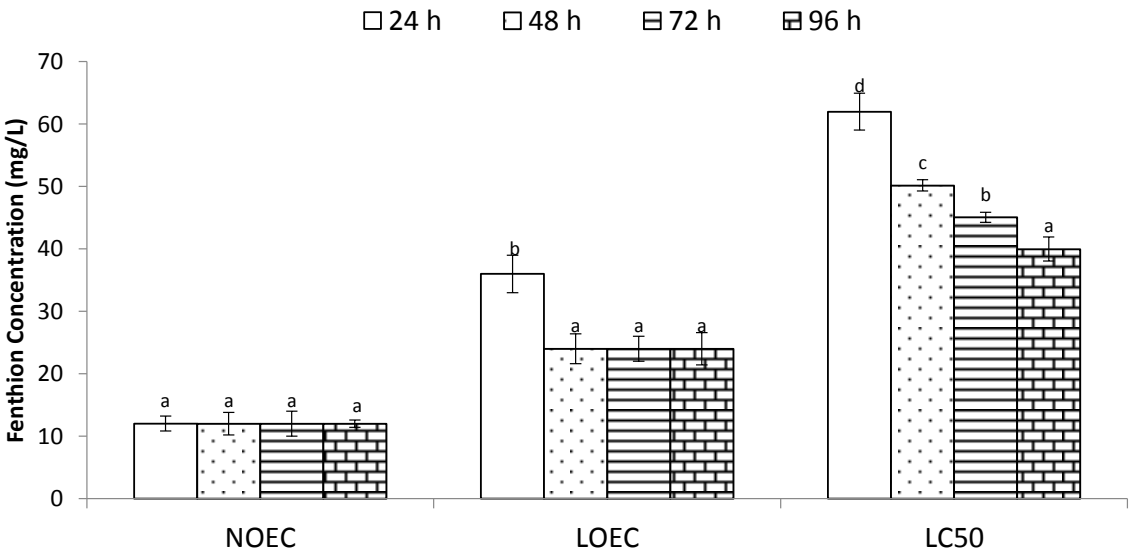

Figure 1. Statistical end points of acute toxicity testing in *Clarias gariepinus* exposed to fenthion for different durations (24, 48, 72 and 96 h).

Table 3. Estimate of safe levels of fenthion at 96 h exposure time in *Clarias gariepinus.*

Pesticide	96h LC_{50} (mg/L)	Method	AF	Safe level (mg/L)
Fenthion	39.97	Hart et al. (1948)*	-	9.96×10^{-1}
		Sprague (1971)	0.1	3.99
		CWQC (1972)	0.01	3.99×10^{-1}
		NAS/NAE (1973)	0.01-0.00001	3.99×10^{-1} - 3.99×10^{-4}
		CCREM (1991)	0.05	1.99
		IJC (1977)	5% of 96 h LC_{50}	1.99

*C = 48 h LC_{50} × 0.03/S^2, where C = presumable harmless concentration and S = 24 h LC_{50}/48 h LC_{50}

> 0.05). LOEC was not significant (p > 0.05) except at 24 h of the exposure where it was significant. The estimated safe levels of fenthion in *C. gariepinus* varied from 3.99 x 10^{-4} to 3.997 (Table 3).

Behavioural responses of *C. gariepinus* exposed to fenthion

The behavioural responses of the test fish were observed at 24 to 96 h of the exposure (Table 4). In the control group, normal behavioural responses such as non-hyperactivity, normal swimming patterns and fin movements were observed. Fish exposed to different concentrations of fenthion displayed behavioural abnormalities in response to the test chemical. At the initial exposure, fish were alert, stopped swimming and remained static in position in response to the sudden changes in the surrounding environment. After some few seconds, fish in the experimental group tried to avoid the test water by swimming rapidly and trying to jump out of the aquaria. Fish in aquaria that had higher concentrations of the pesticide showed abnormal behaviour and tried to avoid the test water by swimming very fast, jumping and displaying erratic with vigorous jerky movements, faster opercula movement, and gulping of air. Skin discoloration was mostly observed in fish that were exposed to the highest concentration of fenthion while it was least in the lowest concentration. After sometime, the fish lost their balance and became exhausted owing to respiratory difficulty. They settled down passively at the bottom of the aquaria and died.

Histopathological observations of gills in *Clarias gariepinus* exposed to fenthion

Histopathological changes in *C. gariepinus* were observed during the experiment (Figures 2 to 4). No alterations were observed in the gills of the control. The

Table 4. Effects of fenthion on the behavioural characteristics of *Clarias gariepinus* at different exposure durations.

Concentration (mg/L).	Equilibrium status	Fin movement	Hyperactivity	Jerky movement	Swimming rate
24 h					
Control	+++	+++	-	-	+++
12	+++	+++	-	-	+++
24	+++	+++	-	-	+++
36	++	++	+	++	++
48	++	++	+	+ ++	++
60	+	+	+	+++	+
72	+	+	+	+++	+
48 h					
Control	+++	+++	-	-	+++
12	+++	+++	-	-	+++
24	+++	+++	-	-	+++
36	++	++	+	+	++
48	+	++	+	++	++
60	+	+	++	+++	+
72	+	+	++	+++	+
72 h					
Control	+++	+++	-	-	+++
12	++	++	-	+	+++
24	+	+	+	++	++
36	+	+	+	++	+
48	+	+	++	++	+
60	-	-	+++	+++	-
72	-	-	+++	+++	-
96 h					
Control	+++	+++	-	-	+++
12	++	++	-	+	++
24	++	++	+	++	++
36	+	+	+	++	+
48	+	+	++	+++	+
60	-	-	+++	-	+
72	-	-	+++	-	-

-, None; +, mild; ++, moderate; +++, strong.

severity and frequency of gill lesions were found to be more pronounced in fish exposed to the highest concentration of fenthion. Histopathological alterations were concentration and duration dependent. The most common pathological changes in the gills of the exposed fish species were fusion of the tip of lamella, deformed lamella, congestion of blood spaces, epithelial lifting, intercellular space variation and hypertrophy of lamella (Table 5).

DISCUSSION

Acute and chronic toxicity tests are mostly used to assess the toxicity of chemicals on non-target organisms (Santos et al., 2010). The 96 h LC_{50} is one of the most important parameters for evaluating the toxic effects of pollutants (Nwani et al., 2015). In the present study, the 96 h LC_{50} values of fenthion for the African catfish, *C. gariepinus* was found to be 39.97 mg/L. This suggests that fenthion is toxic to fish. The observed mortality was dose and time dependent such that as the exposure time increased from 24 to 96 h, the median lethal concentration required to kill the fish was reduced. The 100% mortality observed in fish exposed to 72.0 mg/L of fenthion indicates that the insecticide was very toxic. The 96 h LC_{50} value obtained in the present investigation was higher than the 1.5 mg/L 96 h LC_{50} value of fenthion

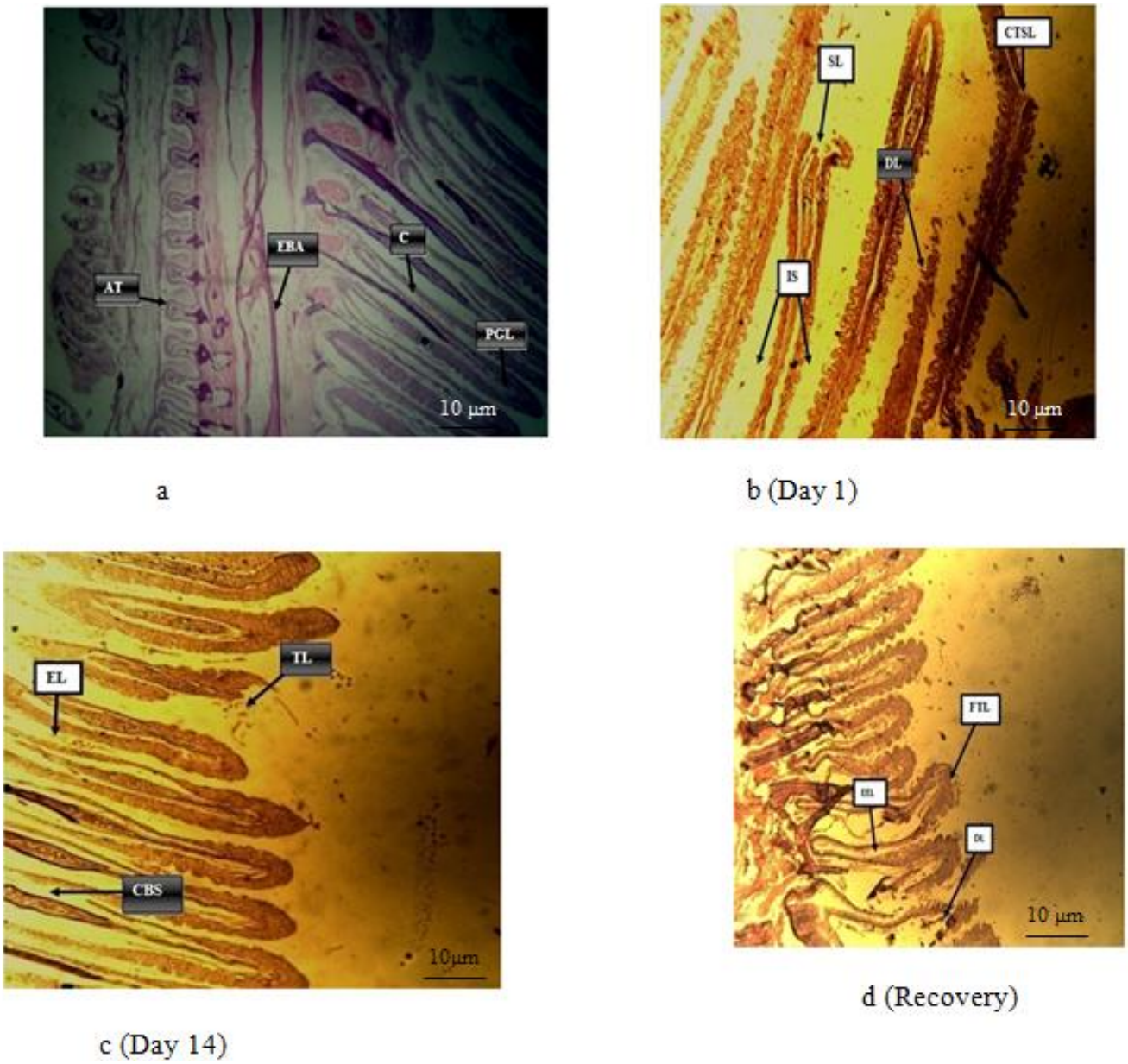

Figure 2. Photomicrograph of gill section of *Clarias gariepinus* of the control (A) showing normal adipose tissue (AT), efferent branchial artery (EBA), cartilage (C) and primary gill lamella (PGL) and fish exposed to 2.0 mg/L (b-d) fenthion showing intercellular spaces (IS), fusion of the tip of secondary lamella (SL), deformed lamella (DL), destruction of the curved tip of secondary lamella (CTSL), epithelial lifting (EL) and congestion of blood spaces (CBS).

previously reported in *C. carpio* (Muralidharan, 2012). The disparity in the toxic potential of the pesticide may be attributed to the difference in age and hardiness of the test species and water quality parameters. According to Johnson and Toledo (1993), the difference in the toxic potential of fenthion can be related to its accumulation, biotransformation and excretion. In general, toxicity of chemicals to aquatic organisms has been reported to be affected by temperature, pH, dissolved oxygen, size and age, strain of species, water quality, concentration and formulation of test chemicals (Nwani et al., 2010; Boran et al., 2012; Rauf and Arain, 2013). The magnitude of toxic effects of pesticides also depends on the length and weight, corporal surface/body weight ratio and breathing rate (Murty, 1986). Oh et al. (1991) also reported that varied inhibition of acetylcholinesterase, detoxification and absorption are factors causing the selective toxicity of pesticides for various species of fish. The safe levels obtained for fenthion in the present study varied from 3.99×10^{-4} to 3.99.

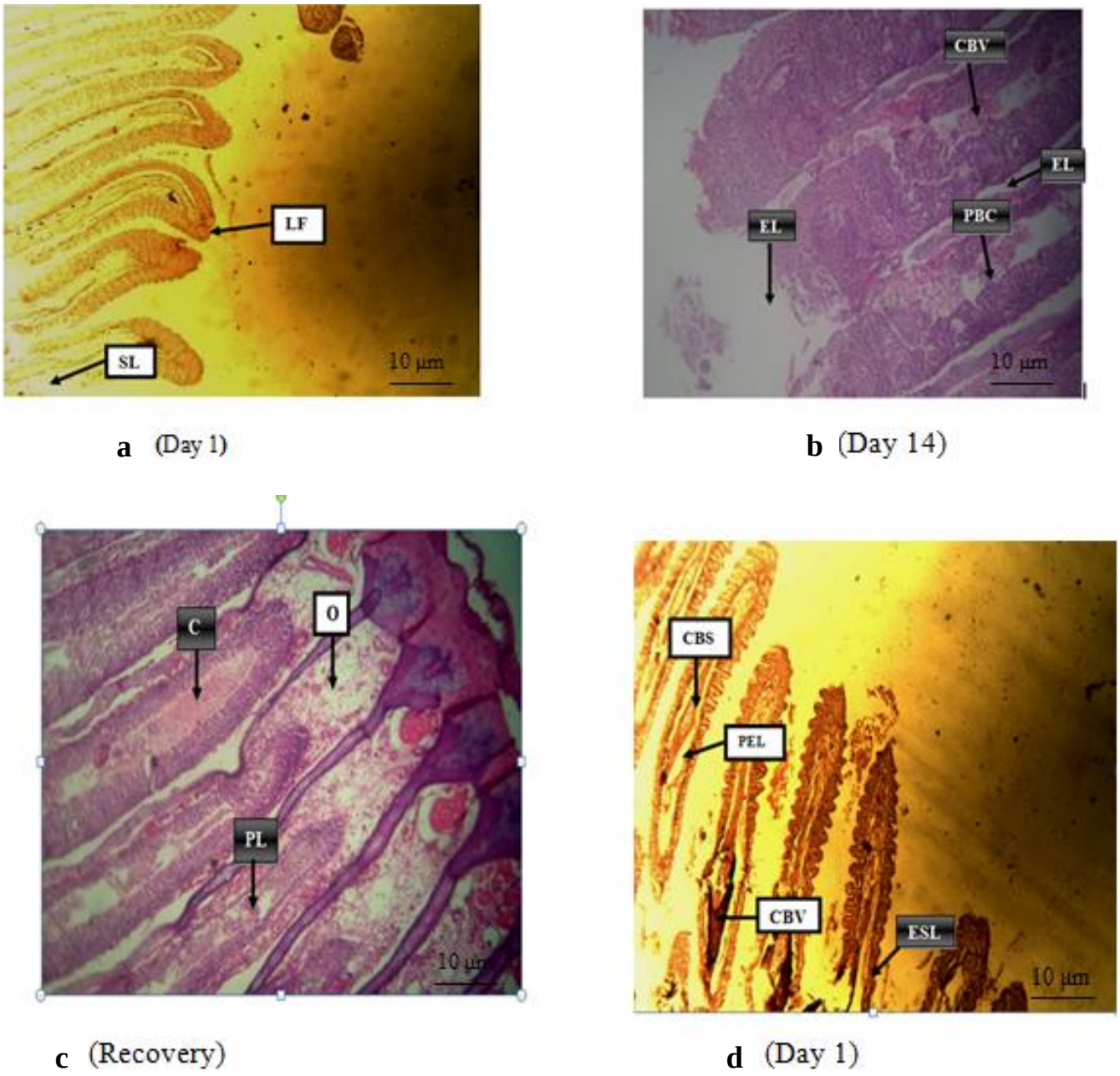

Figure 3. Photomicrographs of gill section of *Clarias gariepinus* in fish exposed to 4.0 mg/L (a-c) fenthion showing severe lamellafusion (LF), proliferation of blood cells (PBCs), epithelial lifting (EL), congestion (C), oedema (O), destruction of primary lamella (PL) and fish exposed to 8.0 mg/L, (d) fenthion congestion of blood spaces (CBS), partial epithelial lifting (PEL), clogged blood vessel and erosion of secondary lamella (ESL).

