African Journal of Biotechnology

Volume 13 Number 10, 5 March, 2014

ISSN 1684-5315

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

Submission of a manuscript implies: that the work described has not been published before (except in the form of an abstract or as part of a published lecture, or thesis) that it is not under consideration for publication elsewhere; that if and when the manuscript is accepted for publication, the authors agree to automatic transfer of the copyright to the publisher.

African Journal of Biotechnology

Table of Contents: Volume 13 Number 10, 5 March, 2014

ARTICLES

Table of Contents: Volume 13 Number 10, 5 March, 2014

academicJournals

Vol. 13(10), pp. 1097-1108, 5 March, 2014
DOI: 10.5897/AJB2013.13316
ISSN 1684-5315 ©2014 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Review

An update on conventional and molecular breeding approaches for improving fiber quality traits in cotton - A review

Kaliyaperumal Ashokkumar[1,2]*, Karuppanasamy Senthil Kumar[3] and Rajasekaran Ravikesavan[3]

[1]Centre for Plant Molecular Biology and Biotechnology, Tamil Nadu Agricultural University, Coimbatore - 641 003, Tamil Nadu, INDIA.
[2]Department of Plant Sciences, University of Saskatchewan, Saskatoon, S7N 5A8, SK, Canada.
[3]Centre for Plant Breeding and Genetics, Tamil Nadu Agricultural University, Coimbatore - 641 003, Tamil Nadu, INDIA.

Accepted 2 January, 2014

The cultivated *Gossypium* spp. represents the most important, natural fibre crop in the world. India is the only country cultivating all the four cultivated species of cotton. Among the *Gossypium* spp., *Gossypium hirsutum* is the most cultivated species in many countries. Breeding for high cotton yield is still the primary goal of cotton breeding programs, but improving fibre quality has become increasingly important. The enhancement of fibre quality traits like fibre length, strength, and fibre fineness is an essential requirement for the modern textile industry. *G. hirsutum* is characterized by its high lint yield while *Gossypium barbadense* has good fibre quality. Through a conventional breeding strategy, introgression of useful alleles for fibre quality from wild species and *G. barbadense* to *G. hirsutum* will be the effective way to improve the fibre quality traits. The identification of the stable quantitative trailt loci (QTLs) affecting fiber traits across different generations will be very helpful in molecular marker-assisted selection to improve fiber quality of cotton cultivars. In this review, we present an overview of the genetics and conventional and molecular breeding techniques that have been used to increase the favorable fibre quality traits in cotton.

Key words: Cotton, fibre quality traits, simple sequence repeat (SSR), restricted fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), quantitative trait loci (QTLs).

INTRODUCTION

Cotton is an important fibre-producing crop, and it plays an important role in the Indian economy. India ranks first in the world in terms of area under cotton cultivation, but occupies third rank in total production. In India, cotton is a major agricultural commodity and a large part of the Indian economy. Worldwide, India is the only country producing that cultivates all four cultivated species of cotton. Cotton production in India is 355 lakh bales during 2011-12 India is the first country throughout the world to exploit hybrid vigor by developing *hirsutum x hirsutum*

*Corresponding author. E-mail: biotech.ashok@gmail.com.

Abbreviations: AFLP, Amplified fragment length polymorphism; **CSR,** complex sequence repeat; **RFLP,** restricted fragment length polymorphism; **RAPD,** random amplified polymorphic DNA; **SRAP,** sequence related amplified polymorphism; **SSR,** simple sequence repeat; **QTL,** quantitative trait loci; **PHB,** polyhydroxybutyrate; **PHA,** polyhydroxyalkanoate; **HPLC,** high performance liquid chromatography; **GC,** gas chromatography; **PCR,** polymerase chain reaction; **MAS,** marker assisted selection.

Table 1. Categories of fibre length in cotton in India (Singh, 2004).

Fibre length			Fibre strength		
Category	**Mean stable length**	**2.5% Span length (mm)**	**Category**	**3.2 mm gauge (g/t)**	**Tenacity (g/tex)**
Short	19.0 and below	Below 20	Very low	Below 17	Below 34.5
Medium	20 - 21.5	20 - 24.5	Low	17-19.5	34.5- 37.4
Superior medium	22 - 24	25 - 27.5	Average	19.6 - 25	37.5 - 43.0
Long	24.5 - 26	28 - 31.5	Good	25.1 - 29.9	43-.1- 47.4
Superior long	27 and above	32 and above	Very good	29.1 & above	47.5 & above

and *hirsutum x barbadense* hybrids which combined high yield potential and superior fibre quality (Alkuddsi et al., 2013).

Fibre quality parameters of cotton, fibre length and fineness have a vital influence the yarn strength. Fibre length is the most important cotton fibre character which determines the amount by which fibres can overlap with one another. The greater overlapping, the easier it is for the fibres to be bound together and result in better yarn strength (Ahmad et al., 2003). Fibre fineness is another important fibre character affecting yarn strength. It contributes to the number of fibres in the cross-section of yarn. The better the fineness of cotton, the more fibres there are per cross-section resulting in higher yarn strength. Broughton et al. (1992) acknowledged that increasing fibre length results in improved yarn strength because a long fibre generates a greater frictional resistance to an external force. Broughton et al. (1992) stated that at high fibre length, the tensile strength of the fibres becomes the controlling factor of yarn strength.

Fibre length and fibre strength properties have influenced textile processing (Kohel, 1999; Amjad, 1999). With this thrust, breeders must always develop new elite cultivars with both high yield and improved quality. One-third of foreign exchange is earned by export of cotton. In total, cotton production contributes to 30% of the Indian agricultural gross domestic product and accounts for 30% of all export earnings. Current modernized spinning mills fibre standards sets mainly based on greater fibre quality, especially strength (Arioli, 2005). Strong fibres survive the rigours of ginning, cleaning, opening, carding, combing and drafting (Guo et al., 2003). Ahuja (2003) also suggested that developing high fibre length and strength cultivars or hybrids is required for current modernized spinning mills. Based on the above, this review summarizes the updated results of conventional and molecular breeding techniques that have been used for the development of favorable fibre quality traits in cotton.

FIBRE QUALITY PARAMETERS

Fibre length

The crucial index of fibre quality in cotton is determined by the spinning performance. Among the fibre properties which contribute most to spinning value are staple length, fibre fineness and strength. The staple length constitutes the basic norm for evaluating quality of cotton in the trade and by the consuming textile industry. Fibre length has been directly correlated with the spinning capacity. The worth of cotton is mainly determined based on the fibre length. Fibre length is generally measured by three ways, as halo length, mean length, and 2.5% span length. Halo length is the length of fibre with attached seed, and it can be measured with the help of halo disc. The mean halo length is the arithmetic mean of the length of all the fibres present within the sample. 2.5% span length is the distance from the clamp on fibre beard to a point up to which only 2.5% of the fibres extend. This is the fibre length representing the majority of the fibres and expressed in millimeter, and measured by the digital fibre graph. Five stable length categories were used for the classification of cotton in India proposed by Singh (2004) (Table 1).

Fibre strength

Of the fibre quality traits, fibre strength is the second most important property of cotton fibre and it determines the yarn strength. The fibre strength is essential for high speed spinning such as rotobar and jet spinning. Fibre strength is generally measured by stelometer. Fibre strength can be determined either on individual fibre or on a bundle fibre. Fibre strength is also known as tensile strength, and expressed as tenacity in gram per tex at 1/8” gauge. Based on the bundle strength and tenacity values, cotton can be classified into five categories (Table 1).

Other fibre quality parameters

Fibre finess is the relative measure of size, diameter and linear density of fibres, which denotes the finess of fibres. It’s also known as micronaire. The ratio between 50 and 2.5% span length is known as uniformity ratio, and it is expressed as percentage. Uniformity ratio denotes the percentage of longer fibres. Fibre elongation percentage is the elongation of the fibre bundle. It is a measure of the

per cent increase in jaw separation of instrument under load.

GENETICS STUDIES OF FIBRE QUALITY IMPROVEMENT IN COTTON

The comparison of *Gossypium hirsutum* cultivars for genetically diverse genotypes for fibre quality traits is essential for developing high fibre quality cultivars. Ashokkumar and Ravikesavan (2011) reported, highest 2.5% span length in *G. hirsutum* cultivar SURABHI (32.90 mm), and the lowest 2.5% span length were found in accession SOCC 11 (23.00 mm) and Copur (2006) and Khan et al. (1989) also observed similar results for fiber length in cotton. The bundle strength was lowest in (18.9 g/tex-1) and highest in (22.9 g/tex-1). Fibre fineness or micronaire is very important characteristic of the fiber quality of cotton and is very useful for textile industry. The comparison of treatment mean indicated that *hirsutum* cultivars varied significantly for fiber fineness. SURABHI had fine fibres (3.4 µg inch-1) SOCC17 registered coarse fibre (4.6 µg inch-1) reported by Ashokkumar and Ravikesavan (2011). Differences between the *hirsutum* cultivars with respect to fiber fineness were also found significant by Copur (2006) and Ehsan et al. (2008). Therefore, genetic studies of gene action and association studies were important for fibre quality traits improvement in cotton.

Gene action

The inheritance of fibre quality characters of fibre properties in cotton may be governed by oligogenes with distinct effect of individual gene, and polygenes with small additive effect of each gene. The inheritance of fibre characters, viz., fibre length and strength is governed by polygenes. In polygenic inheritance, the variation for character is continuous from one extreme to another. The fibre length, fibre strength, fibre fineness and uniformity ratio were mainly governed by additive gene action with some of the degree of dominance. Several studies were reported gene action for fibre quality traits in cotton, and it is summarized in Table 2.

Association studies

In path coefficient analysis, indirect effect of seed cotton yield was influenced positively by ginning outturn, lint index, seed index, 2.5 per cent span length, bundle strength and seed protein. The direct effects of seed cotton yield were influenced in negative direction by uniformity ratio, fibre fineness, and elongation percentage (Ashokkumar and Ravikesavan, 2008). Fibre length and strength were negatively associated with seed cotton yield as reported by Amudha et al. (1996), Gururajan and Sunder (2004) and Ahuja et al. (2006). The reviewed up to date research reports of association analysis for fibre quality traits are summarized in Table 3.

SOURCES OF FIBRE QUALITY TRAITS IMPROVEMENT IN COTTON

Wild species

Wild species are the potential sources of fibre quality in cotton. Besides the four cultivated species of *Gossypium*, 50 species have been reported for fibre quality sources. Among them, four species are cultivated for their spinnable fibre and the remaining 46 species are distributed throughout the tropical and subtropical countries in wild forms. Wild species possess useful genes for fibre quality traits like fibre length and strength in cotton. *Gossypium thurberi* is one of the important sources of fibre strength; it's successfully transformed to the cultivated varieties of *G. hirsutum*. Some wild species can serve as possible donors for fibre quality traits and are presented in Table 4. Meanwhile, the variability available from the cultivated species is limited and has been thoroughly exploited for breeding programmes. Therefore, to develop basic germplasm materials enriched with rare useful genes from wild species through introgression is essential. For example, long stable variety AKA 8401 has been obtained from the cross between *Gossypium arboreum* cultivars and *Gossypium anomalum* (Tayyab, 1990). Phenotypic variation between wild species of cotton is shown in Figure 1.

Induced mutation

Mutations are the potential source of creating genetic variability in the plant breeding material. Since the spontaneous mutations having the extremely low frequency and the induced mutation provides a tool for the rapid creation of variability in crop species. In cotton breeding, while hybridization and selection have stood the test of times, of late mutation breeding techniques has come in handy for improvement of specific characters. Fibre quality traits can also be improved through the use of induced mutations. However, this source is rarely used in the improvement of fibre quality traits. In upland cotton, some of the x ray induced mutants showed better fibre quality than their parents (Thompre and Mehetre, 1982). For example: MCU 7 cotton variety is the x rays irradiation of L1143; it increased the yield and spinning performance.

BREEDING APPROACHES

The breeding methods like introduction, selection, hybridization followed by mass selection, pedigree selection, back cross method, mutation breeding,

Table 2. Gene action for fibre quality traits in cotton.

Fibre character	Gene action	Species	Reference
Fibre length	Additive	*G. hirsutum*	Pavasia, et al. (1989); Mandloi et al. (1998); Muthuswamy et al. (2003); Haq and Azhar (2004); Subramanian et al. (2005)
	Non-additive	*G. hirsutum*	Tuteja et al. (1995); Ahuja and Tuteja (1999); Hassan et al. (2000); McCarthy et al. (2004); Ahuja and Dhayal (2007); Ashokkumar et al. (2010)
	Non-additive	*G. barbadense*	Gururajan and Manickam (2002)
	Additive	*G. arboreum*	Sandhu and Singh (1989)
	Partial dominance	*G. hirsutum*	Krishna Rao (1998)
	Dominance	*G. hirsutum*	Patel et al. (1997)
	Additive and dominance	*G. hirsutum*	Nadarajan and Sree Rangasamy (1992)
	Additive and non-additive	*G.hirusutum*	Gupta (1993)
		G. arboreum	Tomar and Singh (1992)
Fibre fineness	Additive	*G. hirsutum*	Nadarajan and Sree Rangasamy (1990); Amudha et al. (1997); Bharad et al (1999); Mandloi et al. (1998); McCarthy et al. (2004); Subramanian et.al. (2005); Lukonge et al. (2007); Azhar et al. (2004)
	Non-additive	*G. hirsutum*	Krishna Rao, 1998; Hassan et al. (2000); Muthuswamy et al. (2003); Ahuja and Dhayal (2007); Ashokkumar et al. (2010).
	Non-additive	*G. barbadense*	Gururajan and Manickam (2002)
	Additive and dominance	*G. hirsutum*	Nadarajan and Sree Rangasamy (1992); Nageshwara Rao et al. (2002).
Uniformity ratio	Additive	*G. hirsutum*	Nadarajan and Sree Rangasamy,(1990)
	Non-additive	*G. hirsutum*	Muthuswamy et al. (2003); Preetha and Raveendran (2008); Ashokkumar et al. (2010)
	Non-additive	*G. barbadense*	Gururajan and Manickam (2002)
Fibre strength	Additive	*G. hirsutum*	Amudha et al. (1997); Swati et al. (1999); McCarthy et al. (2004; Azhar et al. (2004); Lukonge et al. (2007)
	Additive and non-additive	*G. hirsutum*	Rao and Reddy (2002)
	Non - additive	*G. barbadense*	Gururajan and Manickam (2002)
	Non -additive	*G. hirsutum*	Valarmathi and Jehangir (1998); Ahuja and Dhayal (2007); Hassan et al. (2000); Muthuswamy et al. (2003); Ashokkumar et al. (2010).

heterosis breeding and molecular breeding, among others can be used to breed new varieties/ hybrids combining high yield potential and superior fibre quality. Of them, briefly discussed few breeding techniques utilized for exploitation of fibre quality enhancement in cotton was given under below.

Progeny selection

Superior plants are selected from a heterogeneous population as the basis of their progeny performance is referred association progeny selection. Singh et al. (1988), reported improvement in fibre length and strength in the progenies originally selected from open pollinated Bikaneri Narma. The improvement was more in the progenies isolated from segregating populations of the cross between Bikaneri Narma and Pusa-734. One genotype Pusa GH 95-33-47-2-2 has been registered as germplasm with NBPGR, New Delhi for high fibre length and strength of 26.2 g/tex at 3.2 mm gauge and high

Table 3. Genotypic and phenotypic correlation studies for fibre quality traits cotton.

S/N	Character	Correlation	Reference
A	Seed cotton yield with		
1	Ginning outturn	Positive	Choudhary et al. (1988); Krishna Rao and Mary (1990); Swati Bharad et al. (19990; Kaushik et al. (2003); Ashokkumar and Ravikesavan,(2010)
2	Lint index	Positive	Valarmathi, 1996; Echekwu, 2001; Kowsalya and Raveendran (1996), Murthy et al. (1995); Kaushik et al. (2003); Ashokkumar and Ravikesavan, 2010.
3	Fibre length	Negative	Valarmathi (1996); Swati Bharad et al. (1999); Ahuja et al. (2006); Ashokkumar and Ravikesavan,(2010)
4	Bundle strength	Positive	Swati Bharad et al. (1999)
		Negative	Valarmathi (1996); Rao et al. (2001); Ashokkumar and Ravikesavan (2010)
5	Seed index	Positive	Faqir et al. (1984); Tomar and Singh (1992) Sambamurthy et al. (1995); Ashokkumar and Ravikesavan (2010)
6	Fibre fineness	Positive	Kowsalya and Raveendran (1996), Murthy et al. (1995); Kaushik et al. (2003); Ashokkumar and Ravikesavan (2010)
B	Fibre length with		
1	Fibre strength	Positive	Echekwu, 2001, Basang and Gencer, 2007; Ashokkumar and Ravikesavan, 2010; Magadum et al.(2012).
2	Fibre fineness	Negative	Rajarathinam et al. (1993); Swati Bharad et al. (1999); Basang and Gencer (2007); Ashokkumar and Ravikesavan (2010)
3	Lint index	Positives	Kadambavanasundaram (1980); Magadum et al. (2012).
4	Uniformity ratio	Negative	Rajarathinam et al. (1993); Preetha and Raveendran (2008); Magadum et al. (2012).
C	Fibre fineness with		
1	Strength	Negative	Singh et al. (1990); Basang and Gencer (2007); Ashokkumar and Ravikesavan (2010)
2	Fibre length	Negative	Krishna Rao and Mary (1990)
3	Uniformity ratio	Positive	Rajarathinam et al. (1993)
D	Fibre strength with		
1	Fibre length	Positive	Ashokkumar and Ravikesavan (2010)
		Negative	Larik et al. (1999); Desalegn et al. (2009); Magadum et al.(2012).
2	Fibre fineness	Negative	Singh et al. (1990); Desalegn et al. (2009); Ashokkumar and Ravikesavan (2010); Magadum et al. (2012)
3	Elongation percentage	Positive	Basang and Gencer (2007)
4	Uniformity ratio	Negative	Desalegn et al. (2009)

Table 4. Wild species utilized for fibre quality traits improvement in cotton (Gotmare et al., 2000).

Fibre quality	Species
Fibre length	*G. anomalum, G. stochsii, G. raimondii, G. areysianum and G. longicalyx*
Fibre strength and elongation	*G. stochsii, G. areysianum, G. thurberi, G. anomalum, G. sturtianum, G. raimondii, and G. longicalyx*
Fibre fineness	*G. longicalyx, G. anomalum, and G. raimondii*
Fibre yield	*G. stochsii, G. areysianum, G. anomalum, G. sturtianum, and S. australe*
High ginning	*G. austral*

elongation of 7% (IGNR No. 0.3099). Furthermore, Singh et al. (1988) identified six promising genotypes namely; P56-2, P56-4, P56-6, C4-9-2-1, C4-9-2-1-2 and P4515-1 have been identified for high fibre strength at IARI. These

Figure 1. Genetic variation of the flower in between the cotton wild species *G. hirsutum X G. barbadense G. hirsutum x G. anomalum* Arogya (NISD 2) TCHB 213, MCU 2, MCU 5, Varalaxmi, DCH 32, and HB 224.

genotypes were showed fibre strength ranging from 24.3 g/tex (P56-2) to 29.4 g/tex (P56-4).

Intra and inter-specific hybridization

Hybrids of two genotypes of the same species are referred to association intra specific hybridization. A number of varieties belonging to different cultivated species have been developed through intra specific hybridization. For example, LRA 5166, Suman and DH-286 of *G. hirsutum* were developed from single, three way and double cross, respectively. Variety MCU 5 having high yield potential and spinning to 60 counts and its origin form a multiple cross-involving five parents. Progeny of a cross-between two different species of the same genus are referred association inter specific hybridization (Nijagun and Khadi, 2001). It can be readily made between the cultivated teraploid cottons, that is, *G. hirsutum* and *Gossypium barbadense* and *Gossypium arboreum* and *G. herbaceum.* Interspecific crosses amongst the wild species within a genome, between different genomes and between diploid species and cultivated tetraploid species are successful in varying degrees. Tetraploid species hybridizes are mostly successful for the majority of diploid species. Interspecific crosses between *G. arboreum* and *G. hirsutum* have also been successfully used to develop *G. aboreum* varities with fibre quality at par with, besides high-yield potential. Examples, the varieties like MCU 2 and MCU 5 were developed by introgression from *G. barbadense* and the world first interspecific commercial hybrid Varalaxsmi and DCH-32 (Jayakaksmi) has extra-long stable cotton production and TCHB 213 also have fibre quality with yield were reported by (Gotmare and Singh, 2004) and shown in Figure 2.

Exploitation of heterosis

India is the first country in the world to exploit heterosis in cotton at the commercial level. Inter-specific and Intra specific hybrids have been developed, which combine high yield and superior fibre quality. Besides this, hybrids also have wide adaptability and perform well under varied agro-climatic situations. Inter-specific hybrids between *G. hirsutum* and *G. barbadense* like DCH-32, DHB-105, and HB-224 have fine quality and spin to 80 counts. Ano et al. (1983) reported that *G. barbadense* parent played a dominant role in determining the fibre quality of the hybrids. Inter specific hybrids between *G. hirsutum* and *G. barbadense* have produced high frequency of fibre quality traits (Ano et al., 1983). Therefore, choice of *the G. barbadense* parents is very important in developing *G. hirsutum* x *G. barbadense* hybrids. The summarized up to date research reports of exploitation of heterosis for fibre

Figure 2. Examples of cotton cultivars developed through interspecific hybridization breeding technique for fibre quality.

quality traits are presented in Table 5.

BIOTECHNOLOGICAL APPROACHES

Biosynthesis of cotton fibre

Cotton fibre cells are the tubular outgrowth of single celled trichomes, which arise in near synchrony from the epidermis of the ovule, and it may elongate ate peak rates in excess of 2 mm per day during rapid polar expansion phase of development (Basra and Malik, 1984). There has been a substantial progress in our understanding of cellulose synthesis in developing cotton fibers. However, little is known about the early events controlling fibre cell initiation. Morphologically, the initiation of each fiber cell is associated with the spherical expansion and protrusion of one epidermal cell above the ovular surface during anthesis (Basra and Malik, 1984). Cotton fibre grows in four distinct phases of development viz., fibre initiation, elongation, secondary deposition and maturation and dehydration (Graves and Stewart, 1988). Ultra structural evidence indicates that expansion occurs through a diffusion mechanism, albeit with some bias for deposition of newly synthesized cell wall materials at the tip (Tiwari and Wilkins, 1995). Fibre elongation per se involves the deposition of the primary cell wall via secretory mechanism involving the dictyosomes and a protein synthesis mechanism sufficient to supply the proteins required in the expansion of plasma membrane and tonoplast, while the rate of biosynthesis of degradation.

Fibre modification through Genetic engineering

The fibre properties of cotton arise from the manifestation of thousands of genes. The conventional breeding, part of the gene pool from one cultivar is exchanged with that of another compatible cotton cultivar. Nevertheless, their diversity of fibre quality traits among different cotton cultivars is limited. Hence, recombinant DNA technology and new genetic transformation techniques can over-come this limitation. The critical task is to identity genes that can modify relevant fiber properties. There are several general strategies were used for fibre in cotton.

Table 5. Heterosis, heterobeltiosis and standard heterosis for fibre quality improvement in cotton.

Character	Species	Relative heterosis (%) di	Heterobeltiosis (%) dii	Standard heterosis (%) diii	References
Fibre length (mm)	*G arboreum*	-	-10.9 - 41.5 *	-	Singh and Narayanan (1990a)
		-	-23.0 -26.2*	-	Duhoon (1990)
	G. hirsutum	-	-19.42**-6.86*	-	Siddique (1993)
	G. hirsutum	-	-12.5**-3.4*	2.7 - 28.3	Kumar et al. (1992)
	G arboreum *G. hirsutum*	-16.39** -14.40**	-19.42**-6.86*	-	Siddique and Patil (1994)
	G. hirsutum	(-12.0) - 28.1	(-20.7) - 27.6	(-23.7) - 0.6	Krishnadoss and Kadambavasundaram (1997)
	G. hirsutum	-6.72 to 8.31	-6.23 to 8.21	-7.15 to 8.78	Reddy (2001)
	G. hirsutum	-11.19 to 16.79	-16.29 to 10.60	-11.54 to 16.78	Neelima (2002)
	G. hirsutum	3.70*-4.30*	-0.23- (-7.29)	-	Khan (2002)
	G. hirsutum	-	-	-4.17 to 15.53	Tuteja et al. (2005)
	G. hirsutum	-	-	7.35*- (-20.69**)	Pushpam and Raveendran (2005)
	G. hirsutum	-7.19* to -11.30**	-	-	Preetha and Raveendran (2008)
	G. hirsutum	5.3*-9.6**	-	-	Basal et al. (2011)
Fibre fineness	*G. hirsutum*	10.34* - 24.14**		-	Preetha and Raveendran (2008)
	G. hirsutum	-	-	-12.70*- (-25.40**)	Pushpam and Raveendran (2005)
	G. hirsutum	-0.12 - (-14.40)	-	-	Karademir et al. (2009)
Bundle strength (g/tex)	*G. hirsutum*	-	-	-7.59*- (-18.62**)	Pushpam and Raveendran (2005)
	G. hirsutum	-6.78* - 15.25**	-	-	Preetha and Raveendran (2008)
	G. hirsutum	0.55-24.67	-	-	Karademir et al. (2009)
	G. hirsutum	-5.1* to 9.9*	-	-	Basal et al. (2011)
Uniformity ratio (%)	*G. hirsutum*	-	-	6.21*-8.97**	Pushpam and Raveendran (2005)
	G. hirsutum	0.00-3.10	-	-	Karademir et al. (2009)
	G. hirsutum	1.8* -2.7**	-	-	Basal et al. (2011)
Elongation (%)	*G. hirsutum*			-13.45*-(-30.04**)	Pushpam and Raveendran (2005)
	G. hirsutum	0.53 - (-11.58)	-	-	Karademir et al. (2009)
	G. hirsutum	-0.3 - (-5.5)	-	-	Basal et al. (2011)

*, **, Significant at 5 and 1% probability level, respectively.

One approach is to increase or decrease levels of fibre proteins or enzymes (John and Stewart, 1992). The second strategy is to select potential genes from sources other than cotton. The potential gene polyhydroxybutyrate (PHB), and polyhydroxyalkanoate (PHA) identified from biological sources for fibre modification (Steinbuchel, 1991; John and Keller, 1996). These two genes were engineered for expression in fiber by linking them to fiber-specific promoters and introduced into cotton by particle bombardment. Transformants were identified by expression of the marker gene GUS and further confirmation were done by high performance liquid chromatography (HPLC) and gas chromatography (GC); mass spectra for isolation of PHB were presented in fibre (John and Keller, 1996). These developments will lead to improved fiber quality traits in cotton and enable the textile industry to expand its market share (John, 1997).

Molecular mapping studies for fibre quality traits

To understand the genetic basis of cotton fibre traits for the improvement of fibre quality, a genetic linkage map was constructed in the tetraploid cotton using sequence related amplified polymorphism (SRAP) linkage construction, simple sequence repeat (SSR) and random amplified polymorphic DNA (RAPD) (Lin et al., 2005). A total of 238 SRAP primer combinations, 600 RAPD primers and 368 SSR primer pairs were used to screen polymorphisms. Sixty-nine F_2 progeny from inter specific cross of “Handan 208 X Pima 90” were genotyped with 749 polymorphic markers. The identification of the stable quantitative trait loci (QTLs) affecting fiber traits across different generations will be greatly helpful to be used effectively in molecular marker-assisted selection to improve fiber quality of cotton cultivars in the future. Moreover, Lin et al. (2005) observed a total of 566 loci were assembled into 41 linkage groups with at least three loci in each group, and in total 13 QTL was associated with fibre traits among them for QTL were for fibre length and two for fibre strength.

Shen et al. (2005) used three elite fiber lines of upland cotton as parents, three linkage maps were constructed to tag QTLs for fiber qualities using SSR markers and found 11 QTLs for fiber length, 10 for fiber strength, 9 for fineness and 9 for fiber elongation. Gopalakrishnan et al. (2011) studied RAPD-polymerase chain reaction (PCR) analysis for MCU 5, and its mutant MCU 5LL was initially done with 100 primers. Majority of the primers failed to amplify in both the genotypes and observed the only 20 primers gave the best banding pattern. Shaheen et al. (2013) constructed an intraspecific genetic linkage map of the A-genome diploid cotton with SSR and RAPD markers, by 180 F_2 plants were derived from the cross of 2 *G. arboreum* cotton cultivars. Polymorphisms between the two parents were detected using 1089 pairs of SSR primers and 520 RAPD primers. In total, 34 pairs of SSR and 18 RAPD primers were amplified polymorphic loci of F_2 population. The other molecular studies for fibre quality traits were detailed summarized in Table 6.

ACHIEVEMENTS OF FIBRE QUALITY IMPROVEMENTS IN INDIA

A remarkable success has been achieved in improvement of fibre quality, particularly fibre length and strength of cotton lint during the five decades. MCU 5 is the first extra-long stable variety of *G. hirsutum* and MCU 5 VT, MCU 9, MCU11, and Abhadita are also other extra-long stable varieties. Several *G. barbadense* cultivars have resulted in significant breakthrough in fibre quality improvement in India.

Among them, Sujata and Suvin cultivars resulted in significant capable of spinning 100 counts and 120 counts, respectively, comparable to several Egyptian and Sudan types. This is the distinct landmark in the cotton fibre quality enhancement in India. In addition, inter specific hybrids of Varalaxmi, DCH-32, HB-224; NHB-12, TCHB-123 and Suruthi have the high yield with good fibre quality. *G. herbaceum* cultivars Raichur 51, Sujay, G. Cot 11, 13, 17, 21, and G. Cot 23 varieties had high yielding and medium stable varieties. High yielding and good fibre quality interspecific hybrid is (DH 2, DH 7, DH 9 and Pha 46) milestone of the *G. herbaceum* cotton. The cultivar K 8 is the first long stable variety of *G. arboreum* and K 9, K10, K 11 and AKA 8401 these varieties have fibre length of 25 mm and capable of spinning 36 counts. *G. arboreum* race indicum has been widely used for the improvement of fibre length.

CONCLUSION

India is an only country cultivating all the four cultivated species of cotton. Breeding for high cotton yield is still the primary goal of cotton breeding programmed, but improving fibre quality has become increasingly important. *G. hirsutum* characterized its high lint yield while *G. barbadense* has good fibre quality. In genetic engineering, particle bombardment technology has been developed to introduce and test genes into elite varieties of cotton, without need for regeneration or other tissue culture practices and backcrossing will lead to improved fiber quality traits in cotton and enable the textile industry. Therefore, currently there is an immediate need for a high-density genetic map of cotton anchored with fiber genes to facilitate marker-assisted selection (MAS) for improved fiber traits. Through conventional breeding strategy, introgression of useful alleles for fibre quality from *G. barbadense* to *G. hirsutum* will be the effective way for enhancing fibre length and strength in *G. hirsutum* cultivars.

Table 6. Molecular mapping for fibre quality trait improvement in cotton.

Species	Mapping populations	Markers used	Number of QTLs identified	LOD	QTL in the marker interval (cM)	Total map length coverage (cM)	References
G. hirsutum	F_2, F_2:F_3	SSR	11 QTL for fibre length, 10 QTL for fibre strength 9 QTL for fibre fineness 9 QTL for fibre elongation	≥ 3.0	-		Shen et al. (2005)
G. arboreum	182 F_2	SSR and RAPD	2 QTL for fibre traits	≥ 3.0	-	346	Shaheen et al.(2013)
G. anmolum x G. hirsutum	186 F_2 and F_3	SSR and RAPD	2 QTL for fibre strength	≥ 5.0	4.2	-	Zhang et al. (2003)
G. hirsutum x G. barbadance	F_2	RAPD and RFLP	3 QTL for fibre length 4 QTL for fibre strength 6 QTL for fibre fineness	≥ 2.0	10	-	Kohel et al. (2001)
G. hirsutum	119 F_2:F_3	RFLP	2 QTL for fibre length 3 QTL for fibre strength	-	8.7	700.7	Ulloa and Meredith (2000)
G. hirsutum x G. barbadance	94F_2	AFLP and SSR	7 QTL for fibre traits	≥ 3.0	3.28	5,500	Mei et al. (2003)
G. hirsutum x G. barbadance	183 RILs	SSR and CSR	13 QTL for fibre length, strength and fineness	≥ 2.9	-	1277	Park et al.(2005)
G. hirsutum	F_2, and BC_1	AFLP and SSR	6 QTL for fibre strength	-	10.11	932.9	Chaudhary et al. (2013)

REFERENCES

Ahmad I, Nawaz M, Tayyab M (2003). Influence of Cottonon fibre fineness and staple length upon yarn lea strength. Int. J. Agric. Biol. 5(4):642-644.

Ahuja SL (2003). Inter-relationship and variability analysis in area, production and yield in major Cottonon producing countries of world. J. Cotton Res. Dev. 17(1):75-85.

Ahuja SL, Dhayal LS (2007). Combining ability estimates for yield and fibre quality traits in 4 x 13 lines x tester crosses of *Gossypium hirsutum*. Euphytica 153:87-98.

Ahuja SL, Dhayal LS, Prakash R (2006). A correlation and path coefficient analysis of components in G. hirsutum L. hybrids by usual and fibre quality grouping. Turk. J. Agric. For. 30:317-324.

Ahuja SL, Tuteja OP (1999). Genetic analysis of some quantitative characters in Cotton (*G. hirsutum* L.). J. Indian Soc. Cotton Improv. 24(3):191-194.

Alkuddsi Y, Patil SS, Manjula SM, Patil BC, Nadaf HL, Nandihali BS (2013). Development of heterotic groups (*G. hirsutum* vs *G. barbadense*) based on combining ability and inter specific hybrids performance for yield and fiber quality traits. Cotton Genomics Genet. 4(3):33-44.

Amjad M (1999). Relationship of Cottonon properties and yarn properties. Textech. Millennium Issue. Nat. Col. Text. Engg. Faisalabad, Pakistan. pp. 102-104.

Amudha K, Raveenderan TS, Krishnados D (1996). Path analysis in coloured linted Cotton varieties. Madras Agric. J. 83:693-696.

Amudha K, Raveendran TS, Krishnadoss D (1997). Genetic diversity in coloured linted Cottonon varieties. Madras Agric. J. 84:334-337.

Ano GJ, Fersino, Lacape JM (1983). Comparative study of the performance of 12 F1 hybrids between *G. hirsutum* and *G. barbadense* with their parents. Cotton Fibres Trop. 38:228-239.

Arioli T (2005). Genetic engineering for Cottonon fibre improvement. Pflanzenschutz-Nachrichten Bayer. 58:140-150.

Ashokkumar K, Ravikesavan R (2008). Genetic studies of combining ability estimates for seed oil, seed protein and fibre quality traits in upland Cotton (*G. hirsutum* L.). Res. J. Agric. Biol. Sci. 4(6):798-802.

Ashokkumar K, Ravikesavan R (2010). Genetic studies of correlation and path coefficient analysis for seed oil, yield and fibre quality traits in Cotton (G. Hirsutum L.). Aust. J. Basic Appl. Sci. 4(11):5496-5499.

Ashokkumar K, Ravikesavan R (2011). Morphological diversity and per se performance in upland Cotton (*Gossypium hirsutum* L.). J. Agric. Sci. 3(2):107-113.

Ashokkumar K, Ravikesavan R, Silvas JPK (2010). Combining ability estimates for yield and fibre quality traits in line X tester crosses of upland Cotton (Gossypium hirsutum). Int. J. Biol. 2(1):179 -183.

Azhar M, Khan A, Mahmood N (2004). Combining ability analysis of fibre Characteristics in *Gossypium hirsutum* L. Int. J. Agric. Biol. 6(2):272-274.

Basal H, Canavar O, Khan NU, Cerit CS (2011). Combining ability and heterotic studies through line X tester in local and exotic upland Cotton genotypes. Pak. J. Bot. 43(3):1699-1706.

Basang S, Gencer O (2007). Investigation of some yield and fibre quality characteristics of interspecific hybrid (*Gossypium hirsutum* L. x *G. barbadense* L.) Cotton varieties. Hereditas 144(1):33-42.

Basra AS, Malik CP (1984). Development of the Cotton fibre. Int. Rev. Cytol. 89:65-113.

Broughton RM, Mogahzy YE, Hall DM (1992). Mechanism of yarn failure. Text. Res. J. 62:131-134.

Chaudhary B, Singh J, Tripathi MK, Bhandari HR, Singh RK, Sharma MK (2013). Inheritance studies and quantitative trait loci (QTLs) linked to fibre strength in upland Cotton. Afr. J. Agric. Res. 8(23):2983-2987.

Choudhary PN, Borole DN, Patil SD, Narkhede BN (1988). Path analysis in desi Cotton. J. Maharashtra Agric. Univ. 13 (1):54-55.

Copur O (2006). Determination of yield and yield components of some Cotton cultivars in semi-arid conditions. Pak. J. Biol. Sci. 9(14) 2572-2578.

Desalegn Z, Ratanadilok N, Kaveeta, R (2009). Correlation and heritability for yield and fiber quality parameters of Ethiopian Cotton (*Gossypium hirsutum* L.) estimated from 15 (diallel) crosses. Nat. Sci. 11:1-11.

Duhoon SS (1990). Heterobeltiosis and exploitable heterosis for yield in nine-parental diallel crosses of American Cotton. J. Indian Soc. Cotton. Improv. 15 (2):80-87.

Echekwu CA (2001). Correlations and correlated responses in upland Cotton (*Gossypium hirsutum* L.). Tropicultura 19(4):210-213.

Ehsan F, Ali A, Nadeem, Tahir MA, Majeed A (2008). Comparitive yield performance of new cultivars of Cotton (*Gossypium hirsutum* L.). Pak. J. Life Soc. Sci. 6 (1):1-3.

Faqir MA, Abid AR, Khan MA (1984). Association of yield with various economic characters in Cotton. Pak. Cotton 28:127-134.

Gopalakrishnan N, Prakash AH, Balachandran YL (2011). Temporal Changes in Metabolically Important Enzymes and Solutes act as Trigger for Epidermal Cell Conversion to Fibre Initials in Cotton. World Cotton research conference on Technologies for Prosperity. Edited by, Dr. K.R. Kranthi, Dr. M.V. Venugopalan,Dr. R.H. Balasubramanya, Dr. Sandhya Kranthi,Dr. Sumanbala Singh, and Dr. Blaise. Excel india publsihers, New delhi, India. pp. 43-50.

Gotmare V, Singh P (2004). Cotton improvement through use of wild species in India. International symposium on Strategies for sustainable Cotton production-A global vision, Dharwad. India. pp. 288-291.

Gotmare V, Singh P, Tule BN (2000). Wild and cultivated species of Cotton. CICR Technical bulletin No.5, CICR, Mumbai, Maharashtra, India. p. 1-21.

Graves DA., Stewart JM (1988). Analysis of the protein constituency of developing Cotton fibres. J. Exp. Bot. 39 (1):59-69.

Guo W, Zhang T, Shen X, Yu J, Kohel RJ (2003). Development of SCAR marker linked to major QTL for high fibre strength and its usage in marker assisted selections in upland Cotton. Crop Sci. 43:2252-2256.

Gupta SP (1993). Genetics of seed Cotton yield, boll number, boll weight and halo length in upland Cotton. J. Indian Soc. Cotton Improv. 18 (1):113-115.

Gururajan KN, Manickam S (2002). Genetic divergence in Egyptian Cottonon (*G. barbadense* L.). J. Indian Soc. Cotton Improv. 27(2):86-89.

Gururajan KN, Sunder S (2004). Yield component analysis in American Cotton (*Gossypium barbadense* L). In: International Symposium on Strategies for Sustainable Cotton Production-A Global Vision. Dharwad, Karnataka (India). pp. 201-204.

Haq I, Azhar FM (2004). Genetic basis of varietal differences for seed Cotton yield and its components in *Gossypium hirsutum* L. Int. J. Agric. Biol. 6(5):904-907.

Hassan G, Mahmood G, Razzaq A, Hayatullah (2000). Combining ability in inter-varietal crosses of upland Cotton. Sarhad J. Agric. 16:407-410.

John ME (1997). Cotton improvement through genetic engineering. Crit. Rev. Biotechnol. 17 (3):185-208.

John ME, Keller G (1996). Metabolic pathway engineering in Cotton: Biosynthesis of polyhydroxybutyrate in fiber cells. Proc. Natl. Acad. Sci. USA 93:12768-12773.

John ME, Stewart JMD (1992). Genes for jeans:biotechnological advances in Cotton. TIBTECH 10:165-169.

Kadambavanasundaram M (1980). Heterotic system in cultivated species of Gossypium. An appraisal (Abst). Genetic and crop improvement of heterotic systems. In: Pre-congress scientific meeting of 15th international congress of genetics, TNAU, Coimbatore. p. 20.

Karademir C, Karademir E, Ekinci R, Gencer O (2009). Combining ability estimates and heterosis for yield and fiber quality of Cotton in Line x Tester Design. Not. Bot. Hort. Agrobot. Cluj. 37(2):228-233.

Kaushik SK, Kapoor CJ, Koli NR (2003). Association and path analysis in American Cotton (*Gossypium hirsutum* L.). J. Cotton Res. Dev. 17:24-26.

Khan UQ (2002). Study of heterosis in fibre quality traits of upland Cotton. Asian J. Plant Sci. 1(5):593-595.

Khan WS, Khan AA, Naz AS, Ali S (1989). Performance of six punjab commercial varieties of *Gossypium hirsutum* L. under Faisalabad conditions. Pak. Cotton 33(2):60-65.

Kohel RJ (1999). Cotton germplasm resources and the potential for improved fibre production and quality. In: Basra AS (eds.). Cotton Fibres, the Haworth Press, Inc., NY, USA. p167-182.

Kohel RJ, Yu J, Park YH, Lazo GR (2001). Molecular mapping and characterization of traits controlling fiber quality in Cotton. Euphytica 121:163-172.

Kowsalya R, Raveendran TS (1996). Correlation and path coefficient analysis in Cotton. Madras Agric. J. 83(11):705-707.

Krishna Rao KV (1998). Genetic nature of yield and fibre traits in upland Cotton (*Gossypium hirsutum* L.). J. Indian Soc. Cotton Improv. 23(1):126 - 128.

Krishna Rao KV, Mary TN (1990). Variability, correlation and path analysis of yield and fibre traits in upland Cotton. Madras. Agric. J. 77 (3 & 4):146-151.

Krishnadoss D, Kadambavanasundaram M (1997). Heterosis in intra and inter specific hybrids in tetraploid Cotton. J. Indian Soc. Cotton. Improv. 22(2):110-117.

Kumar C, Joshi P, Bhardwaj RP (1992). Heterosis in Intra *G. arboreum* L. Cotton hybrids. Indian J. Genet. 52 (2):183-186.

Larik AS, Kakar AA, Naz MA, Shaikh MA (1999). Character correlation and path analysis in seed cotton yield (*G. hirsutum* L.). Sarhad J.