However, the large variation in safe levels determined by various methods has resulted in controversy over its acceptability as extrapolation of laboratory data to field data was not always meaningful hence, the difficulty in deciding the acceptable concentration that may be considered 'safe' based on laboratory experiments (Buikema et al., 1982; Pandey et al., 2005). Behavioural changes in fish are the most sensitive indicators of potential toxic effects of pesticide exposure (Banaee et al., 2011; Rauf and Arain, 2013). Clinical symptoms observed during acute exposure of *C. gariepinus* to fenthion in the present study are consistent with the findings of other authors and may indicate fenthion-induced suppressed activity of acetylcholinesterase. The abnormal behaviour exhibited by fish in the experimental groups such as abnormal swimming behaviour are due to inhibition of AChE activity leading to accumulation of acetylcholine in choligenic synapses thus causing hyper

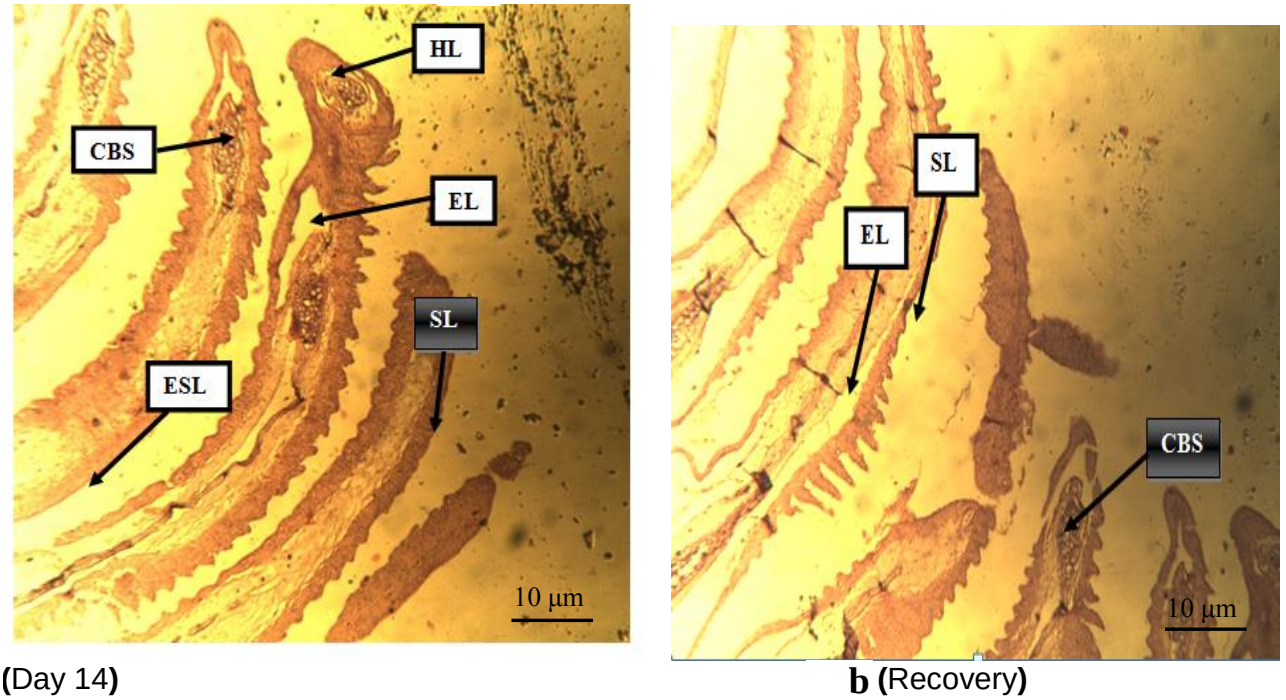

Figure 4. Photomicrograph of gill sections of *Clarias gariepinus* exposed to 8.0 mg/L (a-b) fenthion showing hypertrophy of the tip of lamella (HL), epithelial lifting (EL), congestion of blood spaces (CBS) and destroyed secondary lamella (SL).

Table 5. Summarized histopathological effects in the gill tissues of *Clarias gariepinus* exposed to fenthion and the control.

Conc. (mg/L)	Duration (days)	Congestion of blood spaces	Destruction primary of lamella	Destruction of secondary lamella	Epithelial lifting	hypertrophy	Lamella fusion	oedema
Control	1	0	0	0	0	0	0	0
2.0	1	0	0	0	0	0	0	0
4.0	1	0	0	0	1	0	1	0
8.0	1	3	0	0	2	0	0	0
Control	14	0	0	0	0	0	0	0
2.0	14	1	1	1	1	0	0	0
4.0	14	2	0	0	2	0	2	0
8.0	14	3	0	0	3	3	0	0
Control	Recovery	0	0	0	0	0	0	0
2.0	Recovery	0	1	1	0	0	0	0
4.0	Recovery	3	0	0	0	0	0	1
8.0	Recovery	3	0	0	3	0	0	0

Lesions were scored based on their severity (0: none, 1: mild, 2: moderate, 3: severe).

stimulation. Fenthion may inhibit AChE activity due to its accumulation in the brain tissues. This may lead to impairment of the normal functioning of nerve impulse causing physiological and behavioural modifications that can lead to reduction in survival ability (Muralidharan, 2014a). The behavioural symptoms observed during *C. gariepinus* exposure to fenthion in this study is similar to observations by other authors on the responses of different fishes exposed to organophosphate pesticides. It has been reported that under stress condition, fish become hyperactive, perhaps to get out of the stressful medium and would require an increased amount of oxygen

to meet their energy demand (Alkahem et al., 2011).

Muralidharan (2012) reported that hyperactivity of fish exposed to pollutants could be attributed to impaired gill function and the secretion of increased amount of mucus to coat the body and gills may be attempt to get relief from the irritating pollutants. The secretion of copious mucus by fish could also be a defence mechanism to neutralize the effect of pesticides which gradually cover the body, gills, and buccal cavity. Repeated opening and closing of the mouth and opercula covering accompanied by partially extended fin (coughing) as observed in the present study could be due to clearance of the accumulated mucus debris in the gill region for proper breathing. Similar behavioural changes in *C. gariepinus* exposed to atrazine and chlorpyrifos (Nwani et al., 2013a) and *C. mrigala* exposed to diazinon (Rauf and Arain, 2013) have been reported.

Exposure of organisms to sub-lethal concentrations of pesticides in the environment has been reported to result in biochemical, physiological and histopathological alterations in vital tissues (Anbu and Ramaswamy, 1999). The histological results observed in gill tissues of *C. gariepinus* in the present study indicate that sub-lethal concentrations of fenthion caused moderate to severe alteration in gill architecture which are important organs performing vital functions. Histopathological lesions observed in gill tissues of *C. gariepinus* exposed to fenthion in the present study are similar to reports in *C. carpio* exposed to (Muralidharan, 2014b). Similar pathological lesions in the gill architecture were observed in *Oreochromis niloticus* exposed to dimethoate (Elezaby et al., 2001), *Puntius gonionotus* exposed to paraquat (Cengiz and Unlu, 2006), *Oncorhynchus mykiss* exposed to the fungicide captan (Boran et al., 2012) and in *Gobiocypris rarus* (Yang et al., 2010), *Gnathonemus petersii* (Alazemi et al., 2012) and *C. carpio* (Blahova et al., 2014) exposed to atrazine. Epithelial hypertrophy could be as a result of epithelial detachment as stated by Machado and Fanta (2003). Epithelial lifting increases the distance through which the toxicant reach the blood stream thereby causing impaired oxygen uptake (Kumar et al., 2010) and could result in dysfunction or even non-functional gills and eventually suffocate the fish. Lamella fusion could be a protective mechanism as it reduces the amount of vulnerable gill surface area. According to Olurin et al. (2006) these pathological changes may be a reaction to toxicant intake or an adaptive response to prevent the entry of the pollutants through the gill surface and probably increase capillary permeability. The oedema observed in fish gills exposed to fenthion could be due to acute inflammatory nature of the lesion induced by fenthion. Oedema with epithelial separation was reported by Eller (1971) as additional change in fish gills exposed to toxicants.

Though histological changes observed in the present study and that of Muralidharan (2014b) revealed that the changes were concentration dependent, some authors reported that such structural changes are non-toxicant specific (Meyers and Hendricks, 1985), which could be mere stress response of fish to toxicant exposures (Gabriel et al., 2007). The structural changes observed in tissues throughout the duration of exposure were expected to normalize as reported by Myers et al. (1992) who stated that it is possible for the gill to regenerate and recover from the alterations but no such regeneration and recovery were observed at the post exposure test which shows that the changes observed were non reversible.

Conclusion

Fenthion is toxic to fish and possibly other aquatic organisms. It has the potential to damage the physiology of fish leading to observed changes in behavioural pattern with resultant dose dependent mortality. Histopathological results clearly indicate that sublethal concentrations of fenthion had destructive effects in the gill tissues of *C. gariepinus*. The study shows the significance of behavioural parameters in assessing the risk associated with pesticide exposure to fish. Environmental monitoring programs for the pesticide is necessary in view of the hazard and side effects that might be associated with excessive use on non-target organisms especially fish.

Conflict of interests

The author(s) did not declare any conflict of interest.

Abbreviations: OP, Organophosphate; **AChE,** acetylcholinesterase; **ACh**, acetylcholine; **EPA,** Environmental Protection Agency; **RUP,** restricted use pesticide; **MDA,** melondialdehyde; **$KMnO_4$,** potassium permanganate.

REFERENCES

Agency for Toxic Substances and Disease Registry (ATSDR) (2005). Toxicologic Information about Insecticides Used for Eradicating Mosquitoes (West Nile Virus Control). Department of Health and Human Services: ATSDR. *http://www.atsdr.cdc.gov/consultations/west_nile_virus/fenthion.html*. Accessed: June 25th, 2014.

Alazemi BM, Lewis JW, Andrews EB (2012). Gill damage in the freshwater fish *Gnathonemus petersii* (family: Mormyridae) exposed to selected pollutants: an ultrastructural study. Environ. Technol. 17(2): 225- 238.

Alkahem-Al-Balawi HF, Ahmad Z, Al-Akel AS, Al-Misned F, Suliman, EM, Al-Ghanim KA (2011). Toxicity bioassay of lead acetate and effects of sublethal exposure on growth, haematological parameters and reproduction in *Clarias gariepinus.* Afr. J. Biotechnol. 10: 11039-11047.

Altinok I, Capkin E (2007). Histopathology of rainbow trout exposed to sublethal concentrations of methiocarb, a carbamate pesticide, to rainbow trout. Toxicol. Pathol. 35: 405-410.

Anbu RB, Ramaswamy M (1999). Adaptive changes in respiratory movements of an air breathing fish, *Channa striatus* (Bleeker)

exposed to carbamate pesticide, sevin. J. Ecobiol. 3:11-16.
APHA, AWWA, WPCF (2005). Standard methods for the examination of water and waste water. 21st edition. New York: American Public Health Association.
Auta J (2001). Toxicity of Dimethoate to Juveniles of *Oreochromis nilotius* (Trewavas) and *Clarias gariepinus* (Tengels). Ph.D. Thesis. Ahmadu Bello University, Zaria, Nigeria.
Banaee M, Sureda A, Mirvaghefi AR, Ahmadi K (2011). Effects of diazinon on biochemical parameters of blood in rainbow trout (*Oncorhynchus mykiss*). Pestic. Biochem. Physiol. 99:1-6.
Blahova J, Modra H, Sevcikova M, Marsalek P, Zelnickova L, Skoric M, Svobodova Z (2014). Evaluation of biochemical, haematological and histopathological responses and recovery ability of common carp (*Cyprinus carpio*) after acute exposure to atrazine herbicide. ID 980948. http://dx.doi.org/10.1155/2014/980948.
Boran H, Capkin E, Altinok I, Terzi E (2012). Assessment of acute toxicity and histopathology of the fungicide captan in rainbow trout. Exp. Toxicol. Pathol. 64:175-179.
Buikema JR, Naider-Lehner AL, Cairns JR (1982). Biological monitoring: part IV. Toxicology testing. Environ. Mol. Mutagen 33: 239-262.
Canadian council of Resources and Environmental Ministry (CCREM) (1991). Canadian water quality guidelines, Inland Waters Directorate. Environment Canada: Ottawa, UN, Canada.
Capkin E, Altinok I (2013). Effect of chronic carbosulfan exposure on liver antioxidant enzyme activities in rainbow trout. Environ. Toxicol. Pharmacol. 36:80-87.
Capkin E, Birincioglu S, Altinok I (2009). Histopathological changes in rainbow trout (*Oncorhnchus mykiss*) after exposure to sublethal composite nitrogen fertilizers. Ecotoxicol. Environ. Saf. 72: 1999-2004.
Cengiz EI, Unlu E (2006). Sublethal effects of commercial deltamethrin on the structure of the gills, liver and gut tissues of mosquito fish, *Gambusia affinis*: a microscopic study. Environ. Toxicol. Pharmacol. 21:246-253.
Committee on Water Quality Criteria (CWQC) (1972). A Report of the Committee on Water Quality Research Series. EPA-R3-73-003, US Environmental Protection Agency Report; CWQC: Cincinnati, OH, USA.
Cong NV, Phuong NT, Bayley M (2009). Effect of exposure of diazinon on cholinesterase activity and growth in *Channa striatus*. Ecotoxicol. Environ. Saf. 72: 699-703.
Elezaby MM, El-Serafy S, Heckmann RK, Sharf E, Seddek MM (2001). Effects of some toxicants on the freshwater fish, *Oreochromis niloticus*. J. Egypt. German Soc. Zool. 36:407-434.
Eller LL (1971). Histopathologic lesions in culthroat trout *(Salmo clark)* exposed chronically to the insecticide endrin. Am. J. Pathol. 64 - 321.
Eto M (1979). Organophosphorus Pesticides: Organic and Biological Chemistry. Boca Raton (FL): CRC Press.
Fanta E, Rios FS, Romao S, Vianna ACC, Freiberger S (2003). Histopathology of the fish *Corydoras paleatus* contaminated with sublethal levels of organophosphorus in water and food. Ecotoxicol. Environ. Saf. 54(2):119-130.
Finney YT (1971). Probit Analysis. Cambridge University Press: Cambridge.
Gabriel UU, Amakiri EU, Ezeri GN. (2007).Haematology and gill pathology of *Clarias gariepinus* exposed to refined petroleum oil, kerosene under laboratory conditions. J. Anim. Vet. Adv. 6(3):461-465.
Gernhofer M, Pawet M, Schramm M, Muller E, Triebskorn R (2001). Ultra structural biomarkers as tools to characterize the health status of fish in contaminated streams. J. Aquat. Ecosyst. Stress Recover. 8:241-260.
Hart WB, Weston RF, Dermann JG (1948). An apparatus for oxygenating test solution which fish are used as test animals for evaluating toxicity. Trans. Am. Fish. Soc. 75:288.
International Joint Commission (IJC) (1977). New and Revised Great
Lakes Water Quality Objectives. Great Lake Basin, Windsor; IJC: Ottawa, ON, Canada.
Israel SS, Sam MS (2012). Biochemical changes in certain tissues of *Cirrhinus mrigala* (Hamilton) (Cyprinidae: cypriformes) exposed to fenthion. Int. J. Environ. Sci. 2:1268-1277.
Johnson CM, Toledo MCF (1993). Acute toxicity of endosulfan to the fish *Hyphessobrycon bifasciatus* and *Brachydanio rerio*. Arch. Environ. Contam. Toxicol. 24:151-155.
Jungera P (2014). Fenthion safety summary for veterinary use on dogs, cats, cattle, sheep, goats and swine. Toxicity, poisoning, intoxication, overdose, antidote. Parasitipedia.net. Accessed 18th February 2015.
Kanter A, Celik I (2012). Acute effects of fenthion on certain oxidative stress biomarkers in various tissues of frogs (*Raga ridibunda*). Toxicol. Ind. Health 28(4):369-376.
Kumar M, Prasad MR, Srivastva K, Tripathi S, Srivastva AK (2010). Branchial histopathological study of Catfish *Heteropneustes fossilis* following exposure to purified neem extract, Azadirachtin. World J. Zool. 5(4):239-243.
Machado M, Fanta E (2003). Effects of the organophorous methyl parathion on the branchial epithelium of a freshwater fish *Metynnis eoosevelti*. Braz. Arch. Biol. Technol. 46(3):361-372.
Maduenho LP, Martinez CBR (2008). Acute effects of diflubenzuron on the freshwater fish *Prochilodus lineatus*. Comp. Biochem. Physiol. 148:265-272.
Meyers TR, Hendricks JD (1985). Histopathology. In: Rand, G. M., Petrocellis, R. (Editors). Fundamentals of Aquatic toxicology: Methods and Application. Hemisphere Publishing Corp, Washington, DC.
Muralidharan L (2012). Haemato-Biochemical alterations induced by chronic exposure to fenthion in *Cyprinus carpio*. Trends Fish. Res. 1(3):19-25.
Muralidharan L (2014a). Chronic toxic impacts of fenthion on the profiles of enzymes in the freshwater fish *Cyprinus carpio* (Linn). Int. J. Fish. Aquat. Stud. 1(4):51-56.
Muralidharan L, Pillai S (2012). Chronic effects of organophophorus insecticides. 'fenthion' in melanophore pigments of *Cyprinus carpio* (Linn). ARPN J. Sci. Technol. 2:296-300.
Muralidharan L (2014b). Histopathological studies on carp (*Cyprinus carpio*) exposed to fenthion. Int. J. Adv. Res. 2: 11-26.
Murty AS (1986). Toxicity of Pesticides to Fish. CRC Press Inc Boca Raton.
Myers MS, Olson OP, Johnson LL, Stehr CS, Homt R, Varansi U (1992). Hepatic neoplasm and related lesions and exposure to toxic chemicals in marine fish from U.S West. Environ. Health Perspect. 90:7-15.
NAS/NAE (1973). Water Quality Criteria, EPA-R3–033; US Government Printing Office: Washington, DC, USA.
Nwani CD, Ama UI, Okoh F, Oji UO, Ogbonyealu RC, Agha A (2013a). Acute toxicity of the chloroacetanilide herbicide butachlor and its effects on behaviour of the freshwater fish *Tilapia zilli*. Afr. J. Biotechnol. 12:499-503.
Nwani CD, Ekwueme HI, Ejere VC, Onyeke CC, Chukwuka CO, Onas PS, Nwadinigwe AO (2015). Physiological effects of paraquat in juvenile African catfish *Clarias gariepinus* (Burchel 1822). J. Coast. Life Med. 3(1):35-43.
Nwani CD, Ivoke N, Ugwu DO, Atama C, Onyishi GC, Echi PC, Ogbonna SA (2013b). Investigation on acute toxicity and behavioural changes in a freshwater catfish, *Clarias gariepinus* (Burchell, 1822), exposed to organophosphorous pesticide, Termifos. Pak. J. Zool. 45(4):959-965.
Nwani CD, Lakra WS, Nagpure NS, Kumar R, Kushwaha B, Srivastava SK (2010). Mutagenic and genotoxic effects of carbosulphan in freshwater fish *Channa Punctatus* (Bloch) using micronucleus assay and alkaline single-cell gel electrophoresis. Food Chem. Toxicol. 48: 202-208.
Oh HS, Lee SK, Kim YH, Roh JK (1991). Mechanism of selective toxicity of diazinon to killifish (*Oryzias latipes*) and loach (*Misgurnus anguillicaudatus*). Aquat. Toxicol. Risk Assess. 14: 343-353.
Olurin K, Olojo E, Mbaka G, Akindele A (2006). Histopathological responses of the gill and liver tissues of *Clarias gariepinus* fingerlings to the herbicide, glyphosate. Afr. J. Biotechnol. 5:2480-2487.
Pandey S, Kumar R, Sharma S, Napure NS, Srivastava SK, Verma MS
(2005). Acute toxicity bioassays of mercuric chloride and malathion on air-breathing fish *Channa punctatus* (Bloch). Ecotoxicol. Environ. Saf. 61:114-120.
Peter JV, Cherian AM (2000). Organic insecticides. Anaesth. Intensive Care 28:11-21.