Agric. 15 (4):269-274.
Lin Z, He D, Zhang X, Nie Y, Guo X, Feng C, Stewart J (2005). Linkage map construction and mapping QTL for Cotton fibre quality using SRAP, SSR, and RAPD. Plant Breed. 124:180-187.
Lukonge EP, Labuschange MT, Herselman L (2007). Combining ability for yield and fibre characteristics in Tanzanian Cotton germplasm. Euphytica 161:383-389.
Magadum S, Banerjee U, Ravikesavan R, Thiyagu K, Boopathi NM, Rajarathinam S (2012). Association analysis of yield and fibre quality characters in interspecific population of Cotton (*Gossypium spp*.). J. Crop Sci. Biotechnol. 10:239-243.
Mandloi KC, Koutu GK, Mishra US, Pandey SC, Julka R (1998). Combining ability analysis and inheritance of fibre quality characters in Cottonon. J. Indian Soc. Cotton. Improv. 23 (1):147 - 151.
McCarthy J, Jenkins JN, Wu J (2004). Primitive accession derived germplasm by cultivar crosses as sources for Cotton improvement:II Genetic effects and genotypic values. Crop Sci. 44(4):1231-1235.
Mei M, Syed NH, Gao W, Thaxton PM, Smith CW, Stelly DM, Chen ZJ (2004). Genetic mapping and QTL analysis of fiber-related traits in Cotton. Theor. Appl. Genet. 108:280-291.
Murthy JSVS, Reddy DM, Reddy KHG (1995). Genetic variability, correlation and path analysis in Cotton (*Gossypium hirsutum* L.). J. Indian Soc. Cotton Improv. 20(2):133-138.
Muthuswamy A, Vivekanandan P, Jayaramachandram M (2003). Combining ability and gene action for fibre characters in upland Cotton (*Gossypium hirsutum* L.). J. Indian Soc. Cotton Improv. 28(3):127-131.
Nadarajan N, Sree Rangasamy SR (1992). Genetic analysis of certain fibre characters in *G. hirsutum* L. Indian J. Genet. 52(3):245-251.
Nageshwara Rao G, Shiva Santha Reddy M (2002). Combining ability studies of yield and yield contributing traits using diversified plant types in Cotton. J. Cotton Res. Dev. 16(1):19-23.
Neelima S (2002). Heterosis and combining ability analysis for yield and yield components in Cotton (*Gossypium hirsutum* L.). M. Sc. (Agri.) Thesis, Acharya N.G. Ranga Agricultural University, Rajendranagar, Hyderabad, India.
Nijagun HG, Khadi BM (2001). Progeny analysis of fibre characteristics of DCH 32 an interspecific Cotton hybrid. J. Genet. Breed. 55:209-216.
Park YH, Alabady MS, Ulloa M, Sickler B, Wilkins TA, Yu J, Stelly DM, Kohel RJ, Shihy OM, Cantrell RG (2005). Genetic mapping of new Cotton fiber loci using EST-derived microsatellites in an interspecific recombinant inbred line Cotton Population. Mol. Gen. Genomics 274:428-441.
Patel UG, Patel JC, Patel AD, Nizama JR (1997). Genetic analysis of ginning outturn and fibre length in G. hirsutum L. Cotton. J. Indian Soc. Cotton. Improv. 22(2):127-130.
Pavasia MJ, Badaya SN, Mehta NP, Kukdia MV (1989). Combining ability analysis for yield and other quantitative characters in upland Cotton (*G. hirsutum*). J. Indian Soc. Cotton. Improv. 14:23 - 27.
Preetha S, Raveendran TS (2008). Combining ability and heterosis for yield and fibre quality traits in Line x Tester crosses of upland Cottonon (*Gossypium hirsutum* L.). Int. J. Plant Breed. Genet. 2(2):64-74.
Preetha S, Raveendran TS (2008). Genetic appraisal of yield and fibre quality traits in Cotton using interspecific F_2, F_3 and F_4 population. Int. J. Integr. Biol. 3(2):136-142.
Pushpam R, Raveendran TS (2005). Heterosis and combining ability studies in upland Cotton for fibre characters. Trop. Agric. Res. Exten. 8:65-70.
Rajarathinam S, Nadarajan N, Subramanian S (1993). Genetic variability and association analysis in Cotton (*Gossypium hirsutum* L). J. Indian Soc. Cotton Improv. 18(1):54-59.
Rao GN, Reddy MSS, Shanthi P (2001). Correlation and path analysis of seed Cotton yield and its components in Cotton. J. Cotton Res. Dev. 15:81-83.
Rao GN, Reddy MSS (2002). Combining ability studies of yield and yield contributing traits using diversified plant types in Cotton. J. Cotton Res. Dev. 16(1):19-23.
Reddy AN (2001). Heterosis, combining ability and stability analysis of hybrids for yield and yield components in Cotton (*Gossypium hirsutum* L.). Ph. D. Thesis, Acharya N. G. Ranga Agricultural University, Rajendranagar, Hyderabad, India.
Sambamurthy JSV, Reddy DM, Reddy KHG (1995). Genetic divergence for lint characters in upland Cotton (*G. hirsutum* L.). Ann. Agric. Res. 16:357-359.
Sandhu BS, Singh JRP (1989). Detection of genetic effects using triple test cross analysis in desi Cotton (*G. arboreum* L.). J. Res. Punjab Agric. Univ. 26 (1):10-13.
Shaheen T, Zafar Y, Rahman M (2013). QTL mapping of some productivity and fibre traits in *Gossypium arboreum.* Turk. J. Bot. 37(5):802-810.
Shen X, Guo W, Zhu X, Yuan Y, John Z, Yu, Kohel RJ, Zhang T (2005). Molecular mapping of QTLs for fiber qualities in three diverse lines in Upland Cotton using SSR markers. Mol. Breed. 15:169-181.
Siddique MA (1993). Heterobeltiosis for seed Cotton yield and yield related characters in hirsutum Cotton hybrids. J. Maharashtra Agric. Univ. 18 (3):403-405.
Siddique MA, Patil RA (1994). Heterosis in cross of *Hirsutum* Cotton. J. Indian Soc. Cotton. Improv. 15:104-106.
Singh P (2004). Cotton breeding, Kalyani Pub. New Delhi, India. p. 295.
Singh P, Narayanan SS (1990a). Expression of heterosis in inter-specific crosses of diploid Cottons. J. Indian Soc. Cotton Improv. 15(1):40-42.
Singh TH, Randhawa LS, Singh M (1988). Combining ability studies for lint yield and its components over environments in upland' Cotton. J. Indian Soc. Cotton Improv. 13 (1):11-15.
Singh VH, Singh SS, Verma DS, Faroda AS (1990). Correlations and path coefficient analysis of seed Cotton yield and its components in Cotton. J. Indian Soc. Cotton. Improv. 15:104-106.
Steinbuchel A (1991). Polyhydroxyalkanoic acids. In: Biomaterials: Novel materials from biological sources. Byrom, D., Ed., Stockton Press. pp. 124-213.
Subramanian A, Ravikesavan R, Iyanar K, Thangaraj K, Vindhyavarman P (2005). Combining ability analysis in upland Cotton (*G. hirsutum* L.). Plant Arch. 5 (1):23-28.
Swati Bharad LD, Meshram H, Kalpande V, Khorgade PW (1999). Combining ability studies on fibre and seed characters in colour linted Cotton (*Gossypium hirsutum* L.). J. Indian Soc. Cotton Improv. 24(3):120-124.
Tayyab MA (1990). Use of wild species in heterosis breeding of Cotton. PKV Res. J. 14:1-4.
Thompre MV, Mehetre SS (1982). Cytomorphological studies in Triploid (2n=3x=39) plants in cultivated tetraploid (2n=4x=52) Cottons. Cytologia 47:555-563.
Tiwari SC, Wilkins TA (1995). Cotton (*Gossypium hirsutum*) seed trichomes expand via diffuse growing mechanism. Can. J. Bot. 73:746-757.
Tomar SK, Singh SP (1992). Correlation and path coefficient studies over environments in desi Cotton (*G. arboreum* L.). Indian J. Genet. 52(2):187-191.
Tuteja OP, Kuamr S, Hasan H, Singh M (2005). Heterosis and interrelationship between seed Cotton yield and qualitative characters in upland Cotton *(Gossypium hirsutum* L.). Indian J. Agric. Sci. 75(3):167-171.
Tuteja OP, Senapati BK, Singh AK (1995). Heterosis and combining ability in Desi Cottonon. J. Indian Soc. Cotton Improv. 20(2):129-132.
Ulloa M, Meredith WR (2000). Genetic linkage map and QTL analysis of agronomic and fiber quality traits in an Intraspecific population. J. Cotton Sci. 4:161-170.
Valarmathi M (1996). Genetic studies on yield components and fibre character in inrtaspecific and interspecific hybrids of Cotton. M. Sc. (Agri.) Thesis, TNAU, Coimbatore, India.
Valarmathi M, Jehangir KS (1998). Line x Tester analysis for combining ability in *(Gossypium hirsutum* L.). Madras Agric. J. 85(2):103-105.
Zhang T, Yuan Y, Yu J, Guo W, Kohel RJ (2003). Molecular tagging of a major QTL for fiber strength in Upland Cotton and its marker-assisted selection. Theor. Appl. Genet. 106:262-268.

academicJournals

Vol. 13(10), pp. 1109-1116, 5 March, 2013
DOI: 10.5897/AJB2013.13339
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Genetic diversity of sweet potatoes collection from Northeastern Brazil

Ana Veruska Cruz da Silva[2]*, Luzia Nilda Tabosa Andrade[1], Allívia Rouse Carregosa Rabbani[3], Maria Urbana Corrêa Nunes[2] and Lucas Rezende Pinheiro[3]

[1]Empresa de desenvolvimento agropecuário de Sergipe. Aracaju, Sergipe, 49025-040, Brazil.
[2]Embrapa Coastal Tablelands. Av. Beira-mar, 3250. Aracaju, Sergipe, 49025-040, Brazil.
[3]Universidade Federal de Sergipe. Mestrado em Biotecnologia. São Cristovão, Sergipe, 49000-000, Brazil.

Receive 2 October, 2013; Accepted 24 February, 2014

The sweet potato, *Ipomoea batatas* (L.) Lam has its origin in Tropical America. In Sergipe State (Brazil), its production is very important, and to explore its potential in local agriculture in the State, the Embrapa Coastal Tableland created a collection with 52 accessions located in Umbaúba City. Some accessions were from germplasm belonging to Embrapa vegetables and others from local farmers of Sergipe. Here, we provide the first data on the genetic diversity and structure of sweet potato collection of SPGB using random amplified polymorphic DNA (RAPD) markers. RAPD data were used to determine genetic variability via a model-based Bayesian procedure (structure) and molecular variance analysis (AMOVA). In addition, Shannon index, genetic diversity and Jaccard coefficients were also estimated. RAPD was efficient for the analysis of genetic diversity to identify groups and measure the genetic distance between accessions. The markers showed that the collection had a high level of polymorphism. By UPGMA, we separated three groups of genotypes and identified two reconstructed populations by structure software.

Key words: *Ipomoea batatas*, cultivars, accessions.

INTRODUCTION

Sweet potato [*Ipomoea batatas* (L.) Lam] has storage roots and belongs to the Convolvulaceae family (Zhang et al., 1998). The principal producers are China, Nigeria, United Republic of Tanzania, Uganda and Indonesia (FAOSTAT, 2013).

In Brazil, sweet potato constitutes the seventh largest production of temporary crops and the principal country producing it is Latin America, with approximately 500 tons per year (Cavalcante et al., 2009; IBGE, 2010). Sweet potatoes are consumed because their nutrients (Yoshimoto, 2001) contain beta carotene that prevents vitamin A deficiency in many developing countries (Wang et al., 2010). It is generally accepted that sweet potato has American origin (Zhang et al., 1998). Some species of the genus, *Ipomoea* section *batatas* occur in Brazil which has regions with variation in culture (Ritschel and

*Corresponding author. Email: ana.veruska@embrapa.br. Tel./Fax: (+55) 79 40091362

Huamán, 2002). Studies with genetic markers have shown significant and critical application in the assessment and conservation of genetic variation of sweet potato (Veasey et al., 2008).

Some cultivars, although being the same genetically, often have different names depending on the cultivated area, while different cultivars often have the same name (Daros et al., 2002; Santos et al., 2011). Despite the high genetic variability, changes in consumer habits and the lack of research on culture have contributed to the loss of important genotypes. It is extremely important for the maintenance of sweet potato accessions in Germplasm Banks (GB's), and the subsequent evaluation of different growing regions (Neiva et al., 2011). By having a high genetic variability, sweet potatoes can be selected for numerous purposes (Gonçalves et al., 2011; Neiva et al., 2011). It can be designed for human consumption, animal food and ethanol (Gonçalves et al., 2012).

The primary importance of GBs is that they have the ability to provide genetic variability for improvement of programs and also help to reconcile the conservation of agricultural biodiversity and sustainable development. Wild relatives and crop landraces are important resources for modern cultivars.

The analysis of popu-lation structure provides insight into how diversity is divided within a species, and that may help to define subpopulations of germplasm with high frequencies of particular alleles and allow researchers to explore the relationship between phenotype and genotype in the materials. The information provided is how a specific combination of genes and alleles interacts in varieties and allows breeders to compare the phenotypic effects of genes or chromosome segments that were inherited from a common ancestor and selected in various combinations (Garris et al., 2003).

Cultivars and breeding lines of sweet potato and nearly 26,000 accessions of other *Ipomoea* species are maintained at 83 gene banks in the world (Rao et al., 1994). In Brazil, three GBs of sweet potatoes are referenced in the literature: 1) Embrapa - CNPH created in the 1980s with 324 accessions, located in Brasilia City (Ritschel and Huamán, 2002); 2) Universidade Federal dos Vales do Jequitinhonha e Mucuri (UFVJM) situated in Diamantina City (Minas Gerais state) with 65 accessions (Neiva et al., 2011); and 3) Universidade Federal do Tocantins (Tocantins State) with 20 accessions, created in the 1990s (Martins et al., 2012). We used the Embrapa Coastal Tableland collection, located in Umbaúba City (Sergipe State), the first in the Northeastern region.

In the collection, there are 52 accessions from Embrapa vegetables germplasm bank and local farmers of Sergipe State. The correct identification and variability in the GBs and collections allow the identification of duplicates in order to estimate genetic linkage among accessions and to quantify genetic diversity in the collection for future breeding programs. Morphological traits and biochemical markers have been employed in sweet potato germplasm studies (Ritschel and Huamán, 2002). However, these markers are subject to developmental and environmental variations. Molecular markers are tools used for the detection of variability at the DNA level. Between them, RAPD technique (Random Amplified Polymorphic DNA) is the most accessible (Oliveira et al., 2007). With the help of statistical methods, RAPD technique has been effective in the first method in detecting the diversity of population in various types of specimens (Carvalho et al., 2013). Besides its low cost and speed, this technique has the advantage, even without prior knowledge of the genome, of requiring little amount of DNA for analysis (Goulão et al., 2001). From the genetic characterization, optimization and ordering of groups allows them to complement the classification and prevents erroneous inferences adopted in the allocation of materials within a particular subgroup of genotypes (Costa et al., 2011).

In our study, we related the first GB of sweet potato from the Northeast of Brasil (Sergipe State) and reported the molecular characterization of accessions using RAPD markers.

MATERIALS AND METHODS

Plant material and DNA extraction

DNA was isolated from young leaves as described by Doyle (1991). Fifty-two (52) genotypes were used (Table 1) from Brazilian Agricultural Research Corporation's (Embrapa Vegetable) and local farmers.

RAPD reaction

Seven primers (A02, A04, A08, A09, A19, K20, W04) from Operon (Operon Technologies, USA) and two primers (IDT09, IDT17) from IDT Integrated DNA Technologies (Germany) were used to screen for polymorphisms (Table 2). Each RAPD reaction was performed in 25 µL volume containing 50 ng genomic DNA, 1X PCR buffer (Gibco - BRL, Grand Island, NY, USA), 1.5 mM $MgCl_2$, 0.2 mM dNTP, 1.0 U Taq DNA polymerase (Gibco, Grand Island, NY, USA), 30 ng/µL primer and 20 µL ultrapure water. PCR amplifications were performed using a PTC-100 thermocycler (Programmable Thermal Controller - MJ Research, Inc.) with a cycle comprising 96°C for 5 min for initial denaturation, followed by 35 cycles of denaturation at 96°C for 45 s, 36°C for 45 s for primer annealing, 72°C for 45 s for extension, and one cycle of 72°C for 10 min for final extension (Silva and Martins, 2006).

Electrophoresis and visualization

Fragments were visualized on 1.5% agarose gel (1X TEB - 89 mM TRIS, 89 mM boric acid, 2.5 mM EDTA, pH 8.3) in a horizontal electrophoresis system (Sunrise, Gibco BRL), run at a constant voltage of 100 V for 90 min. The gel was stained with ethidium bromide solution (5 mg/ml) for 15 min. RAPD amplification products were visualized under ultraviolet light using a Gel Doc L-Pix image system (Loccus Biotecnologia, Brazil).

Table 1. 52 sweet potatoes accessions from collection belonging to Embrapa Coastal Tablelands (Umbaúba city, Sergipe State, Brazil, 2012).

Accession	Original location	Accession	Original location
1007	CNPH[1]	1225	CNPH
1189	CNPH	1226	CNPH
1190	CNPH	1227	CNPH
1191	CNPH	1228	CNPH
1192	CNPH	1229	CNPH
1192	CNPH	1230	CNPH
1193	CNPH	1231	CNPH
1194	CNPH	1232	CNPH
1196	CNPH	1234	CNPH
1197	CNPH	24P40	CNPH
1199	CNPH	33P10	CNPH
1200	CNPH	33P53	CNPH
1201	CNPH	P1	CNPH
1202	CNPH	P2	CNPH
1203	CNPH	P3	CNPH
1204	CNPH	P4	CNPH
1206	CNPH	P5	CNPH
1207	CNPH	P6	CNPH
1208	CNPH	P7	CNPH
1209	CNPH	Beauregard	CNPH
1217	CNPH	Italiana	Itabaiana city[2]
1220	CNPH	Olho roxo	Itabaiana city[2]
1223	CNPH	Roxinha	Itabaiana city[2]
1213	CNPH	Ciganinha	Laranjeiras city[2]
1219	CNPH	Laranjeira	Laranjeiras city[2]
1221	CNPH	Rainha	Laranjeiras city[2]
1224	CNPH		

1, Brazilian Agricultural Research Corporation's - Embrapa Vegetable; 2, Cultivars give in by local farmers from Sergipe cities.

Table 2. Primers, total fragments (TF), polymorphic information content (PIC), marker index (MI) among 52 sweet potatoes accessions from collection belonging to Embrapa Coastal Tablelands (Umbaúba city, Sergipe State, Brazil, 2012).

Primer	Sequence 5' - 3'	TF	PIC	MI
A04	AAT CGG GCT G	5	0.24	1.20
A08	GTG ACG TAG G	3	0.28	0.84
A09	GGG TAA CGC C	5	0.10	0.49
A19	CAA ACG TCG G	5	0.25	1.23
IDT09	CCC AAG GTC C	7	0.34	2.36
IDT17	ACC TGG ACA C	10	0.26	2.64
K 20	GTG TCG CGA G	8	0.33	2.67
W04	CAG AAG CGG A	4	0.35	1.41
Total		50	0.38	0.30

Data analysis

RAPD markers were scored as binary matrix. Bootstrap procedure was applied to calculate variance of the genetic distance obtained from markers, and was obtained from 5,000 bootstrap random draws using the DBOOT software (Coelho, 2000).

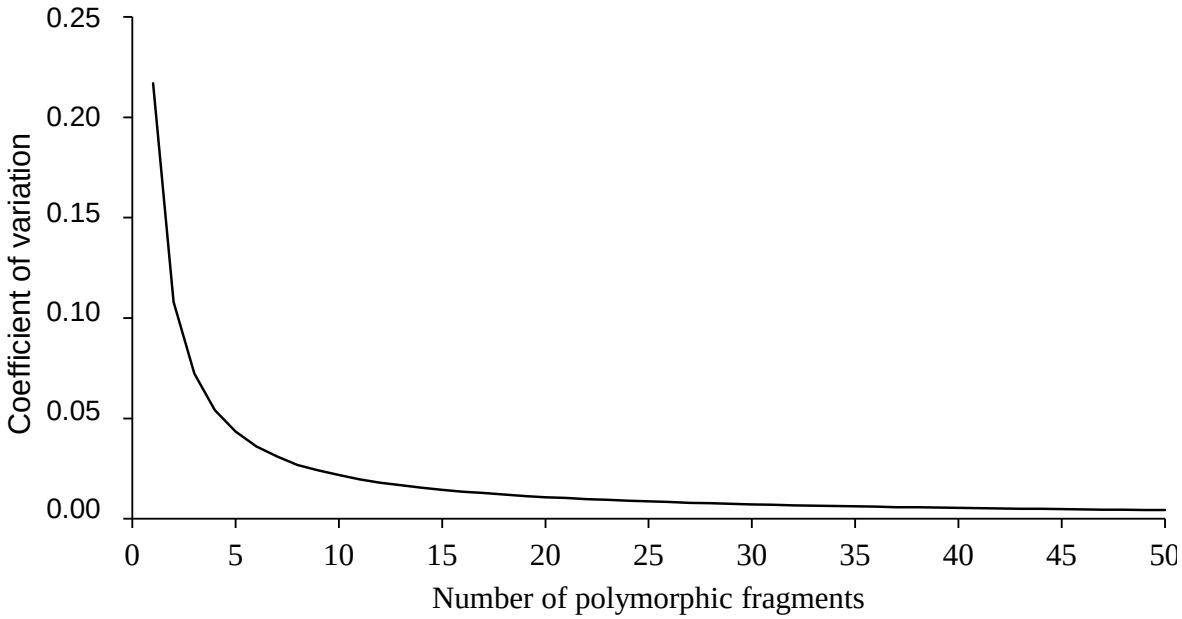


Figure 1. Coefficient of variation for the number of polymorphic RAPD markers among 52 sweet potatoes accessions from collection belonging to Embrapa Coastal Tablelands (Umbaúba city, Sergipe State, Brazil).

Polymorphic information content (PIC) was calculated according to Ghislain et al. (1999) and the marker index (MI) was determined as described in Zhao et al. (2007). A data matrix of the RAPD scores was generated and similarity coefficients were calculated using Jaccard's arithmetic complement index (Jaccard, 1908). A dendrogram was constructed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) cluster algorithm. In order to determine the robustness of the dendrogram, the data were bootstrapped with 5,000 replicates using FreeTree software (Hampl et al., 2001) and TreeView (Page, 1996).

Inferences regarding genetic structure within sweet potato genotypes were made using STRUCTURE version 2.2 (Pritchard et al., 2000; Falush et al., 2007); K was estimated as the number of reconstructed panmictic populations (RPPs) of individuals, using values ranging from 1 to 10 and assuming that the sampled genotypes were from anonymous plants of unknown origin (usepopinfo and popflag set to 0). We set up runs with a 20,000-iteration burn-in period and a Monte Carlo Markov chain (MCMC) of 20,000 iterations, with five repetitions. The program structure estimates the most likely number of clusters (K) by calculating the log probability of data for each value of K (Santos et al., 2011). We assessed the best K-value supported by the data according to Evanno et al. (2005).

The Shannon index (I) and genetic diversity (H) were calculated as described by Lynch and Milligan (1994) and Maguire et al. (2002), using Genalex v.6.3 (Peakall and Smouse, 2006). The same software package was employed to conduct an analysis of molecular variance (AMOVA) (Excoffier et al., 1992; Michalakis and Excoffier, 1996).

RESULTS

The nine primers generated a total of 50 fragments (100% polymorphic). Primers with the highest number of fragments were IDT17 and K20 (10 and 8). The PIC value ranged from 0.10 to 0.34 and MI from 0.49 to 2.67 (Table 2). There is a direct proportional relationship between the number of fragments analyzed and the coefficient of variation (CV) (Figure 1). The results indicate a clear decreasing CV with increasing number of fragments.

Genetic similarity

Genotypes were clustered by UPGMA using the Jaccard similarity (JS) estimated from the binary data of 52 genotypes (Figure 2A). The distribution of genotypes in the clusters showed the separation of the different groups, as well as the high divergence of some genotypes.

The genotypes, 1213, 1190, 33P10 between the 1213 were the most divergent in the SPGB (Sweet Potato Germplasm Bank), with 0.00 of similarity to 58% of sweet potatoes. The main cultivars were divided among sub groups, and the first group differed at 0.2 JS, with 'Olho Roxo' and 'Beauregard' as well as 'Italiana' and 'Laranjeira' varieties. The 'Rainha' was differentiated at 0.3 SJ and isolated.

The 'Ciganinha' and 'Roxinha' differentiated into groups with larger number of genotypes. The genotypes are associated with cultivars. 'Beauregard' created in US in 1980 (Cervantes-Flores et al., 2002) was connected with 1207 and 1209 genotypes.

Genetic structure and diversity

A Bayesian analysis (structure) was conducted to

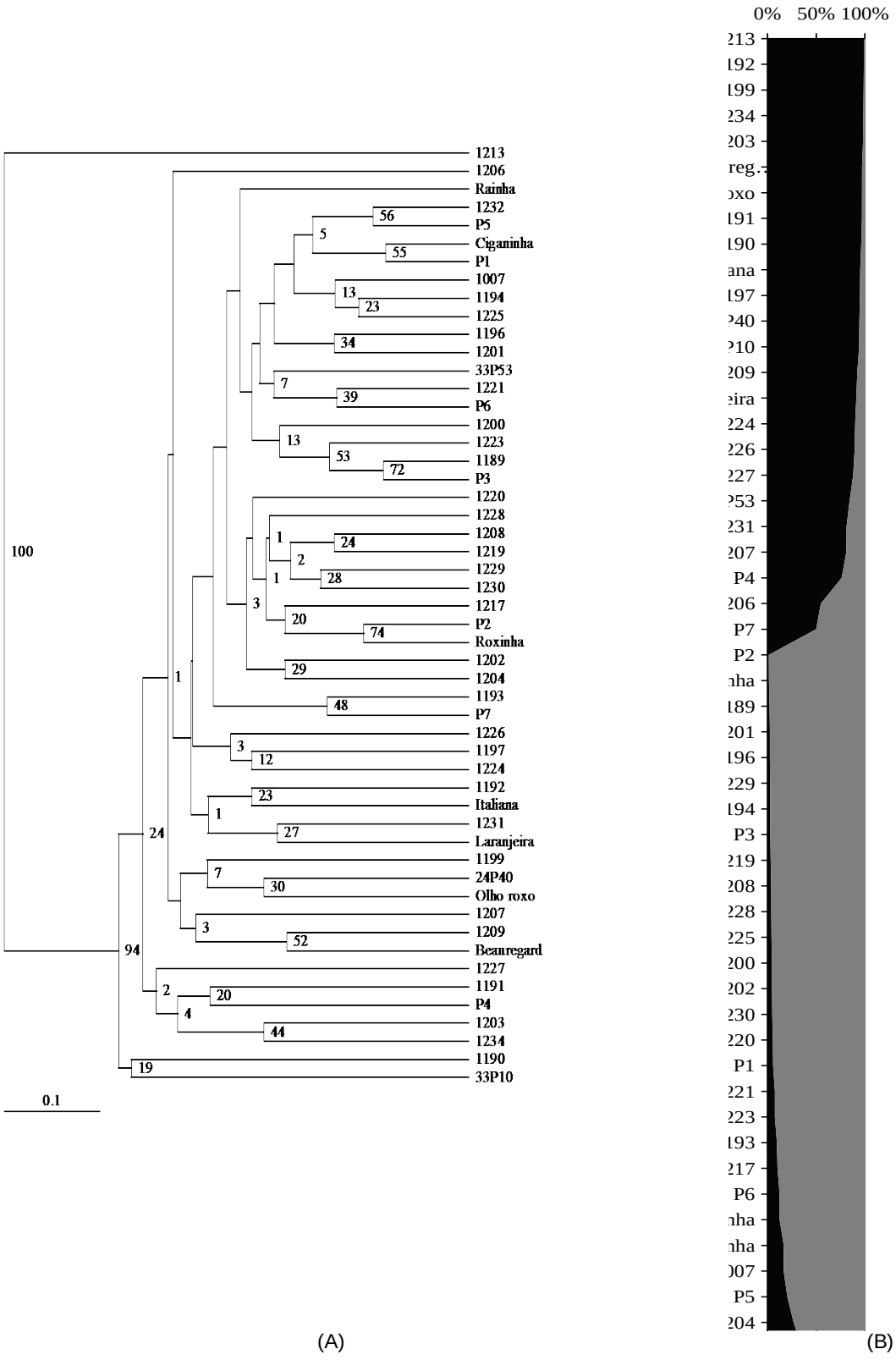


Figure 2. Dendrogram of genetic similarity generated by the Jaccard coefficient using the UPGMA method and bootstrap (A) and the reconstructed populations (B) [RPP1 (■) and RPP2 ( )] defined using structure (Pritchard et al., 2000) to 52 sweet potatoes accessions from collection belonging to Embrapa Coastal Tablelands (Umbaúba city, Sergipe State, Brazil, 2012).

Table 3. Sweet potatoes accessions from germplasm bank belonging to Embrapa Coastal Tablelands (Umbaúba city, Sergipe State, Brazil) for reconstructed populations (RPPs, K = 2) defined by the program structure (Pritchard et al., 2000) using RAPD markers. N, number of genotypes, q*I*, probability to belonging at RPP, I, Shannon Índex, H, genetic diversity.

Population	Principals cultivars	N	q*I*>80%	q*I*<80%	I	H
RPP1	Beauregard, Olho Roxo, Italiana and Laranjeira	24	22	2	0.39	0.25
RPP2	Roxinha, Ciganinha and Rainha	28	26	2	0.44	0.29
Total		52	48	4	0.42	0.27

Table 4. Analysis of molecular variance (AMOVA) for 52 sweet potatoes accessions from germplasm bank belonging to Embrapa Coastal Tablelands (Umbaúba city, Sergipe State, Brazil, 2012) for reconstructed populations (RPPs, K = 2) defined by the program structure (Pritchard et al., 2000).

Source	GF	SS	SM	CV	V%
Between RPPs	1	49.19	49.19	1.58	17%***
Into de RPPs	51	395.38	7.75	7.75	83%
Total	52	444.57		9.33	100%

***, P<0.01. GF, grades of freedom; SS, sum of squares; SM, squares medium, CV, components of variance; V%, variability in percentage.

determine the genetic structure among the sweet potatoes genotypes. This clustering approach assigns individuals to RPPs based on genotype. The program structure estimates the most likely number of clusters (K) by calculating the log probability of data for each value of K, and using ΔK statistics described by Evanno et al. (2005), as recommended by Barnaud et al. (2008) and Santos et al. (2011). The best K for representing the RPP genotypes was K = 2 (RPP1 and RPP2) (Figure 2B). The first RRP (RPP1) included 24 genotypes, of which 22 had a probability of membership (q*I*) > 80% with four cultivars (Beauregard, Olho Roxo, Italiana e Laranjeira). Two genotypes were assigned with a q*I*< 80% (P7 and P2). The second RPP (RPP2) included 29 genotypes, 27 of which had a qI> 80%, including three cultivars (Roxinha, Ciganinha e Rainha), and two genotypes with a qI< 80% (1204 and 1232). SPGB demonstrated similar mean genetic diversity to RPPs. The Shannon index was 0.39 for RPP1, with an increment of RPP2 (0.44). The genetic diversity was 0.25 for RPP1 and 0.29 for RPP2 (Table 3). When an AMOVA was performed with the 52 genotypes grouped by RPPs, genetic differentiation accounted for 17% (Table 4).

DISCUSSION

The polymorphism found was high (100%), and similar values were found in other studies (Costa et al., 2011). In a study on sweet potato, a total of 150 were scored and 145 were polymorphic using 18 RAPD primers (Moulin et al., 2012); and with 15 primers, 86 fragments were produced, which were 100% polymorphic (Zhang et al., 1998). Many studies have reported that DNA fingerprinting techniques are better than phenotypic descriptors for discriminating between related genotypes and for analysis of genetic similarity (Spooner et al., 2005; Solis et al., 2007). According to the study of Moura et al. (2005), there is a point where increased number of fragments does not show a significant increase in experimental accuracy and does not justify the extra effort in labor. From 45 fragments, there is a stabilization of the coefficient of variation, with value less than 10%. This suggests that the results obtained by the fragments used in this study (50) can be used for analysis of diversity. In our results, sweet potato landraces exhibited high variability, with the Jaccard similarity index varying from 0.0 to 0.83. The high level of diversity found in accessions of sweet potato may be associated with spontaneous mutations, knowing that sweet potato presents a high frequency of somatic mutations and is very common in species selection, geographical and environmental factors, which make the species an important genetic resource (He et al., 2006; Love et al., 1978).

Oliveira et al. (2000) also observed high genetic divergence between 51 clones of sweet potato originating from various Brazilian regions. Other studies on amplified fragment length polymorphism (AFLP) markers also detected greater variability (79.8%) in the comparison between two groups (20.2%); one with 14 genotypes from the New Ireland Island and another with 117 genotypes from New Guinea Island, collected in 26 farm plots in four provinces of Papua New Guinea (Fajardo et al., 2002). Genetic diversity indicated by I (0.42) and H (0.27) in SPGB can be considered low with means of low diversity for SPGB, being necessary to insert new accesses to promote increased diversity and more likely to use these resources. It has been suggested that during the spreading of sweet potato accessions from one

country to another, some accessions became known by other new name, which can explain the high similarity of some accessions (McGregor et al., 2001). The average genetic diversity (H) obtained is in agreement with those found for other species like *Solanum tuberosum* (0.49) (Huamán et al., 2000). In contrast to sweet potato, some regions of the globe have a genetic diversity very high as Mesoamerica (0.71), Venezuela-Colombia (0.70) and Peru-Ecuador (0.52). This reflects the richness and evenness of Latin American sweet potato gene pool and center of genetic diversity (Zhang et al., 2000).

The assessing diversity is also important for the construction of a 'core collection' (Zhang et al., 1998); in our case, this makes possible the first preliminary analysis of the first germoplasm bank of Northeastern Brazil and a base for the creation of a 'core collection'. The genotype, 1223 has a commercial production of 155% higher than the national average and 162% higher than the average of Sergipe State (Nunes et al., 2009). Genetically, in the SPGB, three types of sweet potatoes are associated (1192, 1219 and 1226). According to the study of Nunes et al. (2009), the genotypes 1228, 1225 and 1226 have a similar commercial production with 'Italiana'; however, in our study, this genotypes are not interrelated. Knowledge of the genetic variability of sweet potato using RAPD markers may contribute to the development of strategies to guide conservation and management program. We recommend creating a strategic plan for the genotypes with good commercial production characteristics and formation of new commercial varieties. Furthermore, it is important to insert new sweet potatoes for increasing genetic variability in the germoplasm bank.

Conclusion

The genetic variation and genetic relationships among genotypes were efficiently determined using RAPD markers. The discrimination of sweet potatoes from Sergipe State (Brazil) and identification of genotypes more genetically and divergently may contribute to the efforts put in breeding programs and commercial exploitation.

Conflict of Interests

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

REFERENCES

Barnaud A, Triguero G, McKey D, Joly HI (2008). High outcrossing rates in fields with mixed sorghum landraces: How are landraces maintained? Heredity 101:445-452.

Cavalcante M, Ferreira PV, Paixão SL, Costa JG, Pereira RG, Madalena JAS (2009). Potenciais produtivo e genético de clones de batata-doce. Acta Scientiarum. Agronomy 31: 421-426. doi: 10.4025/actasciagron. 313:835

Carvalho SVA, Silva-Mann, RS, Ferreira, RA, Melo, MFV, Souza, DCL (2013). Diversidade genética. In: Gomes, LJ, Silva-Mann, RS, Mattos, PP, Rabbani, ARC (Org.). Pensando a biodiversidade: aroeira (*Schinus terebinthifolius* Raddi). 1ed. São Cristóvão: Edufs, 2013, pp.89-108. doi: 10.7198/8-857822-349-6-01.

Cervantes-Flores JC, Davis EL, Yencho GC (2002). Efficient evaluation of resistance to three root-knot nematode speciesin selected sweetpotato cultivars. HortScience 37:390-392.

Coelho SG (2000). Software DBOOT - Avaliação dos erros associados a estimativas de distâncias/similaridades genéticas através do procedimento de bootstrap com número variável de marcadores, Goiânia, Brasil, Universidade Federal de Goiás 1:1.

Costa TS, Silva AVC, Lédo AS, Santos ARF, Silva Junior JF (2011). Diversidade genética de acessos do banco de germoplasma de mangaba em Sergipe. Pesquisa Agropecuária Brasileira. 46:499-508.

Daros M, Amaral Júnior AT, Pereira TNS, Leal NR, Freitas SP, Sediyama T (2002). Caracterização morfológica de acessos de batata-doce. Horticultura Brasileira, 20:43-47.

Evanno G, Regnaut S, Goudet J (2005). Detecting the number of clusters of individuals using the software Structure: a simulation study. Molecular Ecology, 14: 2611-2620. Doi: 10.1111/j.1365-294X.2005.02553.x

Excoffier L, Smouse PE, Quattro JM (1992). Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics, 131:479-491.

Fajardo DS, La Bonte DR, Jarret RL (2002). Identifying and selecting for genetic diversity in Papua New Guinea sweet potato *Ipomoea batatas* (L.) Lam. germplasm collected as botanical seed. Genetic Resources and Crop Evolution, 49:463-470.

Falush D, Stephens M, Pritchard JK (2007). Inference of population structure using multilocus genotype data: Dominant markers and null alleles. Mol. Ecol. Notes, 7:574-578.

FAOSTAT - FAO Statistical (2013). Food and Agricultural commodities production. Sweet potatoes. Year 2011. Food and Agriculture Organization of the United Nations. < http://faostat.fao.org/site/339/default.aspx >

Garris A, Mccouch S, Kresovich S (2003). Population structure and its effect on haplotype diversity and linkage disequilibrium surrounding the *xa5* locus of rice (*Oryza sativa* L.). Genetics, 165:759-769.

Ghislain M, Zhang D, Fajardo D, Huamann Z, Hijmans RH (1999). Marker-assisted sampling of the cultivated Andean potato *Solanum phureja* collection using RAPD markers. Genetic. Resources Crop Evolution. 46:547-555.

Gonçalves Neto AC, Maluf WR, Gomes LAA, Gonçalves RJS, Silva VF, Lasmar A (2011). Aptidões de genótipos de batata-doce para consumo humano, produção de etanol e alimentação animal. Pesquisa agropecuária Brasileira, 46:1513-1520.

Gonçalves Neto AC, Maluf WR, Gomes LAA, Maciel GM, Ferreira RPD, Carvalho RC (2012). Correlação entre caracteres e estimação de parâmetros populacionais para batata doce. Horticultura brasileira, 30:713-719.

Goulão L, Cabrita L, Oliveria CM, Leitão MJM (2001). Comparing RAPD and AFLP analyis in discrimination and estimation of genetic similarities among apples (*Malusdomestica*Borkh.) cultivars. Euphytica.119: 259-270.

Hampl V, Pavlícek A, Flegr J (2001). Construction and bootstrap analysis of DNA fingerprinting-based phylogenetic trees with a freeware program FreeTree: Application to trichomonad parasites. Inter. J. Syst. Evol. Microbiol. 51:731-735.

He X, Liu Q, Ishiki K, Zhai H, Wang Y (2006). Genetic Diversity and genetic relationships among Chinese Sweetpotato landraces revealed by RAPD and AFLP markers. Breeding Science, 56:201-207.

Huamán ZR, Ortiz DP, Zhang, Rodriguez F (2000). Isozyme analysis of entire and core collections of *Solanum tuberosum* subsp. andigena potato varieties. Crop Science 40:273-276.

IBGE - Instituto Brasileiro de Geografia e Estatística (2010). Produção agrícola municipal: culturas temporárias e permanentes, v. 37. Brazil: Ministério do Planejamento, Orçamento e Gestão.

Jaccard P (1908). Nouvellesrecherchessur la floraledistribuição. Bul. Soc.Science VaudoiseNaturales, 44:223-270.

Jaccard P (1908). Nouvelles recherches sur la distribution florale. Bul. Soc. Vaudoise Sci. Nat. 44:223-270.
Lynch M, Milligan BG (1994). Analysis of population genetic structure with RAPD markers. Mol. Ecol. 3:91-99.
Love JE, Hernandez P, Mahmood M (1978). Performance of 'Centennial' sweet potato mutants. HortScience, 13:578- 579.
McGregor C, Greyling M, Banda J, Laurie S (2001) DNA fingerprinting of sweetpotato (Ipomoea batatas L.): Two case studies in Africa. Acta Horticulturae 546:243-248.
Maguire TL, Peakall R, Saenger P (2002). Comparative analysis of genetic diversity in the mangrove species *Avicennia marina* (Forsk.)Vierh.(Avicenniaceae) detected by AFLPs and SSRs. Theor. Appl. Gen. 104:388-398.
Martins ECA, Peluzio JM, Coimbra RR, Oliveira Júnior WP (2012). Variabilidade fenotípica e divergência genética em clones de batata doce no estado do Tocantins. Ciência Agronômica, 43:691-697.
Michalakis Y, Excoffier L (1996). A generic estimation of population subdivision using distances between alleles with special reference for microsatellite loci. Genetics 142:1061-1064.
Moulin MM, Rodrigues R, Gonçalvez LSA, Sundré CP, Pereira MGA (2012). Comparison of RAPD and ISSR markers reveals genetic diversity among sweet potato landraces (*Ipomoea batatas* (L.) Lam.). Acta Scientiarum Agronomy, 34:139-147.
Moura NF, Chaves LJ, Vencovsky R, Zucchi MI, Pinheiro JB, Morais LK, Moura MF (2005). Seleção de marcadores RAPD para o estudo da estrutura genética de populações de *Hancornia speciosa* Gomez. Bioscience Journal, 21:119-125.
Neiva IP, Andrade Júnior VC, Viana DJS, Figueiredo JA, Mendonça Filho CV, Parrella RAC, Santos JB (2011). Caracterização morfológica de acessos de batata-doce do banco de germoplasma da UFVJM, Diamantina. Horticultura Brasileira 29:537-541.
Nunes MUC, Santos JR, Sousa EF (2009). Produtividade de clones e cultivares de batata-doce com diferentes colorações de polpa em sistema de produção orgânico em Sergipe. Aracaju: Embrapa Tabuleiros Costeiros, p.16. <http://www.cpatc.embrapa.br/publicacoes_2009/bp_52.pdf.>
Oliveira ACB, Sediyama MAN, Sediyama T, Cruz CD (2000). Avaliação da divergência genética em batata-doce por procedimentos multivariados. Acta Scientiarum 22:895- 900, 2000.
Oliveira MSP, Amorim EP, Santos JB, Ferreira DF (2007). Diversidade genética entre acessos de açaizeiro baseada em marcadores RAPD. Ciência e Agrotecnologia 31:1645-1653.
Page RDM (1996). Treeview: An application to display phylogenetic trees on personal computers. Comput. Appl. Biosci. 12:357-358.
Peakall R, Smouse PE (2006). GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. Mol. Ecol. Notes, 6:288-295.
Pritchard JK, Stephens M, Donnelly P (2000). Inference of population structure using multilocus genotype data. Genetics, 155:945-959.
Rao VR, Debouck T, Iwanaga M (1994). The role of international organizations in root and tuber crop conservation. In"The First Ministry of Agriculture, Forestry and Fisheries, Japan International Workshop on Genetic Resources—Root and Tuber Crops" 15-17 March 1994, Tsukuba, MAFF, Japan. p. 7-22.
Ritschel PS, Huamán Z (2002). Variabilidade morfológica da coleção de germoplasma de batata-doce da Embrapa-Centro Nacional de Pesquisa de Hortaliças. Pesquisa Agropecuária Brasileira 37:485-492.
Santos ARF, Ramos-Cabrer AM, Diaz-Hernandez M, Pereira-Lorenzo S (2011). Genetic variability and diversification process in local pear cultivars from northwestern Spain using microsatellites. Tree Genetic and Genome, 7:1041-1056. DOI: 10.1007/s11295-011-0393-3.
Silva AVC, Martins ABG (2006). Identificação de marcas moleculares associadas à ausência de sementes em videira. Ciência Rural, 36:801-806.
Solis JS, Ulloa DM, Rodriguez LA (2007). Molecular description and similarity relationships among native germplasm potatoes (Solanum tuberosum ssp. Tuberosum L.) using morphological data and AFLP markers. Electr. J. Biotechnol. 10:436-443.
Spooner DM, Mclean K, Ramsay G, Waugh R, Bryan GJ (2005). Single domestication for potato based on multilocus amplified fragment length polymorphism genotyping. PNAS 102:14694-14699.
Veasey EA, Borges A, Rosa MS, Queiroz-Silva JR, Bressan EA, Peroni N (2008). Genetic diversity in Brazilian sweet potato (*Ipomoea batatas* (L.) Lam., Solanales, Convolvulaceae) landraces assessed with microsatellite markers. Gen. Mol. Biol. 31:725-733.
Wang Q, Zhang L, Wang B, Yin Z, Feng C, Wang Q (2010). Sweetpotato viruses in China. Crop Protection, 29:110-114. <http://www.sciencedirect.com/science/article/pii/S0261219409002841>.
Yoshimoto M (2001). New trends of proceccing and use of sweet-potato in Japan. Farming Japan 35:22-28.
Zhang D, Ghislain M, Huaman Z, Golmirzaie A, Hijmans R (1998). RAPD variation in sweetpotato (*Ipomoeabatatas* (L.) Lam) cultivars from South America and Papua New Guinea. Genetic Resources and Crop Evolution 45:271-277.
Zhang DP, Cervantes J, Huamán Z, Carey E, Ghislain M (2000). Assessing genetic diversity of sweet potato (*Ipomoea batatas* (L.) Lam.) cultivars from tropical America using AFLP. Genet Resource Crop Evolution, 47:659-665.
Zhao K, Zhou M, Chen L, Zhang D, Robert GW (2007). Genetic Diversity and Discrimination of Chimonanthuspraecox (L.) Link Germplasm Using ISSR and RAPD Markers. Hortscience. 42:1144 - 1148.

academicJournals

Vol. 13(10), pp. 1117-1122, 5 March, 2014
DOI: 10.5897/AJB2013.13347
ISSN 1684-5315 ©2014 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Evaluation of genetic diversity in different genotypes of *Gerbera jamesonii* Bolus using random amplified polymorphic DNA (RAPD) markers

Priyanka Prajapati[1]*, Alka Singh[1], N. L. Patel[2], Diwakar Singh[3] and Vishal Srivastav[3]

[1]Department of Floriculture and Landscape Architecture, ACHF, NAU, Navsari, India.
[2]Department of Fruit Science, ACHF, NAU, Navsari, India.
[3]Department of Plant Molecular Biology and Biotechnology, ACHF, NAU, Navsari, India.