Rauf A, Arain N (2013). Acute toxicity of diazinon and its effects on haematological parameters in the Indian carp, *Cirrhinus mrigala* (Hamilton). Turk. J. Vet. Anim. Sci. 37:535-540.

Roberts RJ (2001). Fish Pathology. Third Edition. Bailliere Tindall, London.

Santos LH, Araujo AN, Fachini A, Pena A, Deleure-Matos C, Montenegro MCB (2010). Ecotoxicological aspects related to the presence of pharmaceuticals in the aquatic environment. J. Hazard Mat. 175:45-95.

Sevgiler Y, Uner N (2010). Tissue-Specific effects of fenthion on glutathione metabolism modulated by NAC and BSO in *Oreochromis niloticus.* Drug Chem. Toxicol. 33(4):348-356.

Sprague JB (1971). Measurement of pollutant toxicity to fish. I. Bioassay methods for acute toxicity. Water Res. 3:793-821.

Wu JC, Hseu YC, Tsai JS, Chen LC, Chye SM, Chingchen S (2011). Fenthion and terbufos induce DNA damage, the expression of tumor-related genes and apoptosis in HEPG2 cells. Environ. Mol. Mutag. 52(7):529-537.

Yang I, Zha J, Li W, Li Z, Wang Z (2010). Atrazine affects kidney and adrenal hormones (AHs) related genes expressions of rare minnow (Gobiocypris rarus). Aquat. Toxicol. 97:204-211.

academicJournals

Vol. 14(25), pp. 2114-2123, 23 June, 2015
DOI: 10.5897/AJB2014.14265
Article Number: F45CA4053886
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Cellular biomarker responses of bagrid catfish, *Chrysichthys nigrodigitatus* in a contaminated coastal ecosystem

Ejimadu C. Uzoma[1]*, Chukwu L. Obinna[1] and Amaeze H. Nnamdi[2]

[1]Aquatic Toxicology and Ecophysiology Laboratory, Department of Marine Sciences, University of Lagos, Akoka-Yaba, Lagos, Lagos State, Nigeria.
[2]Ecotoxicology Laboratory, Zoology Department, University of Lagos, Akoka-Yaba, Lagos, Lagos State, Nigeria.

Received 22 October, 2014; Accepted 26 January, 2015

An assessment of the pollution status of Agboyi creek, a water body associated with various anthropogenic activities was carried out in order to determine responses induced in Catfishes, *Chrysichthys nigrodigitatus* inhabiting it. Cellular biomarkers of stress including the antioxidative stress enzyme, catalase (CAT), lipid peroxidation measured as thiobarbituric acid reactive substance (TBARS), as well as serum enzymes alanine aminotransferase (ALT) and Aspartate aminotransferase (AST) were evaluated in *C. nigrodigitatus* from Agboyi creek and compared to a control (non-polluted) water body, Epe Lagoon. The results of physicochemical assessments of both water bodies showed significant differences (P<0.05) with Agboyi creek having high levels of total suspended solids (TSS), phosphate as well as total dissolved solids (TDS) and heavy metal concentrations which exceeded National Environmental Regulations (NESREA) safe limits. The mean concentration (Mean ± SD) of TBARS in the liver of *C. nigrodigitatus* at Agboyi Creek (2455.43 ± 1440.0 nmol/mg protein) was about twice the levels at Epe Lagoon (1398.31 ± 1446.50 nmol/mg protein). Inhibition of CAT activity was observed in liver samples of catfishes at Agboyi Creek (48.17 ± 60.23 µmol/min/mg protein) compared to Epe Lagoon (87.97 ± 13.00 µmol/min/mg protein). The mean activities of ALT and AST were significantly higher (P<0.05) at Agboyi Creek (12.67 ± 15.04 and 151.67 ± 76.76 U/L) compared to Epe Lagoon (2.33 ± 0.52 and 6.00 ± 3.46 U/L). The present study shows altered biochemical conditions in fishes sampled in the water body impacted by anthropogenic contaminants and suggest that those parameters could be used as reliable biomarkers of contaminant exposure to fish.

Key words: Biomonitoring, water pollution, oxidative stress, fish health.

INTRODUCTION

The increase of population in coastal areas and the rise of our standard of living is driving the efforts to produce more food, thereby increasing domestic, agricultural and industrial activities. These activities generate solid wastes,

*Corresponding author. E-mail: uzo.chris.ejimadu@gmail.com.

sewage and energy which are released into the coastal ecosystem resulting in the disturbance of their delicate balance, threatening the health and existence of living organisms including humans (Chukwu and Ogunmodede, 2005). Despite the fact that the sea has an enormous capacity to buffer the various impacts from human activities degrading it, streams, creeks, rivers, estuaries and swamps have only a little capacity to do so due to their relative small sizes (Odiete, 1999). The aquatic environment provides a sink for many environmental contaminants some of which have the potential to cause oxidative stress in aquatic organisms (Amaeze, 2014). In Nigeria, the lagoons and estuaries have continued to be under intensifying pressure from pollution (Ajao, 1996; Oyewo, 1998; Otitoloju, 2000). Primary concerns are the effects of domestic and industrial effluents on the general health of aquatic life, the maintenance of hitherto viable artisanal commercial fisheries and the safety of humans occupationally exposed to the pollution (Ajao and Fagade, 1990). Apart from mortality, some of the more common and equally serious effects of environmental stressors on aquatic organisms are changes in behavior, growth, and reproduction (Walker et al., 2001).

Fish are considered to be the bioindicators of freshwater/marine pollution because of their ability to respond to pollutants (Adams et al., 1989). They are obligate aquatic animals and require movement of water across their gills and mouth for respiration and feeding, respectively. Their physiological responses to their environment have been employed by investigators to monitor aquatic pollution (Odiete, 1998). Such specific responses described as biomarkers are employed in monitoring changes in status resulting from exposure of living organisms to contamination (Depledge and Hopkin, 1995). Biomarkers effect consist of biochemical and/or physiological changes in organisms exposed to toxicants or varying abiotic conditions and thus represent initial responses to environmental perturbations and contamination (Bengtson and Henshel, 1996; Roy et al., 1996). They are generally regarded as more sensitive than bioindicators, which are employed at higher levels of the biological hierarchy (Stegeman et al., 1992). This is because subtle changes at the individual level is more rapid than at population or community levels and presents an early warning signal for monitoring changes in status of the environment (Doherty, 2014).

The use of biochemical biomarkers in monitoring organism health status is now a regular procedure given their ease of measurement and relationship with concentrations of pollutants to which organisms are exposed to (Otitoloju and Olagoke, 2011). They are often used in monitoring oxidative stress impacts from pollutants by comparing relative cellular enzyme activity with exposure levels. Typically, the activities of antioxidant stress enzymes are increasingly gaining relevance in aquatic ecosystem monitoring. Oxidative stress may result from an increase in reactive oxygen species (ROS), an impairment of antioxidant defense systems or an insufficient capacity to repair oxidative damage. It may ensue when the ability to buffer against ROS is exceeded either by excessive production of ROS or by depletion of antioxidants. This can alter cellular redox-potential and initiate a variety of responses via intracellular pathways (Timbrell, 2000). ROS are produced by the cellular metabolism of aerobic organisms either enzymatic or non-enzymatic reaction which produces oxyradicals. The ROS are formed due to incomplete reduction of oxygen, which may generate the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) or the hydroxyradical (OH^-) as well as other partially reduced molecules (Valavanidis et al., 2006).

The measurement of levels of lipid peroxidation and the activities of antioxidant stress enzymes are often used as complementary procedure in monitoring the extent of oxidative stress in cells. Lipid peroxidation results from the damage of the phospholipid bi-layer of cell membranes and is considered a biomarker of damage (Geffard, 2001). This damage produces a number of by-products, one of which is the aldehyde, malondialdehyde (MDA). MDA is formed from the breakdown of polyunsaturated fatty acids (PUFA) and it serves as a convenient index for determining the extent of lipid peroxidation (Jamil, 2002). Together with levels of MDA, activities of antioxidant stress enzymes such as catalase (CAT) and superoxide dismutase (SOD) are often monitored in aquatic and terrestrial animals in other to determine the extent of stress (Otitolohu and Olagoke, 2011; Esiegbe et al., 2013). These enzymes are produced naturally in the body to counter the onslaught of oxidative stress precursors such as free radicals, singlet oxygen, superoxides, peroxides and other breakdown and reactive products of toxicants (Azqueta et al., 2009). SOD breaks down ROS into peroxides which are further detoxified by CAT into water and oxygen. Inhibition of SOD therefore is linked with CAT because it leads to reduced hydrogen peroxides, the substrate on which the CAT acts (Amaeze et al., 2014). CAT works by breaking down superoxides, helping the body to convert hydrogen peroxide into water and oxygen, thus preventing the formation of carbon dioxide bubbles in the blood (Livingstone et al., 1990). Catalase also uses hydrogen peroxide to break down potentially harmful toxins in the body, including alcohol, phenol, and formaldehyde (Papagiannis et al., 2004).

Serum enzymes such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST), which primarily measures of liver damage, have also been employed to measure damage of organs like kidney and gills (Bernet et al., 2001; Yang and Chen, 2003). ALT is found mainly in the liver, but also in smaller amounts in the kidneys, heart, muscles, and pancreas, with low levels normally found in the blood (Reev et al., 1980).

Most increases in ALT levels however are caused by liver damage (Reev et al., 1980). When the liver is damaged or diseased, it releases ALT and AST into the bloodstream, which increases their peripheral levels. Thus, these proteins are also employed together with other battery of assays as biomarkers in measuring health status of organisms. Fish under stress mobilizes triglycerides and protein to meet an increased demand for energy resulting from increased physical activity, bio-transformation and excretion of xenobiotics (Alkahem et al., 1998). Both the aminotransferases (alanine and aspartate) function as a link between carbohydrate and protein metabolism by the interconversion of strategic compounds like α-ketoglutarate and alanine to pyruvic acid and glutamic acid, a process known as transamination (Knox and Greengard, 1965). Aminotransferases respond to any stress or altered physiological condition (Knox and Greengard, 1965).

The use of a biochemical approaches has been advocated to provide an early warning of potentially damaging changes in stressed fish. Changes in the concentrations and activities of enzyme often directly reflect cell damage in specific organs (Casillas et al., 1983). The measurement of biomarker responses offer to demonstrate that toxicants have entered an organism, are being distributed within the tissue and are eliciting a toxicological effect on biological structures and functions (McCarthy and Shugart, 1990). Organisms' responses are measurements of cellular and physiological processes that are normal components of an organism's attempt to deal with metabolic processes and to maintain a constant internal balance. These processes fluctuate within some normal (homeostatic) range for an organism. If an organism is exposed to a pollutant, it may respond to the exposure by compensatory increases or decreases in one or several of the organism's cellular or physiological processes. Under stress conditions the body mechanisms are altered to combat the effect of the pollutants/ stressors in order to maintain equilibrium in the organism (Siva, 1980). Monitoring of the biomarkers in living organisms including fish is a validated approach and serves as early warning of adverse changes and damage resulting from chemical exposure (van der Oost et al., 1996).

The bagrid catfish (*Chrysichthys nigrodigitatus*) was chosen for the study because of its potential as a bioindicator species being common and often abundant, having a wide distribution in Nigerian coastal ecosystem (Ikusemiju, 1975; Fagade, 1980). This study was conducted to assess the levels of lipid peroxidation and biochemical changes that occur in the liver tissue and serum (enzyme levels) by measuring the levels of TBARS (commonly measured as MDA) and activities of CAT, ALT and AST as biomarkers, in *C. nigrodigitatus* living in the polluted Agboyi Creek. The levels in the catfishes at Agboyi creek when compared with those from non-polluted waters like Epe lagoon could validate their use for water quality assessments.

MATERIALS AND METHODS

Description of study area

Agboyi Creek is located at the western side of Lagos and northern section of Lagos lagoon, (Longitude 6° 35'N; latitude 3° 25'E) (Figure 1). The creek derives its source from Ogun River and receives input from Ogudu Creek towards the middle of its course before it empties into the Lagos lagoon at Oworonshoki. It serves as a major drainage channel for the area, receiving domestic wastes and industrial effluents which are discharged from nearby industries at Maryland, Isheri, Magodo and Ogudu areas of Lagos State (Onyema and Ojo, 2008). It has a length of 7.63 km. It is a major drainage channel for industries located in Ikeja, Agege, Ogudu areas and the municipal water plant at Iju. The sampling station is at the southern end of the creek between the connection to Ogudu Creek and the opening of the Agboyi creek into Lagos Lagoon. Epe Lagoon (Longitude 6° 35'N; latitude 3° 25'E) was selected as a control station given the similarity of both water bodies and does not receive effluents from major industries.

Field sample collection

Six live specimens of adult *C. nigrodigitatus* of an average body weight and length of 148.4 g and 22.9 cm, respectively, were caught from Agboyi Creek (Latitude 06° 34.183'N; Longitude 003° 24.621'E), representing polluted site, and Epe Lagoon (Latitude 06° 34.649'N; Longitude 003° 56.921'E), representing control or unpolluted site, with hook and line. The fishes were placed in 10 L plastic buckets and water from their natural habitat was added. All buckets were kept cool and aerated during return trip back to the laboratory. Surface water samples from both sites were collected using 2 L plastic kegs and transported cool in icepacks to the laboratory for analysis as well. Some physicochemical parameters were determined *in situ* using Horiba U50G Multi water sampler.

Analytical procedures

Surface water physicochemical characteristics

Water temperature, pH, electrical conductivity, dissolve oxygen (DO), total suspended solids (TSS), total dissolved solids (TDS and salinity were measured *in situ*. The chloride ion levels, sulphate, total nitrate, ammonia and phosphate concentrations in the water samples were determined using standard volumetric analytical procedures (APHA, 2005) at the Central Research Laboratory, Chemistry Department, University of Lagos. Water samples measuring up to 500 ml from each site were filtered and digested using concentrated nitric acid before measuring their absorbance relative to respective metallic standards using Perkin Elmer atomic absorption spectrophotometer as reported by (Don-Pedro et al., 2004).

Fish samples preparation and analysis

Blood samples were collected using sterile syringe (5 ml) followed by dissection to remove liver samples. The liver samples were dissected out of the fish through the ventral side. The liver samples

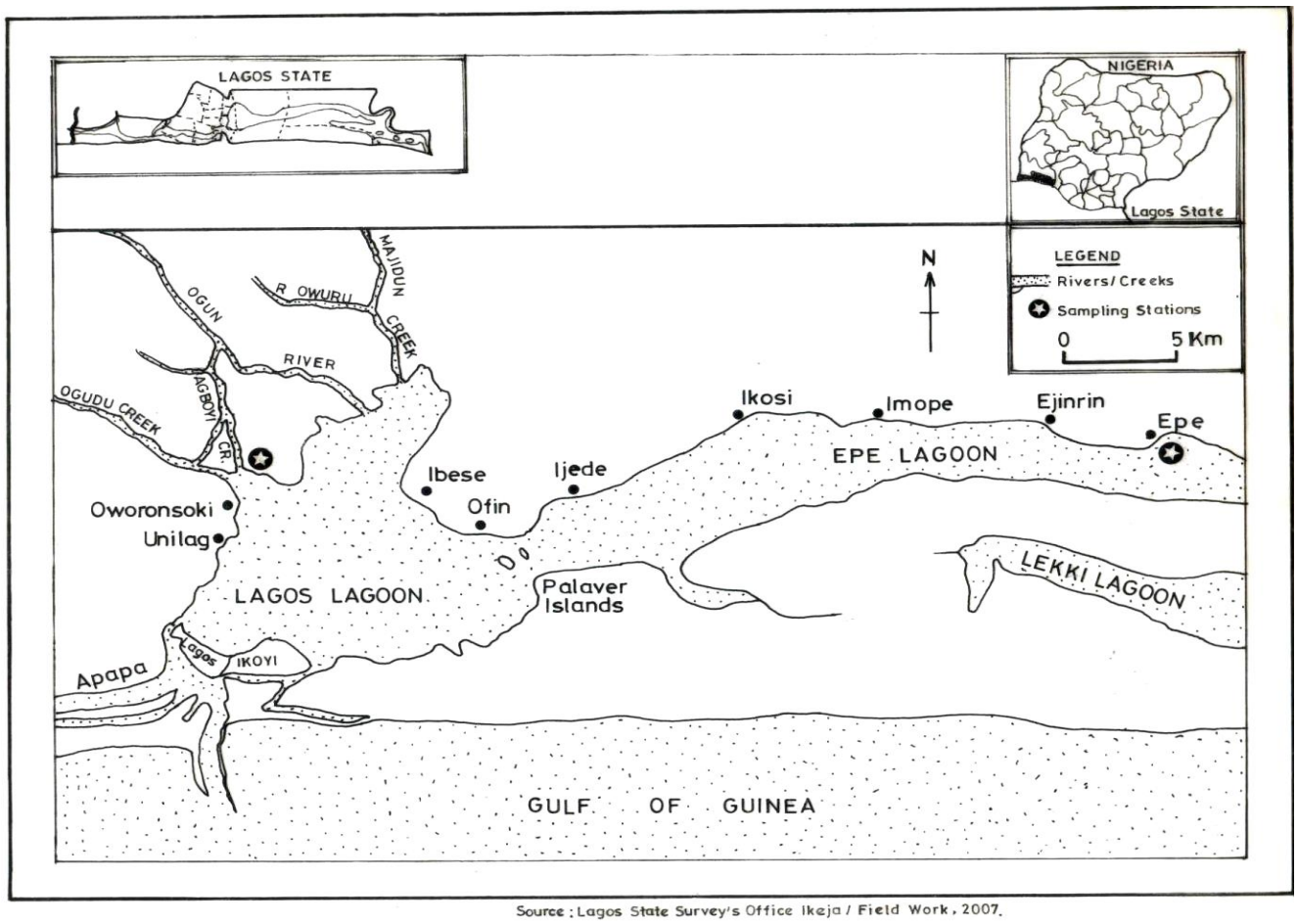

Figure 1. The sampling stations at Agboyi Creek and Epe Lagoon.

were washed with normal saline solution and placed in a small plastic container with tight cover and preserved in a (-20°C) until analysis. The collection, handling and dissection of the fishes were in line with the ethical standard approved by the University of Lagos. Each liver sample was retrieved from the freezer, 0.5 g weighed out and homogenized when cold and dissolved inphosphate buffer saline (1 to 10 w/v, that is, to 0.5 g of liver sample, 5.0 ml of phosphate buffer was added). The homogenate were then centrifuged at 10,000 rpms for 10 min. The supernatant were collected and stored in the fridge for estimation of Catalase (CAT), thiobarbituric acid reactive substances (TBARS) and total protein.

Protein determination

The protein levels were also determined in the supernatant, with bovine serum albumin (BSA) standard (Bradford, 1976). The results were expressed in mg of protein/ml.

Catalase assay

Catalase activity in supernatant was determined according to the method of Aebi (1974) by monitoring the initial rate of disappearance of hydrogen peroxide (initial concentration 10 mmol) at 240 nm (ε = 40 /M/cm) in an OPTIMA SP-3000 PLUS spectrophotometer. The reaction volume containing 50 mM/0.1 ml phosphate buffer at 7 pH, 0.2 ml of supernatant and 1.8 ml of 30 mM H_20_2 or 0.9 ml of 30 Mm H_20_2. Blank were run without supernatant or containing phosphate buffer. The activity of the enzyme was expressed as µmol H_2O_2/min/mg protein.

Malonaldehyde/ TBARS assay

The level of tissue lipid peroxidation was determined by assessment of MDA as total thiobarbituric acid (TBARS)-reactive products (Buege and Aust, 1978). The absorbance was read at 535 nm against blank containing water using OPTIMA SP – 3000 PLUS spectrophotomer. The concentration of the malonaldehyde produced was determined based on the molar extinction coefficient for MDA-TBA of 1.56×10^5 /M/cm. 1 Mm EDTA was added to a 0.5 ml of the supernatant and was mixed with 1.0 M cold 10% (W/V) trichloroacetic acid (TCA) to precipitate protein. The solution was mixed and centrifuged for 10 min at 5,000 g. The supernatants from the TCA extract were combined with the same volume of TBA and heated in boiling water for 15 min. Control sample contained water instead of supernatant. The TBARS values were expressed as nmol/mg protein. Each sample was run twice to enhance accuracy

Table 1. Physicochemical properties of surface water samples from both stations.

S/N	Parameters	Agboyi Creek	Epe Lagoon	(NESREA 2011) Limit
1	Water Temperature (^{o}C)	31.5	29.7	<40
2	pH	7.6	7.4	6.5-8.5
3	Electrical Conductivity (μScm^{-1})	8.500	1.675	-
4	Dissolved Oxygen (ppm)	3.40	4.4	4.0
5	Total Suspended Solids (ppm)	4.170	39	-
6	Total Dissolved Solids (ppm)	4.150.0	1.080	2.000
7	Salinity (‰)	5.8	1.45	-
8	Chloride (ppm)	3.300.0	500.0	
9	Sulphate (ppm)	72.8	62.10	500
10	Nitrate	18.62	3.40	20
11	Ammonia (ppm)	0.10	ND	-
12	Phosphate (ppm)	4.08	0.34	3.5
13	Iron (ppm)	15.72	0.24	0.5
14	Copper (ppm)	4.65	0.001	0.01
15	Manganese (ppm)	2.16	2.4	-
16	Zinc ppm)	65.6	0.001	0.2
17	Lead (ppm)	0.24	ND	0.1
18	Mercury (ppm)	ND	ND	0.01
19	Cadmium (ppm)	ND	ND	0.01

of readings, implying two replicate per fish samples.

Amino transaminase (AST) and alanine amino transaminase (ALT) assay

Blood samples were extracted from six fish samples each from both stations into heparin's sample bottle. The samples were allowed to coagulate at room temperature for 2 h. The serum were obtained by centrifugation of an amount of blood at 3000 rpm for 10 min and were prepared for the analysis of the activities of AST and ALT using Reitman and Frankel (1957) method, with reagents supplied in RANDOX assay kit.

Statistical analysis

Statistical differences between physicochemical properties of water samples from both water bodies and biochemical parameters in the sampled fishes were analyzed by two-way analysis of variance (ANOVA) and significant differences were examined by post hoc analysis using SSPS statistical software version 16.0. Data are presented as mean ± standard deviation (SD).

RESULTS

Physicochemical characteristics of the sampling sites

The physicochemical parameters measured in the surface water of Agboyi creek and Epe lagoon is shown in Table 1. The two way analysis of variance indicated that the differences in the overall physicochemical characteristics for both water bodies did not differ significantly (P= 0.057). The pH values observed at both water bodies were slightly alkaline but were within permissible NESREA limit of 6.5 to 8.5. The surface water conductivity was 5 times higher in Agboyi creek, compared with the value at Epe Lagoon. Similarly, values of salinity, total dissolved solids and total suspended solids at Agboyi creek were higher compared with the same parameters at Epe Lagoon. The total dissolved solids (TDS) value at Agboyi was more than 2 times the NESREA limit for water quality. Agboyi creek also recorded high chloride levels which were more than 6 times the value at Epe lagoon. The nutrient concentrations (that is, sulphate, nitrate and phosphate) at Agboyi creek were also higher when compared with those at Epe Lagoon. The phosphate level at Agboyi was higher than the NESREA limit of 3.5 ppm while the nitrate levels were close to the limit. Some heavy metals measured were detected in both water bodies (Fe, Cu, Mn, Zn and Pb) but were significantly higher at Agboyi creek (F=1.792 Fcrit =5.987, df=1, p=0.23).

Catalase activities in the catfishes

The mean catalase activity in the tissue of *C. nigrodigitatus* in both water bodies were not significantly different (P>0.05) (Figure 2).

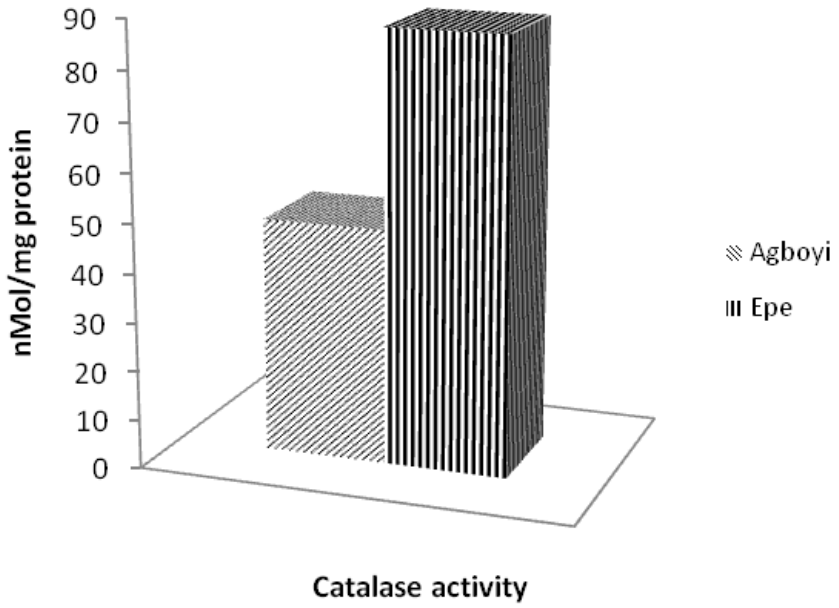

Figure 2. Inhibition of Catalase (CAT) activity in the liver samples of catfishes in Agboyi creek relative to Epe lagoon (Control site). The values are significantly different (F=0.409, Fcrit = 6.608, df =1 p= 0.55).

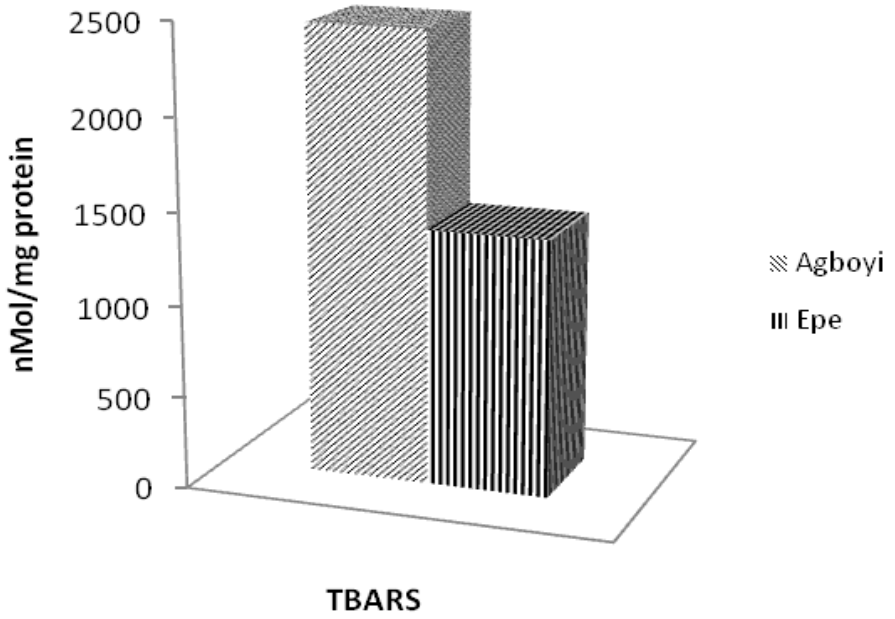

Figure 3. Lipid peroxidation (TBARS) concentration in the liver samples of Catfishes in Agboyi creek and Epe lagoon (Control site). The values are significantly different (F=1.739, Fcrit = 6.608, df =1 p= 0.24).

Lipid peroxidation levels in the catfishes

The mean hepatic content of TBARS as measured with the level of MDA in catfishes at Agboyi creek was 2,455.43± 1,577.42 nmol/mg protein, while level at Epe lagoon samples was 1,398.31± 1,584.64 nmol/mg protein (Figure 3). The t test indicated that the values of MDA in both water bodies were not significantly different

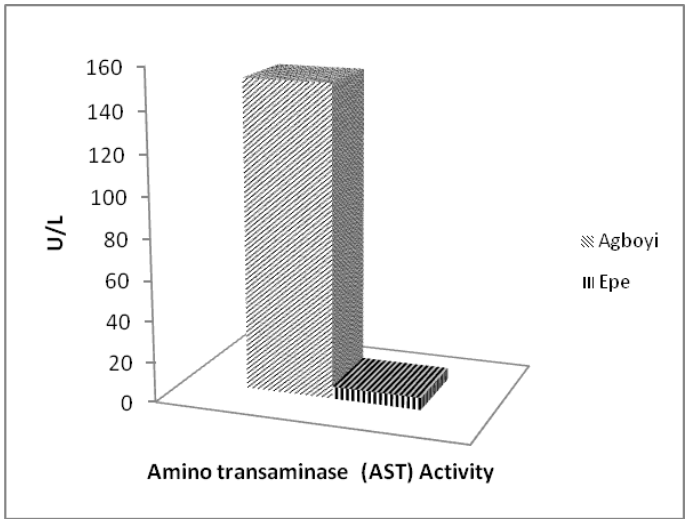

Figure 4. AST activities in the serum of catfishes in Agboyi creek and Epe lagoon (Control site). The values are significantly different (F=20.179, Fcrit = 6.608, df =1 p= 0.01)

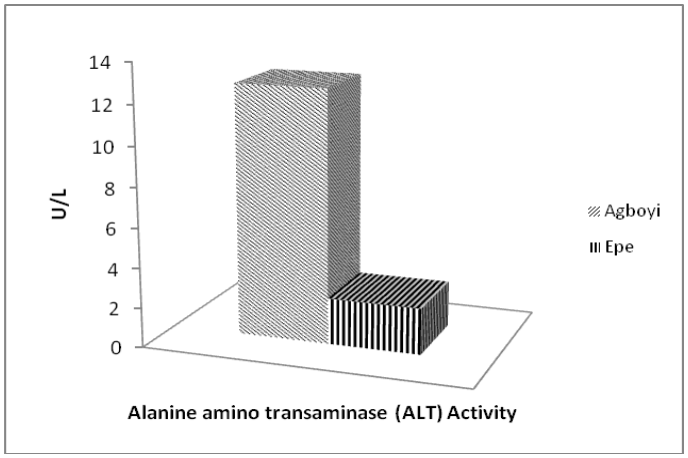

Figure 5. ALT activities in the serum of catfishes Agboyi creek and Epe lagoon (Control site). The values are significantly different (F=1.739, Fcrit = 6.608, df =1 p= 0.24)

(P>0.05).