Accepted 17 February, 2014

Genetic diversity within *Gerbera jamesonii* Bolus is the key to genetic improvement of this important ornamental species. In the present study, genetic diversity of 12 accessions of gerbera was assessed through random amplified polymorphic DNA (RAPD) markers. A total of 40 RAPD primers belonging to OPD, OPE, OPF and OPG series of universal primers set were used out of which, only 10 primers produced clear, reproducible and scorable bands. Ten (10) decamer RAPD primers produced a total of 49 scorable bands from 12 genotypes of *G. jamesonii* Bolus, out of which 42 were polymorphic and seven were monomorphic. The percentage of polymorphism ranged from a maximum of 100.00% by OPE-02, OPE-14, OPF-18, OPG-18, OPG-16 and OPG-17 to a minimum of 50.00% by OPE-08. The RAPD profiles generated were further evaluated for studying the Jaccard's similarity coefficient. The average genetic similarity coefficient for the 12 accessions evaluated by Jaccard index was 0.66 ranging from 0.35 to 0.86. The RAPD amplification data were used to obtain similarity matrix and for generation of dendrogram using unweighted pair group method with arithmetic averages (UPGMA) method. Based on the dendrogram, all 12 genotypes could be distinctly divided into two clusters and the accession CF Orange was found most dissimilar from other accessions. This study shows that DNA based molecular marker RAPD is a powerful, less time consuming and cost effective molecular technique for assessment of genetic diversity among different genotypes of *G. jamesonii* Bolus. The availability of these gerbera RAPD markers would facilitate the use of molecular markers in gerbera breeding and genetic studies.

Key words: *Gerbera jamesonii* Bolus, molecular markers, genetic diversity, RAPD.

INTRODUCTION

Cultivated gerbera (*Gerbera jamesonii* Bolus) is one of the most important cut and pot flowers worldwide, ranking fifth, only after rose, carnation, chrysanthemum, and tulip, in the global cut flower trade (Bhatia et al., 2009; Teeri et al., 2006). It is valued for its unique and attractive flower forms and bright colours. Pioneered in the late ninetieth century in England, gerbera breeding has been very active in Netherlands, Denmark, Germany, United States, Israel and Japan (Kloos et al., 2005; Rogers and Tjia, 1990). Numerous cultivars have been released as cut

*Corresponding author: E-mail: priyanka.p.prajapati@gmail.com.

Abbreviations: RAPD, Random amplified polymorphic DNA; **UPGMA,** unweighted pair group method with arithmetic averages.

flowers, pot flowers, or garden plants. Remarkably, no less than 1,150 gerbera cut flower cultivars were available from the Dutch flower auctions alone (Anonymous, 2001). Although different in important horticultural traits (e.g. flowering time, bloom height, count and longevity), many cultivars show only minor differences in leaf and flower morphology and are difficult to identify correctly based on morphology. This difficulty also exists in distinctness, uniformity and stability (DUS) tests of new gerbera cultivars for registration.

Traditionally, breeding of gerbera was based on hybridization among cultivars and phenotypic selection of novel or improved progeny, followed by clonal propagation of released cultivars. Increasingly, gerbera breeding is shifted toward seed propagated F1 hybrids for better uniformity and lower production costs (Rogers and Tjia, 1990), and incorporation of disease resistance and stress tolerance into new cultivars for better performance and sustainability (Deng and Harbaugh, 2008; Kloos et al., 2005). Molecular markers are being sought as a powerful tool to assist gerbera breeders' pursuit of these new breeding goals. Molecular markers have become an indispensable tool for breeding and cultivar development in many crops (Varshney et al., 2005). They are frequently required for correct identification of cultivars, accurate assessment of genetic relationships and diversity, efficient tagging and mapping of desirable genes, and early selection of superior genotypes. Apart from marker systems such as restriction fragment length polymorphism (RFLP), mini- and micro-satellites, RAPDs have proved to be very useful for the analysis of large numbers of genotypes (Debener et al., 1996).

The main advantage of random amplified polymorphic DNA (RAPD) markers over other molecular markers, in particular to markers involving DNA-DNA hybridization techniques, is the low technical input and small quantity of DNA needed for the analysis. The present study has been planned to evaluate genetic diversity among 12 accessions of cultivated gerbera genotypes using DNA based molecular marker RAPD.

MATERIALS AND METHODS

Plant material

The present investigation was conducted at Department of Plant Molecular Biology and Biotechnology, ASPEE College of Horticulture and Forestry, Navsari Agricultural University, Navsari, Gujarat, India during 2011-12. Twelve (12) genotypes of tissue cultured plantlets viz., Stanza, Fana, CF Gold, Diego, Cherany, CF Orange, Lion, Venezia, Torbin, Jaffana, Kento and Ice Queen, were planted in 2009, two years before the commencement of the present study at greenhouse complex of ACHF, NAU, Navsari. Eight week old plantlets of these 12 genotypes were procured from Germini Agro Pvt. Ltd., Pune, Maharashtra, India.

DNA isolation and RAPD analysis

The genomic DNA was extracted from the young leaves following the cetyldimethyl ammonium bromide (CTAB) method of (Keim et al., 1988) with some modifications. Fresh 10 days old leaves (1 g) from each genotype were powdered in liquid nitrogen using pre-cooled pestle and mortar. The resulting powder was transferred to a 30 ml test tube and extracted for 45 min at 65°C with 5 ml of pre-warmed (65°C) extraction buffer. Equal volume of chloroform: isoamyl alcohol (24:1) was added and transferred in new centrifuge tube. The mixture was centrifuged at 5000 rpm for 10 min at 10°C. This process was repeated and the aqueous phase was transferred into another tube containing equal volume of chilled isopropanol. The sample was incubated at -20°C for 1 h to precipitate nucleic acid. Tubes were centrifuged to collect precipitate. The pellete was washed with 70% ethanol, vacuum dried, dissolved in Tris-EDTA (TE) buffer, pH 8.0, containing RNAase (50 µg ml^{-1}), and incubated for 1 h at 37°C for RNA degradation. Degraded RNA was then removed with equal volume of chloroform: isoamyl alcohol (24:1). DNA was precipitated with equal volume of chilled isopropanol and incubated at -20°C for 30 min, followed by centrifugation at 10,000 rpm for 10 min. The pellete of DNA was washed twice with 70% ethanol, dried at room temperature and resuspended in 100 µl TE buffer.

The reaction volume of 25 µl was subjected to amplification through PCR in a thermal cycler (Eppendorf, Germany) along with a control (without genomic DNA). Prior to amplification, reaction mixture was gently tapped and spun briefly. The genomic DNA was amplified using random primers of OPB, OPC, OPD, OPE, OPF, OPG and OPI series (Operon Tech., California, USA). The PCR reactions for RAPD were carried out in a 25 µl of reaction mixture as described by William et al. (1990). The reaction buffer consisted of 2.5 µl of 10X Taq Buffer with 15 mM $MgCl_2$, 0.4 µl dNTPs mix (2.5 mM each), 0.6 µl Taq DNA polymerase (3 U μl^{-1}), 2 µl genomic DNA (30 ng) and 17.5 µl sterile distilled water. The primers showing polymorphic bands were then used for analyzing the genetic diversity. PCR amplification was performed in a DNA thermal cycler and amplified products were resolved by electrophoresis on 1.5% agarose gel in tris-borate EDTA (TBE) buffer stained with 0.5 µg/ml ethidium bromide and photographs were taken by MultiDoc Digital Imaging System UVP.

Data scoring and analysis

Consistently, well-resolved fragments in the size range of 100 bp to 2.5 kb were manually scored. Each band was treated as a marker. The scoring of bands was done on the basis of their presence (1) or absence (0) in the gel. The genetic associations were evaluated by calculating the Jaccard's similarity coefficient for pair-wise comparisons based on the proportion of shared bands produced by the primers. The similarity matrix was subjected to the cluster analysis of unweighted pair group method with arithmetic averages (UPGMA) and a dendrogram was generated by using NTSYS-pc version 2.1 software (Rohlf, 2000).

RESULTS

Forty (40) RAPD primers (Operon Technologies Inc., Germany) belonging to OPD, OPE, OPF and OPG series of universal primers set were used for initial screening. However, primers from OPD showed no amplification, it may be due to absence of complementary sequence in the genome. Ten primers viz., OPE-02, OPE-08, OPE-13, OPE-14, OPF-18, OPG-03, OPG-10, OPG-13, OPG-16 and OPG-17 were selected for evaluating molecular differences existing among genotypes. The nucleotide sequences of each primer are shown in Table 1. The number of scorable bands for each RAPD primer varied from 2 (OPE-02) to 6 (OPE-08, OPE-13, OPG-03 and OPG-10) as shown in Table 1. Ten (10) RAPD primers produced 49 distinct and scorable bands, with an

Table 1. Details of amplification obtained with different RAPD primers in different varieties of gerbera (*Gerbera jamesonii* Bolus).

Name of primer	Sequence details	Total number of bands	Number of monomorphic bands	Number of polymorphic bands	Polymorphism (%)	Total number of bands amplified
OPE 02	5'-GGTGCGGGAA-3'	2	0	2	100.00	17
OPE 08	5'-TCACCACGGT-3'	6	3	3	50.00	51
OPE 13	5'-CCCGATTCGG-3'	6	1	5	83.33	32
OPE 14	5'-TGCGGCTGAG-3'	5	0	5	100.00	40
OPF 18	5'-TTCCCGGGTT-3'	5	0	5	100.00	42
OPG 03	5'-GAGCCCTCCA-3'	6	0	6	100.00	49
OPG 10	5'-AGGGCCGTCT-3'	6	1	5	83.33	49
OPG 13	5'-CTCTCCGCCA-3'	5	2	3	60.00	41
OPG 16	5'-AGCGTCCTCC-3'	3	0	3	100.00	28
OPG 17	5'-ACGACCGACA-3'	5	0	5	100.00	23
Total		49	7	42	-	372

Table 2. Jaccard's similarity coefficient among different varieties of gerbera (*Gerbera jamesonii* Bolus) based on the RAPD data.

	Stanza	Fana	CF Gold	Diego	Cherany	CF Orange	Lion	Venezia	Torbin	Jaffana	Kento	Ice Queen
Stanza	1.000											
Fana	0.718	1.000										
CF Gold	0.600	0.725	1.000									
Diego	0.634	0.800	0.725	1.000								
Cherany	0.639	0.641	0.488	0.641	1.000							
CF Orange	0.485	0.421	0.457	0.459	0.394	1.000						
Lion	0.667	0.707	0.675	0.707	0.632	0.486	1.000					
Venezia	0.711	0.795	0.763	0.750	0.590	0.486	0.744	1.000				
Torbin	0.692	0.775	0.744	0.821	0.615	0.472	0.816	0.865	1.000			
Jaffana	0.610	0.690	0.659	0.651	0.537	0.472	0.683	0.725	0.707	1.000		
Kento	0.559	0.487	0.571	0.450	0.351	0.429	0.556	0.556	0.500	0.500	1.000	
Ice Queen	0.488	0.610	0.537	0.571	0.415	0.371	0.641	0.600	0.625	0.548	0.625	1.000

average of 4.9 bands per primer. Each primer generated a unique set of amplification products (Figure 1). A perusal of data shown in Table 1 reveals that 10 decamer primers produced a total of 49 scorable bands in the 12 genotypes of *G. jamesonii* Bolus, out of which 42 were polymorphic and seven were monomorphic. The percentage of polymorphism ranged from a maximum of 100.00% by OPE-02, OPE-14, OPF-18, OPG-18, OPG-16 and OPG-17 to a minimum of 50.00% by OPE-08.

The RAPD profiles generated were further evaluated for studying the Jaccard's similarity coefficient (Table 2). The RAPD amplification data were used to obtain similarity matrix (Table 2) and for generation of dendrogram (Figure 2) using UPGMA method. The dendrogram obtained clearly indicated two distinct major clusters I and II. The genotype (CF Orange) found in second cluster was having minimum similarity with other genotypes while rest of the genotypes were laid in first cluster. It is evident from Table

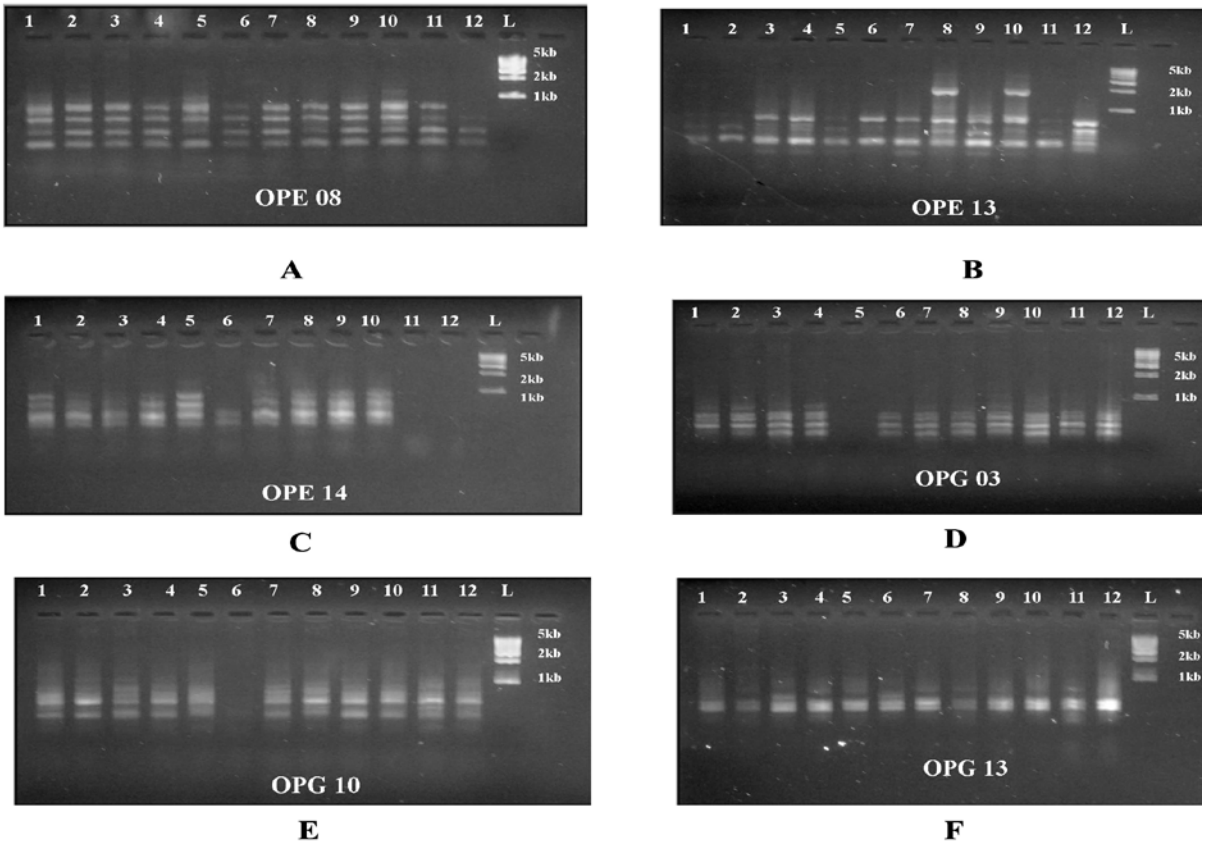


Figure 1. RAPD amplification pattern of 12 different accessions of *Gerbera jamesonii* Bolus using RAPD primers. (Lanes 1-12: 12 accessions of gerbera that is, Stanza, Fana, CF Gold, Diego, Cherany, CF Orange, Lion, Venezia, Torbin, Jaffana, Kento and Ice Queen respectively). A, Amplification pattern with OPE 08; B, amplification pattern with OPE 13; C, amplification pattern with OPE 14; D, amplification pattern with OPG 03; E, amplification pattern with OPG 10; F, amplification pattern with OPG 13.

2 that the lowest genetic similarity coefficient is 0.35 between cluster I and II. Moreover, this major cluster was again divided into two sub-clusters; I (1) and I (2). The sub-cluster I (1) was consisting of nine genotypes (Stanza, Fana, Diego, Venezia, Torbin, Cherany, Lion, CF Gold and Jaffana) while the second sub-cluster I (2) consisting of only two genotypes (Kento and Ice Queen).

The present results show that RAPD can be used for evaluating the molecular variation existing among twelve accessions of gerbera (*G. jamesonii* Bolus). It is evident from the result that some of the primers sequences exhibited 100% polymorphism, which may be more useful to differentiate the gerbera accessions as compared to other primers (Sulan et al., 2002). An UPGMA dendrogram was generated from the Jaccard's similarity values using NTSYS-pc software version 2.1 (Figure 2). Based on this dendrogram, Fana - Diego, Venezia - Torbin and Kento - Ice Queen were found parallel to each other.

DISCUSSION

PCR based markers are gaining much popularity nowadays to assess genetic diversity among horticultural crops. Diversity analysis of any crop species is first step towards crop improvement. The results of present study could assess diversity among 12 accessions of gerbera through RAPD. The absence of genetic variation using RAPD has also been reported in micro-propagated shoots of *Pinus thunbergii* (Goto et al., 1998), *in vitro*-regenerated turmeric (Salvi et al., 2001) and *in vitro*-raised bulblets of Lilium (Varshney et al., 2001). A similarity matrix based on Jaccard's coefficient revealed that the pair-wise varieties have more similarity.

Similar results were also obtained by Sreedhar et al. (2007) during the clonal fidelity analysis of long-term micropropagated shoot cultures of vanilla (*Vanilla planifolia* Andrews) by RAPD and inter-simple sequence repeat (ISSR) markers. Earlier, Reynoird et al. (1993) also did not observe any phenotypic variations during vegetative and reproductive phases among the regenerates of gerbera.

Plants regenerated from adventitious buds around axillary buds or from other well-developed meristematic tissues showed the lowest tendency for genetic variation (Joshi and Dhawan, 2007). Even plants derived from organised meristems are not always genetically true to the type in many crops (Devarumath et al., 2002).

Molecular marker study also revealed that the pairs of

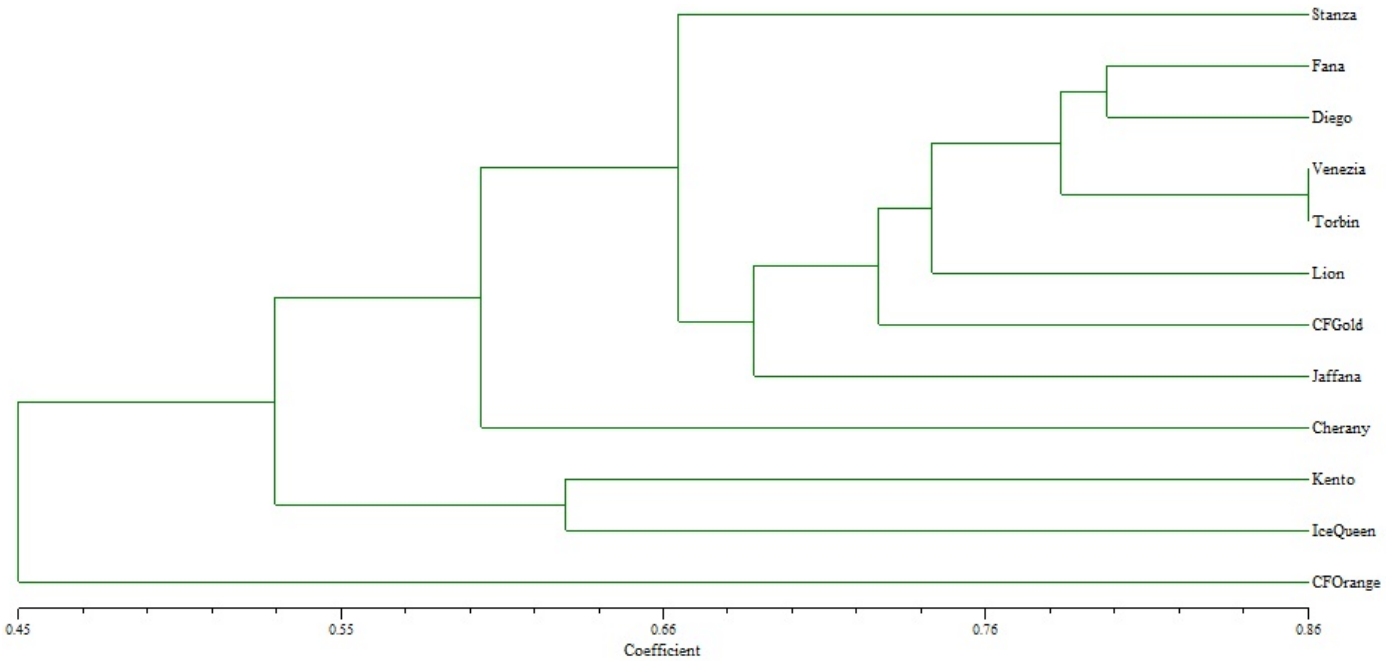


Figure 2. Dendrogram depicting the genetic relationship among different accessions of *Gerbera jamesonii* Bolus based on pooled RAPD data.

the cultivars have very less divergent ability and they are genetically similar to each other. Based on present investigation a good amount of genetic difference was noticed among all the twelve accessions through RAPD molecular marker. Screening of more number of primers is recommended to evaluate the present set of accessions. Moreover, screening of more accessions may also give some divergence. Similar finding was reported by Mo-Suk et al. (1999) in 52 melon lines, Huang and Sun (2000) in *Ipomea* sp., Benedetii et al. (2000) in 5 putative interspecific hybrids of Alstroemeria and Lee et al. (2005) in 55 interspecific hybrids between *Dianthus giganteus* and *Dianthus carthusianorum* and their parents.

In the present study, the average genetic similarity coefficient for the 12 accessions, evaluated by Jaccard index was 0.66 ranging from 0.35 to 0.86. Similar results were reported by Da Mata et al. (2009) for a total of 42 accessions of *G. jamesonii* with 0.55 average genetic similarity coefficients. The banding profiles showed a total of 42 polymorphic and 7 monomorphic bands from twelve accessions of gerbera. The results show that RAPD is a fast, relatively inexpensive and useful technique for genetic divergence characterization between different cultivars of *G. jamesonii* (Rezende et al. 2009).

Conclusion

In summary, significant diversity has been reported among 12 accessions of gerbera through RAPD. Analyses of more number of primers are recommended to evaluate the present set of accessions. Screening of more gerbera accessions with RAPD primers may give further divergence.

ACKNOWLEDGMENTS

Continuous technical support from the Head of Department of Plant Molecular Biology and Biotechnology, ACHF, NAU, Navsari is dully acknowledged.

REFERENCES

Anonymous., 2001. Classic gerbera with a new look. http://www.flowercouncil.org/uk/news/fpnews/Productnieuws_2001/mrt2001_Gerbera.asp?Source, Accessed 4 Feb 2004.

Bhatia R, Singh, KP, Jhang T, Sharma, TR (2009). Assessment of clonal fidelity of micropropagated gerbera plants by ISSR markers. Sci. Hortic. 119:208-211.

Da Mata YL, Inês Segeren M, Fonseca AS, Colombo CA (2009). Genetic divergence among gerbera accessions evaluated by RAPD. Sci. Hortic. 121:92-96.

Debener T, Bartels C, Mattiesh L (1996). RAPD analysis of genetic variation between a group of rose cultivars and selected wild rose species. Mol. Breed. 2:321-327.

Deng Z, Harbaugh BK, 2(008). Progress in breeding for disease resistance and stress tolerance in caladium, gerbera, and lisianthus. Acta Hortic. 766:399-403.

Devarumath RM, Nandy S, Rani V, Marimuthu S, Muraleedharan N (2002). RAPD, ISSR and RFLP fingerprints as useful markers to evaluate genetic integrity of micropropagated plants of three diploid and triploid elite tea clones representing *Camellia sinensis* (China type) and *C. assamica sp. assamica* (Assam- India type). Plant Cell Rep. 21:166-173.

Goto S, Thakur RC, Ishii K (1998). Determination of genetic stability in long-term micropropagated shoots of *Pinus thunbergii* Parl. using RAPD markers. Plant Cell Rep. 18:193-197.

Huang J, Sun SM (2000). Genetic diversity and relationships of sweet potato and its wild relatives in Ipomea series Batatas (Canvolvulaceae) as revealed by intersimple sequence repeat (ISSR) and restriction analysis of chloroplast DNA. Theor. Appl. Genet. 100:1050-1060.

Joshi P, Dhawan V (2007). Assessment of genetic fidelity of micropropagated *Swertia chirayita* plantlets by ISSR marker assay. Biol. Plant. 51:22-26.

Keim P, Olsen TC, Shoe Maker RC (1988). A rapid protocol for isolating soybean DNA. Soybean Genet. Newslett. 15:150-152.

Kloos WE, George CG, Sorge LK (2005). Inheritance of powdery mildew resistance and leaf macrohair density in Gerbera hybrida. Hortic. Sci. 40:1246-1251.

Lee SY, Yae BW, Kim KS (2005). Segregation patterns of several morphological characters and RAPD markers in interspecific hybrids between *Dianthus giganteus* and *D. carthusianorum*. Sci. Hortic. 105:53-64.

Mo-Suk Y, Lm-Sung LL, Go-Gawn D, Ann-Chong M, Kim-Doo H, 1999. RAPD analysis for genetic diversity of melon species. Korean J. Hort. Sci. Tech. 16:21-24.

Reynoird JP, Chriqui D, Noin M, Brown S, Marie D (1993). Plant regeneration from *in vitro* leaf culture of several gerbera species. Plant Cell Tissue Organ Cult. 33:203-210.

Rezende RKS, Paiva LV, Paiva R, Chalfun Junior A, Torga PP, Masetto TE (2009). Genetic divergence among cultivars of gerbera using RAPD markers. Cienc. Rural [online DOI: http://dx.doi.org/10.1590/S0103-84782009005000176]. 39:2435-2440.

Rogers MN, Tjia BO (1990). Gerbera production. Timber Press, Portland.

Rohlf FJ (2000). NTSYS-pc: numerical taxonomy and multivariate analysis system, version 2.1. Exeter Software, Setauket, New York.

Salvi ND, George L, Eapen S (200). Plant regeneration from leaf base callus of turmeric and random amplified polymorphic DNA analysis of regenerated plants. P. Cell Tiss. Organ Cult. 66:113-119.

Sreedhar RV, Venkatachalam L, Bhagyalakshmi N (2007). Genetic fidelity of long-term micropropagated shoot cultures of vanilla (*Vanilla planifolia* Andrews) as assessed by molecular markers. Biotechnol. J. 2:1007-1013.

Sulan L, Pucchao H, Xuequin Z, Peng Z (2002). Inheritence of RAPD markers in an interspecific F1 hybrid of grape between *Vitis auinquangularis* and *V. vinifera*. Sci. Hortic. 93:19-28.

Teeri TH, Elomaa P, Kotilainen M, Albert VA (2006). Mining plant diversity: Gerbera as a model system for plant developmental and biosynthetic research. Bio Essays 28:756-767.

Varshney A, Lakshmikumaran M, Srivastava PS, Dhawan V (2001). Establishment of genetic fidelity of *in vitro* raised *Lilium* bulblets through RAPD markers. In Vitro Cell Dev. Biol. Plant. 37:227-231.

Varshney RK, Graner A, Sorrells ME (2005). Genic microsatellite markers in plants: features and applications. Trends Biotechnol. 23:48-55.

William JGK, Kubelik KJ, Livak KJ, Rafalski JA, Tingey SV (1990). DNA polymorphisms amplified by arbitrary primers are useful genetic markers. Nucleic Acid Res. 18:6531-6535.

academicJournals

Vol. 13(10), pp. 1123-1130, 5 March, 2014
DOI: 10.5897/AJB12.2448
ISSN 1684-5315 ©2014 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Laboratory evaluation of freshly prepared juice from garlic (*Allium sativum L.*) Liliaceae as protectants against the maize weevil, *Sitophilus zeamais* (Motsch.) [Coleoptera: Curculionidae]

Ifeanyi D. Nwachukwu[1,3] and Elechi F. Asawalam[2]*

[1]Department of Plant Physiology, Institute for Biology, RWTH Aachen University, Worringerweg 1, D-52056 Aachen, Germany.
[2]Department of Plant Health Management, Michael Okpara University of Agriculture, Umudike P.M.B 7267 Umuahia, Abia state Nigeria.
[3]Department of Human Nutritional Sciences, University of Manitoba, W542-190 Dysart Road, Winnipeg, Canada MB R3T 2N2.

Accepted 27 September, 2013

Fresh prepared garlic (*Allium sativum* L.) juice, containing the antimicrobial allicin, was evaluated as a possible grain protectant against the maize weevil, *Sitophilus zeamais* (Motsch.). Each experiment was set out in completely randomized design (CRD) with four replications and a control treatment. Adult mortality and percentage weight loss were investigated. There was an observed increase in adult mortality following days of exposure in all treatments. Statistically significant ($P<0.05$) reduced grain loss was also observed in all the treatments when compared with the control. The juice prepared from an indigenous Nigerian garlic cultivar (GUN) was more lethal (causing 93% adult mortality), when applied topically on the freshly emerged *S. zeamais* adults, compared to the juice prepared from a clove of garlic purchased at a supermarket in Germany (GAG). High performance liquid chromatography (HPLC) analysis indicated that the amount of allicin in GUN was 1883.2 µg/ml while that in GAG was 3500.93 µg/ml. This study highlights the potential of *A. sativum* containing allicin for biorational control of maize grains against *S. zeamais* infestation and damage.

Key words: Allicin, *Allium sativum*, biopesticide, biorational control, crop protectant, *Sitophilus zeamais,* stored product.

INTRODUCTION

Maize (*Zea mays L.*) or corn is a major source of dietary carbohydrate as well as the most important cereal in Sub-Saharan Africa (IITA, 2009), while the maize weevil, *Sitophilus zeamais* (L.) (Coleoptera: Curculionidae), is a major pest of stored maize grain in many regions of the world including Nigeria (Adedire, 2001). Although

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

Abbreviations: GUN, Umuahia main market, Nigeria; **GAG,** supermarket in Aachen, Germany; **HPLC,** high performance liquid chromatography; **DADS,** diallyl disulfide; ***TRPA1*,** transient receptor potential ankyrin-1.

synthetic insecticides have long been widely used in the control of insect pests, the indiscriminate application of synthetic products has led to various problems including toxic residues in the treated products, environmental pollution and growing resistance against insecticides by insects and pests (Huang et al., 1997). There is therefore an urgent need to continue the search for eco-friendly, cheap, sustainable and safe plant protection agents that will not contaminate food products in their use as grain protectants in storage systems for small holder farmers. Moreover, because they are often viewed as "mild" on the environment, compounds of biogenic origin are generally more positively regarded compared to substances partially or completely chemically synthesized in laboratories (Slusarenko et al., 2008), and are therefore more likely to gain wider acceptance among farmers in the long run. Crop protection agents of natural origin also have the advantage of possessing novel modes of action against insects and thus have the potential to reduce the risk of cross-resistance while offering new leads for the design of target-specific molecules (Zhou et al., 2012).

There has been a heightened interest in the last few decades in plants like garlic, which have been equipped by evolution to defend themselves against invading pathogens and pests, not only because of environmental concerns trailing the use of chemically synthesized plant protection products, but also because of farmers' and consumers' preference for organic farming strategies and produce, respectively (Nwachukwu et al., 2012; Slusarenko et al., 2008). For many of such plants, protection against pathogens and pests often comes in the form of sulphur-containing secondary metabolites synthesized following external attacks on them (Nwachukwu et al., 2012). Allicin (diallylthiosulfinate), the major antimicrobial substance in garlic, has attracted the attention of investigators because of its widely acclaimed potency. Garlic is known for its positive effect on health particularly the prevention of cardiovascular diseases and certain digestive cancers (Lalla et al., 2013). Previous studies have shown that Garlic also possess some insecticidal, fungicidal, acaricidal, nematicidal and bactericidal properties (Lalla et al., 2013). With a widespread antimicrobial activity comparable to those of common antibiotics like ampicillin (Curtis et al., 2004) and penicillin (Cavallito et al., 1944), it is hardly surprising that this compound has shown activity against some of the world's most notable plant pathogens including *Phytophthora infestans* and *Pseudoperonospora cubensis* (Portz et al*.,* 2008).

Allicin is a phytoanticipin which means that its synthesis, from preformed precursors already present in garlic, occurs prior to any external attack or irritation, and so does not involve any expenditure of energy (Nwachukwu et al., 2012; van Etten et al., 1994). Allicin is formed as a volatile organosulfur compound following the disruption of garlic tissues either by crushing, piercing, or wounding. The substrate alliin (S-allyl-L-cysteine sulfoxide) held in the cytosol prior to tissue disruption reacts with the now liberated vacuolar enzyme, alliinase to give allicin with pyruvate and ammonia as by-products.

Although the search for and use of plant materials with grain protectant ability is not new (Parugrug and Roxas, 2008; Asawalam and Emosairue, 2006; Asawalam et al., 2006; Rajapakse, 2006; Udo, 2005; Arannilewa et al., 2002; Adedire and Ajayi, 1996; Odeyemi, 1993; Lale, 1992), given the growing role of allicin from garlic in crop protection, we decided to evaluate the efficacy of freshly prepared *Allium sativum* juice as protectants of maize grains against infestation by *S. zeamais*. While significant progress has been recorded in the use of such active agents from natural products, it is hoped that concerted efforts by stored product entomologists will lead to greater success in the biorational control of insects.

MATERIALS AND METHODS

Sitophilus zeamais culture

Adult *S. zeamais* was cultured in the laboratory at 27±2°C, 60-65% r.h and 12 h: 12 h light: dark regime. *S. zeamais* was obtained from stocks maintained at the Crop Science Laboratory, Michael Okpara University of Agriculture, Umudike, Nigeria. The food media used was whole maize grains, purchased from Umuahia main market, Abia State Nigeria. Fifty (50) pairs of *S. zeamais* were introduced into 1 l glass jars containing 400 g weevil-susceptible maize grains. The jars were then covered with nylon mesh held in place with rubber bands. Freshly emerged adults of *S. zeamais* were subsequently used for the experiments.

Preparation and application of *Allium sativum*

The *A. sativum* (Garlic) bulbs used for the study were locally purchased from Umuahia main market, Nigeria (GUN) and from a supermarket in Aachen, Germany (GAG). The garlic juice was prepared by blending axillary buds from composite garlic bulbs using a NAKAI Japan Model 1706 Extractor. Prior to high performance liquid chromatography (HPLC) determination of allicin, the juice was introduced into a sterile 50-ml Falcon tube and centrifuged (Megafuge 1.0R; Heraeus Instruments, Osterode, Germany) at 5000 rpm (3000 g) for 10 min to separate the majority of the pulp from the liquid. Remnants of the pulp were then carefully removed from the top of the liquid with a clean spatula. A diaphragm vacuum pump (Vacuubrand GmbH, Wertheim, Germany) was used to separate the remaining pulp from the pure liquid juice under pressure. The pure filtrate was then transferred to a second sterile Falcon tube and sealed preparatory to HPLC analysis.

Fifty gram of clean and uninfested weevil-susceptible Bende white maize variety used for the study were weighed, using an MP Citizen Electronic weighing balance, and subsequently introduced into four sterilized plastic vials. To each plastic vial, 1 ml of each garlic juice type was added and mixed thoroughly by manual agitation of the vials. A control experiment containing no garlic juice was also set up. Five pairs of adult *S. zeamais* were introduced into treated and untreated maize grains. The lids of the plastic vials were perforated in order to maintain aerobic conditions in the vials. Muslin textile

materials were used to secure the top of the plastic vials and served to ensure aeration while preventing entry or exit of insects. The contents of the plastic vials were then shaken gently for proper and uniform mixing. Each treatment was replicated four times. The samples were arranged in a completely randomized design on a laboratory table.

Mortality and damage assessment assays

The number of dead insects in each vial was counted at 7, 14, 21, 28 and 35 days after treatment to estimate mortality. Maize weevil mortality was assessed as

Maize weevil mortality = Number of dead insects/Total number of insects x 100.

Data on percentage adult weevil mortality were corrected using Abbott's formula (Abbott, 1925) thus:

$$P_T = \frac{P_o - P_c}{100 - P_c}$$

Where, P_T, is the Corrected mortality (%); P_O, is the observed mortality (%); P_C, is the control mortality (%).

Weight loss was assessed by re-weighing the grains to determine percentage weight loss. Percentage weight loss was calculated following the method of the FAO (FAO, 1985) as follows

$$\text{Percentage weight loss} = \frac{[UaN - (U + D)]}{UaN} \times \frac{100}{1}$$

Where, U is the Weight of undamaged fraction in the sample; N is the total number of grains in the sample; Ua, is the average weight of undamaged grains and D, is the weight of damaged fraction in the sample.

Contact toxicity test by topical application

The fresh garlic juice samples at 1 ml dosage were applied uniformly to the bottom of the plastic vials and the control was set up in which there was no garlic juice. Five male and five female adult weevils of about 5 days old were introduced separately into each vial. Each treatment was replicated four times and weevil mortality was recorded after 12, 24, 36 and 48 h of exposure. Insects were presumed dead if they remained immobile and did not respond to five jabs with a blunt dissecting probe after an arbitrary 5-min recovery period.

High-performance liquid chromatography (HPLC) determination of allicin in garlic juice

Determination of the amount of allicin in the garlic juice preparations was performed using a JASCO HPLC. The method used was taken from Krest and Keusgen (2002). HPLC-grade water was used to dilute freshly prepared garlic juice in the ratio of 1:10. Thereafter, 1 ml of the diluted sample was introduced into a sterile vial with the injection volume set at 20 µl.

In order to protect the column, the diluted garlic juice was passed through a polyether sulfon membrane (0.2 µm pore size, Steriflip, Millipore), before introduction into the vial and subsequent injection into the HPLC (JASCO Chromatography Data System, with intelligent UV detector, Jasco Labor-u. Datentechnik GmbH, Groß-Umstadt, Germany). 1.5 ml of a 0.05 mg ml−1 solution (in methanol) of butyl-4-hydroxybenzoate was used as internal standard. Using the HPLC software ChromPass (version 1.8.6.1), a mixed gradient elution [solvent A, 30% (v/v) HPLC grade methanol adjusted to pH 2.0 with 85% (v/v) orthophosphoric acid; solvent B, 100% HPLC grade methanol] was performed. Elution spectra were recorded between 200-600 nm with detection at 254 nm for the chromatogram.

Statistical analysis

Data obtained were subjected to analysis of variance (ANOVA) and significant difference (P>0.05) means were separated by using Student Newmans Keul (SNK) test.

RESULTS

Mortality

The effect of fresh garlic juice on the mortality of *S. zeamais* is presented in Figure 1. The results obtained show that fresh GAG juice with a mortality rate of 73% and GUN at 87% mortality rate were significantly more effective in causing *S. zeamais* mortality at 28 DAT compared to the control.

Contact toxicity test by topical application

Upon exposure to adult *S. zeamais*, fresh GUN juice caused 100% mortality, while GAG juice caused 90% mortality (Figure 2) 48 h after topical application. The control led to zero mortality.

Effect on grain weight

There was significant weight loss of the control (Figure 3) when compared with the maize grains treated with garlic juice indicating the effectiveness of the juice in offering protection to the stored maize grains. While the untreated control grains lost over 8% of original weight on average, the grains treated with GAG and GUN only lost a negligible < 0.5% of average weight after 60 days.

High- performance liquid chromatography (HPLC) analysis of *A. sativum*

The HPLC chromatograms depicting the amount of allicin in the freshly prepared garlic juice is shown in Figures 4 and 5. The amount of allicin in GUN and GAG was found to be 1.88 and 3.50 mg/ml, respectively.

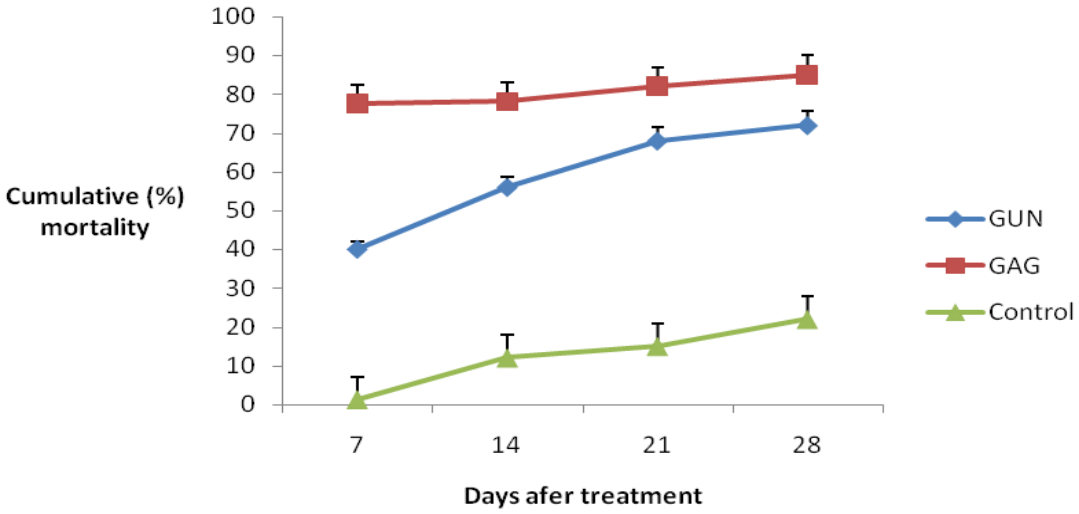


Figure 1. Cumulative mortality of freshly emerged adults of *S. zeamais* after introduction onto maize grains treated with freshly prepared *A. sativum* juice.

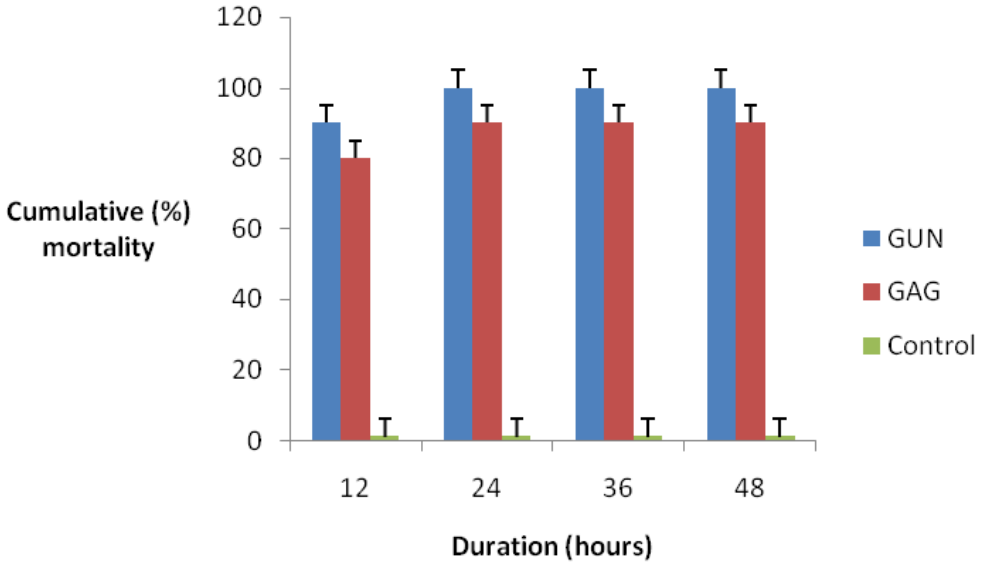


Figure 2. Contact toxicity of fresh garlic juice against *S. zeamais* adult at 48 h after treatment.

DISCUSSION

Over the years, various studies investigating the possible use of agents of biogenic origin as crop protectants have highlighted the potency of natural products as biopesticides (Gonzalez-Coloma et al., 2010; Lee et al., 2004; Isman, 2000; Qi and Burkholder, 1981). For instance, studies examining the fumigant and contact insecticidal effects of 22 plant essential oils against the bean weevil, *Acanthoscelides obtectus* (Regnault-Roger et al., 1993), and of 28 plant essential oils against four adult members of Order *Coleoptera* including the rice weevil *Sitophilus oryzae,* a close relative of *S. zeamais* (Shaaya et al., 1990), have not only added to the impressive body of evidence in literature clearly demonstrating the efficacy of using natural pesticides as biocontrol agents in the open field but also as stored product protectants against pests related to the maize weevil. While none of the two works just cited examined the use of agents from garlic but instead studied the use

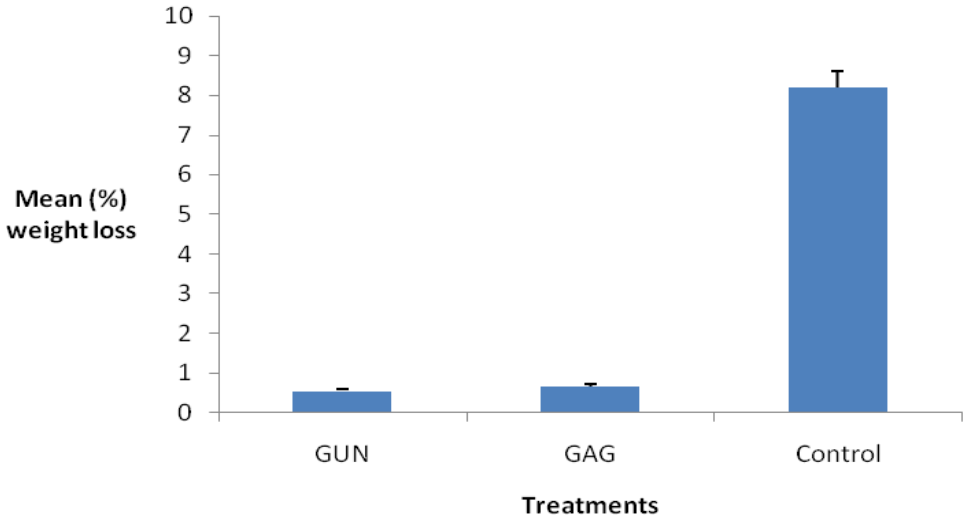
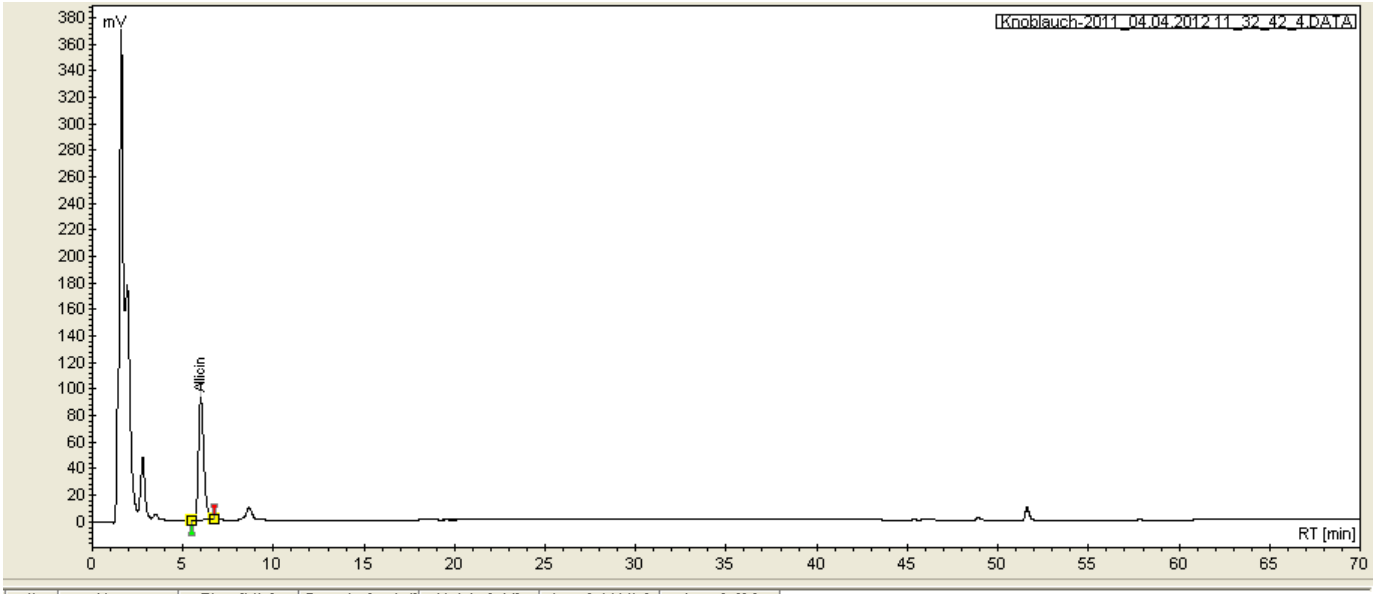


Figure 3. Effect of *A. sativum* juice on weight loss of maize grains.

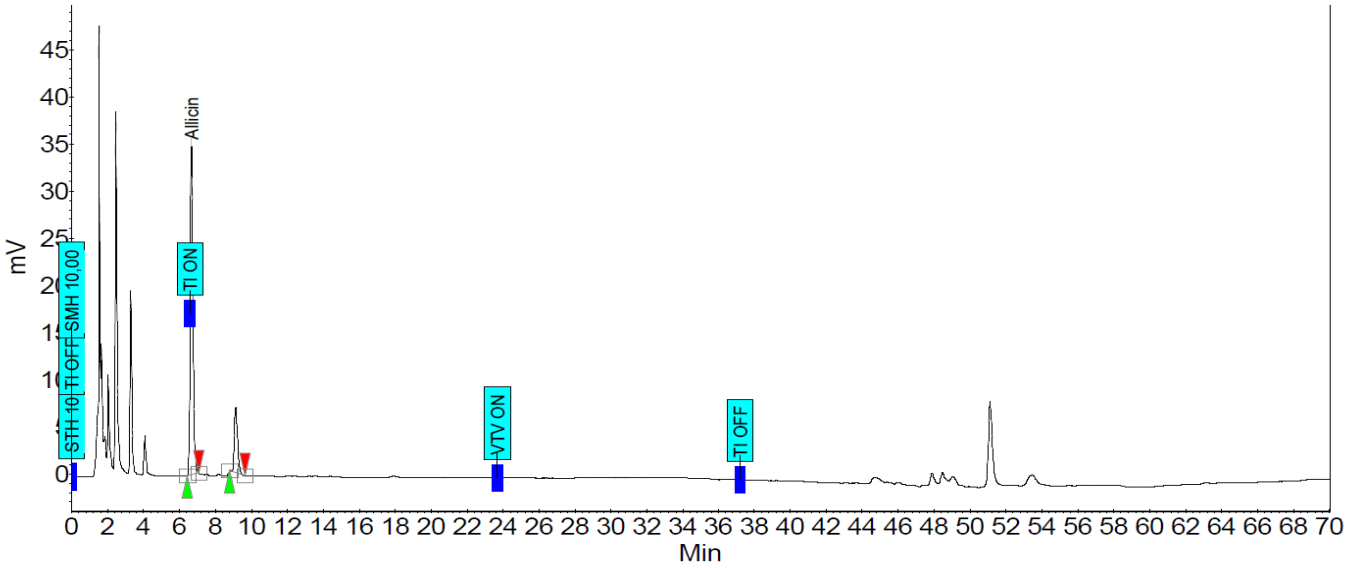


#	Name	Time [Min]	Quantity [ug/ml]	Height [mV]	Area [mV.Min]	Area % [%]
1	Allicin	5,983	188,32	92,2	31,7	100,000
Total			188,32	92,2	31,7	100,000

Figure 4. HPLC chromatogram for GUN allicin determination. The peak for allicin in diluted garlic juice (1:10) was detected at 5.98 min. Amount of allicin was determined to be 1883.2 µg/ml corresponding to an allicin concentration of 11.60 Mm.

of other natural compounds including those from lavender, coriander, lemon and celery, such as α-terpineol, α-terpinene, β-caryophyllen, and carvacrol among others, they provide materials for fascinating comparative analyses of chemical compounds in plants as crop protectants especially as allicin the major biologically active agent in fresh garlic juice is only found in garlic (Cavallito and Bailey, 1944; Cavallito et al., 1944; Jain and Apitz-Castro, 1987).

Research on the potential application of biologically active compounds from garlic abound in literature. For instance, steam-distilled garlic oil has been tested for toxicity against the eggs, larvae and adults of *Tribolium castaneum* and against the adults of *S. zeamais* (Ho et



Peak results :

Index	Name	Time [Min]	Quantity [ug/ml]	Height [mV]	Area [mV.Min]	Area % [%]
1	Allicin	6,658	35,93	35,0	6,1	80,606
Total			35,93	41,9	7,5	100,000

Figure 5. HPLC chromatogram for GAG allicin concentration determination. The peak for allicin in diluted garlic juice (1:100) was detected at 6.66 min. The amount of allicin in the freshly prepared garlic juice was determined to be 3500.93 µg/ml corresponding to an allicin concentration of 21.57 Mm.

al., 1996) while extracts from garlic (and other plants) have been vapourized and used in fumigation tests involving *T. castaneum* and *S. zeamais* (Ho, 2000). The result from this study corroborates Musa (2013) who recorded 100% mortality at 6% w/w in groundnut seeds treated with *A. sativum* clove powder. Ibrahim and Garba (2011) found garlic powder to be effective in the control of maize weevil. Zhao et al (2013) observed that the essential oil of *A. sativum* possessed contact toxicity against overwintering *Cacopsylla chinensis* (Hemiptera:Psyllidae) with an LC_{50} value of 1.42 µg per adult. The two main constituent compounds diallyl trisulfide and diallyl disulfide, exhibited strong acute toxicity against the overwintering *C. Chinensis* with LC_{50} values of 0.64 and 11.04 µg per adult respectively. Feng-Lian et al. (2011) also reported that garlic essential oil diallyl disulfide and diallyl trisulfide inhibited the development of grain moth *Sitotroga cerealella* (Lepidoptera:Gelechidae). Ofuya et al. (2010) proved that fumigation of pods with crushed bulbs of *A. sativum* and *Allium cepa*, showed a toxic effect to *Callosobruchus maculatus.*

Other investigations include those of Hamed et al. (2012), Adedire and Ajayi (2006), and Arannilewa et al. (2006). Each of these works employed garlic essential oils obtained by steam distillation or solvent extraction. However, to the best of our knowledge, no study has been carried out on the use of fresh garlic juice as a biopesticide against *S. zeamais.* A search of the leading electronic database, *Scopus*, returned no hits for each of the search terms “garlic juice weevil”, “garlic juice Sitophilus” and “garlic juice *Sitophilus zeamais*.” Therefore, this work most likely represents a remarkable shift from the conventional approach to studying the use of biologically active compounds from garlic in crop protection. Importantly, given that allicin is volatile and unstable, and upon production rapidly decomposes to other breakdown products like ajoene and the vinyldithiins (Apitz-Castro et al., 1983; Block et al., 1984; Block, 1985; Voigt and Wolf, 1986; Iberl et al., 1990), we propose that allicin could not have been principally responsible for the insecticidal effects reported in previous works investigating the exposure of agricultural pests to garlic essential oil. As comprehensively discussed by Staba et al. (2001), any of a number of treatments/processing of garlic such as freeze drying, steam distillation, oil maceration, ethanolic extraction and low temperature drying, results in the production of

complex mixtures including allicin, diallyl disulfides, dially trisulfides, allyl methyl trisulfides, ajoene, and vinyldithiins.

The results show that although GUN and GAG belong to the same species, they exert slightly different effects on *S. zeamais*- a difference which can be reasonably attributed to their different allicin contents. Our results are similar to the work of Hamed et al. (2012) which recorded mortality rates ranging from 78 -100% following 3-14 days of exposing *Sitophilus oryzae* adults to garlic essential oils. In addition to using garlic essential oils, other similar works such as those by Adedire and Ajayi (2006), and Arannilewa et al. (2006) also employed solvents like petroleum ether and ethanol as extraction vehicles thus making direct and accurate comparisons improbable.

Garlic's pungent smell has been attributed to the presence of organosulfur compounds such as allicin and diallyl disulfide (DADS) in the edible allium (Bautista et al., 2005). It has been suggested that garlic's pungency contributes to its toxicity in weevils by disrupting regular respiratory events (Adedire and Ajayi, 2006). Furthermore, the structures of allicin and DADS are similar to that of allyl isothiocyanate which apart from lending wasabi and other mustard plants their pungency, induces pain and inflammation by activating the transient receptor potential ankyrin-1 (*TRPA1*) ion channel in neuronal cells (Wang and Woolf, 2005; Jordt et al., 2004; Fahey et al., 2001).

Work with neuronal cell cultures have also provided molecular evidence suggesting that allicin is the main sulphur compounds in garlic that excites allyl isothiocyanate-sensitive sensory neurons as well as activate TRPA1 and the related TRPV1 ion channels (Bautista et al., 2005; Macpherson et al., 2005) which are present in pain-sensing neurons. Induction of pain could have significantly contributed to insect mortality by causing considerable stress the maize weevils. Finally, plant essential oils given their lipophilicity are able to penetrate the cuticle of insects (Richards, 1978) thus contributing to lethal effects.

Conclusion

The present findings suggest that freshly prepared garlic juice which has allicin as its main biologically active compound possesses a potentially vital insecticidal effect on *S. zeamais* when compared with the control. Thus, garlic offers significant promise for combating the threat posed by maize weevils to farmers in developing countries. The major thrust of this work is its adaptability for use by small scale farmers plagued by the challenge of not being able to afford conventional pesticides on the market. With no need for the more complex and sophisticated production of essential oils, this work's simplicity expressly lends zest to the overarching essence of providing a quick and easy solution to the problem of pest infestation in third world countries. There is need for further investigations to identify the other garlic juice constituents (apart from allicin) with toxic effects on *S. zeamais*, and to elucidate the precise mechanisms by which they exert their insecticidal effects.

ACKNOWLEDGEMENTS

The research leading to this publication was supported by facility at and funding from Michael Okpara University of Agriculture Umudike Nigeria, the RWTH Aachen University, Germany and the European Community's 7th Framework Programme [FP7/2007-2013] under grant agreement No: 215009.

REFERENCES

Abbott WS (1925). A Method of Computing the effectiveness of an insecticide. J. Econ. Entomol. 18(2):265 - 267.

Adedire CO (2001) Biology, ecology and control of insect pests of stored grains. In: pp 59-94. Ofuya T.I. and Lale, N.E.S. (eds) Pest of stored cereals and pulses in Nigeria. Dave Collins Publications, Nigeria. pp. 59-94

Adedire CO, Ajayi TS (1996). Assessment of insecticidal properties of some plants as grain protectants against the maize weevil, *Sitophilus zeamais* (Motsch.). Nig. J. Entomol. 13: 93-101.

Apitz-Castro R, Cabrera S, Cruz MR, Ledezma E, Jain MK (1983). Effects of garlic extract and of three pure components isolated from it on human platelet aggregation, arachidonate metabolism, release reaction and platelet ultrastructure. Thromb. Res. 32(2):155-69.

Arannilewa ST, EkrakeneT, Akinneye JO (2006). Laboratory evaluation of four medicinal plants as protectants against the maize weevil, *Sitophilus zeamais* Motsch. Afr. J. Biotechnol. 5(21):2032-2036.

Arannilewa ST, Odeyemi OO, Adedire CO (2002). Effects of medicinal plant extract and powder on the maize weevil, *Sitophilus zeamais* Motsch. (Coleoptera: Curculionidae). Ann. Agric. Sci. 3:1-10.

Asawalam EF, Emosairue SO (2006). Comparative efficacy of *Piper guineense* Schum and Thonn and Pirimiphos methyl on [*Sitophilus zeamais* (Motschulsky)]. Trop. Subtrop. Agroecosys. 6:143-148.

Asawalam EF, Emosairue SO, Hassanali A (2006). Bioactivity of *Xylopia aetiopica* (Dunal) A. Rich essential oil constituents on maize weevil *Sitophilus zeamais* (Motschulsky). Electr. J. Environ. Agric. Food Chem. 5(1):1195-1204.

Bautista DM, Movahed P, Hinman A, Axelsson HE, Sterner O et al. (2005). Pungent products from garlic activate the sensory ion channel TRPA1. PNAS 102(34): 12248-12252

Block E (1985). The chemistry of garlic and onions. Sci. Am. 252(3):94-99.

Block E, Ahmad S, Jain MK, Crecely RW, Apitz-Castro R, Cruz MR (1994). (E,Z)-ajoene: a potent antithrombotic agent from garlic. J. Am. Chem. Soc. 106(26):8295-8296.

Cavallito CJ, Bailey JH (1944). Allicin, the antibacterial principle of *Allium sativum*. I. Isolation, physical properties and antibacterial action. J. Am. Chem. Soc. 66(11):1950-1951.

Cavallito CJ, Buck JS, Suter CM (1944). Allicin, the antibacterial principle of *Allium sativum*. II. Determination of the chemical structure. J. Am. Chem. Soc. 66(11):1952-1954

Curtis H, Noll U, Störmann J, Slusarenko AJ (2004). Broad-spectrum activity of the volatile phytoanticipin allicin in extracts of garlic (*Allium sativum* L.) against plant pathogenic bacteria, fungi and Oomycetes. Physiol. Mol. Plant Pathol. 65(2):79-89.

FAO (1985). Prevention of Post-harvest food losses. Training series No.10. Italy, Rome. p. 122.

Feng-Lian Y, Fen Z, Chao-Liang L (2011). Insecticidal activities of garlic substances against adults of grain moth, *Sitotroga cerealella* (Lepidoptera: Gelechidae) Insect Sci. 19(2): 205-212.
Gonzalez-Coloma A, Reina M, Diaz CE, Fraga BM. (2010). Natural Product-Based Biopesticides for Insect Control. In: Comprehensive Natural Products II, Mander L, Liu HW (Eds). Elsevier. Oxford.
IITA (2009). Cereals and legumes systems: Maize. Available at: http://old.iita.org/cms/details/maize_project_details.aspx?zoneid=63andarticleid=273 Accessed on May 20, 2012.
Huang Y, Tan JMW, Kini S, Ho H (1997). Toxic and antifeedant action of nutmeg oil against *Tribolium castaneum* (Herbst) and *Sitophilus zeamais* Motsch. J. Stored Prod. Res. 33(4):289-298.
Iberl B, Winkler G, Knobloch K (1990). Products of allicin transformation: ajoenes and dithiins, characterization and their determination by HPLC. Planta Medica 56(2): 202-211.
Ibrahim ND, S Garba (2011). Use of garlic powder in the control of maize weevil. Proceeding of the 45 Annual conference of Agric. Soc. Nig. pp: 177-181.
Isman MB. (2000). Plant essential oils for pest and disease management. Crop Prot. 19(8-10):603-608.
Jain MK, Apitz-Castro R (1987). Garlic: molecular basis of the putative 'vampire-repellant' action and other matters related to heart and blood. Trends Biochem. Sci. 12:252-254.
Jordt SE, Bautista DM, Chuang HH, McKemy DD, Zygmunt PM, Högestätt ED, Meng ID, Julius D (2004). Mustard oils and cannabinoids excite sensory nerve fibres through the TRP channel ANKTM1. Nature 427(6971):260-265.
Krest I, Keusgen M (2002). Biosensoric flow-through method for the determination of cysteine sulfoxides. Anal. Chim. Acta. 469(2):155-164.
Lale NES (1992). A laboratory study of the comparative toxicity of products from three spices to the maize weevil. Postharvest Biol. Technol. 2(1): 61-64.
Lalla FD, Ahmed B, Omar A, Mohieddine M (2013) Chemical composition and biological activity of *Allium Sativum* essential oils against *Callosobruchus maculatus* J. Environ Sci. Toxicol. Food Technol. 3(1):30-36
Lee B, Annis PC, Tumaalii F, Choi W (2004). Fumigant toxicity of essential oils from the Myrtaceae family and 1, 8-cineole against 3 major stored-grain insects. J. Stored Prod. Res. 40(5): 553-564.
Macpherson LJ, Geierstanger BH, Viswanath V, Bandell M, Eid SR et al (2005). The pungency of garlic: activation of *TRPA1* and *TRPV1* in response to allicin. Curr. Biol. 15(10):929-934.
Musa AK (2013). Influence of plant powders on infestation by Adults and larvae of Khapra beetle, *Trogoderma granarium* Everts (Coleoptera: Dermestidae) in stored groundnut. Aust. J. Basic Appl. Sci. 7(6):427-432.
Nwachukwu ID, Gruhlke MCH, Slusarenko AJ (2012). Sulfur and sulfur compounds in plant defence. Nat. Prod. Commun. 7(3):395-400.
Odeyemi OO (1993). Insecticidal properties of certain indigenous plant oils against *Sitophilus zeamais* Mots. Appl. Entomol. Phytopathol. 60(1 and 2):19-27.
Ofuya TI, Olotuah OF, Ogunsola OJ (2010). Fumigant toxicity of crushed bulbs of two Allium to *Callosobruchus malulatus* (Fabricius) (Coleoptera: Bruchidae) Species. Chilean J. Agric. Res. 70 (3):510-514.
Parugrug ML, Roxas AC (2008). Insecticidal action of five plants against maize weevil, *Sitophilus zeamais* Motsch. Coleoptera: Curculionidae. KMITL Sci. Technol. J. 8(1)21-38.
Portz D, Koch E, Slusarenko AJ (2008). Effects of garlic (*Allium sativum*) juice containing allicin on *Phytophthora infestans* and downy mildew of cucumber caused by *Pseudoperonospora cubensis.* Eur. J. Plant Pathol. 122(1):197-206
Qi IT, Burkholder WE (1981). Protection of stored wheat from the granary weevil by vegetable oils. J. Econ. Entomol. 74: 502-505.
Rajapakse RHS (2006). The potential of plants and plant products in stored insect pest management. J. Agric. Sci. 2(1):11-21.
Richards AG (1978). The chemistry of insect cuticle. In: Biochemistry of insects, Academic Press, New York, U.S.A. pp. 205-232.
Slusarenko AJ, Patel A, Portz D (2008). Control of plant diseases by natural products: Allicin from garlic as a case study. Eur. J. Plant Pathol. 121(3):313-322.
Staba John E, Lash L, Staba Joyce E (2001). A Commentary on the Effects of Garlic Extraction and Formulation on Product Composition. J. Nutr. 131: 1118S-1119S.
Udo IO (2005). Evaluation of the potential of some local spices as stored grain protectants against the maize weevil *Sitophilus zeamais* Motsch (Coleoptera: Curculionidae). J. Appl. Sci. Environ Manage. 9 (1):165-168.
van Etten HD, Mansfield JW, Bailey JA, Farmer EE(1994). Two classes of plant antibiotics: phytoalexins versus "phytoanticipins". Plant Cell. 6(9):1191-1192.
Voigt M, Wolf E (1986). Knoblauch: HPLC-Bestimmung von Knoblauchwirkstoffen in Extrakten, Pulver und Fertigarzneimitteln. Dtsch. Apoth. Ztg. 126:591-593.
Wang H, Woolf CJ (2005). Pain TRPs. Neuron 46(1):9-12.
Zhao NN, Zhang HX, chang Liz, Zhou C, Cheng L, Qi Z, Shi W, Liv ZL, (2013) Evaluation of acute toxicity of essential of garlic (*Allium Sativum)* and it's selected major constituents against over wintering *Cacopsylla chinensis* (Hemiptera: Psyllidae.) J. Econ. Entomol. 106 (3): 1349-1354.
Zhou HN, Zhao NN, Shu Shan D, Yang K, Cheng FW, Zhi LL, Yan JQ (2012). Insecticidal activity of the essential oil of *Lonicera japonica* flower buds and its main constituent compounds against two grain storage insects. J. Med. Plants Res. 6(5):912-917.

academicJournals

Vol. 13(10), pp. 1131-1135, 5 March, 2014
DOI: 10.5897/AJB12.2461
ISSN 1684-5315 ©2014 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Total phenols, flavonoids, anthocyanins, ascorbic acid contents and antioxidant activity of *Rhamnus kurdica* Boiss for flower and leaves in flowering and pre-flowering stages

Mohammad Bagher Gholivand* and Marzieh Piryaei

Faculty of Chemistry, Razi University, Kermanshah, Iran.

Accepted 7 August, 2013

The antioxidant capability, total phenol, total flavonoid, anthocyanins, ascorbic acid contents, and reducing power contents of polar and non-polar extracts for flower and leaves in two stages of growth for *Rhamnus kurdica* Boiss in flowering were evaluated in this work. The polar extraction of flower of *R. kurdica* Boiss had a higher total phenolic content as well as antioxidant activity compared to that of the other subfractions of flower and leaves in the different stages. Antioxidant activities of the samples were determined by three various testing systems namely 2, 2-diphenyl-1-picrylhydrazyl (DPPH), b-carotene/linoleic acid and reducing power assay. In DPPH system, the highest radical scavenging activity was seen by the polar subfraction in flowers of methanol extract [21.04±1.35 (µg/ml)]. Our findings demonstrate that the methanolic extracts of *R. kurdica* Boiss may be suggested as a new potential source of natural antioxidant.

Key word: *Rhamnus kurdica* Boiss, antioxidant, total phenolics, flavonoid, anthocyanins.

INTRODUCTION

Antioxidant activity is essential for life, to counteract the strongly oxidizing environment in which we live (Velioglu et al., 1998). Free radical formation is controlled naturally by compounds known as antioxidants. The damage in biological systems can be cumulative when the concentration of radical species and antioxidants are not in balance (Erkan et al., 2011). Phenolic compounds such as flavonoids, phenolic acids, diterpenes, and tannins have received attention for their high antioxidative activity (Rice-Evans et al., 1996).

Converging evidence from both experimental and epidemiological studies have demon-strated that cereals, vegetables, and fruits contain a myriad of phenolic compounds (Maheshwari et al., 2011). Natural antioxidants are compounds that increase the lifetime period and the nutrition value of food. They are transferred to beverages during the production process from different parts of plants (leaves, fruits, etc). Their beneficial effect on the health of consumers is seen mainly in reducing the concentration of free radicals and in decreasing hypertension (Theodoridis et al., 2011).

To the best of our knowledge, there is no information on the antioxidant properties of *R. kurdica* Boiss. The aim of this work was to evaluate the *in vitro* antioxidant properties of the methanol extracts of *R. kurdica* Boiss for flowers and leaves in two stages by DPPH, β-carotene/linoleic acid, reducing power assays, total phenolics, anthocyanins.

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

MATERIALS AND METHODS

Plant material

The aerial parts of *R. kurdica* Boiss in different stage were gathered before flowering (pre-flowering) and flowering period in summer 2011 in the west of Iran. The aerial parts (leaves and flowers) were dried in shade (at room temperature). The plants were identified and authenticated by the Laboratory of Botanic Ecology of the Razi University. A voucher specimen was deposited at the chemistry herbarium of this laboratory under the code 2010 RKB.

Chemicals

Linoleic acid, 2, 6-di-tert-butyl-4-methylphenol (butylated hydroxytoluene, BHT), 2,2-diphenyl-1-picrylhydrazyl (DPPH, 95%), gallic acid, oxalic acid, ascorbic acid (AA), catechin, PVPP (polyvinylpolypyrrolidone), cyanidin-3-glucoside and β-carotene, were procured from Sigma-Aldrich Chemie (Steinheim, Germany). Analytical grade methanol, and HPLC grade chloroform, standard Folin-Ciocalteu's phenol reagent, anhydrous sodium sulphate, ferric chloride, sodium carbonate, potassium ferricyanide, phosphate buffer solution (PBS), and Tween 40 were obtained from Merck (Darmstadt, Germany).

Preparation of the methanol extract

60 g of the dried leaves in different stage of growth and 20 of powdered flower of *R. kurdica* Boiss were extracted with methanol by using Soxhlet apparatus at 50°C for 18 h. The extract was filtered and concentrated under vacuum at 60°C by using a rotary evaporator (Heidolph, Laborota 4000, Schwabach, Germany), yielding a waxy material for leaves in per-flowering, leaves in flowering and flower were (3.73 g, 6.25%), (4.16 g, 6.93% w/w), and (1.08 g, 5.40%) respectively. These extracts were suspended in water and extracted with chloroform (4 ×100 ml) to obtain 2.11 g (3.51%), 2.97 g (4.95%), and 0.61 g (3.05%) polar and 1.22 g (2.03%), 1.04 (1.73%), and 0.38 g(1.9%) non-polar extracts. The extracts were stored in darkness at 4°C until used within a maximum period of one week.

Antioxidant properties

1, 1-Diphenyl-2-picrylhydrazyl radical (DPPH) scavenging activity

The free radical-scavenging activities of extract were measured by using 2, 2-diphenyl-1-picryl-hydrazyl (DPPH) as described by Sharififar et al. (2007) with some modification. 3 ml of various concentrations of the extract was added to 1 ml of a 0.5 mM methanol solution of DPPH. The mixture was strongly shaken and left to stand at room temperature for 60 min in the dark. Then, the absorbance was measured at 517 nm against a blank. Determinations were made on a Shimadzu UV-visible spectrophotometer; model UV 160 U (Japan). Inhibition of free radical, DPPH, in percent (I %) was calculated according to the formula:

$$I\% = ((A_b \_ A_S)/A_b) \times 100$$

Where, A_b is the absorbance of the control reaction (containing all reagents except the test compound), and As is the absorbance of the test compound.

The sample concentration providing 50% inhibition (IC_{50}) was calculated from the graph of inhibition percentage against sample concentration. Tests were carried out in triplicate. Ascorbic acid (AA) was used as positive control.

β -Carotene linoleic acid assay

The antioxidant activity was evaluated according to the method described by Miller (1971) with some modifications. Briefly, 1.5 ml of β-carotene solution (1 mg/ml in chloroform), 3 ml of linoleic acid solution (10 mg/ml in chloroform), and 1.0 ml of Tween 40 solution (300 mg/ml in chloroform) were pipetted into a 250 ml flask. The chloroform was removed by rotary vacuum evaporator, and 150 ml deionized water was added to the residue and the mixture was shaken to form an emulsion. Three hundred and fifty microliter (350 µl) of test sample in methanol (2 mg/ml) was mixed with 2.5 ml of this reagent, and the emulsion system was incubated for up to 24 h at room temperature. The same procedure was repeated with the synthetic antioxidant, BHT as positive control, and a blank containing only 350 µl of methanol. After this incubation period, absorbances of the mixtures were measured at 490 nm. Antioxidative capacities of the extract were compared with those of BHT and blank.

Reducing power

The reducing power of extract was determined according to the method of Sfahlan et al. (2009) with some modifications. Different concentrations of methanolic extract (polar and nonpolar) of plant in methanol (1.0 ml) were mixed with 2.5 ml of phosphate buffer (200 mM, pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture to stop the reaction, which was then centrifuged at 3000 *g* for 10 min. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and $FeCl_3$ (0.5 ml, 0.1%), and the absorbance was measured at 700 nm: higher absorbance indicates higher reducing power. Ascorbic acid was used as positive control.

Determination of total phenolic contents

Total phenolic contents of the extract and the oil were determined using the Folin-Ciocalteu reagent according to the method of Singleton and Rossi (1965) using gallic acid as standard, with some modifications. The extract solution (0.1 ml) containing 1000 µg of the extract was mixed with 46 ml of distilled water in a volumetric flask and 1 ml Folin-Ciocalteu reagent was added, and the flask was thoroughly shaken. The mixture was allowed to react for 3 min and 3 ml aqueous solution of 2% Na_2CO_3 was added. At the end of incubation of 2 h at room temperature, absorbance of each mixture was measured at 760 nm. The same procedure was also applied to the standard solutions of gallic acid, and a standard curve was obtained. Total phenol contents were expressed as µg gallic acid equivalents per mg of the extract. All tests were carried out in triplicate, and gallic acid equivalent values were reported as X ± SD of triplicates.

Determination of total flavonoids

A modified protocol of that described by Kim et al. (2003) was employed. A 0.1 ml aliquot of methanolic extract, appropriately diluted, was mixed with 0.4 ml distilled water in a 1.5 mL microcentrifuge tube, 0.03 ml of 5% $NaNO_2$ was added and the mixture was allowed to react for 5 min. Following this, 0.03 ml of 10% $AlCl_3$ was added and the mixture stood for a further 5 min. Finally, the reaction mixture was treated with 0.2 ml of 1 M Na_2CO_3 and 0.24 ml distilled water, and the absorbance at 510 nm was obtained against a blank prepared similarly, by replacing extract with distilled water. Total flavonoid content was calculated from a calibration curve using catechin as standard, and expressed as mg catechin equivalents (CTE) per 100 g plant (Dourtoglou et al.,

Table 1. Antioxidant activities and total phenol of *Rhamnus kurdica Boiss* methanol extract in two stage of growth for leaves and flowers.

Sample	DPPH IC_{50} (µg/ml)	β-Carotene bleaching (RAA) (%)	Total phenol contents (µg/mg)
Polar subfraction $^{L-P}$	47.16±3.83	78.84±1.96	212.72± 8.82
Nonpolar subfraction $^{L-P}$	321.49±9.61	34.29±1.43	49.87±2.87
Polar subfraction $^{L-F}$	31.11±3.57	98.06±3.98	252.33±7.18
Nonpolar subfraction $^{L-F}$	287.02±7.47	54.77±1.37	76.30±3.47
Polar subfraction^{F}	21.04±1.35	99.13±3.12	307.38±10.34
Nonpolar subfraction^{F}	98.36±4.02	61.39±0.67	102.51±4.21
BHT	19.5 ± 1.06	100	nd
Ascorbic acid	5.1 ± 0.87	nd	nd

$^{L-P}$Leaves in pre - flowering; $^{L-F}$ leaves in flowering; ^{F}flower. Nd, Not detected.

2006).

Anthocyanins

Total anthocyanin content was measured with the pH differential absorbance method, as described by Cheng and Breen (1991). Briefly, absorbance of the extracts were measured at 510 and 700 nm in buffers at pH 1.0 (hydrochloric acid-potassium chloride, 0.2 M) and 4.5 (acetate acid- sodium acetate, 1 M). Anthocyanin content was calculated using a molar extinction coefficient of 29,600 (cyanidin-3- glucoside) and absorbance of

$$A = [(A_{510} - A_{700})_{pH\ 1.0} - (A_{510} - A_{700})_{pH\ 4.5}]$$

Results were expressed as µg cyanidin-3-glucoside equivalents 100 mg^{-1} fw (Pantelidis et al., 2007).

Ascorbic acid content

Ascorbic acid of the extracts was determined using ascorbic acid as standard, with some modifications. The samples (1 g) and 4 ml oxalic acid (1%) were mixed, homogenised for 1 min, and filtered. Polyvinylpolypyrrolidone (PVPP) (100 g) was added to 2.5 ml of the filtered sample, to remove phenols, and 2-3 drops of H_2SO_4 (25%) were added, to reduce the pH to below 1. Absorbance of the mixture was determined at 254 nm. Results were expressed as mg ascorbic acid (AA) 100 g^{-1} fresh weight (fw) (Pantelidis et al., 2007).

RESULTS AND DISCUSSION

Antioxidant properties

Free radical-scavenging activity

The free radical-scavenging abilities of the methanol extract of *R. kurdica* Boiss and polar and nonpolar fractions (water, chloroform) against DPPH were tested, and the results are presented in Table 1. The polar subfraction for flower of methanol extract provided the highest radical-scavenging activity with the lowest IC_{50} value of 21.04±0.35µg/ml than the other subfractions. The antioxidant activities of the plant extract was also evaluated by the spectrophotometric β-carotene bleaching test. The rate of β-carotene bleaching can be slowed down in the presence of antioxidants (Kulisic et al., 2004). The relative antioxidative activities (RAAs) of the extracts were calculated from the equation, RAA = A sample/A BHT, where A BHT is the absorbance of the control (BHT) and A sample is the absorbance of the extract. The calculated RAAs of the extract are given in Table 1. In the reducing power assay, the presence of antioxidants in the sample would result in the reducing of Fe^{3+}-Fe^{2+} by donating an electron. Amount of Fe^{2+} complex can then be monitored by measuring the formation of Perl's Prussian blue $Fe^{4+}[Fe(CN)6]^{3+}$ at 700 nm. Increasing absorbance at 700 nm indicates an increase in reductive ability. Figure 1a, b, c shows the reducing power of the methanolic (polar and non-polar) extract of *R. kurdica* Boiss as a function of their concentrations. The amounts of total phenolics in the extract for different stage were determined spectrometrically according to the Folin-Ciocalteu procedure and calculated as gallic acid equivalents. Gallic acid is a water-soluble polyhydroxyphenolic compound that can be found in various natural plants. The standard curve equation was, y (absorbance) = 0.0003 ×gallic acid (µg) + 0.00534. The amounts of total phenols found in the plant methanolic extract are shown in Table 1.

Flavonoid

The standard curve equation for determination flavonoids with catechin is y (absorbance) = 0.004× catechin (µg) + 0.0483. The amounts of total flavonoids found in plant methanolic extract for flowers of *R. kurdica* Boiss was 101.17 ± 5.74 µg/mg, for leaves in flowering stage was 86.32 ± 2.98 µg/mg and in pre-flowering stage for leaves was 51.67 ± 1.54 µg/mg.

Anthocyanins

Significant differences in anthocyanin content were

Table 2. Flavonoid, anthocyanins and ascorbic acid contents of *R.kurdica Boiss* methanol extract in two stages of growth for leaves and flowers.

Sample	Flavonoid µg/mg	Anthocyanin (µg 100 mg^{-1} fw)	Ascorbic acid (µg 100 mg^{-1} fw)
Flower	101.17± 5.74	21.53±0.57	25.13±1.59
Leaves in flowering	86.32±2.98	12.36±0.84	16.87±1.02
Leaves in pre - flowering	51.67±1.54	2.27±0.03	14.06±1.07

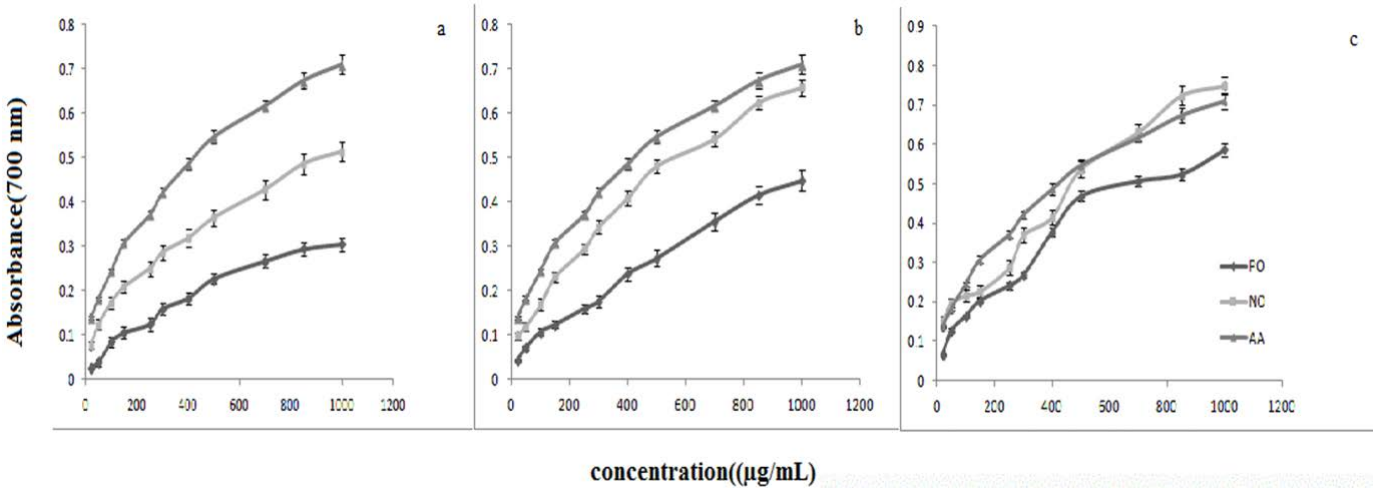


Figure 1. Reducing power of different concentrations of methanolic extract polar and non-polar subfraction. a: leaves in the pre-flowering stage, b: leaves in the flowering stage and c: flowers of *Rhamnus kurdica Boiss* compared to ascorbic acid, (spectrophotometric detection of the Fe^{+3}-Fe^{+2} transformations). Polar (PO) and non-polar (NO) subfraction extract; AA, ascorbic acid.

recorded, since these pigments are responsible for the red and blue color. The nonpolar subfraction in flowering stage contained the highest anthocyanin content expressed as cyanidin-3- glucoside. The results are shown in Table 2. The results show relation between anthocyanins and antioxidant activity.

Ascorbic acid

Significant differences in ascorbic acid content among the different sub-fraction are recorded in Table 2. The flowers subfraction had the highest content of ascorbic acid (25.13 ± 1.59 mg 100 g^{-1} fw).

REFERENCES

Cheng GW, Breen PJ (1991). Activity of phenylalanine ammonialyase (PAL) and concentrations of anthocyanins and phenolics in developing strawberry fruit. J. Am. Soc. Horticult. Sci.116:865-869.

Dourtoglou VG, Mamalos A, Makris DP (2006). Storage of olives (Olea europaea) under CO2 atmosphere: Effect on anthocyanins, phenolics, sensory attributes and in vitro antioxidant properties Idolo Tedesco, Gian Luigi Russo, Filomena Nazzaro, Maria Russo, Rosanna Palumbo. Food Chem. 99:342-349.

Erkan N, Cetin H, Ayranci E (2011). Antioxidant activities of Sideritis congesta Davis et Huber-Morath and Sideritis arguta Boiss et Heldr: Identification of free flavonoids and cinnamic acid derivatives. Food Res. Int. 44:297-303.

Kim DO, Chun OK, Kim YJ, Moon HY, Lee CY (2003). Quantification of polyphenolics and their antioxidant capacity in fresh plums. J. Agric. Food Chem. 516:509-6515.

Kulisic T, Radonic A, Katalinic V, Milos M (2004). Use of different methods for testing antioxidative activity of oregano essential oil. Food Chem. 85:633-640.

Maheshwari DT, Yogendra Kumar MS, Verma SK, Singh VK, Singh SN (2011). Antioxidant and hepatoprotective activities of phenolic rich fraction of Seabuckthorn (Hippophae rhamnoides L.) leaves. Food Chem. Toxicol. 49(9):2422-2428.

Miller HE (1971). A simplified method for the evaluation of antioxidants. J. Am. Oil Chem. Soc. 48:91-97.

Pantelidis GE, Vasilakakis M, Manganaris GA, Diamantidis Gr (2007). Antioxidant capacity, phenol, anthocyanin and ascorbic acid contents in raspberries, blackberries, red currants, gooseberries and Cornelian cherries. Food Chem. 102:777-783.

Rice-Evans CA, Miller NJ, Paganga G (1996). Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Rad. Biol. Med. 20:933-956.

Sfahlan J, Mahmoodzadeh A, Hasanzadeh A, Heidari A, Jamei R (2009). Antioxidants and antiradicals in almond hull and shell (*Amygdalus communis* L.) as a function of genotype. Food Chem. 115:529-533.

Sharififar F, Moshafi MH, Mansouri SH, Khodashenas M, Khoshnoodi M (2007). *In vitro* evaluation of antibacterial and antioxidant activities of the essential oil and methanol extract of endemic Zataria multiflora Boiss. Food Control. 18:800-805.