Serum enzyme levels in catfish from both sampling sites

The mean activities of AST in the serum of *C. nigrodigitatus* were significantly higher at Agboyi creek (12.67 ± 15.04 and 151.67 ± 76.76 U/L, respectively) (Figure 4). ALT activities were also significantly higher at Agboyi when compared to the same enzymes at Epe lagoon (2.33 ± 0.52 and 6.00 ± 3.46 U/L, respectively) (Figure 5).

DISCUSSION

The evidence from the physicochemical assessments of surface water samples from Agboyi creek and Epe lagoon clearly shows some level of deviation from the set limit, with the former having values which differed more from the NESREA standard. Overall, the physicochemical characteristics indicate that Agboyi creek is disturbed as a result of anthropogenic activities along its course. Apart from the activities within the creek such as dumping of solid wastes, sand mining, domestic sewage and effluents discharge from cottage industries in the upstream Ogun river, this creek is also influenced by pollution from the neighbouring Odo Iyalaro creek which receives effluents from the Ogba-Ikeja industrial estates. The continuous input of these contaminants inevitably impacts on its physical parameters and in turn its biota. According to Nwankwo (1995), storm water channels, creeks and creeklets acts as conduits for land based human-induced activities into the coastal waters of Nigeria. The very high level of suspended solids in the creek can be attributed to among other factors, the artisanal sand mining activities prevalent in the area. This raises the turbidity of the water and therefore may interfere with light penetration for photosynthetic activities. Impairment of photosynthesis would ultimately disrupt the ecological process and food web structure of the creek. The high levels of sulphate and phosphates at Agboyi creek, relative to the control, Epe Lagoon and the NESREA standard, are indicative of input of sewage rich in organic matter and perhaps, to some extent, fertilizer runoff from farms in the hinterland. The concentrations of heavy metals, Fe, Cu, Pb and Zn in Agboyi creek, were higher than the NESREA standards. Heavy metals in the Lagos Lagoon and associated creeks have been associated with the discharge of effluents from the adjourning industries (Oyewo, 1998). Essential heavy metals, such as Cu and Zn, are required in various physiological and metabolic processes. However, at excessively high concentrations, they become toxic, damaging the plasma membrane and other cell components, altering enzymes, and producing cytotoxic
species such as reactive oxygen radicals (Lowry et al., 1951). Many studies conducted during recent years show that heavy metals accumulation may lead to oxidative stress, with an overproduction of ROS that can alter various organic molecules such as nucleic acids, proteins and membrane lipids (Sevcikova et al., 2011; Oliva et al., 2012). Effects of pollutants on ecosystems and communities originate from effects on individual organisms and ultimately, all effects of pollutants are the result of the interaction between a foreign chemical and one or several biomolecules in an individual. This interaction may lead to a disturbance in the cell function, which in turn, may be important enough to alter the function of the organ (Hogstrand, 2000).

It is proposed that measurement of antioxidants in fish tissues may prove to be a useful biomonitoring procedure of exposure to aquatic pollutants (Sevcikova et al., 2011). A number of investigators have employed biochemical biomarkers in both *in situ* and *ex situ* conditions to measure the effect of exposure to toxicants (Esiegbe et al., 2013; Doherty, 2014). However, there are so many natural variables that can mask the effects of pollutants, so that it is very difficult if not impossible to attribute a change in an ecosystem to a single chemical or even an effluent (Hogstrand, 2000). The activities of anti oxidative stress enzymes as well as lipid peroxidation levels are the most commonly employed biochemical tools for toxicant effect monitoring (Timbrel, 2000). Different enzymes and non-enzymatic compounds participate in the antioxidant chain in biological systems. Among them, super oxide dismutase (SOD), converts superoxide anion (O^{-2}) to H_2O_2 while CAT reduces H_2O_2 to water (Timbrel, 2000).

With respect to the case - control model for which the disturbed Agboyi Creek and the relatively undisturbed Epe Lagoon were sampled in this studies, lower activities of CAT was observed in liver of catfishes at in the former compared to the later. This tends to indicate a reduced ability to protect cells against H_2O_2 in the Agboyi catfishes relative to those from Epe lagoon as prescribed by Papagiannis (2005). This finding is similar to those reported by Heart (1995), who found significantly lower CAT activity in brown bullhead livers from the contaminated St. Lawrence River than in livers of bullheads from the relatively uncontaminated Lac La Peche. Also, CAT activity was found lower in the contaminated Black River fish, Ohio and higher in the uncontaminated the Old Woman Creek fish, Ohio by McCarthy and Shugart (1990) as reported by Papagiannis (2005).

The levels of malondialdehyde (MDA), a key product of lipid peroxidation determined as a function of TBARS was higher in catfishes from Agboyi creek compared to those from the control water body. Lipid peroxidation, a well-recognized mechanism of cellular injury is used as an indicator of oxidative stress in cells and tissues (Niki, 2008). It can be inferred that the activities of CAT, being inhibited in catfishes from Agboyi creek were not high enough to prevent lipid peroxidation. This phenomenon was also described for *M. edulis* by Pellerin-Massicotte (1997). TBARS increase in liver of .3fish chronically exposed to pollutants has also been reported (Karakoc, 1997). Chronic exposure to contaminants such as agrochemicals have been associated impairment of liver antioxidative enzymes and increased lipid peroxidation, potentially compromising hepatic cell function (Amaeze et al., 2014).

The results from the serum enzyme assays indicate significantly higher levels of AST and ALT in catfishes from Agboyi creek compared to those from Epe Lagoon. Although, the activity of either enzyme particularly AST may also be elevated also in hepatic disease, the elevation of AST and ALT usually reflect some injury to the liver (Dheer et al., 1987). Some workers have illustrated that enzyme pattern in the serum reflects the physiological state of the organ. For instance increase in serum levels of AST and ALT was observed in serum of fish exposed to 2,3,4 –triaminoazo benzene resulting to the hepatocellular damage (Krishan and Veena, 1980). The increase of plasma AST and ALT have been attributed to the hepatocellular damage or cellular degradation by heavy metals, perhaps in liver, heart or muscle (Yamawaki et al., 1986).

Palace et al. (1996) proposed that organic contaminants which cause oxidative stress could also inhibit the activities of protective enzymatic antioxidants. Therefore, continuous increased exposure of the fish to nutrient enrichment, high concentrations of heavy metals as recorded in this study, together with other toxicants inherent in the water body (Agboyi creek) which was not measured could reduce their antioxidant enzyme activity and increase the stress leading to increased lipid peroxidation as well as impairment in protective enzyme functions.

Conclusion

The findings from this study reinforce the relevance of biochemical responses in fish as a tool for aquatic ecosystem monitoring. It also points the unsustainable manner in which water bodies associated with the Lagos metropolis are being used. There is a need to respond to the numerous findings from investigations in the Lagos lagoon and its associated creeks so as to stem the tide of pollution and therefore enhance the quality of life of aquatic biota and improve the utility of the water bodies for various purposes.

Disclosure of conflict of interest

There is no conflict of interests regarding this article.

Abbreviations: CAT, Catalase; **ALT,** alanine aminotransferase; **TDS,** total dissolved solids; **TSS,** total suspended solids; **TBARS,** thiobarbituric acid reactive substance; **AST,** aspartate aminotransferase.

REFERENCES

Adams SM, Shepard KL, Greeley MS Jr., Jimenez BD, Ryon, MG, Shugart LR (1989). The use of bioindicators for assessing the effects of pollutant stress on fish. Mar. Environ. Res. 28:459-464.

Aebi H (1974). Catalase. In Methods of Enzymatic Analysis. 2nd edn. Hans Ulhrich Bergmeyer (Ed), Verlag Chemie Int., FL. 2:673-682.

Ajao EA (1996). Review of the state of pollution of the Lagos Lagoon. Nigerian Institute of Oceanography and Maritine Research (NIOMR) Techechnical Paper No. 106. 19p.

Ajao EA, Fagade SO (1990). A study of the Sediments and Communities in Lagos Lagoon, Nigeria. Oil Chem. Pollut. 7: 85-117.
Al-Kahem HF, Ahmed Z, Al-Akel AS, Shamusi MJK (1998). Toxicity bioassay and changes in haematological paramters of *Oreochromis niloticus* induced by Trichlorfon. Arab. Gulf J. Sci. Res. 16:581-593.
Amaeze NH (2014). Endocrine disruption and cyto-genotoxicity as indicators of pollution in the Lagos Lagoon. Ph.D Thesis, University of Lagos.
Amaeze NH, Onadeko A, Nwosu CC (2014).Comparative acute toxicity and oxidative stress responses in tadpoles of *Amietophrynus regularis* exposed to refined petroleum products, unused and spent engine oils. Afri. J. Biotechnol. 13(45):4251-4258.
APHA-AWWA-WEF (2005). Standard Methods for the examination of water and wastewater, 21st edition.
Azqueta A, Shaposhinkov S, Collins, AR (2009). DNA oxidation: Investigating its key role in environmental mutagenesis with the comet assay. Mutat. Res. 674:101-108.
Bengtson DA, Henshel DS (1996). Environmental toxicology and risk assessment: Biomarkers and risk assessment- vol. V. ASTM, STP 1306, West Conshohocken, PA. 35p.
Bernet D, Schmidt H, Wahli T, Burkhardt-Holm P (2001). Effluent from a sewage treatment works causes changes in serum chemistry of brown trout (*Salmo trutta* L.). Ecotox. Environ. Safe. 48:140-147.
Buege JA, Aust SD (1978). Microsomal lipid peroxidation. Meth. Enzymol. 52:302-310.
Bradford M (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Analyt Biochem. 72:248-254.
Chukwu LO, Ogunmodede OA (2005). Toxicological response and sensitivity of estuarine macro-invertebrates exposed to industrial effluents. J. Environ. Biol. 26(2):323-327.
Depledge MW, Hopkin SP (1995). Methods to assess effects of brackish, estuarine and near-coastal water organisms. In: SCOPE 53 IPCS Joint activity 23. SGOMSE 10. Methods to assess the effects of chemicals on ecosystem. Chichester. John Wiley and Sons Drosophila populations. Genet. 72:469-474.
Dheer JM, Dheer TR, Mahajan CL (1987). Haematological and haematopietic response to acid stress in an air breathing fresh water fish *Channal punctatus*. Bioch. J. Fish Bio 30:577-588.
Doherty VF (2014). Antioxidant enzymes and histopathological biomarkers of exposure to monocyclic hydrocarbons in *Clarias gariepinus* (Catfish) and Eudrilus euginiae (Earthworm). PhD. Thesis, University of Lagos, Nigeria.
Don-Pedro KN, E.O. Oyewo EO, Otitoloju AA (2004). Trend of heavy metal concentrations in Lagos lagoon ecosystem, Nigeria,WAJAE. 5:103-114.
Esiegbe FJ, Doherty FV, Sogbanmu TO, Otitoloju AA (2013). Histopathology alterations and lipid peroxidation as biomarkers of hydrocarbon-induced stress in the earthworm, *Eudrilus eugeniae*. Environ. Monit. Assess. 185 (3):2189-2196.
Fagade SO (1980). The morphology of the Otoliths of the bagrid catfish, *Chrysichthys nigrodigitatus* (Lacepede) and their use in age determination. Hydrobiologia. 71:209-215.
Geffard A (2001). Réponses du biota à la contaminatio polymétallique d'un milieu estuarien, la Gironde, Fr: exposition, imprégnation, induction d'une protéine de détoxication, la métallothionéine, impact au niveau individuel et populationnel. Thèse, Université de Nantes. 127p.
Hogstrand C (2000). Metal body burden and biological sensors as ecological indicators. Environ. Toxicol. Chem. 19:1199-1212.
Ikusemiju K (1975): A comparative racial study of the catfish, *Chrysichthys nigrodigitatus* (Lacepede) from Lagos and Lekki Lagoons, Nigeria. Bull. I.F.A.N. 37:887-898.
Jamil K (2002). Bioindicators and biomarkers of environmental pollution and risk assessment. Science Publishers, Enfield. 56p.
Karakoc FT, Hewer A, Phillips DH, Gaines, AF, Yuregir G (1997). Biomarkers of marine pollution observed in species of mullet living in two eastern Mediterranean harbours. Biomarkers, 2: 303-309. liver metabolism of mutagens and carcinogens in fish living in polluted seawater. Mutat. Res. 262:129-137.
Knox WE, Greengard O (1965). Advan. Enzyme Regul. 3: 247
Livingstone DR, Garcia-Martinez P, Michel X, Narbonne JF, O'hara S, Ribera D, Winston GW (1990). Oxyradical production as a pollution-mediated mechanism of toxicity in the common mussel, *Mytilus edulis* L., and other molluscs. Funct. Ecol. 4:415-424.
Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein Measurement with the Folin Phenol Reagent. J. Biol. Chem. 193:265-275
McCarthy JF, Shugart LR (1990). Biological markers of environmental contamination. In: McCathy, J.F. and Shugart, L.R. (Ed.) Biomarkers of Environmental Contamination. Lewis Publishers. Boca Raton. 314p.
NESREA National Environmental Regulations. (2011). Surface and Groundwater quality National Environmental Standards and Regulations Enforcement Agency, S.I. p. 22.
Nwankwo DI (1995) Euglenoids of some polluted storm-water channels in Lagos, Nigeria. Trop. Freshwat. Biol. 4:29-39.
Odiete WO (1999). Environmental Physiology of Animals and Pollution. Diversified Resources Ltd., Lagos. 261p.
Odiete WO (1998). Ecophysiology: Environment and Adaptation in Animals: Inauragral Lecture. University of Lagos Press, Akoka. 47p.
Oliva M, Vicente J, Gravato C, Guilhermino L, Galindo-Riano D (2012). Oxidative stress biomarkers in Senegal sole, *Solea senegalensis*, to assess the impact of heavy metal pollution in a Huelva estuary (SW Spain): Seasonal and spatial variation. Ecotoxicol. Environ. Saf. 75: 151–162.
Onyema IC, Ojo AA (2008). The zooplankton dynamics and chlorophyll a concentration of a tropical tidal creek in relation to water quality indices. Life Science Journal. 5 (4):7– 14.
Otitoloju AA (2000). Joint action toxicity of heavy metals and their bioaccumulation by benthic animals of the Lagos Lagoon. Ph.D Thesis, University of Lagos, Lagos, Nigeria. 231pp.
Otitoloju, AA, Olagoke O (2011). Lipid peroxidation and antioxidant defense enzymes in Clarias gariepinus as useful biomarkers for monitoring exposure to polycyclic aromatic hydrocarbons. Environ. Monit. Assess. 30:340-346.
Oyewo EO (1998). Industrial sources and Distribution of heavy metals in Lagos Lagoon and their biological effects on estuarine animals. Ph.D.Thesis, University of Lagos. 247p.
Palace VP, Mjewski HS, Klaverkamp JF (1992). Interactions among antioxidant defenses in liver of rainbow trout (*Oncorhyncus mykiss*) exposed to cadmium. Can. J. Fish. Aquat. Sci. 50:156-162.
Papagiannis I, Kagalou I, Leonardos J, Petridis D, and Kalfakakou V. (2004). Copper and Zinc in Four Freshwater Fish Species From Lake Pamvotis (Greece). Environ. Int. 30:357-362.
Pellerin-Massicotte J (1997). Influence of elevated temperature and air-exposure on MDA levels and catalase activities in digestive glands of the blue mussel (*Mytilus edulis* L.). J. Rech. Océanogr. 22:91-98.
Reev J, Goldstein L, Kuhlenkamp J, Kaplowitz N (1980). Effect of salicylates and phenobarbiturate on hepatic glutathionein rats. U.S.A. J. Pharmacol. Exp. Ther. 212:240-245.
Reitman S, Frankel S. (1957). Colormetic assay of Alanine and Aspartate aminotransferase Amer J. Clin. Path 28:56.
Roy S, Lindström-Seppä P, Hänninen O (1996). Integrative approach to aquatic environment biomonitoring. In: Richardson, M. (Ed.), Environmental Xenobiotics. Taylor and Francis, London. pp. 123-142.
Stegeman JJ, Brouwer M. Di Giulio RT, Förlin L, Fowler BA, Sanders BM, Van Veld PA (1992). Molecular responses to environmental contamination: enzyme and protein systems as indicators of chemical exposure and effect. In: Huggett RJ, Kimerle RA, Merhle PM, Bergman HL. (Eds), Biomarkers: biochemical, physiological and histological markers of anthropogenic stress. A special publication of SETAC, Lewis Publishers, Chelsea, MI, pp. 235-335.
Timbrell, J. (2000): Principles of Biochemical Toxicology. 3rd Edition. Taylor and Francis. 394p.
Valavanidis A, Vahogianni T, Dassenakis M, Scoullos M (2006). Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. Ecotoxicol. Environ. Safe. 64:178-189.