Singleton VL, Rossi JA (1965). Colorimetry of total phenolics with phosphomolybdic phosphotungstic acid reagents. Am. J. Enol. Viticul. 16:144-158.

Theodoridis G, Las kov M, ker kov V, Tegou A, Giantsiou N, Jandera P (2006). Molecular imprinting of natural flavonoid antioxidants: Application in solid-phase extraction for the sample pretreatment of natural products prior to HPLC analysis. J. Sep. Sci. 29:2310-2321.

Velioglu YS, Mazza G, Gao YL, Oomah BD (1998). Antioxidant activity and total phenolics in selected fruits, vegetables and grain products. J. Agric. Food Chem. 46:4113-4117.

academicJournals

Vol. 13(10), pp. 1136-1142, 5 March, 2014
DOI: 10.5897/AJB2013.12228
ISSN 1684-5315 ©2014 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Optimization of up-flow anaerobic sludge blanket reactor for treatment of composite fermentation and distillation wastewater

G. A. Amin[1]* and L. Vriens[2]

[1]Department of Agricultural Microbiology, Faculty of Agriculture, Cairo University, Giza, Giza, Egypt
[2]Waterleau, Radioweg 18, 3020Herent (Leuven), Belgium.

Accepted 5 June, 2013

Optimization of up-flow anaerobic sludge blanket (UASB) reactor operation for treatment of a composite fermentation and distillation wastewater was achieved using a locally available thickened municipal sludge instead of imported commercial anaerobic granulated sludge. Over the first 12 days, a fed batch start-up operation was maintained and anaerobic stable sludge granules with 11.2% of extra cellular polymers (ECP) were successfully developed and further used for long-term continuous operation. Two types of granules were developed within the reactor but with very different characteristics. Granules grown in the bottom part of UASB reactor were more compact and tense than those that occurred in the upper part. The latter were fragile, irregular in shape and with much lower methanogenic activities. Bottom granules were dominated by both *Methanosarcina* spp. and *Methanosaeta* spp. whereas upper granules harbored only *Methanosarcina* spp. During continuous anaerobic treatment of composite fermentation and distillation wastewater with organic load of 24 g.l^{-1} of chemical oxygen demand (COD), a removal efficiency of up to 84% was achieved. Moreover, biogas was produced with a production rate of o.52 m^3/Kg COD removed.

Key words: Composite wastewater, up-flow anaerobic sludge blanket (UASB), anaerobic biological treatment, biogas, granulated anaerobic sludge, industrial wastewater.

INTRODUCTION

Fermentation wastewaters have a very high organic load compared to municipal wastewater (Shi et al., 2012). Their treatment to reduce chemical oxygen demand (COD) and biological oxygen demand (BOD) is essential prior to disposal on land or water bodies. The up-flow anaerobic sludge blanket (UASB) reactors have been proven for efficient anaerobic treatment systems for petroleum refinery wastewater (Rastegar et al., 2011), poultry wastewater (Glatz et al., 2011), dairy wastewater (Gotmare et al., 2011), and vitamin C biosynthesis wastewater (Shi et al., 2012). Such anaerobic treatment involves biodegradation of organic substances by exo-enzyme producing micro-organisms and simultaneous production of methane by methanogenic bacteria.

Compared with other treatment processes, USAB reactors have the advantage of their ability to retain high bio-mass with high void volume, because no support material is externally supplied (Mrunalini et al., 2013),

*Corresponding author: E-mail: gamilamin2007@yahoo.com.

Abbreviations: ECP, Extra-cellular polymers; **COD**, chemical oxygen demand; **BOD**, biochemical oxygen demand; **PVC**, polyvinyl chloride; **GLSS**, gas-liquid-solid separator; **TS**, total solids; **VFAs**, volatile fatty acids; **VS**, volatile solids.

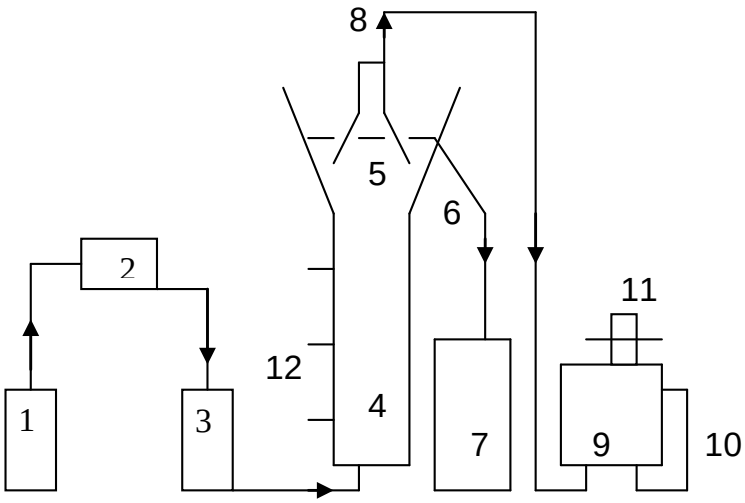


Figure 1. Schematic diagram for experimental set-up of the UASB reator. I, Waste water reservoir; 2, feeding pump; 3, heat exchange unit; 4, UASB reactor; 5, three-phase separator; 6, effluent overflow; 7, effluent collector; 8, gas outlet; 9, gas collector; 10, side-arm measuring device; 11, gas discharge valve; 12, sampling ports.

their independence from mechanical mixing of reactor contents (Ghangrekar and Kahalekar, 2003), and their ability to cope with perturbances from temperature fluctuations and high organic loading rates (Nidal 2008; Kovacik et al., 2010).

The successful operation of an UASB reactor depends on the use of highly flocculated compact sludge aggregates called granules (Najafpour et al., 2006; Narihiro et al., 2009). These anaerobic sludge granules can be supplied as commercial product or possibly formed during the start-up of UASB reactors by self immobilization of anaerobic bacterial cells, a process which is correlated with the production of extra-cellular polymers (ECP) by these cells (Schmidt and Ahring, 1994; Yoochatchaval et al., 2008).

Several researchers (Emiliano et al.2006; Vlyssides et al., 2008; Shi et al.2012) have suggested that the loose structure of filaments of methanogenic bacteria, *Methanosaeta* spp. cells and the clamps formed by *Methanosarcina* spp. cells, are the most crucial factors affecting stability of the granules. In absence of commercial preparations of anaerobic granulated sludge, development of anaerobic sludge granules starting from a locally available thickened sludge and their utilization for continuous anaerobic treatment of industrial wastewater, produced by various fermentation industries, were the objectives of the present study.

MATERIALS AND METHODS

Wastewater

The wastewater used throughout this investigation was obtained from The Company of Sugar and Integrated Industries, Hawamdia complex, Giza, Egypt. The final wastewater stream comprises of wastewaters coming from yeast, ethyl alcohol and distillation industries.

Bioreactor

The UASB reactor was made of polyvinyl chloride (PVC). The working volume of the reactor was 12 litres, consisting of 10.8 litres column portion with 15 cm in ID and 68 cm in height, and a 1.2 litre Gas-liquid-solid separator (GLSS). Three sampling ports were located at 15, 30 and 45 cm from the reactor bottom. The schematic representation of the experimental set-up is shown in Figure 1. The reactor operated in upflow mode. The biogas was collected from the top of the reactor to a flexible water-filled gasholder fitted with measurement systems for both gas and liquid flows. A top opening in the gasholder was used to discharge the biogas every time the gasholder was completely filled.

Development of stable anaerobic granular sludge

In view of the absence of locally sold commercial preparations of granular anaerobic sludge, sludge obtained from a gravity thickener at municipal wastewater treatment plant at Zenien, Giza was used to seed the reactor. Two liters of the thickened sludge with 2.8% total solids (TS) and 76% volatile solids (VS) were introduced, into UASB reactor, and stored for two weeks in order to improve its anaerobic activities. For further improvement of sludge settling capacity and methanogenic characteristics, the reactor was operated in fed-batch upflow mode at a loading rate of 0.5 g COD $litre^{-1}$. day^{-1} of diluted wastewater with a COD content of 12 000 ppm . Samples were collected over 12 days from both the lowest and the highest sampling ports and analyzed.

Characteristics of the anaerobic sludge granules

Compared to most probable number (MPN) technique and other cultivation methods, microscopic examination was proven as a

Table 1. Chemical composition of industrial wastewater.

Parameter	Value	Unit
pH	4.2	Số
COD	24300	ppm
BOD	15000	ppm
Total Nitrogen	430	ppm
Total phosphorous	128	ppm
BOD/COD	0.61	-

reliable technique for anaerobic granular sludge examinations particularly those concerning with methanogenic populations (Yoochatchaval et al., 2008; Shi et al., 2012). Therefore, microscopic examination was used in this study to study different microbial groups contributed if sludge granules at the bottom and upper part of the bioreactor.

Optimization of continuous operation of the UASB reactor

Composite fermentation and distillation wastewater was fed into the reactor continuously at a rate of 4 litres day^{-1} (retention time three days) with a stepwise increase in COD content (organic loading rates of 4, 6 and 8 g $COD.litre^{-1}$ day^{-1}). The pH of wastewater was adjusted at 7.5 ± 0.2 before being passed through an external heat exchange unit in order to control influent temperature at 35 ± 0.5. The organic loading rate was increased when the previous steady state was reached and maintained for three volume turnovers at least. Samples were taken from reactor effluent every 24 h and analyzed.

Analytical methods

The effluent from the UASB reactor was analyzed for residual COD, pH, and total volatile fatty acids (VFAs) and BOD in raw wastewater and the volatile solids (VS) of the granules according to the American Public Health Association (APHA) (1989). Acetate and propionate were determined gchromatographically (Amin et al., 1983). After thermal extraction of sludge granules at 70°C in a shaking water bath for 4 h, the water soluble ECP were separated by centrifugation at 9500 g for 10 min and analyzed for polysaccharides using the phenol/sulphuric acid method of Dubois et al. (1956), protein as Kjeldahl nitrogen (Schmidt and Ahring, 1994) and lipids by gas chromatography (Arneborg et. al., 1993), after methylation by KOH and methanol.

RESULTS AND DISCUSSION

Chemical composition of wastewater

Table 1 illustrates the chemical composition of wastewater. Clearly, wastewater contains the three major nutrients (nitrogen, carbon and phosphorous) in excess amounts than required for anaerobic metabolism. The COD: N: P ratio was 1000: 17: 5, which is considerably higher than that cited in literature (1000: 13: 3, Mrunalini et al., 2013) for optimum anaerobic metabolism. Certainly, this could be considered to be an advantage as no external nutrient addition was required throughout the whole investigation. Only pH of the wastewater had to be controlled as mentioned above.

Start-up of UASB reactor

Conversion of COD in UASB reactor

A fed-batch operation of UASB reactor was commenced after two weeks from seeding with municipal thickened as described above. Samples were taken and analyzed over 12 days. The performance of the reactor is shown in Figure 2 and Table 2. As operation proceeded, both consumption of COD and production of VFAs increased with acetic and propionic acids being the major components. Acetic acid was consumed much faster than propionic acid.

Up to 1910 ppm of propionic acid were detected on day 5 with only 230 ppm of acetic acid. Despite the high COD removal efficiency of 85%, biogass yield was very low, only 0.13 litres/g consumed COD (Table 2). This is most probably due to accumulation of high concentration of VFAs (2550 ppm). From days 5 to 9, biogas production was markedly improved as a result of breakdown of VFA indicating stimulation of methanogenic microorganisms. The best performance was observed on day 9 with a COD removal efficiency of 88% and the highest biogas yield of 0.66 liters/g consumed COD. The total VFA concentration decreased down to 1540 ppm. With further fed-batch operation, a total different performance characterized by a dramatic deterioration in COD removal efficiency was noticed. Both residual COD and concentration of VFA, particularly propionic acid increased sharply in reactor effluent and biogas production rate decreased (Table 2). Similarly several investigators have reported that propionate oxidation to acetic acid was the rate-limiting step of anaerobic methane production from various fermentative substrates (Harada et al., 1996; Kovacik et al., 2010).

The lowest COD removal efficiency of only 33% was recorded on day 12 with the highest concentration of propionic acid (2400 ppm). Acidification of reactor contents must have taken place as pH of reactor effluent decreased down below 4 (Figure 1). This resulted in a partial inhibition of methanogenic activity of developed sludge as biogas yield decreased to 0.44 litres/g consumed COD.

Development of anaerobic granular sludge within UASB reactor

It is well known that, both cell-to-cell adhesion and formation of anaerobic sludge granules are very much correlated with microbial excretion of ECP. Therefore, the amounts and chemical composition of ECP of the developed sludge granules from the bottom and the

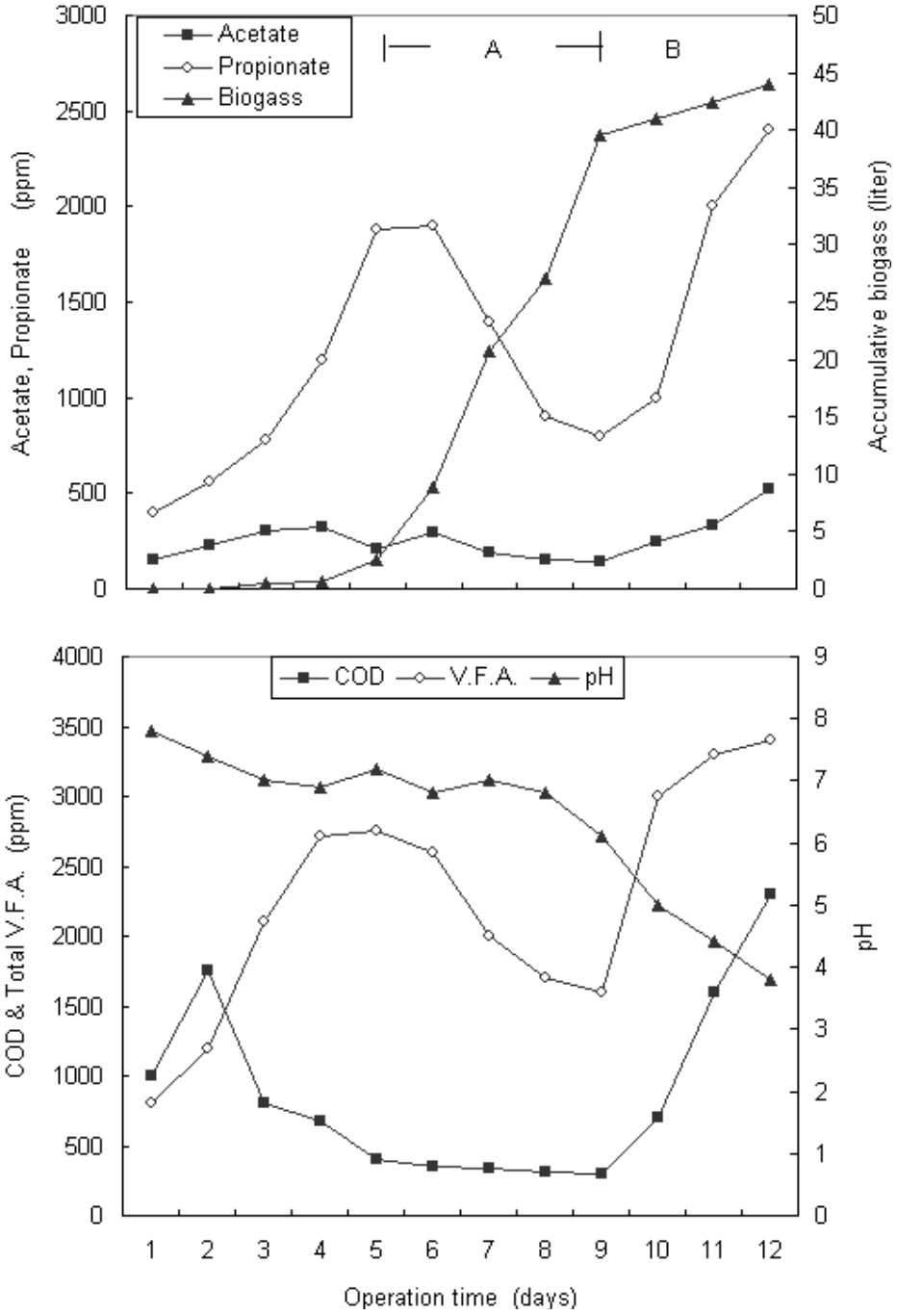


Figure 2. Performance of UASB reactor during fed-batch operation. A and B are operation stages between days 5, 9 and 12, respectively. COD value taken after excluding the amount of COD consumed in VFAs synthesis.

Table 2. Kinetic parameters on performance of UASB during fed-batch operation.

Parameter	Day 5	Day 9	Day 12
Biogas yield (liters biogas/g COD $_{cons.}$)	0.13	0.64	0.46
Concentration of VFAs (g/l)	2. 50	1.46	3.00
Removal Efficiency (%)	85.0	88.0	36.0

Table 3. Chemical composition of extra-cellular polymers (ECP) of granules formed in UASB reactor during fed-batch operation.

Type of granule	Concentration mg/g of VSS				
	Protein	Poly-saccharide	Lipid	Protein/polysaccharide	ECP
Upper granules	67.85	8.19	0.74	8.3	76.78
Bottom granules	90.09	15.3	0.204	5.9	105.59

upper part of UASB reactor was determined over the last four days of fed-batch operation and related to the dramatic change in reactor performance. Table 3 shows the results. It is very clear that granules developed in the bottom part of UASB were very much different from those formed in the upper part. The total amount of ECP was higher in former granules compared with those formed in the upper part being 105.6 and 76.8 $mg.g^{-1}$ (VS), respectively. The reported percentages of ECP in different anaerobic granular sludge are between 0.6 and 20% of VS, depending on type of wastewater, extraction and analytical methods for ECP (Emiliano et al., 2006; Gotmare et al., 2011). The obtained results show also that in both types of granules, protein contributes the major part of ECP followed by polysaccharides. The granules grown in the bottom part of the reactor had markedly higher contents of protein and polysaccharides per gram VS in their ECP compared to granules formed in the upper part.

It is suggested that the implemented fed-batch operation mode might have created an up-flow staged sludge-bed system similar to that reported by Yetilmezsoy and Sakar (2008) with various compartments along the upflow direction of the UASB reactor. The wastewater fed daily at reactor bottom might have generated a zone for fermentation of complex substances in the bottom part, which gradually becomes lower in the following upper parts, leading to more acetogenic and methanogenic substrates, such as propionic and acetic acids, in the upper part (compartment) of UASB reactor. This must be considered to explain the similarity between the obtained results and those reported by Schmidt and Ahring (1994) who found that changing the feed of an UASB reactor from a sugar-containing wastewater to a synthetic wastewater containing acetate, butyrate and proionate resulted in a decrease in both protein and polysaccharides content of the ECP of sludge granules. More recently, Kovacik et al. (2010) reported major changes in microbial diversity in biogranules in response to changes in nature and concentration of organic load fed into UASB reactor.

Lipids have also been detected in ECP of bottom and upper part granules with much lower amounts than protein and polysaccharides. However, granules grown in the bottom part had significantly lower amounts of lipids compared to upper part granules (0.204 and 0.740 $mg.g^{-1}$ VS, respectively). Similarly, Schmidt and Ahring (1994) found that granules with low content of ECP had higher amount of lipids compared to granules with high ECP content. The authors suggested that higher excretion of lipids could be a way of compensating for the lower production of protein and polysaccharides in anaerobic sludge granules.

Characteristics of the anaerobic sludge granules

Dolfing (1986) reported that microscopic examination gave up to 50% more methanogens than MPN techniques. Besides, all cultivation methods are selective and non-cultivable strains cannot be counted. In the present study, microscopic observations showed that bottom granules contained both easy detected auto fluorescent *Methanosarcina* coccoids and gas- vacuolated bamboo-shaped *Methanosaeta* filamentous rods, whereas only *Methanosarcina* cells dominated granules formed in the upper part. Narihiro et al. (2009) pointed out that *Methanosarcina* spp., unlike *Methanosaeta* spp., have the capacity to use acetate, butyrate and propionate.

Methanosaeta spp. is known to grow only on acetate. This might be considered to explain the presence of *Methanosarcina* dominated granules in the upper part of UASB reactor, when acidic conditions were developed. Microscopic examination showed also that granules grown in the bottom part of UASB reactor were more compact, and tense than those occurred in the upper part. The latter were fragile and irregular in shape.

Optimization of continuous operation of UASB reactor

In view of the above-mentioned results, it was decided to start feeding wastewater in a continuous mode in an attempt to washout acidic wastewater from the reactor, get red of fragile sludge granules in the upper part and maintain those actively growing granules in the bottom part of the USAB reactor. Optimization of the UASB reactor continuous operation for anaerobic treatment of industrial wastewater having higher organic loads was also considered. Wastewater was fed into the reactor continuously at a rate of 4 $litres.day^{-1}$ (retention time of three days) with a stepwise increase in COD content

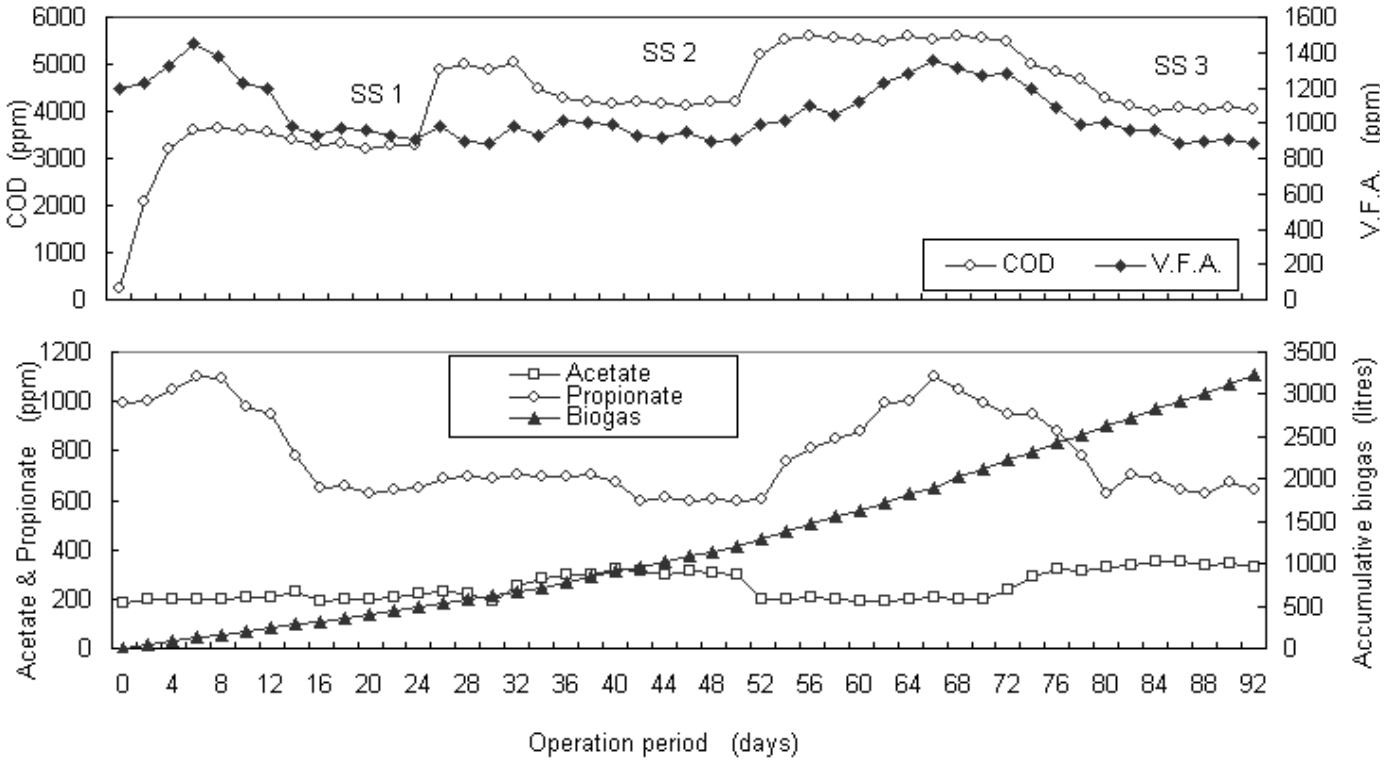


Figure 3. Performance of UASB reactor during continuous operation using industrial wastewater with different organic loading loading rates. SS1, steady state at 4 g COD/L/d; SS 2, steady state at 6 g COD/L/d ; SS 3, steady state at 8 g COD/L/d. COD values taken after excluding COD consumed in VFA synthesis.

Table 4. Removal efficiency and biogas production by UASB reactor during steady state conditions of anaerobic treatment of industrial wastewater with different organic loads.

Operation parameter	Steady state at organic loading rate (g COD. Litre^{-1}.d^{-1})		
	4	6	8
Final discharge COD (ppm)	3275	4170	4067
Bioreactor productivity liters biogas /g consumed COD	0.48	0.52	0.52
Liters biogas / liter of reactor volume / day	1.66	2.80	4.08
mg V.F.A. / g consumed COD	3.79	2.40	1.92
Removal efficiency (%)	73	77	84

(organic loading rates of 4, 6 and 8 g COD.litre^{-1}. day^{-1}). The organic loading rate was increased when the previous steady state was reached and maintained for three volume turnovers at least. Samples were taken from reactor effluent every 24 h and analyzed for residual COD, VFAs, acetate, propionate and biogas. Figure 3 and Table 4 show the results. For each loading rate transit increases in COD and VFA content of the reactor effluent, particularly propionate, were observed followed by a steady state. After adaptation period (the first 2 weeks), UASB reactor reached the first steady state with 73% removal efficiency of the COD. However, biogas production yield was only 0.48 liters. g^{-1} consumed COD most probably due to the high production of VFAs (3.79 mg .g^{-1} consumed COD. As loading rate increased COD, removal efficiency and biogas production yield increased in directly proportional way to give the highest biogas yield and COD removal efficiency of 0.52 liters. g $^{-1}$ consumed COD and 84%, respectively. The production of VFAs decreased to 1.9 mg. g^{-1} consumed COD. With regard to pollution control, the results show that efficiency of COD removal using UASB reactor compares very favorably with those reported by other investigators (60 to 80% by Ghangrekar and Kahalekar (2003); 59-76% by Bertin et al. (2004); 75% by Gotmare et al. (2011)). Moreover, continuous operation of UASB reactor showed

that after a short transit increase, propionate concentration stabilized during each steady state at about 650 ppm level. This demonstrates the markedly improved activity of acetogenic and methanogenic bacteria, and a satisfactory balance between different microbial populations in sludge granules. This was never happened with previous fed-batch operation.

Conclusion

The successful conversion of locally obtained thickened sludge into actively growing anaerobic sludge granules with high methanogenic activity during the start-up operation provides alternative means of an effective seeding to methane reactors in absence of the expensive commercial preparations of anaerobic granulated sludge. Work is in progress to optimize a two-stage anaerobic aerobic system for more removal of organic pollutants from highly polluted industrial wastewaters, in order to reached final effluent with characteristics required by recent environmental law.

REFERENCES

American Public Health Association (APHA) (1989). Standard methods for the examination of water and wastewater, 17th ed. Washington, D, C.

Amin G, Van den Eynde E,Verachtert H (1983). Determination of by-products formed during the ethanolic fermentation, using batch and immobilized cell systems of *Zymomonas mobilis* and *Saccharomyces bayanus*. Eur. J. Appl. Microbiol. Biotechnol. 18:1-5.

Arneborg N, Salskov-Iversen A, Mathiasen T (1993). The effect of growth rate and other growth conditions on the lipid composition of *Escherichia coli*. Appl. Microbiol. Biotechnol. 39:353-357.

Bertin L, Chiara CM, Ruzzi MM, Fava F (2004). Performance and microbial features of a granular activated carbon packed-bed biofilm reactor capable of an efficient anaerobic digestion of olive mil wastewaters. FEMS Microbiol. Ecol. 48:423-424.

Dolfing J (1986). Granulation in UASB reactors. Water Sci. Technol. 18:15-25.

Dubois M, Gilles K, Hamilton J, Robers P, Smith F (1956). Colorimetric method for determination of sugars and related substances. Anal. Chem. 28:350-356.

Emiliano E, Díaz JM, Stams RA, José LS (2006). Phenotypic Properties and Microbial Diversity of Methanogenic Granules from a Full-Scale Upflow Anaerobic Sludge Bed Reactor Treating Brewery Wastewater. Appl. Environ. Microbiol. 72:4942-4949.

Ghangrekar MM, Kahalekar UJ (2003). Performance and Cost Efficacy of Two- stage Anaerobic Sewage Treatment IE (I). Journal. EN 84: 16-22.

Glatz P, Zhihong M, Belinda R (2011). Handling and Treatment of Poultry Hatchery Waste: A Review. Sustainability 3:216-237.

Gotmare M, Dhoble RM, Pittule AP (2011). Biomethanation of Dairy Waste Water Through UASB at Mesophilic Temperature Range". Int. J. Adv. Eng. Sci. Technol. 8(1):1-9.

Harada H, Uemura S, Chen A, Jayadevan J (1996). Anaerobic treatment of a recalcitrant distillery wastewater by thermophilic UASB reactor. Bioresource Technol. 55:215-221.

Kovacik WP, Scholten JCM, Culley D, Hickey R, Zhang W, Brockman FJ (2010). Microbial dynamics in upflow anaerobic sludge blanket (UASB) bioreactor granules in response to short-term changes in substrate feed. Microbiology 156:2418–2427.

Mrunalini MP, Vijay S K, Sunanda V K, Girish SK (2013). Review on application of UASB technology for wastewater treatment. Inter. J. Adv. Sci. Eng. Technol. 2(2):125-133.

Najafpour GD, Zinatizadeh AA, Mohamed AR, Isa MH, Nasrollahzadeh H (2006). High-rate anaerobic digestion of palm oil mill effluent in an upflow sludge-fixed film bioreactor. Process Biochem. 41:370-379.

Narihiro T, Terada T, Kikuchi K, Iguchi A, Ikeda M, Yamauchi T, Shiraishi k, Kamagata Y, Nakamura k, Sekiguchi Y (2009). Comparative analysis of bacterial and archaeal communities in Methanogenic sludge granules from upflow anaerobic sludge blanket reactors treating various food-processing, high-strength organic wastewaters. Microbes Environ. 24:88-96.

Nidal M (2008).High strength sewage treatment in a UASB reactor and an integrated UASB-digester system. Bioresour. Technol. 99:7531–7538.

Rastegar SO, Mousavi SM, Shojaosadati SA, Sheibani S (2011). Optimization of petroleum refinery effluent treatment in a UASB reactor using response surface methodology. J Hazard. Mater. 197:26-32.

Schmidt JE, Ahring BK (1994). Extra-cellular polymers in granular sludge from different up-flow anaerobic sludge blanket (UASB) reactors. Appl. Microbiol. Biotechnol. 42:457-462.

Shi R, Zhang Y, Yang W, Xu H (2012). Microbial community characterization of an UASB treating increased organic loading rates of vitamin C biosynthesis wastewater. Water Sci. Technol. 65(2):254-61.

Vlyssides A, Barampouti EM, Mai S (2008). Determination of granule size in a UASB reactor. J. Environ. Manage. 86:660-664.

Yetilmezsoy K, Sakar S (2008). Development of empirical models for performance evaluation of UASB reactors treating poultry manure wastewater under different operational conditions. J. Hazard. Mater. 153:532-543.

Yoochatchaval W, Ohashi A, Harada H, Yamaguchi T, Syutsubo K (2008). Characteristics of granular sludge in an EGSB reactor for treating low strength wastewater at 20deg.C. Inter. J. Environ. Res. 2 (4):319-328.

academicJournals

Vol. 13(10), pp. 1143-1146, 5 March, 2014
DOI: 10.5897/AJB12.2328
ISSN 1684-5315 ©2014 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Studies on serum macro and micro minerals status in repeat breeder and normal cyclic Nili-Ravi buffaloes and their treatment strategies

Muhammad Saleem Akhtar[1]*, Abdul AsimFarooq[1], Laeeq AkbarLodhi[2], SayyedAun Muhammad[3], M. MazharAyaz[1], Mushtaq Hussain Lashari[1], Saeed Murtaza[1], Irtaza Hussain[1], Muhammad Irshad[1], Maqbool Hussain[1] and Muhammad AsifRaza[4]

[1]Faculty of Veterinary Sciences, BahauddinZakariya University, Multan, Pakistan.
[2]University of Agriculture, Faisalabad, Pakistan.
[3]College of Veterinary and Animal Sciences, Jhang, Pakistan.
[4]Animal Husbandry in the Tropics and Subtropics, University of Kassel-Witzenhausen, Germany.

Accepted 25 March, 2013

The present study was carried out with the objective to know the calcium (Ca), inorganic phosphorus (P), magnesium (Mg), copper (Cu), iron (Fe) and zinc (Zn) concentrations in serum of repeat breeder and normal cyclic buffaloes during oestrus. On the day of estrus, blood samples were collected from 35 buffaloes with a history of repeat breeding (RB) and 35 normal cycling (NC) buffaloes for mineral estimation. In the second part of the study, 35 repeat breeder (RBS) buffaloes were treated with a mineral mixture given orally for 10 days at the dosage rate of 150 g per day whereas other 35 repeat breeder buffaloes were given no mineral mixture (RBC). The overall pregnancy rate as well as 1st, 2nd and 3rd service pregnancy rate was calculated. The serum calcium, inorganic phosphorus, magnesium, copper, iron and zinc concentrations were significantly lower (P<0.01) in RB buffaloes as compared to NC buffaloes. Sodium concentrations differed non-significantly between repeat breeder and normal cyclic buffaloes. Repeat breeder buffaloes (RBS) when fed orally 150 g per day of the mineral mixture for 10 days, the 1st, 2nd and 3rd service pregnancy rates were 42, 25 and 20%, while, overall pregnancy rate in these animals was 87%; whereas in repeat breeder control buffaloes, the overall pregnancy rate was 21%. In conclusion, the concentrations of macro and micro minerals were significantly lower in repeat breeder buffaloes and mineral mixtures should be added in the food stuff to improve reproductive efficiency of repeat breeder buffaloes.

Key words: Buffalo, repeat breeder, minerals, pregnancy rate.

INTRODUCTION

Livestock is an important sector of agriculture in Pakistan contributing about 55.1% of the agricultural value added as well as 11.5% to gross domestic product. Repeat breeding is one of the most important reproductive problem in buffalo which anguish fertility and results to massive economic losses to buffalo farmers. Typical repeat breeding is defined as the animal that did not conceive after three or more consecutive inseminations, despite it comes normally in heat and shows clear estrus signs with no clinical detectable reproductive disorders

*Corresponding author. E-mail: drsaleem46@hotmail.com. Tel: 0092314-7890797.

(Yusuf et al., 2010).

Augmented competence for milk production has been related through a decreased fertility in lactating dairy cows (Butler, 2000) by alterations in reproductive physiology (Wiltbank et al., 2006), causing more number of services per conception (Lucy, 2001). Consequently, the incidence of repeat breeding has increased (Dochi et al., 2008). The incidence varies from 15 to 32% and is higher in adult buffaloes up to the third parity (Noakes et al., 2009). The different risk factors associated with repeat breeding includes abnormal recommencement of postpartum ovarian cycles, lower parity and shorter days in milk at first artificial insemination (AI) (Yusuf et al., 2010).

Normal levels of several minerals may affect the reproductive performance of ruminants. Differences in the level of trace minerals are related with different reproductive disorders (Jain, 1993). Excess or deficiency of minerals can cause repeat breeding in cattle (Das et al., 2002). The blood picture may have potential in describing the problematic situation and diagnosing deficiency conditions.

At present, there is scanty evidence on blood mineral levels during the oestrus in repeat breeding Nili-Ravi buffaloes. We speculated that variations in levels of different minerals in blood may contribute on the occurrence of repeat breeding in buffaloes. The current study was carried out with the objective to know the calcium (Ca), inorganic phosphorus (P), magnesium (Mg), copper (Cu), iron (Fe) and zinc (Zn) concentrations in serum of repeat breeder and normal cyclic Nili-Ravi buffaloes during oestrus and the application of some field treatment trials.

MATERIALS AND METHODS

Animals

The current study was completed on Nili-Ravi buffaloes selected from private farms located around Multan district during the period from September 2010 to March 2011. A total of 35 buffaloes with a history of repeat breeding (RB) and 35 normal cycling (NC) buffaloes were utilized. The ages of experimental animals were between five to nine years with body weight from 400 to 550 kg. The animals were raised under stall-fed conditions, fed sufficient green forages and given *ad libitum* water. Before the start of experiment, palpation of the reproductive tract per rectum was conducted and buffaloes that had been bred three or more times without conception and free from any abnormalities in their ovaries and uteri on palpation were diagnosed as repeat breeder.

Blood sampling for mineral estimation

Blood samples were collected from repeat breeder (RB) and normal cyclic (NC) buffaloes on the day of estrus. The serum was stored at -20°C till analyses. The spectrophotometric method was adopted for the determination of calcium, magnesium, phosphorus and sodium. The micro minerals (Cu, Fe and Zn) were measured with the help of atomic absorption spectrophotometer (AA-5).

Treatment, insemination and pregnancy diagnosis

In the second part of the study, 35 repeat breeder (RBS) buffaloes were treated with a mineral mixture given orally for 10 before breeding days at the dosage rate of 150 g per day whereas other 35 repeat breeder buffaloes were given no treatment and served as repeat breeder control (RBC). Each 1000 g of mineral mixture contained calcium (155 g), phosphorus (135 g), magnesium (55 g), sodium (45 g), iron (1000 mg), zinc (3000 mg), manganese (2000 mg), copper (600 mg), cobalt (10 mg), iodine (40 mg) and selenium (3 mg) (L.S. Minerals, Nawan Laboratories Pvt Ltd., Karachi-Pakistan).

Estrus detection of RBS and RBC buffaloes was done two times everyday (morning and evening) through visual observation by means of a vasectomized teaser buffalo bull. Buffalo which stood to be mounted by teaser bull was considered to be in estrus. The artificial insemination (AI) was done almost 12 h subsequently to the beginning of estrus with frozen thawed semen in 0.50 ml straw having 30 million spermatozoa. Animals which returned to estrus were again inseminated at the following estrus. Pregnancy diagnosis was done after 60 days of insemination in all those buffaloes that do not come back to estrus. The overall pregnancy rate as well as 1^{st}, 2^{nd} and 3^{rd} service pregnancy rate was calculated for RBS and RBC buffaloes.

Statistical analysis

The mean (± SE) for different tmacro (calcium, phosphorus, magnesium, sodium) and micro (copper, iron and zinc) minerals of two experimental groups of buffaloes were computed. Completely randomized design (Steel et al., 2006) was applied with $P<0.01$ level of significance.

RESULTS

The serum means (± SE) for calcium, inorganic phosphorus, magnesium, sodium, copper, iron and zinc in repeat breeder and normal cyclic buffaloes are presented in Table 1.

The serum calcium, concentrations were significantly lower ($P<0.01$) in repeat breeder (RB) buffaloes as compared to normal cyclic (NC) buffaloes. Similarly, mean serum inorganic phosphorus and magnesium concentrations were also significantly lower in RB buffaloes as compared with NC buffaloes. Sodium concentrations differed non-significantly between repeat breeder and normal cyclic buffaloes. Among micro-minerals, the concentrations of copper, iron and zinc were significantly lower ($P<0.01$) in RB buffaloes when compared with NC buffaloes.

Thirty five (35) repeat breeder buffaloes were given orally as 150 g per day of the mineral mixture for 10 days and then at estrus, these animals were artificially inseminated with semen of a fertile bull. In repeat breeder (RBS) buffaloes treated with mineral mixture, the first service pregnancy rate was 42% while the second and third service pregnancy rates were 25 and 20%, whereas in repeat breeder control (RBC) buffaloes, the first service pregnancy rate was 6% while the second and third service pregnancy rates were 6 and 9%. The overall

Table 1. Mean (±SE) values for different minerals in the serum of repeat breeder and normal cyclic buffaloes.

Group	Calcium (mg/dl)	Inorganic phosphorus (mg/dl)	Magnesium (mg/dl)	Sodium (mmol/l)	Copper (µg/dl)	Iron (µg/dl)	Zinc (µg/dl)
Repeat breeder (n=35)	7.46^{a}±0.29	3.71^{a}±0.19	2.13^{a}±0.03	138.2±1.22	64.3^{a}±1.09	353.81^{a}±1.96	139.82^{a}±1.67
Normal cyclic (n=35)	9.22^{b}±0.33	5.56^{b}±0.11	2.18^{b}±0.04	139.9±1.69	72.5^{b}±1.35	367.49^{b}±1.31	172.19^{b}±1.46

Values sharing different superscripts in the same column differed significantly (P<0.01).

pregnancy rate in repeat breeder buffaloes treated with mineral mixture was 87% and it was 21% in repeat breeder control buffaloes.

DISCUSSION

Repeat breeding is among reproductive disorders which hinder favourable productivity in buffaloes (Sah and Nakao, 2006). In the present study, mean calcium concentrations were significantly lower (P<0.01) in RB buffaloes than NC buffaloes. These findings are in agreement with the results of many other workers (Chaurasia et al., 2010; Singh and Pant 1998; Chandrakar 1999). El-Shahata and Maatyb (2010), also reported that calcium plays a key part in improving the number and size of ovarian preovulatory follicles, and the ovulation rate.

In comparison with NC buffaloes, the concentrations of serum inorganic phosphorus were significantly lower (P<0.01) in RB buffaloes. Lower inorganic phosphorus concentration in repeat breeder buffalo have also been reported in many other studies (Chaurasia et al., 2010). Burleet al. (1995) pointed out significantly lower inorganic phosphorus concentrations in repeat breeder crossbred cows.

In this study, repeat breeder Nili-Ravi buffaloes had significantly lower (P<0.01) serum magnesium concentrations when compared with normal cyclic buffaloes. Magnesium deficiency in repeat breeder buffalo have also been reported earlier (Das et al., 2009). Though magnesium do not play any unwavering function in reproduction, yet it is involved in numerous enzymatic reactions catalysed by ATP linked enzymes. Moreover, magnesium affects the absorption of calcium and phosphorus (Sharma et al., 2004). Thus, magnesium imbalance can affect reproduction secondarily. The results of the present study specified that the lower calcium, inorganic phosphorus and magnesium concentrations might be associated to the ovulatory disturbances in repeat breeder buffaloes.

Serum copper, iron and zinc concentrations were significantly (P< 0.01) lower in repeat breeder buffalos compared to normal cyclic animals. Ahmad et al. (2010) also reported lower micro-minerals (Cu, Fe and Zn) in repeat breeder buffaloes. The lower concentrations of copper, iron and zinc found in this study coincide with the findings of other scientists (Das et al., 2009; Ceylan et al., 2008) working on repeat breeders buffalo cows.

The lower levels of serum iron (Fe) have been associated with altered usual gonadal activity by decreasing follicular growth and fertility (Ceylan et al., 2008). The lower concentrations of micro minerals in the present study might be associated to the ovulatory disturbances in repeat breeder buffaloes.

Repeat breeder buffaloes (RBS) of the present study when fed orally 150 g per day of the mineral mixture for 10 days, the 1st, 2nd and 3rd service pregnancy rates were 42, 25 and 20%, respectively while the overall pregnancy rate in these animals was 87%. Improvements in pregnancy rates after mineral mixture supplementation have been reported in Egyptian buffalo (Ahmed et al. 2010). The increase in pregnancy rate in our findings is also in accordance with those of Sah and Nakao (2006) who reported that 64.6% of repeat breeder buffaloes came to estrus and 58.4% conceived within one month after supplementation with vitamin/mineral mixture for three weeks.

In conclusion, this study demonstrates that the concentrations of macro (Ca, P and Mg) and micro (Cu, Fe and Zn) minerals were significantly lower in repeat breeder buffaloes which are perhaps essential for the normal ovulatory processes. Also, mineral mixtures should be added in the food stuff to improve reproductive efficiency of repeat breeder buffaloes.

REFERENCES

Ahmed WM, El-khadrawy HH, Hanafi EM, Ali AH, Shalaby SA (2010). Clinical perspective of repeat breeding syndrome in buffaloes. J. Am. Sci. 6:661-666.

Burle PM, Mangle NS, Kothekar MD,Kalorey DR (1995). Blood biochemical profiles during various reproductive states of Sahiwal and Jersey x Sahiwal cattle.Livest.Advis.20:13-20.

Butler WR (2000).Nutritional interactions with reproductive performance in dairy cattle. Anim. Reprod. Sci.61:449-457.

Ceylan A, SerinI, AksitH,Seyrek K (2008). Concentrations of some elements in dairy cows withreproductive disorders. Bullet. Vet. Inst. Pulawy 52:109-112.

Chandrakar D (1999). Studies on microbial and biochemical profile with therapeutic measures in repeat breeder cross-bred cows. M. V. Sc. & A. H. Thesis, G.K.V.V. Raipur.

Chaurasia R, Kushwaha HS, Chaurasia D, Gendley MK, Santra AK (2010). Comparative studies of certain macro minerals during various reproductive states in buffaloes. Buff.Bullet. 29(4):291-298.

Ceylan A, Serin I, Aksit H, Seyrek K (2008). Concentrations of some elements in dairy cows with reproductive disorders. The Bulletin of the Vet.Inst.In Pulway, 52:109-112.

Das JM, Dutta P, Deka KC, Biswas RK, Sarmah BC, Dhali A (2009). Comparative study on serum macro and micro mineral profiles during oestrus in repeat breeding crossbred cattle with impaired and normal ovulation. Livest. Res. Rural Dev. 21, http://www.lrrd.org/lrrd21/5/das21072.htm.

Das S, Bandopadhya SK, Basu S, Ghosh BB, Dattagupta R (2002). Blood mineral profile of normal cyclic and repeat breeder crossbred cows under rural condition. Ind. J.Anim. Reprod. 23:167-169.

Dochi O, Takahashi K, Hirai T, Hayakawa H, Tanisawa M, Yamamoto Y, Koyama H (2008). The use of embryo transfer to produce pregnancies in repeat-breeding dairy cattle. Theriogenology 69:124-128.

El-Shahata KH, Maatyb AMA (2010). The effect of dietary supplementation with calcium salts of long chain fatty acids and/ or l-carnitine on ovarian activity in Rahmani ewes. Anim. Reprod. Sci. 117(1):78-82.

Jain NC (1993). Essentials of Veterinary Hematology. Wiley, Medical 417 p.

Lucy MC (2001). Reproductive loss in high-producing dairy cattle: where will it end? J. Dairy Sci. 84:1277-1293.

Noakes DE, Parkinson TJ, Gary CW (2009). Veterinary Reproduction and Obstetrics, Saunder Elsevier, 9th Edition, 833.

Sah SK, Nakao T (2006). Characteristics of repeat breeding buffaloes in Nepal. J. Reprod. Dev. 52:335-341.

Sharma MC, Joshi C, Saxena N, Das G (2004). Role of minerals in reproductive performance of livestock. Livest. Intl. 8:5-10.

Singh M, Pant HC (1998). Blood biochemical profile of normal and repeat breeder cows in Himachal Pradesh. Ind. J. Anim.Reprod.19:156-157.

Steel RGD, Torrie JH, Dickey DA (2006). Principles and Procedures of Statistics. A biometrical approach. 3rd Ed., McGraw Hill Co. New York, USA.

Wiltbank M, Lopez H, Sartori R, Sangsritavong S, Gumen A (2006). Changes in reproductive physiology of lactating dairy cows due to elevated steroid metabolism. Theriogenology. 65:17-19.

Yusuf M, Nakao T, Long ST, Yoshida C, KoikeK,Hayashi A (2010). Reproductive performance of repeat breeders in dairy herds. Theriogenology 73:1220-1229.

academicJournals

Vol. 13(10), pp. 1147-1153, 5 March, 2014
DOI: 10.5897/AJB2013.13240
ISSN 1684-5315 ©2014 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Heavy metal, proximate and microbial profile of some selected commercial marine fish collected from two markets in south western Nigeria

Ogundiran, M. A.[1]*, Adewoye, S. O.[1], Ayandiran, T. A.[1] and Dahunsi, S. O.[2]

[1]Department of Pure and Applied Biology, Ladoke Akintola University of Technology, Ogbomoso, Nigeria.
[2]Department of Biological Sciences, Landmark University, Omun-Aran, Nigeria.

Accepted 17 December, 2013

The study on the elemental, proximate and microbial composition of fresh samples of *Scomber scombrus*, *Gadus macrocephalus*, *Saclina pilchradus* and *Jack mackerel* was determined to gain the knowledge of the risk and benefits associated with indiscriminate consumption of marine fishes. Wet digestion was done for the samples and was analyzed for minerals, heavy metals and microorganisms. The species examined contained appreciable concentrations of moisture, protein, lipids and ash content suggesting that the fish species could be used as a good source of minerals. Heavy metals analyzed were above tolerable limits; therefore, it can be suggested that taste, size, freshness and other related external appearances should not be the only factor to be considered in making choice for marketing and consumption of marine and freshwater fishes in Nigeria.

Key words: *Scomber scombrus*, *Gadus macrocephalus*, *Saclina pilchradus*, *Jack mackerel*, elemental, proximate, microbial, heavy metals.

INTRODUCTION

Malnutrition (protein energy malnutrition, micronutrient deficiencies and over nutrition) is a public health problem in developing countries. Chronic malnutrition in Nigeria according to the 2008 Nepal Demographic and Health Survey Fact Sheet (NDHS) was 38% (NPC and ICF Macro, 2009). The nutrient composition of locally available foods/diets are used to estimate the adequacy of dietary intake of population groups, determine diet-disease relationships, health and nutritional status, and for achieving dietary intake goals (Ene-Obong et al., 2013).

Fish and fish products are highly nutritious with protein content of 15 to 20% and are particularly efficient in supplementing the cereal and tuber diets widely consumed in Africa (Fagbenro et al., 2005). It was further reported that in Nigeria, fish are regarded as a major food item contributing a total of 40% to dietary protein. It is also a preferred and reliable source of animal protein with balanced amino-acids, vitamins and essential minerals for healthy human growth. Fish allows for protein improved nutrition in that it has a high biological value in terms of high protein retention in the body (Anthony and Akinwumi, 1991).

Fish is a highly proteinous food consumed by a larger percentage of populace because of its availability and palatability (Foran et al., 2005). Also, when compared to other protein sources like goat and chicken meat, it is safer, healthier and is also known to be an excellent source of protein from amino acid composition and protein digestibility (Astawan and Ikan, 2004). Fish is also one of the main sources of protein in developing countries (Louka et al., 2004). In Nigeria, fish is eaten fresh and smoked and form a much cherished delicacy that cut across socio-economic, age, religions and

*Corresponding author. E-mail: Kelv2dav@yahoo.co.uk. Tel: +2348034237739, +23408167485017.

educational barriers (Adebayo et al., 2008).

According to the study of Adekoya and Miller (2004), fish and fish products constitute more than 60% of the total protein intake in adults especially in rural areas. The marine fish is generally cheaper and more abundant when compared with fresh water fishes, which are relatively more expensive in Nigeria. The major constituents of fish are moisture, protein and fat with minerals occurring in trace amount (Holland et al., 1993). All living organisms require these mineral elements and some of these biochemical attributes at moderate levels; but when they exceed metabolic demand or requirement, they tend to become accumulated in tissues of organisms such as fish which can only metabolize these substances to lesser extents because most of the heavy metals are non-biodegradable (Lenntech, 2006). Nutrient content varies with fish species. There are limited data on the nutritional composition of fish species which are commonly consumed by the poor in developing countries of Asia and sub-Saharan Africa (Oladipo and Bankole, 2013).

In a study by Adeniyi et al. (2012), the nutritional composition of three fish species namely catfish (*Clarias gariepinus*), electric fish (*Malapterurus electricus*) and tilapia fish (*Tilapia guineensis*) were analyzed. Oladipo and Bankole (2013) carried out a study on the nutritional and microbial quality of fresh and dried *C. gariepinus* and *Oreochromis niloticus.* Emmanuel et al. (2011) carried out a comparative analysis of the proximate composition of *Tarpon atlanticus* and *C. gariepinus* from culture systems in South-Western Nigeria. Fawole et al. (2007) carried out a study on the proximate and mineral composition in some selected fresh water fishes in Nigeria and discovered the presence of heavy metals (such as zinc, copper, lead, cadmium, nickel and arsenic) present in the order Zn > Ni > As > Cu > Pb > Cd). Olagunju et al. (2012) evaluated the nutrient composition of *Tilapia zilli*, *Hemisynodontis membrenacea*, *Clupea harengus* and *Scomber scombrus* consumed in Zaria. Omotosho (1995) did a comparative analysis of the chemical composition of *Dasyatis margarita* (Gunther) with *T. zilli* (Gervias) and *C. gariepinus* (Burchell) from Nigerian waters. Fish has the potential to be considered as a balanced food and can therefore be expected to provide relief from malnutrition; Sanker et al. (2013) investigated the chemical composition and nutritional value of Anchovy (*Stolephorus commersonii*) caught from Kerala coast, India. They reported that the proximate composition of anchovies compares well with the general composition of fish reported earlier by Gopakumar (1997), Ninan (2003), Devi (2006) and Mohan et al. (2008).

Fish mineral and metal contents may vary according to the surrounding environment (Ambedkar and Muniyan, 2011; Sen et al., 2011). Heavy metal contamination may have devastating effects on the ecological balance of the recipient environment and a diversity of aquatic organisms (Ashraj, 2005; Vosylene and Jankaite, 2006; Farombi et al., 2007). Heavy metals have long been recognized as serious pollutants of the aquatic system. The heavy metals that are toxic to many organisms at very low concentrations and are never beneficial to living beings are Hg, Cd and Pb (Dural et al., 2006). The fish is often exposed to various microorganisms. A number of these microorganisms are naturally present in the aquatic environment, and some of them enter water via animal excreta, agricultural runoff, industrial and human wastes (Adams and Thompson, 2006; Almedia et al., 2009; Altinok et al., 2008).

MATERIALS AND METHODS

Sample collection

The fish samples used for this study were collected from two different major markets in Ogbomoso, Oyo State, Nigeria.

Sample preparation

The fish samples were thoroughly washed with tap water and distilled water to remove any adhering contaminants and drained under folds of filter paper. The fish sample was dissected with a knife and the intestines, guts and bones were removed. The muscle samples were then homogenized into a fine mesh with an electric food blender and thereafter, stored in a deep freezer (- 18°C) prior to analysis.

Metal analysis

Three specimens from each species were used for the analysis. The tissues were oven dried at 70 to 73°C until constant weight was obtained. The specimens were then ground to fine powder and stored in desiccators in order to avoid moisture accumulation before digestion. The digestion procedure was carried out as described by Kotze et al. (2006). Twenty milliliter (20 ml) of concentrated nitric acid (55%) and 10 ml of perchloric acid (70%) was added to approximately 1 g tissue (dry mass) in a 100 ml Erlenmeyer flask. The digestion was done on a hotplate (200 to 250°C) until the solutions were clear. The solutions were then filtered through an acid resistant 0.45 mm filter paper and made up to 50 ml each with distilled water. The samples were stored in clean glass bottles prior to the determination of the metal concentration using an inductively coupled plasma mass spectrophotometer (ICP-MS). A standard sample, consisting of tuna homogenate (sample IAEA-350) from the International Atomic Energy Agency Marine Environment Laboratory, was prepared and used as a control in accordance with the afore-mentioned procedures with every set of samples, to ensure accuracy of data through comparison.

Analytical standards were prepared from Holpro stock solutions. Prior to use, all glassware were soaked in a 2% Contrad soap solution (Merck chemicals) for 24 h, rinsed in distilled water, acid-washed in 1 m HCL for another 24 h and rinsed again in distilled water.

Proximate analysis

Moisture content analysis: Moisture content of fish fillets was determined according to the method of AOAC (2000). The samples

Table 1. Mean and standard error of heavy metal composition of fish species collected from the two markets.

Sample	Fe	Pb	Ni	Cd	Zn	Cu
Scomber scombrus	3.1561±0.1230	0.4571±0.0163	0.0256±0.0116	0.4883±0.0123	0.2724±0.0048	0.2766±0.0030
Sardina pilcladus	5.6259±0.0052	0.1265±0.0029	0.3141±0.0036	0.1937±0.0001	0.3531±0.0001	0.2394±0.0004
Jack mackerel	0.8658±1.000	0.0141±0.0001	0.0135±0.0100	0.0234±1.0040	0.0964±0.0002	0.0161±5.7705
Gadus macrocephalus	2.3714±1.000	0.2024±0.0001	0.1011±1.000	0.0298±1.0000	0.3448±1.0000	0.1271±0.000

were dried in moisture dish in an oven at 105°C until constant weights was obtained.

Ash content analysis: Ash content of fish fillets was determined according to Association of Official Analytical Chemists. Pre-dried samples obtained from moisture content analysis were ashed in furnace at 550°C overnight.

Crude protein analysis: Crude protein content of fish fillets was determined according to the method of AOAC (2000). Briefly, 1 g of sample was weighed into digestion tubes. Two Kjeltabs Cu 3.5 (catalyst salts) were added into each tube. About 20 ml of concentrated sulphuric acid (H_2SO_4) was carefully added into the tube and then shaken gently. Digestion procedure was carried out. Digested samples were cooled for 10 to 20 min. Distillation procedure was then performed using distillation unit and the distillate was titrated with 0.025N sulphuric acid (H_2SO_4) until the end point changes from green to pink. Volume of acid required in the titration was recorded. Blank was prepared with the exclusion of sample. The percentage of protein content was calculated according to the following equation:

$$\% \text{ Nitrogen} = \frac{0.014 \times VD \times N \times 100 \times TV}{\text{Weight of sample} \times AD}$$

% Protein = %N × F

Where, VD is the Volume of digest; N is the normality of acid; TV is the titre value; AD is the aliquot of digest and F is the conversion factor for nitrogen to protein (6.25).

Fat content analysis: Crude fat was obtained by exhaustively extracting 2.0 g of each sample in a Soxhlet apparatus using petroleum ether (b.p. 40 to 60°C) as the extractant.

Carbohydrate content analysis: Carbohydrate content was calculated based on difference calculation:

Carbohydrate = 100% - (%moisture + %ash + %crude protein + %fat).

Microbial analysis

The microbial content of the fish samples were enumerated by standard plate count technique using 0.1 ml aliquots of appropriate dilution pour plated onto Nutrients agar, MacConkey, Mannitol salt agar and Salmonella-Shigella agar for bacteria. Potato dextrose agar (PDA) plus chloramphenicol was used for fungi isolation and enumeration. All plates for bacteria isolation were incubated at 37°C for 24 to 48 h while PDA plates were incubated at room temperature for 3 to 5 days. Individual colonies were purified and identified by morphological and biochemical techniques (Jott et al., 1994). In the case of fungal isolates, the microscopic and macroscopic features of the hyphal mass, morphology of cells and spores, nature of the fruiting bodies, among other criteria were used for identification (Tsuneo, 2010).

Statistical analysis

Analysis of the data was carried out using the excel worksheet package.

RESULTS

Metal composition

The mean values for the heavy metal composition of the fish species is shown in Table 1. In *S. scombrus*, Fe had the highest concentration of 3.1561 while other metals were found in trace quantities with the lowest being Ni (0.0256). *Sardina pilcladus* had the highest metal concentration of 5.6259 for Fe and the lowest is 0.1265 for Pb. In *Jack mackerel*, the highest concentration was 0.8658 for Fe while the lowest value of 0.0135 was obtained for Ni.

For *Gadus macrocephalus*, Fe had the highest value of 2.3714 while Cd had the lowest value of 0.0298. The overall order of heavy metal concentration in all the fish species is Fe > Cd > Pb > Zn > Ni > Cu.

Moisture, protein, carbohydrate and lipid composition

Table 2 shows the mean values for the proximate composition of the fish species. The highest moisture content was found in *G. macrocephalus* with value of 81.4233 while the lowest was recorded for *S. pilcladus* with 57.5700. For the protein content of the fishes, *S. pilcladus* had the highest value of 26.3866 while the lowest value of 11.7233 was recorded for *G. macrocephalus. G. macrocephalus* had the highest ash content of 1.4866 among the four fish species while *S. scombrus* recorded the lowest value of 1.1133. Considering the lipid content of the fish species, *S. scombrus* had the highest value of 10.2133 while *G. macrocephalus* recorded the lowest value of 2.0866. For the carbohydrate content, *S. pilcladus* recorded the highest value of 7.1233 while *S. scombrus* had the lowest value of 2.5133.

Table 2. Mean and standard error of proximate composition of fish species collected from the two markets.

Sample	Moisture content (%)	Protein content (%)	Ash content (%)	Lipid content (%)	Carbohydrate content (%)
Scomber scombrus	63.3866±0.5398	23.0900±0.0100	1.1133±0.0057	10.2133±0.0057	2.5133±0.0152
Sardina pilcladus	57.5700±0.3732	26.3866±0.0057	1.3033±0.0057	7.8133±0.0057	7.1233±0.0057
Jack mackerel	72.8566±0.0208	16.7833±0.0057	1.4166±0.0115	6.0866±0.0057	2.8833±0.0057
Gadus macrocephalus	81.4233±0.0057	11.7233±0.0057	1.4866±0.0057	2.0866±0.0057	3.3033±0.0057

Table 3a. Bacterial count in fish species collected from the two markets.

Fish species	× 10^3 cfu ml^{-1}
Scomber scombrus	4.0
Sardina pilcladus	3.0
Jack mackerel	7.0
Gadus macrocephalus	10.8

Table 3b. Fungal count in fish species collected from the two markets.

Fish species	× 10^3 cfu ml^{-1}
Scomber scombrus	1.0
Sardina pilcladus	1.0
Jack mackerel	1.0
Gadus macrocephalus	1.0

Microbial analyses

The microbial profile of the four fish species reveal that *G. macrocephalus* had the highest bacterial concentration of 10.8 × 10^3 cfu ml^{-1} while *S. pilcadus* had the lowest bacterial count of 3.0 × 10^3 cfu ml^{-1}. All the fish species were found to have recorded the same values (1.0 × 10^3 cfu ml^{-1}) of fungal count (Table 3a and b). Table 4 shows the different microorganisms isolated from each fish species. *S. scombrus* was found to have *Staphylococcus aureus*, *Escherichia coli*, *Lactobacillus plantarum* and *Pseudomonas florescence.* From *G. macrocephalus*, *Clostridium botulinum* and *S. aureus* were isolated. *S. aureus*, *Salmonella* species, *Shigella* species and *E. coli* were isolated from *Sardina pilcladus* while from *J. mackerel*, *P. florescence* and *Flavobacterium* species were both isolated. Table 5 shows the antibiotic resistivity of the bacterial isolates to chloramphenicol, ampicillin and tetracycline, respectively. The highest resistance to chloramphenicol (32 mm) was exhibited by *P. florescence* isolated from *J. mackerel* while the lowest (12 mm) was exhibited by *Shigella* species isolated from *S. pilcladus.*

For Ampicillin, the highest resistance (40 mm) was exhibited by *S. aureus* isolated from *S. scombrus* while the lowest (25 mm) was exhibited by *P. florescence* isolated from *S. scombrus.* The highest resistance to tetracycline (50 mm) was exhibited by *E. coli* isolated from *S. pilcladus* while the lowest (29 mm) was exhibited by *E. coli* isolated from *G. macrocephalus.*

DISCUSSION

In the present study, heavy metals were found to have bioaccumulated in the tissues of the fishes under study following different patterns. Such pattern has been observed in a number of other studies, covering several fish species (Dural et al., 2006; Storelli et al., 2006; Ploetz et al., 2007; Rashed, 2001; Pyle et al., 2006; Agah et al., 2009). Muscle has been considered to have metal accumulating potential (Erdogrul and Erbilir, 2007; Uysal et al., 2009; Bervoets and Blust, 2003; Dahunsi et al., 2012). Also, Kotze et al. (2006) and Senthil et al. (2008) reported significant bioaccumulation of metals in fish muscle. The high value recorded for Fe in the fish muscles may be due to its availability in the water and feeds they consume. This trend agrees with other studies where elevated amount of Fe was found in fish tissues (Yilmaz et al., 2007; Dural et al., 2006; Uysal et al., 2009; Dahunsi et al., 2012). The accumulation pattern of Cd and other metals studied conformed closely with the work done by Vinodhini and Navayanan (2008) where they carefully observed the trend of bioaccumulation of heavy metals in various organs of the fresh water fish *Cyprinus carpio* (common carp) exposed to heavy metal contaminated water system. Cadmium was found in this study to have low levels of bioaccumulation in the body muscle investigated. Cadmium is considered in other works to have the highest concentration in liver.

Ivan et al. (2011) reported that it reached 40 to 100 times greater concentrations in muscle than those found by Agusa et al. (2004) in five Caspian sturgeon species. An increased Cd levels in these fishes is worrying, especially considering the fact that it could be very hazardous for fish genetic material according to the study of Alibabic et al. (2007) and it is one of the most toxic heavy metals, even at relatively low concentrations (Dural et al., 2006; Fianko et al., 2007; Yilmaz et al., 2007).

Table 4. Isolated bacteria from fish species collected from the two markets.

Fish	Organism
Scomber scombrus	*Staphylococcus aureus* *Escherichia coli* *Lactobacillus plantarum* *Pseudomonas florescence*
Gadus macrocephalus	*Clostridium botulinum* *Staphylococcus aureus*
Sardina pilcladus	*Staphylococcus aureus* *Salmonella species* *Shigella species* *Escherichia coli*
Jack mackerel	*Pseudomonas florescence* *Flavobacterium species*

Table 5. Antibiotic sensitivity of bacteria isolates collected from the two markets.

Fish	Organism	Chl (mm)	Amp (mm)	Tet (mm)
Scomber scombrus	*Staphylococcus aureus*	23	40	45
	Escherichia coli	20	38	40
	Lactobacillus plantarum	26	35	38
	Pseudomonas florescence	20	25	35
Gadus macrocephalus	*Clostridium botulinum*	16	30	49
	Staphylococcus aureus	22	28	39
	Escherichia coli	19	26	29
Sardina pilcladus	*Staphylococcus aureus*	26	29	45
	Salmonella species	32	38	47
	Shigella species	12	26	40
	Escherichia coli	21	36	50
Jack mackerel	*Pseudomonas florescence*	32	37	49
	Flavobacterium species	25	34	40

Chl, Chloramphenicol; Amp, ampicillin; Tet, tetracycline.

These metals can remain in the body like other heavy metals for a long period of time and can bioaccumulate for many years after exposure to low levels (Groundwork, 2002).

A fish contaminated with these metals can find its way into man's food chain, resulting in biomagnifications of such heavy metal and this becomes harmful to man's health. Generally, virtually all the heavy metals analyzed were found to exceed National Environmental Standard and Regulation Enforcement Agency (NESREA) (2007) and WHO (2011) standards. For the proximate composition of the fishes, moisture of a given samplesimply refers to the water content of that sample. The fish moisture content indicates that the percentage moisture in fish muscles was within the acceptable level (60 to 80%) in all the samples which could be due to the stable water levels in the environmental location where the fish were raised. The percentage of water is also a good indicator of its relative content of energy, protein and lipid. The high moisture content is a disadvantage in that it increases the fishes' susceptibility to microbial spoilage, oxidative degradation of polyunsaturated fatty

acids and consequently decreases in the quality of the fishes for longer preservation time in agreement with Omolara and Omotayo (2008).

The protein levels shows that all the fish species are good sources of protein. The relatively high to moderate percentage crude protein may be attributed to the fact that fishes are good source of pure protein, but the differences observed in values obtained could also be as a result of fish consumption or absorption capability and conversion potentials of essential nutrients from their diets or their local environment into such biochemical attributes needed by the organisms body (Adewoye and Omotosho, 1997). The observed range of ash content in the fishes indicates that the species is a good source of minerals such as calcium, potassium, zinc, iron and magnesium.

Ash is a measure of the mineral content of food item. It is the inorganic residue that remains after the organic matter has been burnt off. Generally, lipids are soluble in ether; hence, they are ether extractable. They serve as source of energy during starvation and fasting. According to the study of Ackman (1989), generally, fish can be grouped into four categories according to their fat content: lean fish (< 2%), low fat (2 to 4%), medium fat (4 to 8%) and high fat (> 8%). These marine fishes had a high lipid content; hence, their classification as high fat fishes. This indicates that the fishes are better sources of lipid in the body when consumed (Osibona et al., 2009). The appreciable values of carbohydrate could be due to the presence of elements like calcium and potassium in their diets. The microorganisms isolated from the fishes under study are an indication of the nature of the aquatic environment where they were reared. This conforms with previous studies that a number of these microorganisms are naturally present in the aquatic environment, and some of them enter water via animal excreta, agricultural runoff, industrial and human wastes (Adams and Thompson, 2006; Almedia et al., 2009; Altinok et al., 2008).

Also, it has been reported that the spread of antibiotic resistant microorganisms in the environment is recognized as an important public health issue which has attracted the attention of physicians concerning their future ability to treat infectious diseases (Mukherjee and Chakraborty, 2007).

Conclusion

This study therefore, showed that marine fishes are good sources of minerals. Thus, it is right to say that, the mineral elemental contents of each species is a function of the availability of these elements in their local environment, diet absorptive capability and as well as their preferential accumulation. However, it was discovered that, some hazardous heavy metals and pathogenic microbes recorded high values which may pose danger to the humans consuming such fishes. Also, this present work has elucidated more on the importance of marine fishes as good sources of protein and minerals.

REFERENCES

Ackman RG (1987). Nutritional composition of fats in seafoods. Prog. Food Nutr. Sci. 13:161-241.

Adams A, Thompson KD (2006). Biotechnology offers revolution to fish health management. Trends Biotechnol. 24:201-205.

Adebayo-Tayo BC, Onilude AA, Patrick UG (2008). Mycofloral of smoke-dried fishes sold in Uyo, Eastern Nigeria. World J. Agric. Sci. 4(3):346-350.

Adekoya BB, Miller JW (2004). Fish cage culture potential in Nigeria - An overview. National Cultures. Agric. Focus 1(5):10.

Adeniyi SA, Orjiekwe CL, Ehiagbonare JE, Josiah SJ (2012). Nutritional composition of three different fishes (*Clarias gariepinus, Malapterurus electricus* and *Tilapia guineensis*). Pak. J. Nutr. 11(9):793-797.

Adewoye, S.O. and Omotosho, J. S.(1997). Nutrient Composition of some freshwater Fishes in Nigeria. BioSci. Res. Commun.11(4)333-336.

Agah H, Leermakers M, Elskens M, Fatemi SMR, Baeyens W (2009). Accumulation of trace metals in the muscle and liver tissues of five fish species from the Persian Gulf. Environ. Monit. Assess. 157:499-514.

Agusa T, Kunito T, Tanabe S, Pourkazemi M, Aubrey D (2004). Concentrations of trace elements in muscle of sturgeons in the Caspian Sea. Mar. Pollut. Bull. 49:789-800.

Alibabic C, Vahcic N, Bajramovic M (2007). Bioaccumulation of metals in fish of Salmonidae family and the impact on fish meat quality. Environ. Monit. Assess. 131:349-364.

Almedia A, Cunha A, Gomes N, Alves E, Costa L, Faustino M (2009). Phage therapy and photodynamic therapy: Low Environmental impact Approaches to Inactivate Microorganisms in Fish Farming plants. Mar. Drugs 7(3):263-313.

Altinok I, Capkin E, Kayis S (2008). Development of multiplex PCR assay for simultaneous detection of five bacterial fish pathogens. Vet. Microbiol. 131(3-4):332-8.

Ambedkar G, Muniyan M (2011). Bioaccumulation of some Heavy Metals in the selected five freshwater fish from Kollidam River, Tamilnadu, India. Adv. Appl. Sci. Res. 2(5):221-225.

Anthony OR, Akinwumi JA (1991). Supply and distribution of fish in Ibadan, Nigeria. Geogeogr. J. 14(2):16.

AOAC, (2000). Official Methods of Analysis. 17th ed. Gaithersburg, Maryland, USA, AOAC International, 2000.

Ashraj W (2005). Accumulation of heavy metals in kidney and heart tissues of *Epinephelus microdon* fish from the Arabian Gulf. Environ. Monit. Assess. 101 (1-3):311-316.

Astawan M, Ikan Y (2004). Sedap dan bergizi. Penerbit Tiga Serangkai. Solo.

Bervoets L, Blust R (2003). Metal concentrations in water sediment and gudgeon (*Gobio gobio*) from a pollution gradient: relationship with fish condition factor. Environ. Pollut. 126:9-19.

Dahunsi SO, Oranusi SU, Ishola RO (2012). Differential bioaccumulation of heavy metals in selected biomarkers of *Clarias gariepinus* (Burchell, 1822) exposed to chemical additives effluent. J. Res. Environ. Sci. Toxicol. 1(5):100-106.

Devi VR (2006). Influence of ionic strength on conformation and functional properties of fish muscle proteins. M. FSc. Dissertation, Central Institute of Fisheries Education, India.

Dural M, Goksu MZI, Ozak AA, Derici B (2006). Bioaccumulation of some heavy metals in different tissues of *Dicentrachus labrax* L., 1758, *Sparus aurata* L., 1758, and *Mugil cephalus* L., 1758 from the Camlik lagoon of the eastern coast of Mediterranean (Turkey). Environ. Monit. Assess. 118:66-74.

Emmanuel BE, Oshionebo C, Aladetohun NF (2011). Comparative analysis of the proximate composition of *Tarpon atlanticus* and *Clarias gariepinus* from culture systems in South-Western Nigeria. Afr. J. Food Agric. Nutr. Dev. 11(6):5344-5359.

Ene-Obong HN, Sanusi RA, Udenta EA, Williams IO, Anigo KM, Chibuzo EC, Aliyu HM, Ekpe OO, Davidson GI (2013). Data

collection and assessment of commonly consumed foods and recipes in six geo-political zones in Nigeria: Important for the development of a national food composition database and dietary assessment. Food Chem. 140:539-546.
Erdogrul O, Erbilir F (2007). Heavy metals and trace elements in various fish samples from Sir Dam Lake, Kahramanmaras, Turkey. Environ. Monit. Assess. 130 373-379.
Fagbenro OA, Akinbulumo MO, Adeparusi OE, Raji AA (2005). Flesh yield, waste yield, proximate and mineral composition of four commercial West African freshwater food fishes. J. Anim. Vet. Adv. 4(10):848-851.
Farombi EO, Adelowo OA, Ajimoko YR (2007). Biomarkers of oxidative stress and heavy metal levels as indicators of environmental pollution in African Cat fish (*Clarias gariepinus*) from Nigerian Ogun river. Int. J. Environ. Res. Public Health 4(2), 158-165.
Fawole OO, Ogundiran MA, Ayandiran TA, Olagunju OF (2007). Proximate and mineral composition in some selected fresh water fishes in Nigeria. Internet J. Food Saf. 9:52-55.
Fianko JR, Osae S, Adomako D, Adotey DK, Serfor-Armah Y (2007). Assessment of heavy metal pollution of the Iture Estuary in the central region of Ghana. Environ. Monit. Assess. 131:467-473.
Foran JA, Carpenter DO, Hamilton MC, Knuth BA, Schwager SJ (2005). Risk based consumption advice for farmed Atlantic and wild pacific salmon contaminated with dioxins and dioxin-like compounds. Environ. Health Perspect. 33:552-556.
Gopakumar K (1997). In: Biochemical composition of Indian food fish. Central Institute of Fisheries Technology. Harris WS, "n-3 fatty acids and serum lipoproteins: human studies". Am. J. Clin. Nutr. 65:1645S-54S.
Groundwork (2002). Retrieved from: http://www. Groundwork.org.za/chemical-profiles.htm.
Holland B, Brown J, Buss DH (1993). Fish and fish products; the third supplement to McCance & Widdowson's "The composition of foods" 5th edition, HMSO, London.
Ivan J, Željka VJ, Gorin C, Zoran G, Ljubinko J, Stefan S, Mirjana L (2011). Determination of differential heavy metal and trace element accumulation in liver, gills, intestine and muscle of sterlet (*Acipenser ruthenus*) from the Danube River in Serbia by ICP-OES. Microchem. J. 98:77-81.
Jott JG, Krleg NR, Sneath PHA, Stanley JT, Williams ST(1994). Bergey's manual of Systematic Bacteriology, 9th Edition. William and Wilkins CO., Baltimore, Maryland. pp. 786.
Kotze PD, Preez HH, Van-Vuren JHJ (2006). Bioaccumulation of copper and Zinc in *Oreochromis mossambicus* and *Clarias gariepinus*, from the Olifants River, Mpumalanga, South Africa. Dept. of Zoology, Rand Afrikans University press ISSN 0378-4738 = Water SA Vol. 25 No. 1, 2006 South Africa.
Lenntech (2006). Lenntech Water Treatment and air purification holding B.V. Retrieved June 2006, from http://www.lenntech.com/feedback2.htm
Louka N, Juhel F, Fazilleau V, Loonis P (2004). A novel colorimetry analysis used to compare different drying fish processes. Food Control 15:327-334.
Mohan M, Ramachandran D, Sankar TV, Anandan R (2008). Physicochemical characterization of muscle proteins from different regions of mackerel (Rastrelliger kanagurta). Food Chem. 106:451-457.
Mukherjee S, Chakraborty R (2007). Conjugation potential and class 1 integron carriage of resident plasmids in river water copiotrophs. Acta Microbiol. Immunol. Hung. 54:379- 397.
NESREA (2007). National Environmental Standards and Regulations Enforcement Agency (establishment) act, 2007.
Ninan G (2003). In: Chemical composition of fish and shellfish. J. Joseph, P. T. Mathew, A. C. Joseph, & V. Muraleedharan (Eds.), Productdevelopment and seafood safetyIndia:Central Institute of Fisheries Technology. pp. 15-32.
NPC, ICF Macro (2009). National Population Commission, Nigeria. Nigerian Demographic and Health Survey, 2008, Abuja, Nigeria. National Population Commission, Federal Republic of Nigeria, Abuja, Nigeria. Available from:<pdf.usaid.gov/pdf-docs/PNADQ923.pdf> Retrieved 27.11.11.
Oladipo IC, Bankole SO (2013). Nutritional and microbial quality of fresh and dried *Clarias gariepinus* and *Oreochromis niloticus.* Int. J. Appl. Microbiol. Biotechnol. 1:1-6.
Olagunju A, Mohammad A, Mada SB, Mohammed A, Mohammed HA, Mahmoud KT (2012). Nutritional composition of *Tilapia zilli, Hemisynodontis membranacea, Clupea harengus* and *Scomber scombrus* consumed in Zaria. World J. Life Sci. Med. Res. 2:16.
Omolara OO, Omotayo OD (2008). Preliminary Studies on the effect of processing methods on the quality of three commonly consumed marine fishes in Nigeria. Biokemistri J. 21:1-7.
Omotosho, J.S (1995). Comparative analysis of the chemical composition of *Dasyatis margarita* (Gunther) with *Tilapia zilli* (Gervias) and *Clarias gariepinus* (Burchell) from Nigerian waters. *Bioscience Research Communications.* 8(3):215-219.
Osibona AO, Kusemiju K, Akande GR (2009). Proximate composition and fatty acids profile of the African Catfish *Clarias gariepinus*. *Acta* SATECH 3(1):85-9.
Ploetz DM, Fitts BE, Rice TM (2007). Differential accumulation of heavy metals in muscles and liver of a marine fish, (King Mackerel, *Scomberomorus cavalla*, Cuvier) from the Northern Gulf of Mexico, USA. Bull. Environ. Contam. Toxicol. 78:124-127.
Pyle GG, Rajotte JW, Couture P (2006). Effects of industrial metals on wild fish populations along a metal contamination gradient. Ecotox. Environ. Saf. 61, 287-312.
Rashed MN (2001). Monitoring of environmental heavy metals in fish from Nasser Lake. Environ. Int. 27:27-33.
Sanker TV, Amandan R, Mthew S, Asha KK, Lakshmanan PT (2013). Chemical composition and nutritional value of Anchovy (*Stolephorus commersonii*) caught from Kerala Coast, India. Eur. J. Exp. Biol. 3(1):85-89.
Sen I, Shandil A, Shrivastava VS (2011). Study for determination of heavy metals in fish species of the river Yamuna (Delhi) by inductively coupled plasma-optical emission spectroscopy (ICP-OES) Adv. Appl. Sci. Res. 2(2):161-166.
Senthil MS, Karuppasamy K, Poongodi S, Puranesurin (2008). Bioaccumulation Pattern of Zinc in Freshwater Fish *Channa punctatus* (Bloch) after chronic exposure. Turk. J. Fish. Aquat. Sci. 8:55-59.
Storelli MM, Barone G, Storelli A, Marcotrigiano GO (2006). Trace metals in tissues of Mugilids (*Mugil auratus, Mugil capito* and *Mugil labrosus*) from the Mediterranean Sea. Bull. Environ. Contam. Toxicol. 77:43-50.
Tsuneo (2010). Pictorial atlas of soil for seed fungi:Morphologies of cultural fungi for key to species. Third Edition. CRC Press.
Uysal K, Kose E, Bulbul M, Donmez M, Erdogan Y, Koyun M, Omeroglu C, Ozmal F (2009). The comparison of heavy metal accumulation ratios of some fish species in Enne Darne Lake (Kutahya, Turkey). Environ. Monit. Assess. 157:355-362.
Vinodhini, R., Navayanan, M., 2008. Bioaccumulation of heavy metal in organs of fresh water fish *Cyprius capio* (Common carp). *Int. J. Environ. Sci. Tech*, 5(2), 178 -182.
Vosyliene MZ, Jankaite A (2006). Effect of heavy metal model mixture on rainbow trout biological parameters. Ekologija 4:12-17.
WHO (World Health Organization) (2011). Guideline for Drinking Water Quality. 4th edn., NLM Classification:WA 675, World Health Organization, Geneva, Switzerland. ISBN: 978 924 1548151. pp. 307-433
Yilmaz F, Ozodemir N, Demirak A, Tuna AL (2007). Heavy metal level in two fish species *Leuscius cephalus* and *Lepomis gibbosus*. Food Chem. 100:830-835.

academicJournals

Vol. 13(10), pp. 1154-1159, 5 March, 2014
DOI: 10.5897/AJB10.611
ISSN 1684-5315 ©2014 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

A modeling using the maximum growth capacity of *Hantzschia amphioxys* in the Homa Lagoon

Banu Kutlu* and Baha Buyukisik

Faculty of Fisheries, Tunceli University, 62000, Tunceli, Turkey.

Accepted 29 November, 2013

Homa lagoon found in the Izmir bay, Aegean Sea, is important because it is the last lagoon in which fishing activities are carried out. *Hantzschia amphioxys* species, on which this study was carried on, are benthic diatoms and they are isolated from Homa Lagoon. The Monod equation expressing the nutrient dependency of the growth rates can also be used for carrying capacity (with different parameter values; chl-a max, K_s^l). Temporal changes of the calculated maximum chl-a concentrations (carrying capacity) was 115 µg chl-a/L by determining the hyperbolical relations between the maximum biomass levels (biomass carrying capacity) and nutrient concentrations obtained from the phytoplankton growth graphs.

Key words: *Hantzschia amphioxys*, chl-a max, Homa lagoon.

INTRODUCTION

Fossil records of diatoms were found about 185 years ago (Rothpletz, 1896; Gersonde and Harwood, 1990). Diatoms are found in a wide variety of habitats including ice and soil, and they are also commonly found in both seas and freshwaters (Werner, 1977). Diatoms are the main components of phytoplankton organisms for many aquatic ecosystems.

They are named microphytobenthos in coastal areas. Moreover, they are found in high rates in the tide zones of many bays. Benthic diatoms contribute to 50% of total primary production (Perrissinotto et al., 2000; Montani et al., 2003). It is reported that diatoms have an important role in global biogeochemical cycle of nitrogen and carbon (Serodio, 2003). Furthermore, coastal waters are characterized by abundance of diatoms (Nelson et al., 1995).

In coastal waters, wave, wind, entrance of freshwater, etc. processes cause short term variations in hydrodynamic and chemical conditions of upper water column. In the subsequent process, increase of primary production is observed (Fouilland et al., 2007). Production of microphytobenthos has hourly, weekly, monthly, and seasonally changing characteristics in the tide zones of bays (Shaffer and Onuf, 1985; Pinckney and Zingmark, 1991; Smith and Underwood, 1998; Serodio and Catarino, 2000).

Microphytobenthos group is affected by environmental factors such as nutrient concentration, light intensity, salt and water temperature (Thomas, 1996; Dempster and Sammerfeld, 1998; Thessen et al., 2005).

Lagoon fields cause strong water and tide movements cause the rich water found at the bottom to flow out or cause the poor water found outside to flow in. Since water is shallow at these regions, the nutrients originating from the regeneration at the bottom are used more by benthic algae (Kutlu, 2000). Diatoms are the main

components of the phytoplanktons which also serve as an important nutrient source for all aquaculture (St. John et al., 2001). Therefore, they are very important for lagoon fields.

*Corresponding author. E-mail: banukutlu@tunceli.edu.tr or kutlubanu@gmail.com. Tel: +90.94282131784.

Although, the number of the kinetic studies for the Hantzschia genus, which includes the *Hantzschia amphioxys* species, is very few, the number of studies on the *H. amphioxys* species, which forms the subject of this study, is scarce. There is no study present about the isolation of the *H. amphioxys* species and determination of the kinetic parameters on its discontinuous mono culture.

The purpose of this study was to research the maximum growth capacity and the carrying capacity of the *H. amphioxys* species in the medium with nutrient limited discontinuous culture system and optimum temperature.

MATERIALS AND METHODS

H. amphioxys was isolated from the surface sea water of the Homa Lagoon, which was filtered before f/2 was added and replicate cultures were maintained, from Izmir Bay (Aegean Sea) by using the dilution method under conditions presenting with a 11 ± 0.5°C temperature, ‰ 33 salinity, 24 L: 0D day length, 52 µmol photon.m^2s-1 light intensity; 40 W day light fluorescent and f/2 enrichment medium. Turner Design 10AU field fluorometre was equipped with *in vivo* Chl-a filter set and calibrated with the culture of *H. amphioxys* using Hatch -Lange Model DR4000 spektrophotometer and three coloromatic method (Strickland and Parsons, (1972).

The culture medium prepared was incubated under constant (25, 45, 52, 58 and 65 µmol/m^2s) light intensities at 6, 12, 15, 16.5, 18, 20 and 25°C temperatures and the exponential phase was monitored. The measurements were estimated as the amount of chl a using the Turner Designs 10-AU Field Floumeter (Guillard, 1975; Guillard and Rather 1962; Brand and Guillard, 1981). The chlorophyll based specific growth rates were calculated following Guillard (1973):

$$\mu = 1/(t_2-t_1)*\log_2 (N_2/N_1)$$

Firstly, their growth rates in response to temperature were plotted. Each temperature value in the chart represented the experimental point for five different light intensities. The optimum growth rate was obtained under the conditions of 1.711 day^{-1} and 18°C and 52 µmol/m^2s light intensity. This result was defined as µopt. A curve was fitted that matched the experimental points in the chart. In order for this curve to include growth inhibition, the growth rates were obtained. Vollenweider (1965)'s formula describing the photosynthesis/light curves was modified and used to represent temperature curves (µ= µ max*f(T)*h(T)). h (T)= is a function describing inhibition. The whole Equation is as follows:

$$\mu = \mu_{max} * \frac{aT}{\sqrt{1+(aT)^2}} * \frac{1}{(\sqrt{1+(\alpha T)^2})^2}$$

Maximum Chl-a value in each growth curve (Chl-a/time) was recorded as biomass carrying capacity (BCC). Relationships (like µ/properties, BCC/temperatures, BCC/I, BCC/nutrient concentrations) were investigated statistically. The maximum biomass carrying capacity (BCC_{max}) and the half-saturation constants were calculated with the help of the Michaelis-Menten Equation. All the half saturation constants for BCC/I curves (for seven different temperatures) were not statistically significant ($p = 0.55$). But the calculated values of BCC max were statistically significant ($p<0.005$), expect for 16.5 and 20°C.

The maximum biomass carrying capacity (BCCmax) and the half-saturation constants were calculated with the help of the Michaelis-Menten Equation. All the half saturation constants for BCC/ I curves (for seven different temperatures) were not statistically significant ($p = 0.55$) but the calculated values of BCCmax were statistically significant ($p<0.005$), except for 16.5 and 20.0°C.

RESULTS

Temperature

The research was made on seven different temperature values (6, 12, 15, 16.5, 18, 20 and 25°C) with the purpose of determining the optimal temperature of the *H. amphioxys* species used in the study. Test temperatures of the research were chosen considering the seasonal properties of the Homa Fishery.