Van der Oost R, Goksøyr A, Celander M, Heida H, Vermeulen NPE (1996). Biomonitoring of aquatic pollution with feral eel (*Anguilla anguilla*): II. Biomarkers: pollution-induced biochemical responses. Aquat. Toxicol. 36:189-222.

Yamawaki K, Hashimoto W, Fujii K, Koyama J, Ikeda Y, Ozaki H (1986). Hematological changes in carp exposed to low cadmium concentration. Bull of the Japanese. Soc. Sci. Fish. 59 (3):459- 466.

Yang J, Chen H (2003). Serum Metabolic Enzyme Activities and Hepatocyte Ultrastructure of Common Carp after Gallium Exposure. Zool. Stud. 42(3):455-461.

academicJournals

Vol. 14(25), pp. 2124-2129, 23 June, 2015
DOI: 10.5897/AJB2014.14256
Article Number: 6EA3FB553887
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Expression of GFSKLYFamide-like neuropeptide in the digestive system of the sea cucumber *Holothuria scabra* (Echinodermata)

Abayomi Ajayi[1,3*] and Boonsirm Withyachumnarnkul[2,3,4]

[1]Department of Anatomy, Faculty of Medicine, Kogi State University, Anyigba, PMB 1008, Anyigba, Nigeria.
[2]Department of Anatomy, Faculty of Science, Mahidol University, Rama VI Road, Bangkok 10400, Thailand.
[3]Centre of Excellence for Shrimp Molecular Biology and Biotechnology, Faculty of Science, Mahidol University, Rama VI Road, Bangkok 10400, Thailand.
[4]Aquatic Animal Biotechnology Research Center, Surat Thani Campus, Surat Thani 84100, Thailand.

Received 19 October, 2014; Accepted 17 June, 2015

Neuropeptides are key mediators of physiological processes in animals and a considerable amount of information has been accumulated on their diversity and functions across phyla. However, progress in echinoderm neurobiology has been much slower than others. The sea cucumber *Holothuria scabra* is an economically important tropical echinoderm species in which a neuropeptide is yet to be identified. This immunohistochemical study utilized antibody raised against GFSKLYFamide neuropeptide together with confocal laser scanning microscopy. GFSKLYFamide-like immunoreactivity was shown to be localized in the stomach and small intestines of *H. scabra*. This is the first report that provides evidence for the presence of GFSKLYFamide neuropeptide in the digestive tract of this species.

Key words: GFSKLYFamide, SALMFamides, immunohistochemistry, sea cucumber, *Holothuria scabra*.

INTRODUCTION

The sea cucumber *Holothuria scabra*, also called sandfish, is an echinoderm species in the class Holothuroidea, order Aspidochirotida, family Holothuriidae and genus *H. Metriatyla scabra* (Jaeger, 1833). Other classes in the echinoderm phylum are starfish (Astroidea), sea urchin (Echinoidea), sea lilies (Crinoidea) and brittle stars (Ophruroidea) (Hyman, 1955). *H. scabra* is a premium sea cucumber species that is widely consumed due to its medicinal value but there is a threat of extinction on the natural population due to overexploitation in some parts of Asia (Bussarawit and Thongtham, 1999). Consequently, efforts have been made to culture *H. scabra*, however, there is little knowledge about its biology, particularly the neurohormonal pathway (Cobb, 1988). Neuropeptides are key mediators of physiological processes in animals and a considerable amount of information has been accumulated on their diversity and functions across phyla (Strand, 1999). Echinoderms are of phylogenetic importance to chordates in that they are deuterostomes. They exhibit some remarkable biological phenomena

*Corresponding author. E-mail: abajayi2003@yahoo.com.

such as evisceration and regeneration, the mediation of which may involve neuropeptides. Although, research in echinoderm neurobiology has advanced at a much slower pace compared with that of other phyla, the discovery of SALMFamide peptides, which are related to the FMRFamide-related peptides family, in echinoderm species has been very encouraging (Cobb, 1988; Elphick et al., 1991; Walker et al., 2009).

The tetrapeptide FMRFamide neuropeptide was isolated from the ganglia of a mollusc, the clam *Macrocallista nimbosa* by virtue of its cardioexcitatory effect and became the first known member of the family of FMRFamide-related peptides (Price and Greenberg, 1977). FMRFamide-related peptides are a large collection of neuropeptides found in vertebrates and invertebrates (Price and Greenberg, 1989; Walker et al., 2009). They are identified by the possession of a C-terminal -RFamide (-Arg-Phe-NH_2) amino acid sequence and are often coded for by multiple genes. In the *Caenorhabditis elegans* for example, at least 22 genes are known to encode FMRFamide-related peptides (Li et al., 1999). They have been implicated in roles that include cardiovascular function, modulation of muscle contraction, control of locomotor activity, water balance, neuroendocrine and neuromodulatory activities (Dockray, 2004). The effect of FMRFamide neuropeptide itself could be excitatory or inhibitory on cardiac rhythm and contractility in molluscs, depending on the species under investigation. It activates non-cardiac muscles in molluscs (Greenberg and Price, 1979). In rats, FMRF-amide excites brain stem neurons (Gayton, 1982) and induces the elevation of blood pressure (Mues et al., 1982). An FMRFamide-gated sodium channel, which was the first peptide-gated ion channel was reported in snails (Cottrell et al., 1990; Cottrell, 1997; Lingueglia et al., 1995), and it was shown in rats that brain FMRFamide-activated sodium channels may be involved in the mechanism of salt-sensitive hypertension through the regulation of the brain renin-angiotensin system (Nishimura et al., 2000).

The SALMFamide peptides are the first neuropeptides to be fully characterized and sequenced from any echinoderm and they share some similarities with FMRFamide in that they were initially discovered based on radioimmunoassay using antibodies against FMRFamide (Díaz-Miranda et al., 1992; Elphick et al., 1991), and the presence of a Famide (-Phe-NH_2) at their carboxyl terminal (Walker, 1992). Though it was noted that the FMRFamide immunoreactivity earlier observed in echinoderms could have been due to other peptide families (Díaz-Miranda et al., 1992), the proposed criteria by Price and Greenberg (Price and Greenberg, 1989) for identifying any FMRFamide-related peptide seemed to have been met, at least in part, by SALMFamides. Interestingly, none of the newly characterized peptides possess the penultimate Arginine (-RFamide) motif that is signature of FMRFamide-related peptides hence, the notion that SALMFamides represent a new family of neuropeptides in Echinoderms, and the name 'SALMFamide' was first proposed by Elphick et al. (Elphick et al., 1991). The first fully sequenced echinoderm SALMFamide neuropeptides were identified about two decades ago (Elphick et al., 1991). The two peptides, GFNSALMFamide (S1) and SGPYSFNSGLTFamide (S2), were isolated from the starfish *Asterias rubens* and *Asterias forbesi* both in the class Asteroidea. They were designated SALMFamides because of the conserved "SALMF" at the C-terminal of their amino acid sequence since they lack the penultimate –R motif that characterizes FMRFamide-related peptides. A number of SALMFamide peptides were also identified in other echinoderm species (Elphick and Thorndyke, 2005). These include GFNSALMFamide (S1), SGPYSMTSGLTFamide (MagS2), AYHSALPFamide (MagS3), and AYQTGLPFamide (MagS4) which were isolated from the starfish, *Marthasterias glacialis* (Yun et al., 2007). Interestingly, S1 was discovered in two species of starfish. GFSKLYFamide and SGYSVLYFamide were isolated from the digestive tract of the sea cucumber, *Holothuria glaberrima* and they constitute the first neuropeptides to be isolated from any sea cucumber species (Díaz-Miranda et al., 1992). However, it is yet to be determined if the isolated peptides from the sea cucumber *H. glaberrima* are also expressed in other sea cucumber species as is the case with the expression of S1 in starfish.

Our study of the sea cucumber *H. scabra* is premised on it being a tropical species with high economic value. Moreover, adequate knowledge of the neurohormonal pathway of this species is important for its aquaculture and conservation. Of particular interest is the digestive system, which is critical for effective nutrition and growth of *H. scabra* under culture conditions. Unfortunately, a neuropeptide or peptide hormone is yet to be identified in this species. Hence, this project aims to determine the presence and distribution of GFSKLYFamide neuropeptide in the stomach and small intestine of *H. scabra*.

MATERIALS AND METHODS

Animals

Adult sea cucumber *H. scabra* were sourced from the Andaman Sea at Koh Jum Island in Krabi province of Thailand. Six adult *H. scabra* used in this study have an average weight of 123 g (62 to 175 g) and were collected at different times between May, 2009 and April, 2010. They were maintained in filtered natural sea water within a temperature range of 28 to 31°C and salinity of about 32 ppt at the Shrimp Genetic Improvement Centre, Chiya, Suratthani before being transferred to the laboratory in Bangkok in oxygenated sealed plastic bags.

Antibody

Polyclonal antibody against GFSKLYFamide was generously provided by Professor García-Arrarás (University of Puerto Rico, USA). The antibody was raised (as described by Díaz-Miranda et al., 1995), using 63 g of synthetic GFSKLYFamide coupled to 15 mg BSA with 0.3% glutaraldehyde. The reaction was stopped by the addition of 1 M Glycine, and the mixture dialyzed. Aliquot of the dialysate BSA-GFSKLYFamide conjugate was emulsified with complete Freund's adjuvant and injected into two rabbits with half of the emulsion each, subcutaneously and intraperitoneally. Two boosters of the aliquot mixed with incomplete Freund's adjuvant were given after the initial injection and sera was collected 7 and 14 days after each injection, preabsorbed with BSA and assayed by immunohistochemical reactivity on sections of sea cucumber intestine and by dot blot.

Immunohistochemistry

Indirect immunofluorescence method was used to analyze the occurrence of GFSKLYFamide-like immunoreactivity in the digestive tract of *H. scabra* using frozen sections. Stomach and intestinal tissues of *H. scabr*a were processed for immunohistochemical analysis according to a previously described method (Ajayi and Withyachumnarnkul, 2013). Briefly, animals were anaesthetized in ice for about 30 min before dissection. Dissected tissues were fixed immediately in 4% paraformaldehyde for 5 to 24 h at 4°C, washed three times in PBS for 10 min each and cyoprotected in 30% sucrose overnight. Sections of 7 µm thickness were cut with a cryostat (LIECA CM 1850), mounted on poly-L-lysine-coated slides and permeabilized in PBS-Triton X-100 (0.1%) for 5 min before blocking with normal goat serum (1:50 in PBS) for 1 h. Sections were incubated overnight at room temperature with primary antibody (1:1000 in PBS) followed by three washes in PBS-Tween 20 (0.05%) for 10 min each. This was followed by 1 to 2 h incubation in Alexa 488-conjugated goat anti-rabbit IgG (1:500 in PBS) at room temperature. Sections were then washed 3 times, 10 min each, in PBS-Tween 20. Slides were incubated in TOPO-3 (1:500 in PBS) at room temperature for 1 h then rinsed in PBS-Tween 20 (0.05%) and mounted in buffered glycerol (pH 8.6). Tissues were examined and photographs taken with an Olympus Confocal laser scanning microscope (FV 1000). Images were processed using OLYMPUS FLOVIEW 1.7b viewer and Adobe Photoshop CS3. Preabsorption control was done by substituting the primary antibody with PBS or preabsorbed antibody. Working dilutions of GFSKLYFamide antibody (1: 1000 in PBS) were preabsorbed overnight at 4°C with 100, 50 and 10 ng/µl of synthetic GFSKLYFamide peptide.

RESULTS

In this study, portions of the stomach and descending intestine were examined for immunoreactivity to GFSKLYFamide antiserum. Histological features of the stomach and small intestine of *H. scabra* are similar to those of other sea cucumber species. Histologically, the mucosa of the digestive tract of echinoderms consists of pseudostratified luminal epithelium, adjacent to which there is a layer of internal connective tissue. The connective tissue layer is followed by a muscle layer and a layer of visceral coelomic epithelium. A basal lamina separates the internal connective tissue layer from the luminal epithelium and from the muscle layer. The muscle layer is usually subdivided into an inner circular muscle layer and an outer longitudinal layer. External connective tissue layer is associated with the coelomic epithelial layer (Garcia-Arraras et al., 2001; Hyman, 1955). Results show the presence of GFSKLYFamide-like immune-reactivity in the stomach and small intestines of the sea cucumber *H. scabra*. In the stomach, GFSKLYFamide-like immunoreactivity was localized within the submucosal and muscular layers. Immunoreactive cell bodies could also be seen in the submucosal layer (Figure 1). The small intestine expresses intensely stained immunoreactive fibers in mucosa as well as the coelomic epithelium, adjacent to the muscle tissue (Figure 2).

DISCUSSION

The digestive system of *H. scabra* consists of mouth, esophagus, stomach, ascending intestine, descending small intestine, large intestine, rectum, cloaca and anus, supported by a mesentery (Hyman, 1955; Bai, 1971). In this study, only the stomach and small intestines of *H. scabra* were examined for immunoreactivity against GFSKLYFamide neuropeptide. Results indicate wide-spread distribution of GFSKLYFamide-like immune-reactivity in the stomach and small intestines of the sea cucumber *H. scabra* suggesting that GFSKLYFamide neuropeptide might be present in the digestive tract of *H. scabra*. The expression of GFSKLYFamide-like immunoreactivity in the digestive tract of this species, as shown in this study, also provides evidence in support of the potential role of GFSKLYFamide neuropeptide in the digestive functions *H. scabra*. Although, this is the first report of the occurrence of GFSKLYFamide-like immunoreactivity in *H. scabra*, the neuropeptide has previously been reported in the sea cucumber *H. glaberrima*. In earlier report, GFSKLYFamide-like immunoreactivity was observed to be present in the radial nerve cords as well as in the digestive, haemal, respiratory and reproductive systems; in the tentacles; and in tube feet of *H. glaberrima* (Díaz-Miranda et al., 1995). Although, the present study only examined the digestive system, there are some similarities in the expression of GFSKLYFamide immunoreactivity in both species. It was observed that in the intestine of *H. glaberrima*, GFSKLYFamide immunoreactive nerve fibres were found mainly within a dense nerve plexus overlying and in close contact with smooth muscle cells of the intestine. In the stomach, GFSKLYFamide immunoreactivity was observed within the internal connective tissue as well as in the muscular and external connective tissue. These observations are consistent with the pattern of expression of GFSKLYFamide-like

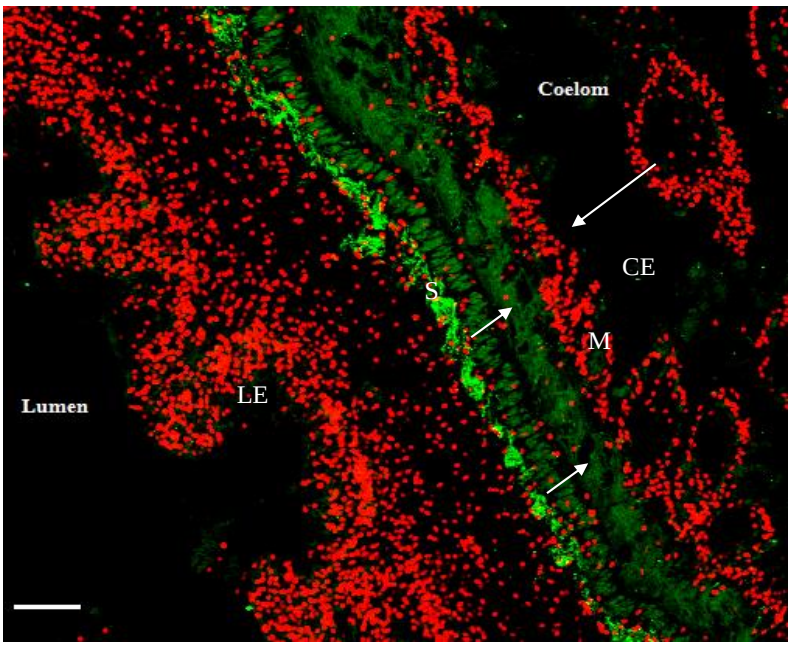

Figure 1. GFSKLYFamide-like immunoreactivity in the stomach of *H. scabra*. Immunoreactivity (green), as indicated by arrows, is present in the submucoca layer, muscular layer and in the coelomic lining of the serosa. Nuclei were counterstained with TOPO-3 (red). LE = Luminal epithelium of the mucosa; S = Submucosa; M = muscular layer; CE = coelomic epithelium of the serosa layer. Scale bar = 50 µm

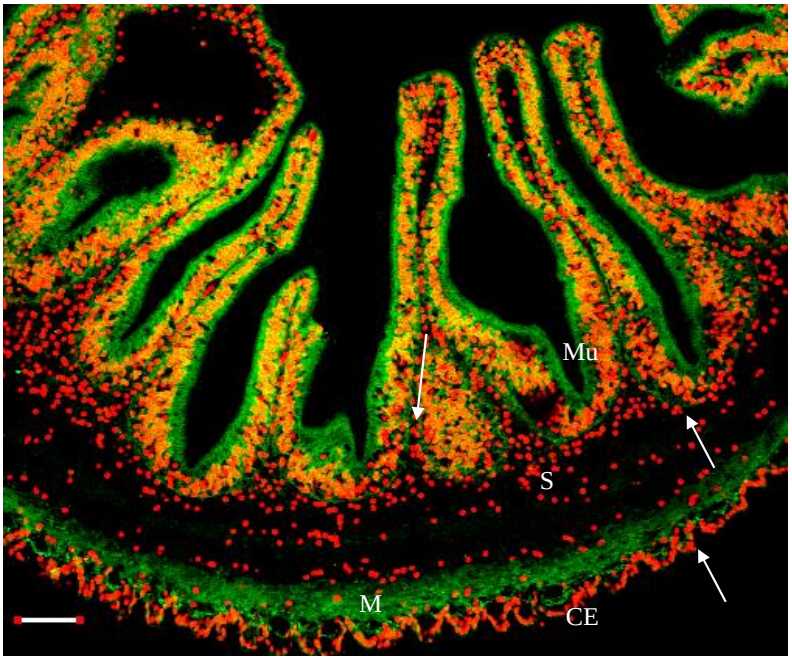

Figure 2. GFSKLYFamide-like immunoreactivity in the small intestine of *H. scabra*. Immunoreactivity (green) is indicated by arrows on the mucosa, submucosal, muscular and in the coelomic epithelium of the serosa lining. Nuclei were counterstained with TOPO-3 (red). Mu = Mucosa; S = submucosa; M = muscular layer; CE = coelomic epithelium of the serosa layer. Scale bar = 50 µm.

immunoreactivity in *H. scabra*. One important difference between the two species though, is the presence of a distinct stomach in *H. scabra* which was said to be absent in *H. glaberrima* (Díaz-Miranda et al., 1995). Since the isolation of the SALMFamide neuropeptide, GFSKLYFamide, alongside SGYSVLYFamide, from the sea cucumber *H. glaberrima* we have begin to gain some insight into the activity of SALMFamides and their global expression in the echinoderm phylum. In *H. glaberrima*, GFSKLYFamide neuropeptide has been observed to cause relaxation of intestinal muscles (Díaz-Miranda and García-Arrarás, 1995). SALMFamide peptides act as inhibitory neuromuscular transmitters in starfish (Elphick and Melarange, 2001). They may act as inhibitory transmitters not only in the starfish neuromuscular system but also more generally in other parts of their nervous systems because S1 inhibits secretion of the hormone gonad-stimulating substance from the radial nerves in the starfish *Asterina pectinifera* (Mita et al., 2004). Furthermore, SALMFamides have been observed to be involved in feeding behavior (Dockray, 2004; Melarange et al., 1999).

Investigations into the expression of SALMFamides in the sea cucumber *Stichopus japonicus* reveal the existence of two peptides, GYSPFMFamide and FKSPFMFamide (Ohtani et al., 1999). These peptides along with twenty others were shown to influence muscle activity in *S. japonicus* although GYSPFMFamide and FKSPFMFamide were the only peptides found to have a direct relaxing effect on muscle (Iwakoshi et al., 1995; Ohtani et al., 1999). Analysis of the genome of the sea urchin *Strongylocentrotus purpuratus* also reveals a SALMFamide gene. The gene encodes seven putative SALMFamide neuropeptides; PPVTTRSKFTFamide, DAYSAFSFamide, GMSAFSFamide, AQPSFAFamide, GLMPSFAFamide, PHGGSAFVFamide and GDLAFAFamide. These peptides were named SpurS1, SpurS2, SpurS3, SpurS4, SpurS5, SpurS6 and SpurS7, respectively. SpurS1, SpurS1 and SpurS3 have the C-terminal sequences -TFamide or -SFamide, which are identical or similar to the C-terminal region of the starfish SALMFamide S2 (Elphick and Thorndyke, 2005). The expression of GFSKLYFamide-like immunoreactivity in the digestive tract of *H. scabra* is remarkable as it underscores the potential physiological roles of the GFSKLYFamide-like peptide in the digestive system of *H. scabra*. Understanding for example, the potential effects of GFSKLYFamide neuropeptide on gastric motility and functions of digestive enzymes in *H. scabra* is vital, considering the economic importance of this species (Bai, 1971).

In conclusion, results from this study indicate that GFSKLYFamide-like immunoreactivity is localized within the stomach and small intestines of the sea cucumber *H. scabra*. This is to the best of our knowledge that, it is the first report providing evidence for the presence of GFSKLYFamide neuropeptide in the digestive tract of *H. scabra*. However, further studies on the structure and activity of the peptide(s) producing GFSKLYFamide-like immunoreactivity in *H. scabra* is warranted.

Conflict of interests

The authors did not declare any conflict of interest.

ACKNOWLEDGEMENT

Authors wish to thank Prof. García-Arrarás (Univ. of Puerto-Rico, USA) for his generosity in providing the GFSKLYFamide polyclonal antibody used in this study and Prof. Maurice Elphick (Queen Mary, University of London) for his contributions to this work and help with antibody. This work was supported by Centre of Excellence for Shrimp Molecular Biology and Biotechnology (Centex Shrimp), Mahidol University, Thailand.

REFERENCES

Ajayi A, Withyachumnarnkul B (2013). Presence and distribution of FMRFamide-like immunoreactivity in the sea cucumber *Holothuria scabra* (Jaeger, 1833). Zoomorphology 132(3):285-300.

Bai MM (1971). Regeneration in the holothurian, *Holothuria scabra* Jager. Indian J. Exp. Biol. 9:467-471.

Bussarawit S, Thongtham N (1999). Sea cucumber fisheries and trade in Thailand. Paper presented at International Conference on the Conservation of Sea Cucumber in Malaysia: Their taxonomy, ecology and trade. Kwalalumpur, Malaysia.

Cobb JL (1988). Neurohumors and neurosecretion in echinoderms: A review. Comp. Biochem. Phys. C 91(1):151-158.

Cottrell GA, Green KA, Davies NW (1990). The neuropeptide Phe-Met-Arg-Phe-NH_2 (FMRFamide) can activate a ligand-gated ion channel in Helix neurons. Plug. Arch. Eur. J. Phy. 416(5):612-614.

Cottrell GA (1997). The first peptide-gated ion channel. J. Exp. Biol. 200: 2377-2386.

Díaz-Miranda L, Price DA, Greenberg MJ, Lee TD, Doble KE, García-Arrarás JE (1992). Characterization of two neuropeptides from the sea cucumber Holothuria glaberrima. Biol. Bull. 182:241-247.

Díaz-Miranda L, Blanco RE, García-Arrarás JE (1995). Localization of heptapeptide GFSKLYFamide in the sea cucumber *Holothuria glaberrima* (Echinodermata): A light and electron microscopic study. J. Comp. Neurol. 352: 626-640.

Díaz-Miranda L, García-Arrarás JE (1995). Pharmacological action of the heptapeptide GFSKLYFamide in the muscle of the sea cucumber Holothuria glaberrima (Echinodermata). Comp. Biochem. Phys. C 110: 171-176.

Dockray GJ (2004). The expanding family of –RFamide peptides and their effects on feeding behavior. Exp. Physiol. 89: 229 – 235.

Elphick MR, Price DA, Lee TD, Thorndyke MC (1991). The SALMFamides: A new family of neuropeptides isolated from an echinoderm. Proc. R. Soc. B 243: 121-127.

Elphick MR, Melarange R (2001). Neural control of muscle relaxation in echinoderms. J. Exp. Biol. 204: 875 – 885.

Elphick MR, Thorndyke MC (2005). Molecular characterisation of SALMFamide neuropeptides in sea urchins. J. Exp. Biol. 208: 4273-4282.

Garcia-Arraras JE, Rojas-Soto M, Jimenez LB, Diaz-Miranda L (2001).

The enteric nervous system of echinoderm: Unexpected complexity revealed by neurochemical analysis. J. Exp. Biol. 204:865- 873.
Greenberg MJ, Price DA (1979). FMRFamide, a cardioexcrtatory neuropeptide in molluscs: An agent in search of a mission. Amer. Zool. 79:163-174.
Gayton RJ (1982). Mammalian neuronal actions of FMRFamide and the structurally related opioid Met-enkephalin-Arg^{6}-Phe^{7}. Nature 298:275 – 276.
Hyman LH (1955). The invertebrates: Echinodermata, vol. 4. New York: McGraw-Hill.
Iwakoshi E, Ohtani M, Takahashi T, Muneoka Y, Ikeda T, Fujita T, Minakata H, Nomoto K (1995). Comparative aspects of structure and action of bioactive peptides isolated from the sea cucumber *Stichopus japonicus*. In: Ohno M. (ed.) *Peptide Chemistry*: Protein Research Foundation, Osaka, pp. 261-264.
Li C, Kim K, Nelson LS (1999). FMRFamide-related neuropeptide gene family in *Caenorhabditis elegans*. Brain. Res. 848: 26 – 34.
Lingueglia E, Champigny G, Lazdunski M, Barbry P (1995). Cloning of the amiloride-sensitive FMRFamide peptide-gated sodium-channel. Nature 378: 730-733.
Melarange R, Potton DJ, Thorndyke MC, Elphick MR (1999). SALMFamide neuropeptides cause relaxation and eversion of the cardiac stomach in starfish. Proc. R. Soc. B. 266:1785-1789.
Mita M, Oka H, Thorndyke MC, Shibata Y, Yoshikuni M, Nagahama Y (2004). Inhibitory effect of a SALMFamide neuropeptides on secretion of gonad-stimulating substance from the radial nerves in the starfish Asterina pectinifera. Zool. Sci. 21:299-303.
Mues G, Fuchs I, Wei ET, Weber E, Evans C, Barchas JD, Chang JK (1982). Blood pressure elevation in rats by peripheral administration of Tyr-Gly-Gly-Phe-Met-Arg-Phe and the invertebrate neuropeptide, Phe-Met-Arg-Phe-NH2. Life. Sci. 31:2555-2561.
Nishimura M, Ohtsuka K, Takahashi H, Yoshimura M (2000). Role of FMRFamide- activated brain sodium channel in salt-sensitive hypertension. Hypertension 35:443-450.
Ohtani M, Iwakoshi E, Muneoka Y, Minakata H, Nomoto K (1999). Isolation and characterisation of bioactive peptides from the sea cucumber, *Stichopus japonicus*. In: Shimonishi, Y. (ed.). Peptide Science – Present and Future. Kluwer, UK, Academic Publishers, pp. 419-420
Price DA, Greenberg MJ (1977). Structure of a molluscan cardioexcitatory neuropeptide. Science 197 : 670-671
Price DA, Greenberg MJ (1989). The hunting of the FaRPs: The distribution of FMRFamide-related peptides. Biol. Bull. 177:198-205.
Walker RJ (1992). Neuroactive peptides with an RFamide or Famide carboxyl terminal. Comp. Biochem. Phys. C. 102(2):213-222.
Walker RJ, Papaioannou S, Holden-Dye L (2009). A review of FMRFamide- and RFamide-like peptides in metazoa. Invertebr. Neurosci. 9 (3-5): 111 – 153.
Strand FL (1999). *Neuropeptides: Regulators of physiological processes*. MA: MIT Press. Cambridge
Yun S-S, Thorndyke MC, Elphick MR (2007). Identification of novel SALMFamide neuropeptides in the starfish *Marthasterrias glacialis*. Comp. Biochem. Phys. A. 147:536-542.

academicJournals

Vol. 14(25), pp. 2130-2134, 23 June, 2015
DOI: 10.5897/AJB2015.14654
Article Number: 318C15153888
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Compound serum and hemin free medium for cultivation of *Leishmania tarentolae:* A recombinant protein expression system

Jalali Akram[1], Bandehpour Mojgan[1,2,3] and Kazemi Bahram[1,2,3]*

[1]Department of Biotechnology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran.
[2]Cellular and Molecular Biology Research Center, School of Medicine, Shahid Beheshti University of Medical sciences, Tehran, Iran.
[3]Department of Biotechnology. School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical sciences, Tehran, Iran.

Received 21 April, 2015; Accepted 15 June, 2015

Serum free cultivation of *Leishmania* is cost-effective and improves large scale production of well-defined parasite material. Moreover, the production of recombinant pharmaceutical proteins requires cultivation of the host in a culture medium free of animal materials, so several culture media for *Leishmania tarentolae* expression system have been introduced. Some investigations have established the development of a serum-free, but hemin containing medium, based on yeast extract and buffer salts. Hemin is a substance of animal origin also interferes with nickel ions on Ni-NTA resin. In this study, *L. tarentolae* from Iranian lizard, cultivated in a compound serum and hemin free medium (LBR medium) and the growth parameters were determined. Here we report that LBR medium could obtain high maximal cell density of 1.8 × 108 cells ml-1 equivalent to that of hemin containing medium in our conditions. This compound medium was confirmed by successful expression of a 28 kDa his-tagged protein. With knowledge of the results, the easy-preparing culture medium could be used as a new culture medium for the production of recombinant proteins in *L. tarentolae*.

Key words: *Leishmania tarentolae*, serum free cultivation of *Leishmania*, protein expression, *Leishmania* culture media.

INTRODUCTION

The protozoan parasites of the genus *Leishmania* have a two-stage life cycle (Peters and Killick-Kendrick, 1987). Actively motile flagellated cells form, known as promastigotes is found in the sand flye's alimentary tract. The intracellular aflagellate amastigote form lives in mammalian host within a vacuole with lysosomal features known as parasitophorous vacuole (Galvao-Quintao et al., 1990; Sacks, 1989). *Leishmania tarentolae* is a parasite of the gecko *Tarentolae annularis* (Wallbanks et al., 1985) and has been introduced as a novel eukaryotic expression system for the production of recombinant proteins with mammalian-like post translational modification pattern (Fritsche et al., 2007).

The protozoan is non-pathogenic to humans (Breitling

*Corresponding author. E-mail: bahram_14@yahoo.com. Tel: 09123268535.

Table 1. Ingredients and preparation of nutrient media used for cultivation of *Leishmania tarentolae.*

Medium	Ingredients	Preparation
LBR	LB (40%), BHI (40%), $RPMI_{1640}$ (20%)	LB (25 g/l) and BHI (37 g/l) powders dissolved and autoclaved $RPMI_{1640}$ (100 g/l) dissolved and filtered Hemin was added to BHI (5 mg/l)
BHI-hemin	Brain Heart infusion + hemin	FBS was added to LBR (10%)
LBR-FBS	LB (40%), BHI (40%), $RPMI_{1640}$ (20%) + 10% FBS	FBS was added to BHI (10%)
BHI-FBS	Brain Heart infusion + FBS	

et al., 2002). The advantages of this system are easy handling, the higher growth rate and cultivation in lower cost medium compared to mammalian cells (Phan et al., 2009). Promastigotes are mainly cultivated in liquid media to which animal serum, blood or hemin is added (Limoncu et al., 2004). A commonly used medium is Brain Heart Infusion (BHI), partially supplemented with serum or hemin (Cox and Hardegree, 1976; Fritsche et al., 2007). The media supplemented with animal substances have a risk of contamination with viruses or prion proteins responsible for bovine spongiform encephalopathy (Grillberger et al., 2009).

In the current study, we tried to develop a nutrient medium free of animal substances for cultivation of *L. tarentolae* as a host for the production of recombinant proteins as therapeutic agents. The growth parameters of the wild type organism and an expressing type, such as the rate of cell division (r), time of doubling (t), and maximal cell density (Nmax) were determined.

MATERIALS AND METHODS

The experiment was performed on a wild and an expressing type of *L. tarentolae* from Iranian lizard. For expressing type, a ~ 28 kDa secretory protein was designed. The gene of interest was cloned into pLEXSY-neo2 (Jenabioscience, Germany) between *SalI* and *NheI* restriction sites. The signal peptide sequence of the *lmsap1* gene for secreted acid phosphatase (*lmsap1*) of *Leishmania mexicana* was used. After electroporation of the host (1300v/5ms) the transfected cells were selected by neomycin.