Exponential growth phase of the *H. amphioxys* species was reached quicker than expected at 6°C in discontinuous culture system. At 12°C in the discontinuous culture system of the *H. amphioxys* species, it was observed that the species did not enter the lag phase and they went into the exponential growth phase. When the temperature was raised to 18°C, the *H. amphioxys* species was observed to enter the lag phase in the 1st day and in the following days it was observed to enter the exponential growth phase.

When the temperature was raised to 20°C, the cells of the *H. amphioxys* species were observed to enter the lag phase in the 1st day. When the effects of the discontinuous culture system and nutrient limited conditions on the growth of *H. amphioxys* are examined at the intensity of light, which is the highest temperature, it was observed that the species enter the growth phase faster than expected like all temperatures.

The carrying capacity of the lagoon water for the *H. amphioxys* species changed in a polynomial relation with the temperature (Figure 1). When the temperature decreased below 10°C, the maximum chl-a value obtained rapidly decreased. It was found to be constant between 10 and 20°C, and again an increase was observed between 20 to 25°C.

Statistically calculated carrying capacity parameters

Nitrate

Potential maximum chl-a values for nitrate were found to be 158.83 ± 113.7 µg chl-a/L and the half-saturation coefficients (K_s^l) were found to be 190.11 ± 184.7 (Figure 2). According to the results of the statistical analysis made by the least squares method, the variation in the nitrate concentration gives the carrying capacity of water to be 98%.

For the *H. amphioxys* species, in adequately high concentrations, nitrate substantially increased the carrying capacity of water with regards to reactive phosphate (Table 1 and Figure 2).

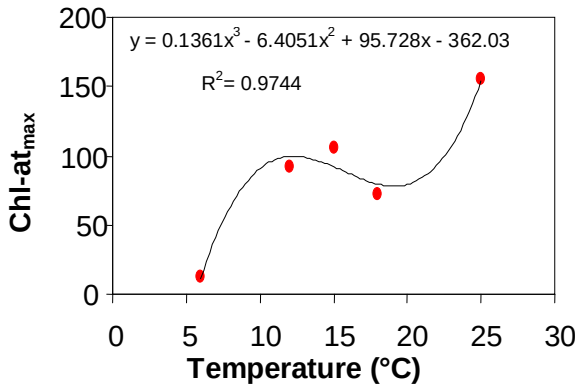


Figure 1. Relationship of potential carrying capacity (in light and nutrient saturation) versus temperature.

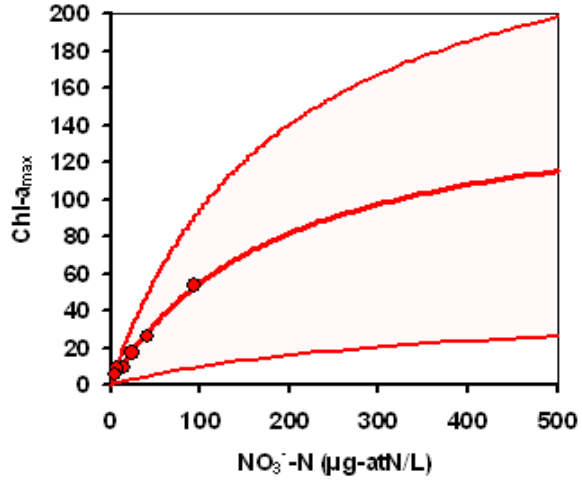


Figure 2. NO_3^--N chla max carrying capacity of the *H.amphioxys* species R^2=0.98 r=0.991

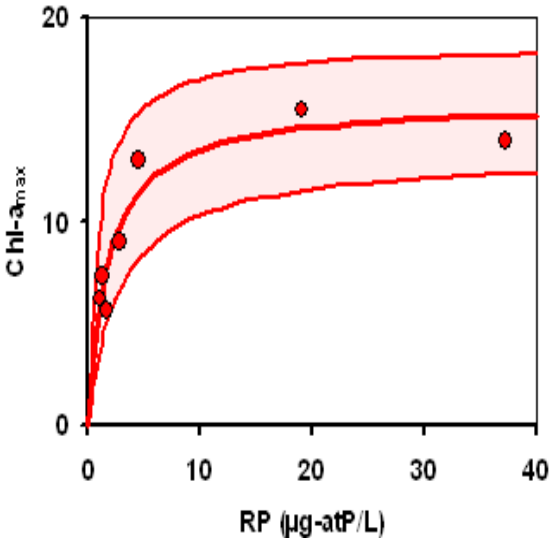


Figure 3. RP chla max carrying capacity of the *H. amphioxys* species. R^2 = 0.90 r = 0.951.

Reactive phosphate

Potential maximum chl-a values for reactive phosphate were found to be 15.90 ± 2.73 µg chl-a/L and the half-saturation coefficients (K_s^l) was found to be 1.798 ± 0.974. According to the results of the statistical analysis made by the least squares method, the variation in the phosphate concentration gives the carrying capacity of water as 90% (Figure 3).

Temporal changes of the calculated maximum chl-concentrations (carrying capacity) are given in Figure 4. In Figure 4, the curve found in the upper part shows the effect of temperature on the carrying capacity, and the carrying capacity of lagoon water for the species in question reaches the maximum value of 115 µg chl-a/L from June to the end of September. However, the observed values (in situ) were substantially low (Figure 4). Moreover, the *in situ* chl-a concentrations denote the biomass of the community. Biomass of the *H. amphioxys* species is less in this total biomass. The Monod equation expressing the nutrient dependency of the growth rates can also be used for carrying capacity (with different parameter values; chl-a max, K_s^l). Nutrients substantially decrease the carrying capacity of lagoon water all year around and while the nitrogenous compounds are the carrying capacity limiting nutrients, they are also found to be limiting the growth rate (Table 1).

Medium concentrations of nutrients decrease the carrying capacity of the lagoon water for the *H. amphioxys* species below 5 µg/L (<5 µg/L). *In situ* chl-a values are smaller than the carrying capacity that is limited by the nutrients. This is due to the contributions of the possible loss terms, which are advective transportation and grazing, because they are statistically

calculated from the values obtained from the carrying capacity. Therefore, they do not include grazing and advective transportation components.

DISCUSSION

The fact that lagoons are shallow and deep, water mixes with water column results in the nutrients' turning back into the medium very fast. The rich deep water's rapidly transferred to the surface means a significant increase in the amount of production. The temporal variations of the growth rate and the maximum chl-concentrations that the species can reach were calculated, considering the temporal variations of the temperature and nutrient concentrations in the lagoon. With the water temperature decreasing to 7°C in February and December, substantial negative effects of water temperature was found. Limiting effects of nutrients have been clearly observed. Small increases were observed in growth rates in March, April, July, September and October. Growth rates substantially decreased in other months. In response to temperature and more significant nutrient concentrations, the carrying capacity of the lagoon water increased in March, July,

Table 1. Temporal changes of the calculated maximum chl-a concentrations (carrying capacity).

Month	*In situ* nutrient concentrations				Temperature (°C)	Chl-a tmax	Chl-a max *in situ*			Limiting nutrient	
	Ammonium (µg at/l)	Nitrate (µg at/l)	Phosphate (µg at/l)	Silica (µg at/l)			NO_3	PO_4	Liebig		Riley
February	2.35	0.27	0.40	1.20	7	38.86	0.05	7.07	0.005	N	0.05
March	2.94	7.26	0.98	13.15	15	72.22	2.66	25.44	2.66	N	2.40
April	11.14	6.65	0.82	19.05	18	45.13	1.52	14.15	1.52	N	1.38
May	12.31	4.03	0.62	13.55	22	30.01	0.62	7.72	0.62	N	0.58
June	1.27	6.50	0.21	1.72	26.3	92.95	3.07	9.72	3.07	N	2.34
July	2.93	8.05	0.69	4.90	26.5	98.87	4.02	27.42	4.02	N	3.50
August	41.43	1.60	1.68	140.38	27	115.06	0.96	55.49	0.96	N	0.95
September	7.11	15.75	0.37	8.77	25	61.72	4.72	10.42	4.72	N	3.25
October	2.73	10.57	0.36	7.16	16.5	58.60	3.09	9.80	3.09	N	2.35
November	3.48	11.68	0.08	11.92	14.5	76.34	4.42	3.18	3.18	RP	1.85
December	1.70	1.36	1.80	24.72	8	60.53	0.43	30.28	0.43	N	0.42
January	2.65	1.96	0.81	7.28	12	89.41	0.91	27.77	0.91	N	0.88
February	0.91	10.77	0.08	0.64	13.5	83.37	4.47	3.63	3.63	RP	2.00

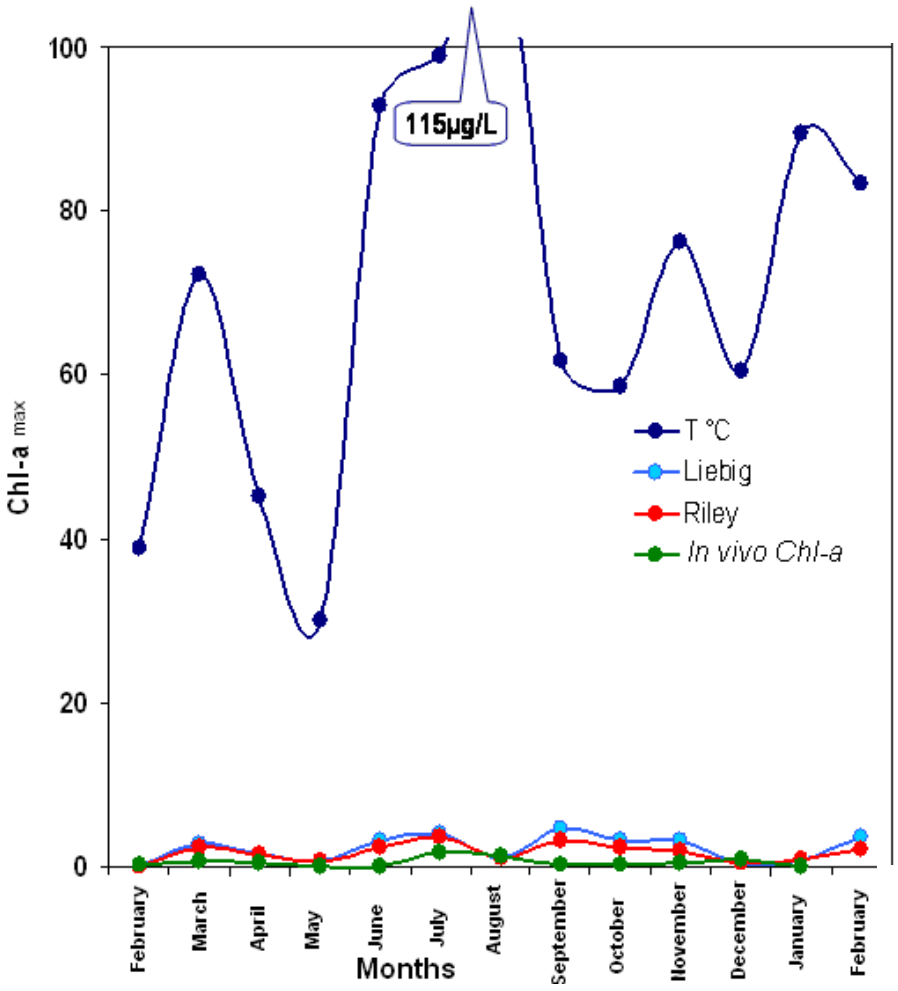


Figure 4. Temporal changes of the calculated maximum chl-a (carrying capacity).

September, October and November. However, in March and July, while the *in situ* chl-a values were observed to be lower than the values determined from the model, small increases was observed.

Reproduction of the *H. amphioxys* species all the year around in the lagoon is limited by *in situ* nutrient concentrations and by the temperature in very cold months of winter. They can be observed in the lagoon in March, July and September. The period we found shows analogy with the study made by Sabanci (2008). Nutrient limited growth for this species has been reactive limited phosphate in February, while it was limited by nitrogenous compounds in the other months. Due to the lipid synthesis observed in silicate limited growth, the surface layer of the sediment will be rich in lipids. This situation shows that, they can have a great role in nutrition of sea bream seed fish, which is a demersal fish and gastropods (Xing et al., 2008) with bottom mud.

A detailed research has not been made yet on the benthic diatoms, which have a great role in nutrition of crustaceans (Pena de la, 2007) and gastropods (Xing et al., 2008). With this study, by revealing the growth kinetics of the benthic diatoms, contribution will be made to dynamic applications related to nutrition of crustaceans and fish.

With this study, including application of the carrying capacity to the Homa Lagoon, it can be concluded that the carrying capacity can be also applied to fish breeding farms (fisheries). At the same time, in demersal fish farming, lipid production of the *H. amphioxys* species at the bottom would have positive contributions on the species nourished in net cages, which also cover the bottom.

By uptake of mineralization products in the sediment by these species, eutrophication will be prevented to some extent (by keeping the produced nutrients in the sediment by algae). Our goal was to determine the growth rate of all nutrients and the maximum levels of the phytoplankton biomass (the maximum biomass carrying capacity) in the case of how full growth can be reached and at what level the nutrient restrictive growth keeps the biomass (biomass carrying capacity) and the nutrient(s) that sustain year long growth. By determining the hyperbolical relations between the maximum biomass levels (biomass

carrying capacity) and nutrient concentrations obtained from phytoplankton growth graphs, we tried to apply flexible models which will restrict the levels phytoplankton can reach.

Concentrating on the well-known importance of N as a limiting nutrient (Carthy and Carpenter, 1983; Dugdale and Goering, 1967), those models tracked the supply of nitrate (NO_3^-) via upwelling and mixing, its uptake by phytoplankton and biological regeneration of N as ammonium (NH_4^+) in surface waters.

Other modeling efforts, beginning with the pioneering work of Walsh (1975) have explicitly considered growth limitation by light, and by two or more nutrients. They have simulated the cycling of up to four elements (C, N, P and Si) in surface waters, and allowed for the depletion of up to four nutrients (NO_3, NH_4^+, PO_4 and $Si(OH)_4$) to limiting concentrations. Almost all, recent multielement models (Dugdale and Wilkerson, 1998; Pondaven et al., 1998; Spitz et al.,2001) have included two or more phyto-plankton groups with qualitatively different nutrient requirements, such as diatoms (which take up $Si(OH)_4$ and require Si for growth) and non-siliceous picoplankton (which do not take up $Si(OH)_4$). This study experimented four different nutrient groups and nutrient requirements of *H. amphioxys* were determined, and a simple model was comprised of nutrient requirements of phytoplankton in the Homa Lagoon.

Mongin et al. (2003) showed that this has the effect of causing chlorophyll a to be limited by cellular N when N limits phytoplankton growth (for example, in surface waters in summer) and by cellular C when light limits growth (for example, within the DCM). In our study, it was found that the *H. amphioxys* species in the community in the Homa Lagoon was N restrictive for all the study period except two months.

REFERENCES

Brand LE, Guillard RR, Murphy S (1981). A method for the rapid and precise determination of acclimated phytoplankton reproduction rates. J. Plankton Res. 3:193-201.

Dempster TD, Sommerfeld MR (1998). Effects of environmental conditions on growth and lipid accumulation in Nitzschia communis (Bacillariophyceae), J. Phycol. 34:712-21.

Dugdale RC, Goering JJ (1967). Uptake of new and regenerated forms of nitrogen in primary productivity. Limnology and Oceanography. 12:196-206.

Dugdale RC, Wilkerson FP (1998). Silicate regulation of new production in the equatorial Pacific upwelling. Nature 391:270-273.

Fouilland E, Raymond GEL, Ken J, Slater J, Calleja A (2007). The response of a planktonic microbial community to experimental simulations of sudden mixing conditions in temperate coastal water importance of light regime and nutrient enrichment, J. Exp. Mar. Biol. and Ecol. 35:211-225.

Gersonde R, Harwood DM (1990). Lower cretaceous diatoms fom odg leg 113 site 963 Weddel Sea. Part1, Vegettaive Cells. In Barker PF, Kennet JP et. al., (Eds) Proceedings of the Ocean Drilling Program. Vol.113. Ocean Drilling Program, College station, Teexas.pp.403-25.

Guillard RL, Rhther JH (1962). Studies of marine Plantonic diatoms I. Cyclotella nana Hustedt and Detonula conferuacea Cleve. Can. J. Microbial, 8:229-239.

Guillard RRL (1973). Culture of phytoplankton for feeding marine invertebrates in: Smith, W.L. and M.H. Chanley (eds), Culture of Marine Invertebrate Animals, Plenum pres, New York, NY.

Guillard RRL(1975). Culture of phytoplankton for feeding marine invertebrates in: Smith, W.L. and M.H. Chanley (eds), Culture of Marine Invertebrate Animals, Plenum pres, new York, N.Y.Journal of Marine Systems 17:587-619.

Kutlu B (2000). Simulated community culture on water of Izmir Bay (Homa Lagoon), Mba thesis, Izmir, 91pp.

Mongin M, Nelson D, Pondaven P, Brzezinski M,Trieguer P (2003). Simulation of upper-ocean biogeochemistry with a flexible-composition phytoplankton model: C, N and Si cyclingin the western Sargasso Sea. Deep-Sea Research I 50: 1445-1480

Montani S, Magni P, Abc N (2003). Seasonal and interannual patterns of intertidal microphytobenthos in combination with laboratory and areal production estimates, Mar.Ecol.Prog.Ser.249:79-91.

Nelson DM, Trequer P, Brzezinski MA, Leynaert A, Pena de la MR (2007). Cell growth and nütrient value of the tropical benthic diatom, Amphora sp., at varying levels of nütrients and light instensity, and different culture locations. J. Appl. Phycol. 19:647-655.

Perrissinotto R, Nozais C, Kibirige I, (2000). Spatio-temporal Dynamics of phytoplankton and microphytobenthos in a South African temporarily-open estuary, Estuar. Coast. Shelf. Sci. 55: 47-59.

Pinckney J, Zingmark RG (1991). Effects of tidal stage and sun angles on intertidal benthic microalgae productivity mar. Ecol. Prog. Ser. 76:819

Pondaven P, Fravalo C, Ruiz-Pino D, Treguer, P Queguiner B, Jeandel C (1998). Modelling the silica pumping the permanently open ocean zone of the Southern Ocean.

Rothpletz A (1896), Uber die flyysch-fuco iden und eniqe andre fossilse algen, sowie uber liasische, Diatomeen Fubrend Horrenchwamme, Z.D Sch, Geol. Ges. 48:854-914.

Sabancı ÇF (2008). Homa Lagoon (Izmir Bay, Aegean Sea) Epipelic Intertidal zone, epiphytic and epilithic diatom kommunitelerinin taksnomik environment as a factor to be studied and its relationship. Phda thesis, Izmir,

Serodio J (2003). A chlorophyll fluorescence index to estimate short-term rates of photosynthesisi by intertidal microphytobenthos, J. Phycol. 39:33-46.

Serodio J, Catarino F (2000). Modelling the primary productivity of intertidal microphytobenthos: time scales of variability and effects of migratory rhythms Mar. ecol. Prog. Ser. 192:13-30.

Shaffer GP, Onuf CP (1985). Reducing the error in estimating annual production of benthic microflora: hourly to monthly rates, patchiness in space and time, Mar. Ecol. Prog. Ser. 26:221-31.

Smith DJ, Underwood GJC (1998). Expolymer production by intertidal epipelic diatoms, Limnol. Oceanogr. 43:1578-1591

Spitz HY, Moisan JR, Abbott MR (2001). Configuring an ecosystem model usingdata from the Bermuda Atlantic Time Series (BATS). Deep-Sea Res. II 48:1733-1768.
St. John MA, Clemmesen C Lund, Koster T (2001). Diatom production in the marine environment: Implications for larval fish growth and Condition. Ices J. Mar. Sci. 58:1106-13.
Strickland, J.D.H., Parsons, T.R., 1972, A practical handbook of seawater analysis, fisheries research boards of Canada. Bull, 167, Ottawa. p. 310.
Thessen AE, Dortch Q, Parsons ML, Mossion S (2005). Effects of salinity on pseudo-nitzshia species (Bacillariophyceae) growth and distribution. J. Phycol. 41:21-9.
Thomas JH (1996). Effects of temperature and illuminance on cell division rates of three species of tropical oceanic phytoplankton, Appl. Phycol. 2:17-22.
Vollenweider, R. A. 1965, Calculation models of photosynthesis-depth curves and some implications regarding day rate estimates in primary production measurements. Mem. Ist. Ital. Idrobiol., 18Suppl.:425-457.
Walsh JJ (1975). A spatial simulation model of the Peru upwelling ecosystem. Deep-Sea Research I. 22:201-236.
Werner D (1977). Silicate metabolism, in werner, d (Ed.), The biology of Diatoms, Botanical Monograph. Vol.13. university of California Pres, Berkeley, pp.110-49.
Xing R, Wang C, Cao X, Chang Y (2008). Settlement, growth and survival of abalone, Haliotis discus hannai, ina response to eight monospecific benthic diatoms, J. Appl. Phycol. 20:47-53.

academicJournals

Vol. 13(10), pp. 1160-1168, 5 March, 2014
DOI: 10.5897/AJB2013.12214
ISSN 1684-5315 ©2014 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

The effects of different concentrations of probiotic *Saccharomyces cerevisia* on growth performance and survival rate of rainbow trout (*Oncorhynchus mykiss*), fry and resistance against salinity

M. Pooramini[1], A. Kamali[1], A. Hajimoradloo[1], M. Alizadeh[2], R. Ghorbani[1], R. Hatami[3]* and S. Haghparast[1]

[1]Gorgan University of Agricultural Sciences and Natural Resources, Faculty of Fishery, Gorgan, Iran.
[2]Yazd Fisheries Research Center, Yazd, Iran.
[3]Isfahan University of Technology, Isfahan, Iran.

Accepted 11 September, 2013

In the present study, a yeast strain *Saccharomyces cerevisia* var. *elipsoidous*, acting as probiotic, was administered to rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792) fry during a period of 21 days and the effects of the yeast on improvement of growth and resistance against environmental stress were evaluated with respect to fish fed on yeast free feed (control group). The control treatment consisted of a standard commercial diet, and the treatments consisted of the control diet supplemented with 0, 1, 5 and 10% yeast (w/w). The results demonstrate the beneficial effects of probiotics on the characteristics of rainbow trout, as the Specific Growth Ratio (SGR), body weight gain (%BWG) and protein conversion ratio (PER) in 5% yeast-fed fish were significantly (P<0.05) enhanced by probiotic administration. On the contrary, no effect on the fry growth performance, mortality, condition factor (CF), food conversion ratio (FCR) and histological assessment was shown. A significant (P<0.05) increase in lipid content of the carcass was detected in diets with probiotic compareD to 0% and the control treatments. Ash and protein contents of the carcass increased and decreased with an increase in yeast amount, respectively. Challenge with different levels of salinity (10 and 15 ppt) after 24 h revealed 100% survival in treatments containing yeast as probiotic, and difference with control group was significant (p<0.05) indicating that *S. cerevisia* could enhance the resistance against salinity stress. Addition of yeast in concentration of 5% to the diet is recommended during the early period of rainbow trout fry farming to achieve the best results on growth performance and feed efficiency.

Key words: *Saccharomyces cerevisia* var. *elipsoidous*, *Oncorhynchus mykiss*, probiotic, survival and growth rate, carcass quality.

INTRODUCTION

Today, increase in demand for fish products means using intensive methods for more production, accompanied with an increase in infectious agents. This rearing condition can be a source of stress for fish, making them susceptible to disease and triggering high mortality. Due to various adverse effects of antibiotics, their use is

*Corresponding author. E-mail: Rezvan.hatami_iut@yahoo.com.

forbidden in several countries. To date, probiotics are considered a good alternative to the use of antibiotics and in particular, in fish larviculture (Rollo et al., 2006). Probiotics, which are micro-organisms or their products with health benefit to the host, have found use in aquaculture for improving the health of their host and increasing growth rate. The range of probiotics examined for use in aquaculture has encompassed many micro-organisms such as bacteria, bacteriophages, yeasts and unicellular algae (Irianto and Austin, 2002). In several studies, the effects of probiotics on survival and growth rate of fish larvae and crustacea such as digestibility coefficients of nutrients, decreasing food conversion ratio and increase tolerance to stress have been studied (Rengpipat et al., 1998; Ali, 2000; Heizhao et al., 2004; Himabindu et al., 2004; Taoka et al., 2006a). It has been shown that the use of probiotics can increase the amount of given food necessary for animal optimal growth, by which the expense of fish farming might be reduced (Lara-Flores et al., 2003).

Oncorhynchus mykiss has a promising market potential in Asia as well as other areas in the world. Many probiotics have been proposed to improve health quality of rainbow trout (Irianto and Austin, 2002). The stains used were generally antagonistic to pathogens (Jöborn et al., 1997; Robertson et al., 2000), and an important feature was the ability to colonize in fish gut (Jöborn et al., 1997; Nikoskelainen et al., 2001). Moreover, the immune system of rainbow trout is stimulated by several probiotic (Irianto and Austin, 2002).

Andlid et al. (1995) suggested that yeast of *Saccharomyce cerevisae* var, *boulardii* isolated from rainbow trout might also improve the health quality by their colonization potential. This probiotic yeast represented positive effects on rainbow trout metabolism, as it increased muscle lipids and red pigmentation, and also improved the resistance of the fish to *Yersinia ruckeri* (Quentel et al., 2005). The larval rearing period of many fish species, is considered as critical in their life history. To succeed in larval rearing, availability of suitable food that is readily consumed and efficiently digested should be mainly regarded to provide the required nutrients that can support their optimal growth and healthy well (Tovar-Ramirez et al., 2002; Waché et al., 2006).

Although, beneficial effects of probiotics are well known in aquaculture; there is inadequate information about best concentrations of different probiotics used for enhancement of survival, increase in body resistance to stress and infectious diseases and improvement in nutritional parameters such as feed efficiency and feed conversion ratio.

Therefore, more investigation is needed for safe and effective use of probiotic. Therefore, this study was conducted to investigate the effect of *S. cerevisiae* var. *elipsoidous* as a probiotic strain on survival, growth parameters and carcass quality of rainbow trout fry at start feeding.

MATERIALS AND METHODS

Fish fry preparation

A batch of 1500 uniformly sized yolk-sac (*O. mykiss*) alevin (80 ± 26.27 mg) was obtained from the Reproduction Center of *O*ncorhynchus *mykiss,* Gorgan, Iran. In this center, all the eggs were incubated and hatched in spring water (9.34 ± 0.04°C). Alevins were transferred to the Reproduction and Culture Complex of Sturgeon Fish, Gorgan, North of Iran and stored in a Fiberglass tank until yolk-sac absorption. One day before start feeding, the fry (with initial weight and length 127/95 ± 16/73 mg and 24/86 ± 1/38) were randomly divided into 15 groups (five treatments and threereplications of 100 individuals) in a period of 21 days.

Farming condition

Each group was placed into an identical 35 L tank with micro-mesh screens in two sides. Freshwater velocity was set at 0.5 L/min; system aeration was done with compressed air, using a number of narrow pipes, connected to bubbliers. Holding tanks were cleaned daily to avoid pollution caused by overfeeding or aggregation of diet particles. Water quality was regularly monitored. Temperature was measured daily (n = 29) and maintained at 9.3°C during the experiment. Oxygen concentration varied between 7.8 and 8.6 mg/L (determined in the morning once a week). Total ammonia-nitrogen [$(NH_4^+ + NH_3)$-N] was always maintained below 0.5 mg/L and pH value from 8 to 8.2. Residual chlorine level was determined weekly and stayed below 0.05 mg/L. The photoperiod for this indoor experiment was set at a 12 L: 12 D cycle (light period from 08.00 am to 8.00 pm) and light intensity was kept at 40 lux at each tank surface. Physical and chemical variables were maintained as constant as possible by continual renewals of oxygenated water, and removing dead fry and food left-overs by siphoning each morning before feeding; dead fry were removed twice daily and counted.

Food preparation and feeding

The artificial food was Biomar- optimal Start, 0.5 mm in size. All groups were fed 5 times per day. The feeders were operational each time for 5 min. The daily ration was adjusted according to fry weight after 7 to 14 days of rearing (Lovell, 1989). Each ration was about 5% of dry body weights per day. The experimental food, mixed with yeast, was prepared with probiotic yeast *S. cerevisiae* strain (Institut Daxal, Italy) that was obtained as commercial preparations. The active dried yeast preparations were powdered by grinding and sifting through a 100 µm-meshed screen, and then suspended in fish oil. The amounts of powder were adjusted in the oily suspensions to obtain a final concentration of ca.10^6 colony forming units (CFU) of yeast per gram of experimental food, then the pellets were coated with the shaken suspensions (32 ml/kg) (Waché et al., 2006).

Feeding treatments

Five different feeding experiments were conducted on three identical groups of fish fry. In all treatment groups, fish characteristics were determined at a specific time; the characteristics of first, second and third groups of every feeding treatment were studied at the end of the first, second and third weeks, respectively. Five feeding treatments were: (1) artificial food covered with fish oil (without yeast) (D_0); (2) artificial food mixed with 1% *S. cerevisiae* (D_1); (3) artificial food mixed with 5% *S. cerevisiae* (D_5); (4) artificial food mixed with 10% *S. cerevisiae* (D_{10}) and (5) only artificial food, called control experiment (C).

Measurement of growth parameters

Growth measurements and chemical analysis occurred when the fish fry were starved for 6 h. Fish samples of all groups were weighted every week. The weight of 30 fish of every group was measured using a digital scale to the nearest 0.1 mg. Total lengths of all samples were measured using a caliper to the nearest 0.01 mm. The amount of food given per group was recorded weekly and used to calculate feed efficiency ratios. Mortality was calculated according to the number of survived fish at the end of experiment. All fish in each tank were pooled for weighing and growth evaluation as follows:

Condition factor (CF) = $W/L^3 \times 100$ (Lagler et al., 1962)

Where W is the Fish wet weight (g) and L is the fish length (cm).

Body weight gain percentage (%BWG) = $BW_f - BW_i / BW_i \times 100$ (Ghosh et al., 2003)

Where BW_f is the Final weight (g) and BW_i is the initial weight (g).

Specific growth rate (%SGR) (% body weight/day) = $LnW_f - LnW_i / (t_2 - t_1) \times 100$ (Helland et al., 1996)

Where W_f is the Final weight (g), W_i is the initial weight (g), $(t_2 - t_1)$: duration of the experiment in days.

Food concersion ratio (FCR) = $F / W_f - W_i$ (Helland et al., 1996)

Where F is the Feed fed (dry weight in g)

Protein efficiency ratio (PER) = $BW_f - BW_i/AP$ (Helland et al., 1996)

Where AP is the Applied protein.

Proximate composition analysis

To determine proximate composition, 200 fish at first week and 50 fish per each replicate tank after 4 weeks of feeding were taken and water content, crude protein, crude lipid and ash were analyzed using AOAC method (1992). These calculations were conducted in duplicate. Normal procedures have been used, for example, water content was measured by drying samples at 105°C overnight, protein levels were evaluated measuring Kjeldahl nitrogen, lipid was analyzed by ether extraction using a Soxhlet system. Ash content was measured in samples that had been mineralized at 550°C for 5 h in a muffle furnace. Results were expressed as a percentage of the total body dry weight.

Test of salinity stress

20 fish fry of each replicate were sampled and affected by salinity levels of 10 and 15 ppt in air blower-equipped tanks. The mortality and survival rates of fish were recorded after 24 h starving.

Histological assessment

To determine any significant effect of yeast-enriched feeding diets on alimentary tract, rainbow trout fry in each treatment were sampled for histological sections at the end of experimental period. Fish samples were stored in formalin solution (4%) and then eviscerated to remove their digestive tract. Paraffined blocks of fish alimentary tract were prepared and then sliced by microtom to give slices of 4 to 5 µ. Slices were stained by the coloration methods of hematoxiline and eosine and then studied under optical microscope (Mumford, 2004).

Statistical analysis

The results are presented as mean values followed by the SD. The significance of differences was determined using ANOVA, followed by Duncan's test to compare the means of the samples for multi group comparisons, with a statistical software package SPSS 12.0 for windows. Differences were considered significant at $p<0.05$.

RESULTS

Growth parameters

Survival rate showed no significant differences among various treatments ($P>0.05$). The highest and the lowest cumulative mortality of fry were found in feeding diets of D_5 and D_{10}, respectively (Figure 1). The maximum average length was found in fish samples fed with diet C after 3 weeks rearing, indicating significant differences ($P<0.05$) with the feeding diet of D_1 treatment (Table 2). Results indicating the differences between the fish groups with different diets during the experiment period were not always similar to the differences observed after three weeks, at the end of the experiments. D_{10} -fed fish samples indicated the highest significant average length at the end of weeks 1 and 2 than other samples ($P<0.05$). The highest weight was observed in treatment D_5 at the end of weeks 3, not showing significant differences with other diets ($P<0.05$). At the end of weeks 1 and 2, the highest weight was detected in feeding diet of D_{10} while no significant differences was observed in diets of D_0 and C ($P<0.05$) (Table 2). Significant differences ($P<0.05$) were also observed in weight gain of fish samples fed with feeding diet of D_5 and samples in other diets during 3 weeks of the experiment. The specific growth rate of D_5 and D_1 diets showed no significant difference; whereas, fish samples fed with the former diet indicated significant differences with other treatments ($P<0.05$). At the end of the rearing period, no significant difference was observed in condition factor of fish samples in all treatments (Table 3).

After three weeks rearing, the lowest FCR was observed in treatments of D1 and D_5, showing a significant difference with diet D_{10} ($P<0.05$). The highest PER at the end of week 3 was found in feeding diet of D_5, which was significantly different ($P<0.05$) from other treatments with exception of diet D_1.

Carcass proximate composition

The chemical composition analysis of trout fry carcass of each experiment (Table 1) showed that the highest amount of crude protein was observed in samples of D_1 and C treatments than samples in other feeding diets ($P<0.05$). Lipid content was lowest in C treatment

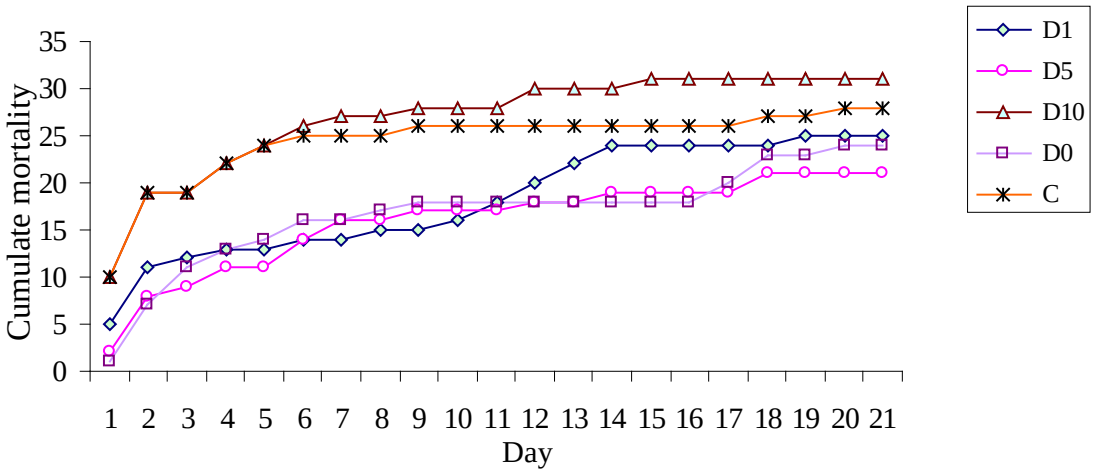


Figure 1. Cumulate mortality of *Oncohynchus mykiss* fry (mean per diet). No significant differences were observed among various treatments.

Table 1. Proximate analysis of trout fry carcass during rearing period, expressed as g kg^{-1} dry weight (mean±SD).

Parameter	Starting rearing period	Final rearing period				
		Feeding diets				
		D_0	D_1	D_5	D_{10}	C
Humidity	869.6	857.1±05.7^{ab}	865.2±9.3^{b}	846.1±6.6^{a}	847.3±6.8^{a}	865.2±9.3^{b}
Proteins	705.7	738.6±3.6^{a}	767.7±8.1^{b}	747.5±2.6^{a}	746.2±4.6^{a}	758.7±4.6^{b}
Lipids	118.8	120.6±1.2^{bc}	120±1.5^{b}	122.7±1.4^{c}	121.1±1.3^{bc}	114.6±0.9^{a}
Ash	93.8	103.3±1.2^{d}	94.4±1.2^{a}	100.8±1.1^{c}	104.3±1^{d}	98.6±1.5^{b}

Values with different superscripts are significantly different in each row (P<0.05).

Table 2. Result of biometry of *Oncohynchus mykiss* fry during rearing period.

Diet	Length / weight	D_0	D_1	D_5	D_{10}	C
End of week 1	Length (mm)	30±0.10^{b}	28.5±0.76^{a}	28.9±0.49^{a}	30.1±0.46^{b}	29.3±0.62^{ab}
	Weight (mg)	256.8±0.29^{a}	242.2±11.0^{a}	238.2±18.4^{a}	258.7±5.9^{a}	239.4±13.7^{a}
End of week 2	Length (mm)	34.7±0.10^{bc}	33.3±0.64^{a}	33.8±0.51^{ab}	35.1±0.63^{c}	34.7±0.61^{bc}
	Weight(mg)	378.9±14.0^{bc}	339.1±19.8^{a}	345.9±18.8^{ab}	399.8±14.8^{c}	374.3±22.4^{bc}
End of week 3	Length (mm)	38.52±1.24^{b}	37.0±0.63^{a}	38.2±0.38^{ab}	38.0±0.51^{ab}	37.7±0.57^{ab}
	Weight (mg)	515.5±5.55^{b}	487.0±21.4^{a}	523.0±18.0^{b}	517.4±12.8^{b}	514.2±8.91^{b}

Values with different superscripts are significantly different in each row (P>0.05).

(11.46%). The highest amount of ash in carcass of fry was found in treatments of D_{10} and D_0, which showed significant differences with other treatments (P<0.05). Fry samples fed with feeding diets of D_1 and C indicated the highest water content (P<0.05).

Histological assessment

Based on our study, no significant difference among various treatments was observed in histological assessment of digestive tract. Histological evaluations in different parts of digestive tract were as follows:

Esophagus

Epithelial with pavement cells was detected in all treatments. As fish fry grow, their esophagus was abundant of phlegm wrinkles (Figure 3).

Table 3. Average final weight (g), percent body weight gain (BWG) per day, specific growth ratio (SGR), condition factor (CF), food conversion ratio (FCR) and protein efficiency ratio (PER). Values are expressed as mean± standard deviation (n = 3).

Treatment index	D_0	D_1	D_5	D_{10}	C
Average final weight (g)	515.4 ± 5.55^{b}	487.0 ± 21.4^{a}	523.0 ± 18^{b}	517.4 ± 12.8^{b}	514.2 ± 8.9^{b}
BWG%	36.1 ± 4.30^{ab}	43.7 ± 4.2^{bc}	51.3 ± 3.5^{c}	29.6 ± 6.6^{a}	37.6 ± 6.0^{ab}
SGR%	4.38 ± 0.46^{ab}	5.00 ± 0.38^{bc}	5.90 ± 0.30^{c}	3.67 ± 0.72^{a}	4.66 ± 0.70^{ab}
CF%	0.90 ± 0.08^{a}	0.95 ± 0.04^{a}	0.92 ± 0.03^{a}	0.94 ± 0.02^{a}	0.96 ± 0.02^{a}
FCR	1.18 ± 0.15^{ab}	0.97 ± 0.10^{a}	0.82 ± 0.06^{a}	1.47 ± 0.32^{b}	1.14 ± 0.18^{ab}
PER	1.44 ± 0.17^{ab}	1.79 ± 0.17^{bc}	2.04 ± 0.14^{c}	1.21 ± 0.27^{a}	1.60 ± 0.25^{ab}

Values with different superscripts are significantly different in each row (P<0.05).

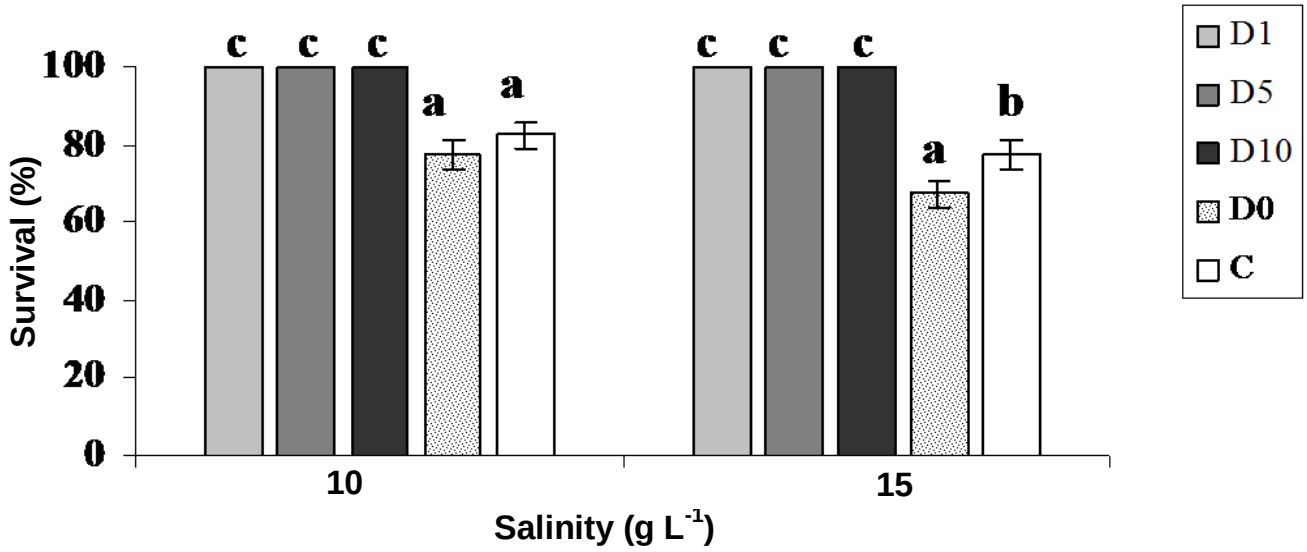


Figure 2. Survival rate in each treatment after challenging with salinity levels of 10 and 15 g L^{-1}.

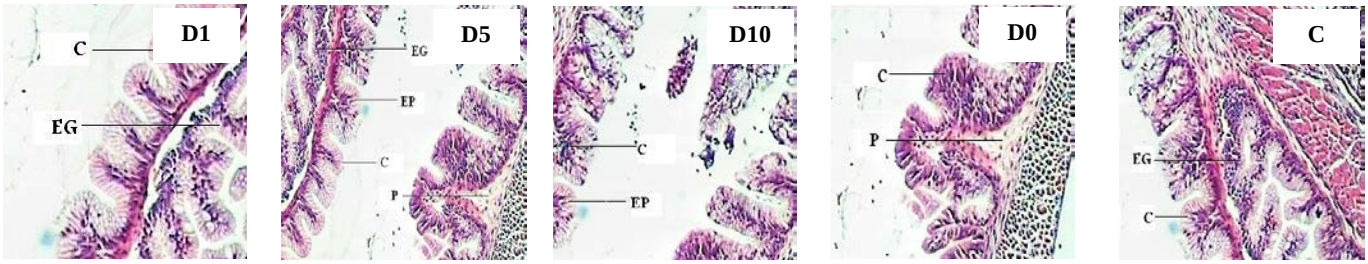


Figure 3. Posterior linear section of esophagus in rainbow trout fry at the end of week 3 (400x). EG, Esophagus glands; EP, epithelial; C, cilia; P, parin. D1, D5, D10, D0 and C implies feeding diets containing 1, 5 and 10% of yeast, fish oil, and the control, respectively.

Stomach

Plenty of wrinkles and secreting glands were observed in phlegm of fry stomach. In all treatments, connective tissue, muscle layers and a number of gland cells were found (Figures 4 and 5).

Salinity challenging

Results of salinity challenged at 10 and 15 ppt revealed that feeding with probiotic yeast *S. cerevisiae* strain had significant effects on survival rate of rainbow trout fry (Figure 2).