The two types, were cultivated in 25 and 75 cm^2 plastic cell culture flasks containing 10 and 50 ml nutrient broth for static and agitated cultures respectively. The agitated groups were cultivated in 75 cm^2 shaker flasks containing 50 ml nutrient broth at 26°C and 70 rpm on a shaker incubator. Primarily frozen cells were cultivated in each of the examined media and sub-passages were grown in new media. All of the groups were grown in the dark. Inoculations from the logarithmic phase growing agitated pre-culture were done. The inoculums were centrifuged (at 1000 × *g*, 20°C, and 5 min) and the pellets adjusted to 1×10^8 parasites/ml. The same ratio of the volume and count of parasites were inoculated into flasks containing each of examined media, so all of the studied groups were initiated at a cell density of 1×10^7 ml^{-1} and counted over 18, 42, 63, 89 h (Graph 1). All of the cultures were adjusted to pH of 7.5.

Medium preparation

A compound of three culture media was examined. LB medium (Mricomedia, EU) and BHI (Sisco, India) were dissolved and autoclaved, and $RPMI_{1640}$ (Gibco, Germany) dissolved and filtered. The LBR medium was prepared from each of the media with a concentration of 40, 40, and 20% sequentially (Table 1). Hemin (Jenabioscience, Germany) was added to BHI with a final concentration of 5µg l^{-1} from a sterile stock solution. Culture medium was supplemented with 10% of FBS (Gibco, Germany). To avoid bacterial contamination, 50 IU ml^{-1} Penicillin (Gibco, Germany) and 50µg ml^{-1} Streptomycin (Gibco, Germany) was supplemented to all of the culture media. For the selection of electroporated cells, 50 µg/ml Neomycin (Sigma, Germany) was added.

Leishmania growth assessment

Parasite growth was monitored daily and every 2-3 days the medium was changed. Continuous subcultures were maintained up to 10 days of cultivation.

Qualification and quantification of promastigotes were assessed by microscopic observation of the appearance and mobility of the parasite. Counting of fixed organisms was done using a haemocytometer. pH was measured by the pH meter (Milwaukee, MW 101, Romania).

Statistical analysis

SPSS. 17 were used to calculate the data. The evaluating of differences between groups was done using student's t-test. $P< 0.05$ was accepted as statistically significant. Mean specific growth rate (r) was calculated from the Equations below:

dN/dt = rN
Nt = No e^rt
tdouble = ln (2)/ r

RESULTS

In agitated cultures, the growth rate of all the groups was significantly higher than the statics, ($p<0.05$) due to better distribution of oxygen in agitated cultures. The maximum growth rate of agitated cultures was during 55-65 h (Figure 1) and of the statics was during 72-96 h. In the

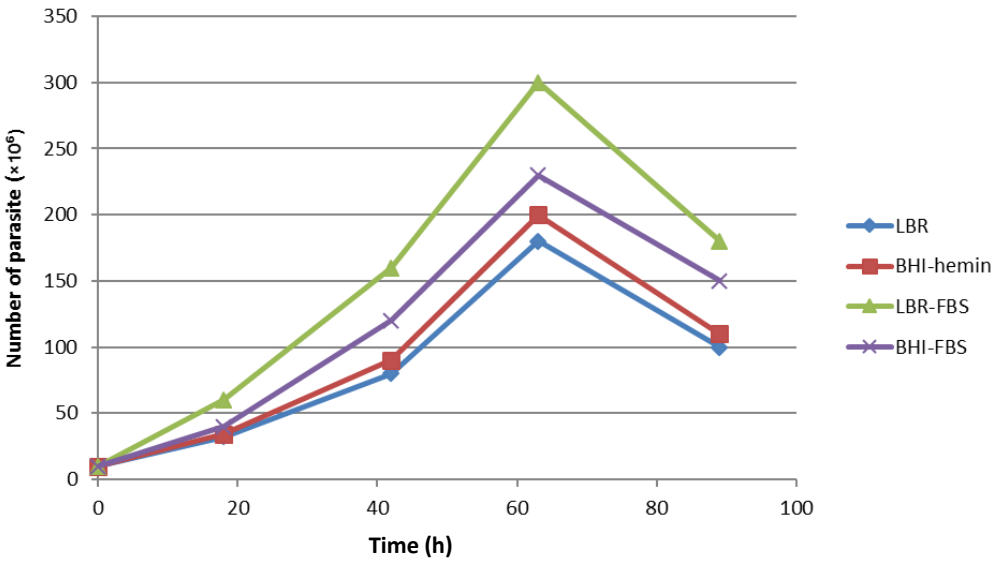

Figure 1. Reproduction of wild and expressing type of *Leishmania tarentolae* in various culture media from agitated cultures.

Table 2. Growth parameters of wild and expressing type of *Leishmania tarentolae* during exponential phase in various nutrient media.

Type of culture	medium	Nmax ×10^6 [cells/ml]	r [h^{-1}]	t double [h]	*P value*
Agitate	LBR	180	0.066	10.5	
	BHI-hemin	200	0.069	10	*P*=0.07
	LBR-FBS	300	0.099	7	
	BHI-FBS	230	0.077	9	*P*=0.001
Static	LBR	120	0.046	15	
	BHI-hemin	125	0.049	14	*P*=0.28
	LBR-FBS	200	0.063	11	
	BHI-FBS	150	0.053	13	*P*=0.003

agitated cultures there was no significant differences between Nmax of BHI-Hemin and LBR media (p=0. 07), as it reached 2×10^8 and 1.8×10^8 ml^{-1}, respectively, while in the groups of FBS supplemented media was significantly different (p= 0.001). The Nmax increased to 2.3×10^8 ml^{-1} for BHI-FBS, but 3×10^8 ml^{-1} for LBR-FBS medium. These results were observed in the static cultures with differences in maximal growth rate (Table 2).

The doubling time of agitated cultures in LBR and BHI-hemin was 10.5 and 10 h with mean specific growth rate of 0.066h^{-1} and 0.069 h^{-1}, respectively, while in LBR-FBS medium was 7 h with mean specific growth rate of 0.099 h^{-1}.

There were no growth differences between wild and expressing type of the parasite (Figure 1). The produced protein secreted into LBR medium was purified by Ni-NTA affinity chromatography (QIAGEN, Germany). We could not purify the protein secreted into BHI-hemin medium (Figure 3). The yield of the produced protein was 2 mg/l, related to the number of parasites/ml, so there were no differences between BHI-hemin and LBR medium according to the same yield of parasites/ml grown in both media.

In all of them the pH shifted down from 7.5 to 6. Although the cells could grow at pH 8 and more which declined to pH 6 during the metabolism, but the better growth rate was achieved at pH 7.5. The comparison between the prices of media related to the number of parasites/ml showed BHI-hemin costs 5 times and FBS-BHI more than 5 times of LBR medium.

Summarizing, the results showed that the growth rate

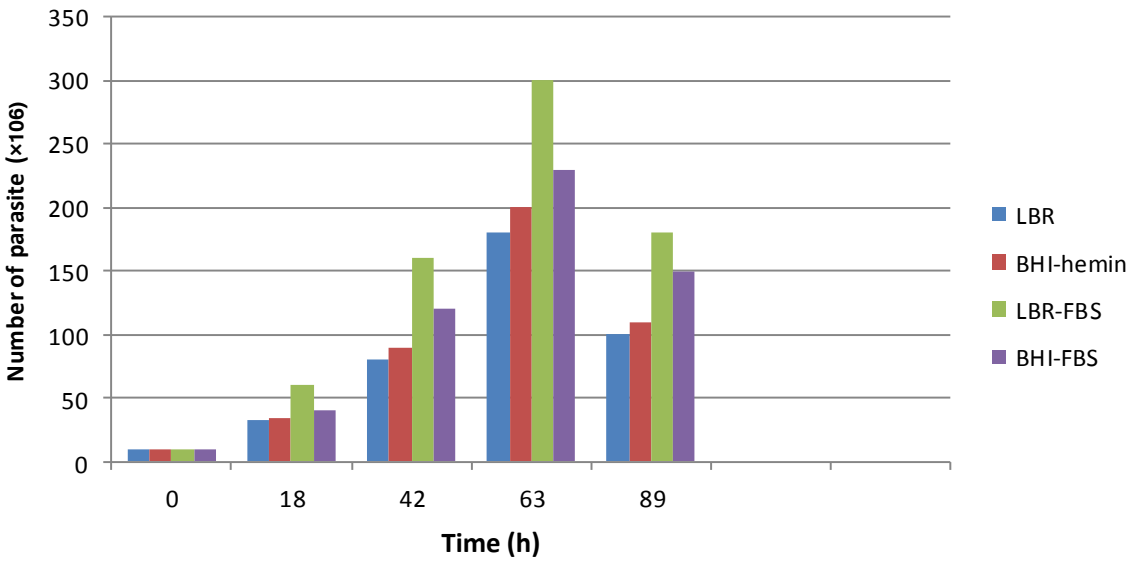

Figure 2. Growth behavior of wild and expressing type of *Leishmania tarentolae* in various nutrient media from agitated culture.

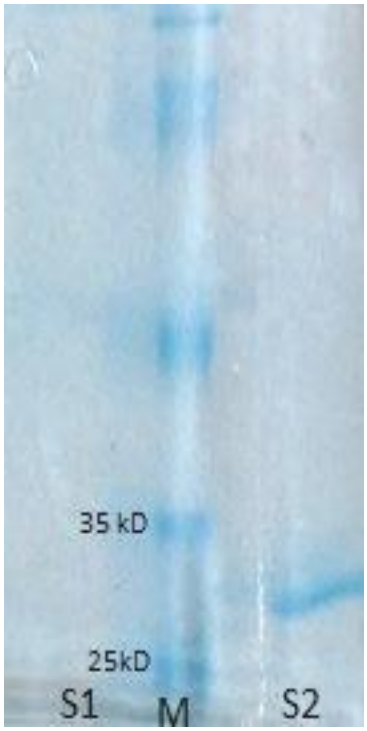

Figure 3. SDS-PAGE of The purified protein from BHI-hemin (S1) and LBR (S2).

and Nmax in the LBR and BHI-Hemin media were similar. Although the two FBS supplemented media have increased the growth rate and Nmax but FBS supplemented LBR achieved significantly better parameters (Figure 2).

DISCUSSION

Many liquid culture media have been established for the *in vitro* cultivation of *Leishmania* species. Fetal bovine serum (FBS) is the main part and the most expensive ingredient of *Leishmania* culture media. Several substances have been used to replace the FBS for successful cultivation of *Leishmania,* which originated from animals (Nasiri et al., 2012). Also hemin have been introduced as an alternative substance to produce a large amount of the parasite (Fritsche et al., 2007).

L. tarentolae has been developed as a novel eukaryotic protein expression system (Basile and Peticca, 2009). Using serum or other animal derived components (like hemin) in cultures is problematic, because of high cost, macromolecule contamination, risk of virus or prion contamination (Van der Valk et al., 2004) particularly when it is used to produce a therapeutic protein (Daniell et al., 2005).

So the cultivation of the host requires media free of animal substances for therapeutic purposes (Grillberger et al., 2009) as well as ability of increasing cell density resulted in higher protein production (Klatt et al., 2013).

Some studies have developed a serum free, but hemin supplemented media (Fritsche et al., 2007). Hemin is a substance of animal origin (Fritsche et al., 2007) also, as stated in the instructions of his-selection from Sigma-Aldrich; iron may displace nickel ions from the resin in the Ni-NTA affinity chromatography during the purification of secretory his-tagged proteins and therefor increases the number of non-specific proteins binding to the column.

We demonstrated that an easy-preparing compound medium (LBR) could obtain the same growth rate and cell density of hemin containing BHI medium without animal

materials. The maximal cell density of 1.8×10^8 $ml^{-1}$for Iranian lizard *Leishmania* cultivated in LBR was achieved. Besides, the LBR supplemented with FBS could significantly improve maximal cell density of the parasite than BHI-FBS. Investigation of Fritsche et al. (2007) on the *L. tarentolae* laboratory strain p10 (Jena Bioscience, Germany) reported a maximal cell density of 8.5×10^8 to 1×10^9 ml^{-1}, cultivated in LEXSY BHI and hemin (Fritsche et al., 2007). We could not achieve such cell density despite the use of BHI supplemented with LEXSY hemin. Meehan et al. (2000) displayed maximum cell density using BHI-hemin medium of 2×10^8 ml^{-1} (2000), which is consistent with our results. Moreover, we could successfully grow an expressing type of the parasite and purify a secretory his-tagged protein using Ni-NTA affinity chromatography, so using LBR medium for the production of recombinant proteins in *L. tarentolae* is more economical. The advantages of this system like easy handling, high similarities of posttranslational protein modifications to mammalian cells, and also ability to grow in animal-substance free media, according to our results, is to be considered (Klatt et al., 2013).

Conclusion

In conclusion the serum and hemin free LBR medium can be used for the cultivation of *L. tarentolae* as an alternative to BHI-hemin medium and can also apply to FBS supplemented media to obtain better growth parameters.

Conflict of interests

The author(s) did not declare any conflict of interest.

ACKNOWLEDGMENT

This article was extracted from the PhD thesis of Akram Jalali. We would like to thank all of our colleagues at Biotechnology Department laboratory of Shahid Beheshti University of Medical Science.

REFERENCES

Basile G, Peticca M (2009). Recombinant protein expression in Leishmania tarentolae. Mol. Biotechnol. 43(3):273-278.

Breitling R, Klingner S, Callewaert N, Pietrucha R, Geyer A, Ehrlich G, Hartung R, Müller A, Contreras R, Beverley SM (2002). Non-pathogenic trypanosomatid protozoa as a platform for protein research and production. Protein Expr. Purif. 25(2):209-218.

Cox C, Hardegree M (1976). Diphtheria and tetanus toxoids: results of assays for the presence of bovine antigens. J. Biol. Standard. 4(2):81-89.

Daniell H, Chebolu S, Kumar S, Singleton M, Falconer R (2005). Chloroplast-derived vaccine antigens and other therapeutic proteins. Vaccine 23(15):1779-1783.

Fritsche C, Sitz M, Weiland N, Breitling R, Pohl HD (2007). Characterization of the growth behavior of Leishmania tarentolae–a new expression system for recombinant proteins. J. Basic Microbiol. 47(5):384-393.

Galvao-Quintao L, Alfieri S, Ryter A, Rabinovitch M (1990). Intracellular differentiation of Leishmania amazonensis promastigotes to amastigotes: presence of megasomes, cysteine proteinase activity and susceptibility to leucine-methyl ester. Parasitology 101(01):7-13.

Grillberger L, Kreil TR, Nasr S, Reiter M (2009). Emerging trends in plasma-free manufacturing of recombinant protein therapeutics expressed in mammalian cells. Biotechnol. J. 4(2):186-201.

Klatt S, Hartl D, Fauler B, Gagoski D, Castro-Obregón S, and Konthur Zn. 2013. Generation and Characterization of a Leishmania tarentolae Strain for Site-Directed in Vivo Biotinylation of Recombinant Proteins. J. Proteome Res. 12(12):5512-5519.

Limoncu M, Özbilgin A, Balcioğlu İ, Özbel Y (2004). Evaluation of three new culture media for the cultivation and isolation of Leishmania parasites. J. Basic Microbiol. 44(3):197-202.

Meehan HA, Lundberg RA, Connell GJ (2000). A trypanosomatid protein specifically interacts with a mammalian iron-responsive element. Parasitol. Res. 86(2):109-114.

Nasiri V, Karimi G, Dalimi A, Paykari H, Ghaffarifar F (2012). Effects of Sheep and Mouse Urine on the Growth Pattern of Leishmania major Promastigotes. BioMed Res. Int. 2013:748592-748592.

Peters W, Killick-Kendrick R (1987). The leishmaniases in biology and medicine. Vol. I. Biology and epidemiology. Vol. II. Clinical aspects and controlcontinued: Academic Press Inc.(London) Ltd.

Phan H-P, Sugino M, Niimi T (2009). The production of recombinant human laminin-332 in a Leishmania tarentolae expression system. Protein Expr. Purif. 68(1):79-84.

Sacks DL (1989). Metacyclogenesis in Leishmania promastigotes. Exp. Parasitol. 69(1):100-103.

Van der Valk J, Mellor D, Brands R, Fischer R, Gruber F, Gstraunthaler G, Hellebrekers L, Hyllner J, Jonker F, Prieto P (2004). The humane collection of fetal bovine serum and possibilities for serum-free cell and tissue culture. Toxicol. *in vitro* 18(1):1-12.

Wallbanks K, Maazoun R, Canning E, Rioux J (1985). The identity of Leishmania tarentolae Wenyon 1921. Parasitology 90(01):67-78.

African Journal of Biotechnology

Related Journals Published by Academic Journals

- *Biotechnology and Molecular Biology Reviews*
- *African Journal of Microbiology Research*
- *African Journal of Biochemistry Research*
- *African Journal of Environmental Science and Technology*
- *African Journal of Food Science*
- *African Journal of Plant Science*
- *Journal of Bioinformatics and Sequence Analysis*
- *International Journal of Biodiversity and Conservation*

academicJournals