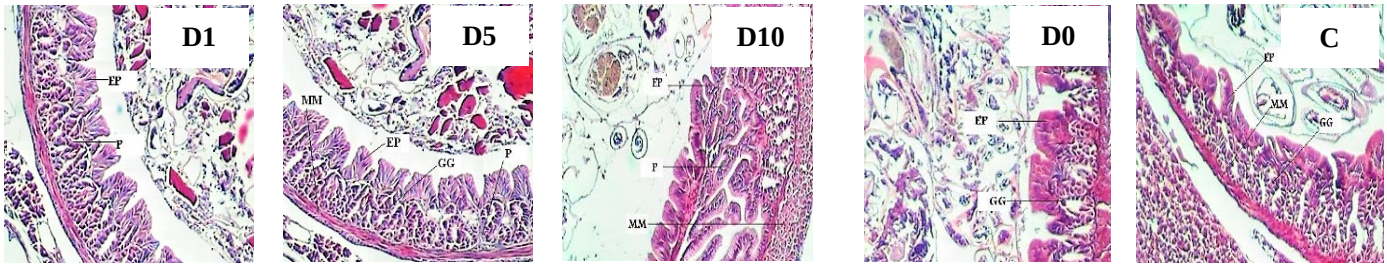


Figure 4. Cross section of stomach in rainbow trout fry at the end of week 3 (400x). EP, Epithelial; GG, gastric glands, P, parin; MM, mucosal muscle. D1, D5, D10, D0, and C implies feeding diets containing 10, 50 and 100 g kg^{-1} of yeast, fish oil, and the control, respectively.

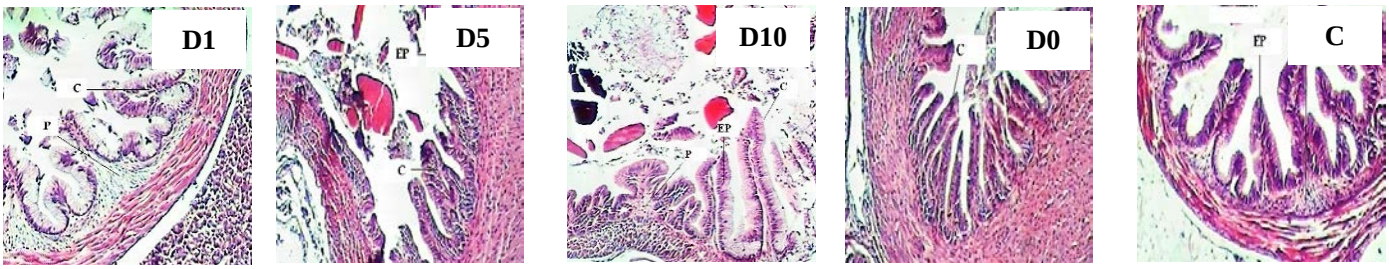


Figure 5. Linear section of intestine in rainbow trout fry at the end of week 3 (400x). C, Cilia; EP, epithelial; P, parin. D_1, D_5, D_{10}, D_0, and C implies feeding diets containing 10, 50 and 100 g kg^{-1} of yeast, fish oil, and the control, respectively.

DISCUSSION

Growth parameters

In the present study, fry of *O. mykiss* fed with different levels of *S. cerevisiae* had a higher growth rate than those fed with normal artificial food and artificial diet covered by fish oil at the first week (C and D_0). Research on the effects of strain of dietary *S. cerevisiae* and rearing conditions in rainbow trout revealed that supplementation of trout starter diet with *S. cerevisiae* may be particularly useful to increase fish growth. Moreover, differences in temperature strongly affect fish growth and metabolism (Waché et al., 2006). Noh et al. (1994) and Lara-Flores et al. (2003) demonstrated greater growth in fry fed with diets containing a probiotic supplement than those fed with the control diet without supplement. The intestinal colonization of early feeding fry with yeast may have some effect on development, for example, by accelerating the maturation of the digestive system. In older fish, dietary yeast may stimulate metabolism and growth (Gatesoupe, 2007). In the present research, the best FCR, SGR and BWG values were observed in diets D_1 [artificial food mixed by 1% *S. cerevisiae* (w/w)] and D_5 [artificial food mixed by 5% *S. cerevisiae* (w/w)] suggesting that these feeding diets improved feed utilization even under stress conditions. Similar results have been reported for *S. cerevisiae* use in diets prepared for Israeil carp (Noh et al., 1994) and Nile tilapia (Lara-Flores et al., 2003). In a similar study, the diet supplemented with yeast produced the best growth performance and feeding efficiency. This was attributed to an increase in the alkaline phosphatase activity, suggesting that yeast is an appropriate growth-stimulating additive in tilapia cultivation (Lara-Flores et al., 2010).

In practical terms, this means that the use of probiotics can decrease the amount of food necessary for optimal growth of the animal by which the expense of fish farming might be reduced. The PER results indicate that supplementing diet with 5% *S. cerevisiae* (D_5) significantly improved protein utilization in *O. mykiss*. This contributes to better protein use for growth, an important quality given that protein is the most expensive feed nutrient component. Similar results were found by Laura-Flores et al. (2003) who reported that supplementing diets with probiotics significantly improved protein utilization in tilapia. In this study, mortality was low and the survival rates within groups fed with *S. cerevisiae* enriched foods did not show any significant differences with other groups at the end of the experiment. Similar effects have been reported for *S. cerevisiae* in diets for *O. mykiss* by Waché et al. (2006). These improvements found when using supplemented diets suggested that the addition of probiotics improved diet and protein digestibility, which may explain the better growth and

feed efficiency seen when using supplemented diets (Lara-Flores et al., 2003).

Proximate composition of carcass

Carcass chemical composition measurements have been used as a reliable index to estimate nutritional conditions and growth of fish larvae (Rengpipat et al., 1998; Hevroy et al., 2005). In the present trial, trout fry in control treatment had the highest and the lowest water and lipid contents, respectively, while yeast-enriched feeding diets revealed no significant difference in lipid content. Moreover, the highest rate of protein was detectable in feeding diets of C and D_1 and fish samples in other treatments showed no significant difference. Aubine et al. (2005) demonstrated that dietary supply of the yeast increased muscle lipids and red pigmentation.

Histological assessment

Based on our study, no significant difference among various treatments was observed in histological assessment of digestive tract. The development of new probiotic strains aims at more active beneficial organisms. In the case of novel microorganisms and modified organisms, the question of their safety and the risk to benefit ratio have to be assessed (Salminen et al., 1998; Ringø et al., 2007a). Today, it is generally accepted that the gastrointestinal (GI) is one of the major routes of infection in fish (Birkbeck and Ringø, 2005). The histological effect of exposing the GI tract of Atlantic salmon to high levels of the *Carnobacterium divergens* was investigated by light and electron microscopy. Results from LM-investigations in that study showed no apparent histopathological changes of the epithelium in the intestine, after exposure of *C. divergens* (Kristiansen et al., 2011). In a similar study, histological investigation of intestine of Atlantic salmon following exposure to one probiotic strain (*C. divergens*), assessed by light and electron microscopy, showed a similar appearance to intact intestinal epithelium (Ringø et al., 2007b). Results of several other studies confirmed lack of histological changes, neither tissue damage nor manifest inflammation provoked by probiotic administration (Picchietti et al., 2007; Harper et al., 2011).

Salinity stress

According to this study, results reveal that feeding with probiotic yeast *S. cerevisiae* strain had significant effects on survival rate of rainbow trout fry in salinity challenging. In feeding studies, useful information on larval requirements to essential components can be achieved by salinity challenging when assessing their physiological condition (Dhert et al., 1992a, 1992b). Challenge tests are proposed as meaningful tools for assessing fish quality in the aquaculture industry, environmental resources management and in research (Wedemyer and McLeay, 1981). The concept is based on the presumptions that stress loading above the acclimation capacity of an organism will weaken it and reduce performance in growth, survival and reproduction, and that the reduction in performance can be quantified by assessing tolerance to reference stressors (Wedemyer et al., 1981). Our results showed 100% survival in treatments containing yeast as probiotic, and there was a significant difference with control group ($p<0.05$). Therefore, probiotic *S. cerevisia* has been shown to enhance the resistance against salinity stress ($P<0.05$). Results of a study investigated the influences of probiotic *Bacilli* sp. on resistance of Persian Sturgeon Larvae against challenge tests including salinity experiment, and showed that the mortality of larvae fed with probiotic was significantly lower than the control group (Faramarzi et al., 2012). Additional of *S. cerevisiae* yeast (0.1%) to feeding diets of Tilapia fry improved their growth and declined the effect of environmental stressors (Lara-Flores et al., 2003). Similar results were obtained by Lara-Flores et al. (2010) who reported that all the probiotic-supplemented diets resulted in growth higher than that of the control diets, suggesting that the addition of probiotics mitigated the effects of the stress factors. This resulted in better fish performance, with better growth results in the diets supplemented with the yeast.

Recent studies have clearly demonstrated the beneficial effects of these feed additives on immune system modulation, stress tolerance and growth rate of farmed fish (Carnevali et al., 2004; Picchietti et al., 2007; Dimitroglou et al., 2011). One possible explanation is that yeast probiotics provide *β-glucans* and nucleotides that stimulate the immune system of fish (Kulkarni et al., 1986). Results of a study conducted on probiotic benefits of stress tolerance indicated that probiotics supplied in the rearing water and the diet of fish enhanced the stress tolerance and the non-specific immune system of Japanese flounder, providing them a higher resistance against stress conditions and pathogens (Taoka et al., 2006b). In the present experiment, growth rate of trout fry in three groups fed with yeast-enriched feeding diets was observed fine but evidently addition of yeast in concentration of 5% to the diet is recommended during the early period of rainbow trout fry farming to achieve the best results on growth performance and feed efficiency. Pulverization of yeast suspension in the diet resulted in lower growth rate. This result is similar to that of Tovar- Ramirez et al. (2002). It can be assumed that the process of incorporation of the pulverized yeast changed some physical properties of the micro particles; a decrease of buoyancy was observed and the sprayed particles sank faster. In this case, feed ingestion by fry could be reduced. Yeast incorporation during raw

material mixing should be considered in further experiments.

ACKNOWLEDGEMENTS

The present study was supported by the Iranian Academy of Fisheries Sciences, Gorgan University of Agricultural Sciences and Natural Resources (UASNR 45163). We would like to thank the personnel of Sturgeon Reproduction and Culture Complex of Marjanii, Gorgan, North of Iran, for their unsparing help in experimental supplies.

REFERENCES

Ali A (2000). Probiotics in fish farming:Evaluation of a bacterial mixture. Ph.D. Thesis, University of Agricultural Sciences, Umea, Sweden.

Andlid T, Vazquez- Juarez R, Gustafsson L (1995). Yeast colonizing the intestine of rainbow trout (*Salmo gairdneri*) and turbot (*Scophthalmus maximus*). Microb. Ecol. 30:321-334.

AOAC (1992). Official Methods of Analysis of Association of Official Analytical Chemists, 14th ed. AOAC, Arlington, p. 3413.

Aubin J, Gatesoupe FJ, Quentel C, Labbé L, Forraz M (2005). Ofimer probiotic study on rainbow trout. III. Flesh quality assessment of rainbow trout (*Oncorhynchus mykiss*) submitted to probiotic treatment with *Saccharomyces cerevisiae* var. *boulardii*. EAS Special Publication No. 35.

Birkbeck TH, Ringø E (2005). Pathogenesis and the gastrointestinal tract of growing fish. In: Microbial Ecology in Growing Animals. Holzapfel, W. And Naughton, P. (Eds.). Elsevier. Edinburgh. UK. pp. 208-234.

Carnevali O, Zamponi MC, Sulpizio R, Rollo A, Nardi M, Orpianesi C, Silvi S, Caggiano M, Polzonetti AM, Cresci A (2004). Administration of probiotic strain to improve sea bream wellness during development. Aquacult. Int. 12(4-5):377-386.

Dhert P, Lavens P, Sorgeloos P (1992a). A simple test for quality evaluation of cultured fry of marine fish. Mededel. Fac. Landb. Wet. Rijksuniv. Gent. 57(4b):2135-2142.

Dhert P, Lavens P, Sorgeloos P (1992b). Stress Evaluation: A tool for quality control of hatchery- produced shrimp and fish fry. Aquacult. Europe. 17(2):6- 10.

Dimitroglou A, Merrifield DL, Carnevali O, Picchietti S, Avella M, Daniels C, Güroy D, Davies S (2011). Microbial manipulations to improve fish health and production--a Mediterranean perspective. Fish Shellfish Immunol. 30(1):1-16.

Faramarzi M, Jafaryan H, Roozbehfar R, Jafari M, Rashidi Y, Biria M (2012). Influences of Probiotic Bacilli via Bioencapsulated Daphnia magna on Resistance of Persian Sturgeon Larvae against Challenge Tests. Global Veterinaria. 8(4):421-425.

Gatesoupe FJ (2007). Live yeasts in the gut: Natural occurrence, dietary introduction, and their effects on fish health and development. Aquaculture. 267(1-4):20-30.

Ghosh K, Kumar SK, Kumar RA (2003). Supplementation of an isolated fish gut bacterium, *Bacillus circulans*, in: Formulated diets for Rohu, *Labeo rohita*, fingerlings. Aquacult. Bamidgeh. 55 (1):13-21.

Harper GM, Monfort M, Saoud IP, Emery M, Mustafa S (2011). An ex vivo approach to studying the interactions of probiotic *Pediococcus acidilactici* and *Vibrio* (*Listonella*) *anguillarum* in the anterior intestine of rainbow trout (*Oncorhynchus mykiss*). J. Aquac. Res. Dev. ISSN:2155-9546.

Heizhao ZL, Zhixun G, Yingying Y, Wenhui Z, Zhuojia JL (2004). Effect of dietary probiotics on apparent digestibility coefficients of nutrients of white shrimp, *Litopenaeus vannamei* Boon. Aquacult. Res. 35:1441-1447.

Helland SJ, GrisdaleHelland B, Nerland S (1996). A simple method for the measurement of daily feed intake of groups of fish in tanks. Aquaculture. 139:157-163.

Hevrøy EM, Espe M, Waagbo R, Sandnes K, Rund M, Hemre G (2005). Nutrition utilization in Atlantic salmon (*Salmo salar* L.) fed increased level of fish protein hydrolyses during a period of fast growth. Aquacult. Nutr. 11:301-313.

Himabindu KV, Narottam PS, Kamal KJ (2004). Effect of feeding Lactobacillus-based probiotics on the gut microflora, growth and survival of post larvae of *Macrobrachium rosenbergi*. Aquacult. Res. 35:501-507.

Irianto A, Austin B (2002). Probiotics in aquaculture. J. Fish. Dis. 25:633-642.

Jöborn A, Olsson JC, Westerdahl A, Conway PL, Kjelleberg S (1997). Colonization in the fish intestinal tract and production of inhibitory substances in intestinal mucus and faecal extracts by *Carnobacterium* sp. strain K1. J. Fish. Dis. 20:383-392.

Kristiansen M, Merrifield DL, Vecino JLG, Myklebust R, Ringø E (2011). Evaluation of Prebiotic and Probiotic Effects on the Intestinal Gut Microbiota and Histology of Atlantic salmon (*Salmo salar*). J. Aquac. Res. Dev. 2011, S1.

Kulkarni AD, Fanslow WC, Rudolph FB, Buren CT (1986). Effect of dietary nucleotides on response to bacterial infections. J. Parenter Enteral Nutr. 10(2):169-171.

Lagler KF, Bardach JE, Miller RR (1962). In Ichthyology:John Wiley and Sons. Inc., 1971, New York. pp. 288-238.

Lara-Flores M, Olivera-Castillo L, Olvera-Novoa MA (2010). Effect of the inclusion of a bacterial mix (*Streptococcus faecium* and *Lactobacillus acidophilus*), and the yeast (*Saccharomyces cerevisiae*) on growth, feed utilizationand intestinal enzymatic activity of Nile tilapia (*Oreochromis niloticus*). Int. J. Fish. Aquac. 2(4):93-101.

Lara-Flores M, Olvera-Novoa MA, Guzmàn-Méndez BE, López-Madrid W (2003). Use of the bacteria *Streptococcus faecium* and *Lactobacillus acidophilus*, and the yeast *Saccharomyces cerevisiae* as growth promoters in Nile tilapia (*Oreochromis niloticus*). Aquaculture. 216:193-201.

Lovell T (1989). Nutrition and feeding of fish. Van Nostrand Reinhold Publishers. New York. pp. 185- 203.

Mumford SL (2004). Histology of finfish. USFWS, Olympia Fish Health Center. Olympia. Washington.

Nikoskelainen S, Ouwehand A, Salminen S, Bylund G (2001). Protection of rainbow trout (*Oncorhynchus mykiss*) from furunculosis by *Lactobacillus rhamnosus*. Aquaculture. 198(3-4):229-236.

Noh SH, Han K, Won TH Choi YJ (1994). Effect of antibiotics, enzyme, yeast culture and probiotics on the growth performance of Israeli carp. Korean J. Anim. Sci. 36:480-486.

Picchietti S, Mazzini M, Taddei AR, Renna R, Fausto AM, Mulero V, Carnevali O, Cresci A, Alelli L (2007). Effects of administration of probiotic strains on GALT of larval gilthead seabream:Immunohistochemical and ultrastructural studies. Fish Shelfish Immunol. 22(1-2):57- 67.

Quentel C, Gatesoupe FJ, Aubin J, Lamour F, Abiven A, Baud M, Labbé L, Forraz M (2005). Ofimer probiotic study on rainbow trout. I: Resistance against *Yersinia ruckeri* and humoral immune response of rainbow trout (*Oncorhynchus mykiss*) submitted to probiotic treatment with *Saccharomyces cerevisiae* var. *boulardii*. EAS Special Publication No. 35.

Rengpipat S, Phianphak W, Piyatiratit S, Menasaveta P (1998). Effect of probiotic bacterium on black tiger shrimp, *Penaeus monodon*, survival and growth. Aquaculture. 167:301-313.

Ringø E, Myklebust R, Mayhew TM, Olsen RE (2007a). Bacterial translocation and pathogenesis in the digestive tract of larvae and fry. Aquaculture. 268:251-264.

Ringø E, Salinas I, Olsen RE, Nyhauq A, Myklebust R, Mayhew TM (2007b). Histological changes in intestine of Atlantic salmon (*Salmo salar* L.) following in vitro exposure to pathogenic and probiotic bacterial strains. Cell Tissue Res. 328(1):109-116.

Robertson PAW, O'Dowd C, Burrells C, Williams P, Austin B (2000). Use of *Carnobacterium* sp. as a probiotic for Atlantic salmon (*Salmo salar* L.) and rainbow trout (*Oncorhynchus mykiss* Walbaum). Aquaculture. 185:235-243.

Rollo A, Sulpizio R, Nardi M, Silvi S, Orpianesi C, Caggiano M, Cresci A, Carnevali O (2006). Live microbial feed supplement in aquaculture for improvement of stress tolerance. Fish Physiol. Biochem. 32:167-177.

Salminen S, Von-Wright A, Morelli L, Marteau P, Brassart D (1998). Demonstration of safety of probiotics - a review. Int. J. Food. Microbiol. 44:93-106.

Taoka Y, Maeda H, Jo JY, Kim SM, Park SI, Yoshikawa T, Sakata T (2006a). Use of live and dead probiotic cells in tilapia *Oreochromis niloticus*. Fisheries Sci. 72(4):755-766.

Taoka Y, Maeda H, Jo JY, Jeon MJ, Bai SC, Lee WJ, Yuge K, Koshio S (2006b). Growth, stress tolerance and non-specific immune response of Japanese flounder (*Paralichthys olivaceus*) to probiotics in a closed recirculating system. Fish. Sci. 72(2):310-321.

Tovar-Ramirez D, Zambonino J, Cahu C, Gatesoupe FJ, Vazquez-Juarez R, Lésel R (2002). Effect of live yeast incorporation in compound diet on digestive enzyme activity in sea bass (*Dicentrarchus labrax*) larvae. Aquaculture. 204:113-123.

Waché Y, Auffray F, Gatesoupe FJ, Zambonino J, Gayet V, Labbé L, Quentel C (2006). Cross effects of the strain of dietary *Saccharomyces cerevisiae* and rearing conditions on the onset of intestinal microbiota and digestive enzymes in rainbow trout, *Onchorhynchus mykiss*, fry. Aquaculture. 258:470-478.

Wedemyer GA, McLeay DJ (1981). Methods for determining the tolerance of fishes to environmental stress. In Pickering, A.D. ed. Stress and fish. Academic press. New York. pp. 247-275.

academicJournals

Vol. 13(10), pp. 1169-1173, 5 March, 2014
DOI: 10.5897/AJB12.1724
ISSN 1684-5315 ©2014 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Effect of cypermethrin toxicity on enzyme activities in the freshwater fish *Cyprinus carpio*

Khalid Abdullah Al-Ghanim

College of Sciences and Humanity Studies, Alkharj 11942, Salman bin Abdul-Aziz University, Kingdom of Saudi Arabia.

Accepted 22 June, 2012

Cyprinus carpio **a freshwater fish, was exposed to lethal concentration (7.5 µg/L) for one, three, five, seven and nine days and, sublethal concentration (1.5 µg/L) for 1, 7, 14, 21 and 28 days of cypermethrin, respectively to observe the enzyme activity in functionally three different tissues; that is, muscle, gill and liver. The activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and glutamate dehydrogenase (GDH) were increased in all the tissues with an increase in exposure time of cypermethrin. Though, under sublethal concentration of cypermethrin for 14, 21 and 28 days, a decreasing trend was observed in all the three tissues. The increased levels of amino transferase might be attributed to tissue damage under toxic stress in *C. carpio*. It has been concluded that the usefulness of the enzymes as biomarkers of cypermethrin toxicity appeared to be concentration and tissue dependent and can be effectively used to assess the impact of the agrochemical on the fish.**

Key words: Cypermethrin, *Cyprinus carpio*, enzymes activity, aspartate aminotransferase (AST), alanine aminotransferase (ALT), glutamate dehydrogenase (GDH).

INTRODUCTION

In agriculture, indiscriminate use of different pesticides to prevent the crop from pest peril has increased in the developing countries (Santhakumar and Balaji, 2000). The pesticides, even when applied to restricted areas are washed and carried away by rains and floods to large water bodies like ponds and rivers and change the water chemistry (Bhalchandra et al., 2001). It may be highly toxic, not only to fishes but also to other organisms, including man (Madhab et al., 2002).

In recent years, synthetic pyrethroids have been developed for major uses in agriculture and public health purposes. The current commercial products were evolved from the natural pyrethrins, which possess high insecticidal potency, low mammalian toxicity and very short persistence. These are highly toxic to fish and some aquatic invertebrates (Coats et al., 1989). Cypermethrin is being increasingly used as the active ingredient in many dips that are used to prevent and treat ticks, lice, and scrab on sheep and as a treatment against infestation by the parasitic sea louse in aquaculture. The sources of contamination of river courses occur as a result of the direct use of pyrethroid-based dips and also from the processing of sheep skin, wood industry and knitwear manufacture. The environmental concentrations of cypermethrin are often below those that are lethal to many freshwater teleost (McLeese et al., 1980; Stephenson, 1982; Ansari and Kumar, 1988; Philip et al., 1995). Studies have been done on the effects of a synthetic pyrethroid and cypermethrin on fish (Polat et al., 2002; Das and Mukherjee, 2003; David et al., 2004), but

E-mail: shahidmahboob60@hotmail.com.

Abbreviations: AST, Aspartate aminotransferase; **ALT**, alanine aminotransferase; **GDH**, glutamate dehydrogenase.

there is little information on the comprehensive effects of cypermethrin during exposure and post exposure period. Earlier reports are available on the sublethal effects of cypermethrin on lipids, free fatty acids, metabolites and enzymes of protein and carbohydrate metabolism of fish during exposure and recovery phase (Begum 2005a, b).

Shivaknmar (2005) reported that the activity of aspartate and alanine amino transferases (AST and ALT), may serve as strategic links between protein and carbohydrate metabolisms, which is known to alter under several physiological and pathological conditions. Reddy and Venugopal (1990) stated that glutamate dehydrogenase (GDH), a mitochondrial enzyme, catalysis the oxidative deamination of glutamate, provides α-ketoglutarate to the Krebs cycle. This enzyme has several metabolic functions with great physiological significance. It is closely associated with the detoxification mechanisms of tissues. GDH in extra-hepatic tissues could be utilized for the channeling of ammonia released during proteolysis for its detoxification into urea in the liver. Hence, the activities of AST, ALT and GDH are considered as sensitive indicators of stress (Gould et al., 1976).

Enhancement in GDH activity in the tissues provided ketoglutarate and reduced nucleotides, which may fulfill the energy requirements during toxicity manifestations (Chandravathy and Reddy, 1994). The regulatory roles of GDH enzyme as observed in mammalian models in checking the deamination process reported earlier (Philip et al., 1988; Ramana et al., 1990; Reddy and Venugopal, 1990; Reddy and Yellama, 1991; David, 1995; Deva, 2000 and Shobha Rani et al., 2001; Prashanth and Neelagund, 2008).

Glutamate dehydrogenase (GDH), a mitochondrial enzyme, catalysis the oxidative deamination of glutamate, provides α-ketoglutarate to the Krebs cycle (Reddy and Venugopal, 1990). Therefore, the present study was aimed at investigating the effect of the synthetic pyrethroid, cypermethrin on aspartate aminotransferase, alanine amino transferase and glutamate dehydrogenase activity in the economically important freshwater fish, *C. carpio*.

MATERIALS AND METHODS

Collection and maintenance of fish

Healthy and active *C. carpio* fingerlings were procured from the Fisheries Department. Fish were brought to the laboratory in large aerated crates, acclimated for 30 days in large fiber tanks (7×4 × 2 m) and fed with commercial dry feed pellets.

In the laboratory, the fish were held in 100 L glass aquaria (120 × 45 × 80 cm) containing dechlorinated tap water for acclimatization for a period of 20 days at 22±1°C. The physio-chemical characteristics of the tap water were followed as described in APHA (1998). Water was renewed every day and a 12 to 12 h photoperiod was maintained during the acclimatization and test periods. The fish were fed regularly with commercial fish food pellets during the acclimatization and test tenures, but feeding was stopped two days before the exposure to test medium for acute toxicity test.

Preparation of stock and acute toxicity test

Technical grade cypermethrin (95%) was obtained from Merck. After the normal process of acclimatization, a group of 10 fish each was transferred to the aquaria. Stock solution of lethal cypermethrin (7.5 µg/L) and sub-lethal concentration (1/5th of LC_{50} that is, 1.5 µg/L for 96 h) added into the water in the aquaria. Predetermined exposure of lethal concentration of fish was given for one, three, five, seven and nine days and sub-lethal concentration 1, 7, 14, 21 and 28 days according to Finney (1971). Each treatment was replicated three times. Control and challenged fishes were sacrificed at the end of each day in the laboratory. At the end of the experiment, the fish were killed with a blow on the head and gill, liver and muscle tissue were excised and immediately transferred to the deep freezer prior to analysis. Total protein content was estimated by the method of Lowry et al. (1951), amino acids by Moore and Stein (1954), and ammonia with the Nessler reagent as described by Bergmeyer (1965); Prashanth and Neelagund (2008).

Aspartate aminotransferase and alanine aminotransferase were assayed by the colorimetric method of Reitman and Frankell (1957). Alanine aminotransferase activity was expressed as µM pyruvate formed/mg protein/h and the AST activity as µM oxaloacetate formed/mg protein/h. Glutamate dehydrogenase was assayed by the method of Lee and Hardy (1965). GDH activity was expressed as µM formozan formed/mg protein/h. The experiments were repeated for seven times to get concurrent values.

Statistical analysis of data

The data obtained was analyzed statistically by following Duncan's multiple range test (Duncan, 1955).

RESULTS AND DISCUSSIONS

The present results show that cypermethrin-induced alterations are time dependent, tissue-specific and they point to disrupt activity. GDH, AST and ALT enzymes has shown significant elevation in all tissues after lethal and sub lethal exposure (Tables 1 to 3). A progressive increase was noticed in the activities of ALT, AST and GDH in all the organs of the fish exposed to cypermethrin. It might be due to the active trans-deamination of amino acids for the incorporation of ketoacids into the tricarboxylic acid (TCA) cycle to release necessary energy required for the synthesis of new proteins (Sreedevi et al., 1992; Sivaramakrishna and Radhakrishnaiah, 1998). The elevation in these enzymes was probably due to the utilization of amino acids during this cycle. The elevation in transaminases suggests the existence of the heavy drain on metabolites during cypermethrin stress. Awasthi et al. (1984) proposed that stress conditions in general induce elevation in the transamination pathway. The present results are in line with the findings of above mentioned studes. Involvement of alternate pathways like aminotransferase reactions are also possible due to inhibition of oxidative enzymes like isocitrate dehydrogenase and cytochrome C -oxidase, a situation demonstrated by Ghosh (1989) in *Labeo rohita*

Table 1. GDH activity (µM glutamine/mg protein/h) in the organs of fish, *Cyprinus carpio* on exposure to the lethal and sub lethal concentrations of cypermethrin.

Organ	Control	Exposure period in days									
		Lethal					Sublethal				
		1	3	5	7	9	1	7	14	21	28
Gill	0.130^{J}	0.146^{I}	0.159^{H}	0.182^{G}	0.198^{F}	0.233^{E}	0.257^{C}	0.274^{B}	0.288^{A}	0.252^{D}	0.231^{E}
SD±	0.003	0.004	0.006	0.005	0.007	0.006	0.009	0.010	0.008	0.009	0.007
Muscle	0.176^{H}	0.186^{G}	0.209^{F}	0.242^{C}	0.256^{B}	0.276^{A}	0.211^{F}	0.233^{B}	0.244^{C}	0.238^{D}	0.229^{E}
SD±	0.001	0.002	0.001	0.003	0.003	0.004	0.002	0.003	0.005	0.002	0.001
Liver	0.421^{F}	0.449^{E}	0.557^{C}	0.631^{B}	0.671^{A}	0.404^{F}	0.445^{E}	0.553^{D}	0.559^{C}	0.401^{G}	0.336^{H}
SD±	0.003	0.004	0.005	0.003	0.003	0.002	0.004	0.003	0.006	0.007	0.005

Values are means ± SD (n=6) for a tissue. Values in a column followed by the same letter are not significantly different ($P < 0.05$) from each other according to Duncun's multiple range (DMR) test.

exposed to cypermethrin. The changes in the activities of the amino transferases would often be reflected in nitrogen metabolism and interdependent biochemical reactions. The increased levels of amino transferase might be attributed to tissue damage under toxic stress in *C. carpio*. Similar findings were reported by Raju and Ramna, (1985). AST, a key enzyme of nitrogen metabolism and energy mobilization in invertebrates, is often used as a biochemical indicator of stress (Shobha Rani et al., 2001; Reddy and Venugopal, 1990). We are of the view that the increase in AST and ALT levels indicate that *C. carpio* was under toxic stress. The amino acids appear to be mobilized to get transamination to 2-keto acids, for use in the production of energy rich compounds (David, 1995; Rajamannar and Manohar, 1998; Deva, 2000).

Enhancement of GDH activity in the tissues provided ketoglutarate and reduced nucleotides, which may fulfill the energy requirements during toxicity manifestations (Chandravathy and Reddy 1994). GDH is also known to play a crucial role in ammonia metabolism and is known to be affected by a variety of effectors (Shakoori et al., 1976; David, 1995). After several metabolic functions with great physiological significance and known to be closely associated with the detoxification mechanisms of tissues, GDH in extrahepatic tissue could be utilized for its ultimate detoxification to urea in the liver. In the present study the significant elevation in the activities of these enzymes in the organs of fish exposed to the lethal concentration of cypermethrin was probably due to greater association of oilgomers of these enzymes in response to toxic stress. This shows that oxidative deamination contributes to higher ammonia production. The high levels of ammonia produced is not eliminated but is salvaged through GDH activity, which is utilized for amino acid synthesis through transaminases (David, 1995; Deva, 2000 and Prashanth 2003; Begum, 2004). The GDH elevation in all tissues (Table 1) also suggests the possibility of a need for α-ketoglutarate to the TCA cycle for the liberation of energy. In the present study, the GDH activity showed a progressive enhancement in all tissues (gill, muscle and liver), throughout the exposure, suggesting a need for α-ketoglutarate.

The regulatory roles of this enzyme as observed in mammalian models in checking the deamination process were reported earlier (Moorthy et al., 1984; Philip et al., 1988; Ramana et al., 1990; Reddy and Venugopal, 1990; Reddy and Yellama, 1991; David, 1995; Deva, 2000; Shobha et al., 2001; Prashanth and Neelagund, 2008). GDH catalyzes the reversible reaction of oxidative deamination of glutamate to α-ketoglutarate and ammonia (Begum and Vijayaraghavan, 1998) and plays an important role in the catabolism and biosynthesis of amino acid. GDH activity was enhanced in muscle and liver tissues for 28 days of cypermethrin toxicity, which indicates increased deamination of glutamate and formation of ammonia. It has been observed that the sublethal exposure of cypermethrin produced less change in the protein metabolism. It has also been noticed that the liver, gill and muscle were affected and the disturbances were found to be more in those tissues than that of muscle tissue.

Increased activities of AST (Table 2) and ALT (Table 3) in the study indicate that there may be an active transamination of amino acids, possibly to provide keto acid in the TCA cycle. The steady rise in the activities of GDH, AST and ALT in the organs of *C. carpio* exposed to sublethal concentrations of cypermethrin (Tables 1 to 3) may be due to the synthesis of these enzymes under sub acute cypermethrin stress. The increase in these enzyme activities could be helpful to the fish for structural reorganization of proteins and incorporation of keto acids into the TCA cycle to favor gluconeogenesis or energy production. The increase in transaminases can also link to the formation of urea (Ramna and Ramamurthi, 1983). The gradual increase in the activities of AST, ALT and GDH lead to metabolic compensation and allow the

Table 2. The aspartate aminotransferase (AST) activity (µM oxaloacetate /mg protein/h) in the organs of fish, *Cyprinus carpio* on exposure to the lethal and sub lethal concentrations of cypermethrin.

Organ	Control	Exposure period in days									
		Lethal					Sublethal				
		1	3	5	7	9	1	7	14	21	28
Gill	1.462^{I}	1.68^{F}	1.825^{F}	1.973^{E}	2.177^{D}	2.431^{C}	2.720^{B}	2.882^{A}	2.015^{H}	1.732^{G}	1.574^{H}
SD±	0.006	0.010	0.007	0.006	0.053	0.0557	0.002	0.004	0.050	0.007	0.009
Muscle	2.205^{J}	2.555^{I}	2.942^{G}	3.221^{F}	3.543^{D}	3.620^{C}	3.885^{B}	3.917^{A}	3.392^{E}	3.002^{G}	2.723^{H}
SD±	0.008	0.012	0.015	0.020	0.018	0.022	0.027	0.021	0.028	0.019	0.017
Liver	2.467^{H}	2.884^{G}	3.221^{F}	3.478^{E}	3.886^{C}	4.123^{B}	4.234^{A}	4.112^{B}	3.946^{C}	3.657^{D}	3.23^{F}
SD±	0.030	0.040	0.055	0.047	0.063	0.074	0.079	0.081	0.066	0.007	0.008

Values are means ± SD (n=6) for a tissue. Values for a tissue in a column followed by the same letter are not significantly different ($P < 0.05$) from each other according to Duncun's multiple range (DMR) test.

Table 3. The alanine aminotransferase (ALT) activity (µM pyruvate formed /mg protein/h) in the organs of fish, *Cyprinus carpio* on exposure to the lethal and sub lethal concentrations of cypermethrin.

Organ	Control	Exposure period in days									
		Lethal					Sublethal				
		1	3	5	7	9	1	7	14	21	28
Gill	1.662^{I}	1.889^{H}	2.112^{G}	2.477^{B}	2.876D	3.112^{C}	3.237^{B}	3.431^{A}	3.214^{B}	2.750E	2.554^{F}
SD±	0.009	0.011	0.014	0.019	0.018	0.023	0.026	0.029	0.022	0.027	0.018
Muscle	4.575^{J}	4.984^{I}	5.886^{F}	6.324^{D}	6.778^{B}	6.931^{A}	6.114^{D}	6.448^{C}	5.994^{E}	5.576^{G}	5.341^{H}
SD±	0.022	0.029	0.034	0.039	0.041	0.044	0.033	0.051	0.052	0.047	0.061
Liver	6.131^{K}	6.778^{J}	7.723^{H}	8.886^{E}	9.997^{B}	10.234^{A}	9.886^{C}	9.236^{D}	8.687^{F}	7.965^{G}	6.876^{I}
SD±	0.056	0.060	0.550	0.623	0.675	0.723	0.765	0.663	0.559	0.721	0.645

Values are means ± SD (n=6) for a tissue. Values for a tissue in a column followed by the same letter are not significantly different ($P < 0.05$) from each other according to Duncun's multiple range (DMR) test.

animal to adapt to the imposed toxic stress. The increase in GDH activity at the sub lethal concentration (Table 1) could lead to increased production of glutamate in order to eliminate ammonia. To have an insight into the role of these enzymes in the altered metabolism of cypermethrin intoxicated fish, the activities of both AST and ALT were investigated in the present experiment. Elevated levels of AST and ALT indicate the enhanced transamination of amino acids, which may provide keto acids to serve as precursors in the synthesis of essential organic elements. It is likely that toxic stress imposed by cypermethrin might be one of the factors for the observed activities of AST and ALT in the present study.

Conclusion

It can be concluded from the current study that the sublethal exposure of cypermethrin produced less change in the protein metabolism. It has also been observed that the liver, gill and muscle were affected and the stress was found to be more in liver and gill than that of muscle tissue. With respect to the toxic effects on exposure to sublethal concentration of cypermethrin, the fish tries to withstand the toxic effects imposed by the pesticide by modulating their physiological and metabolic response towards proper utilization of enzymes and proteins for synthetic processes but after 28 days exposure recovery was not possible. These kinds of studies will help to determine remedial measures to be taken at appropriate times in a polluted organism, particularly fish and thus prevent ill effects in fish consumers.

REFERENCES

Ansari BA, Kumar K (1988). Cypermethrin toxicity effect on the carbohydrate metabolism of a Indian catfish, *Heteropneustes fossilis*. Sci. Total. Environ. 72:161-166.

APHA (1998). Standard methods for the examination of water and

wastewater. 20th Edn. American Public Health Association, Washington, DC.
Awasthi M, Shaw P, Dubale MS, Gadhia P (1984). Metabolic changes induced by organophosphates in the piscine organs. Environ. Res. 35:320-325.
Begum G (2004). Carbofuran insecticide induced biochemical alterations in liver and muscle tissue of the fish *Clarias batrachus* (Linn.) and recovery response, Aquat. Toxicol. 66:83-92.
Begum G (2005a). In vivo biochemical changes in liver and gill of *Clarias batrachus* during cypermethrin exposure and following cessation of exposure. Pest. Biochem. Physiol. 82:185-196.
Begum G (2005b). Toxicity of cypermethrin on total lipids and free fatty acids in liver, muscle, kidney and ovary of *Clarias batrachus* (L) and recovery response. Toxicol. Environ. Chem. 87:253-260.
Begum G, Vijayaraghavan S (1998). Toxicity of rogor on total free amino acids and glutamate dehydrogenase in hepatic and muscle tissues of a fish. Ecol. Environ. Conserv. 4:235-238.
Bergmeyer HUC (1965). Aminotransferases and related enzymes. In: Bergmeyer, H.U.C., Bernt, E., eds. *Methods of Enzymatic Analysis*. vol. II. New York: Academic Press. pp. 735-739, 760-764.
Bhalchandra WB, Lomte VS (2001). Acute toxicity of pesticides carbaryl and endosulfan to freshwater Bivalves, *Parreysia cyclindrica*. Pollut. Res. 20:25-29.
Chandravathy MV, Reddy SLN (1994). In vivo recovery of protein metabolism in gill and brain of a freshwater fish, *Anabas scandens* after exposure to lead nitrate. J. Environ. Biol. 1:75-82.
Coats JR, Symonick DM, Bradubry SP, Dyer SD, Timson LK, Achison GJ (1989). Toxicity of synthetic in aquatic organisms on over view. Environ. Toxic. and Chem. 8: 671-679.
Das BK, Mukherjee SC (2003). Toxicity of cypermethrin in *Labeo rohita* finger-lings: biochemical enzymatic and haematological consequences. Comp. Biochem. Physiol. C. 134:109-121.
David M (1995). Effect of fenvlaterate on behavioural, physiological and biochemical aspects of freshwater fish*, Labeo rohita*. Ph.D. thesis, S. K. University, Anantapur India.
David M, Mushigeri SB, Shivakumar R, Philip GH (2004). Response of *Cypri-nus carpio* (Linn) to sublethal concentration of cypermethrin: alterations in protein metabolic profiles. Chemosphere 576:347-352.
Deva PR (2000). Fenvalerate induced changes in the protein metab-olism of freshwater fish, Tilipa mossambica (Peters). Ph. D. thesis, S. K. University, Anantapur India.
Duncan DM (1955). Multiple range of multiple tests Biometrics. 42:1-42
Finney DJ (1971). Probit Analysis. 3rd ed. Cambridge University Press, London,
Ghosh TK (1989). Influence of cypermethrin on the oxidative metabolism of fish *Labeo rohita.* Proc. Indian Acad*. B*. 55:115-120.
Gould E, Collier, RS, Karolous JJ, Givenus H (1976). Heart transaminase in the rock crab, Cancer irroratus exposed to cadmium salts. Bull. Environ. Contam. Toxicol. 15: 635-643.
Lee YL, Hardy HA (1965). Influence of thyroid hormone on L-glycerophosphate dehydrogenase and other dehydrogenases in various organs of rats. J. Biol. Chem. 240:1427-1452.
Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ (1951). Protein measurement with folin-phenol reagent. J. Biol. Chem. 193:265-275.
Madhab P, Bandyopadhaya, Ajit KA (2002). Xenobiotic impact on sensitivity in *Anabas testudineus* (Bloch). J. Ecobiol. 14:117-124.
McLeese DW, Metcalfe CD, Zitko V (1980). Lethality of permethrin, cyper-methrin and fenvalerate to salmon, lobster and shrimp. Bull. Environ. Contam. Toxicol. 25:950-955.
Moore S, Stein WH (1954). A modified ninhydrin reagent for the photometric determination of amino acids and related compounds. J. Biol. Chem. 211:907-913.
Moorthy KS, Moorthy KS, Kasi Reddy, B, Swami SK, Chetty CS (1984). Changes in respiration and ionic content in tissues of freshwater mussel exposed to methylparathion toxicity. Toxicol. Lett. 21:287-291.
Philip GH, Reddy PM, Srideve G (1995). Cypermethrin induced invivo alterations in the carbohydrate metabolism of freshwater fish, *Labeo rohita*. Exotoxicol. Environ. Saf. 31:173-178.
Philip HGP, Malla RR (1988). Changes in the protein metabolism in liver and kidney of *Mus booduga* Gray after oral BHC Feeding. Bull. Envion. Contam. Toxicol. 41:822-827.
Polat H, Erkocs F, Viran R, Kocak O (2002). Investigation of acute toxicity of β-cypermethrin on guppies *Poecilia reticulata.* Chemosphere 49:39-44.
Prashanth MS (2003). Cypermethrin induced physiological and biochemical and histopathological changes in freshwater fish, *Cyprinus carpio*. Ph.D thesis, Karn-ataka university, Dharwad.
Prashanth MS, Neelagund SE (2008). Impact of Cypermethrin on enzyme activities in the freshwater fish *Cirrhinus mrigala* (Hamilton) Caspian J. Environ. Sci. 6: 91-95
Rajamannar K, Manohar L (1998). Sublethal toxicity of certain pesticides on carbohydrates,proteins and amino acids in *Labeo rohita* (Hamilton). J. Ecobiol. 10:185-191.
Raju TN, Ramana RJV (1985). Variations in amino transferases during nitrate stress. Ind. J. Comp. Anim. Physio. 3:29-32.
Ramana RKV, Surendranath P, Kodavanti PRS (1990). Levels of Trans-minase in tissues of the peneaid prawn, *Metapenaeus monoceros* (Fabricius) following sub lethal kelthane exposure. Bull. Environ. Contam. Toxicol. 44: 114-120.
Reddy ATV, Yellama K (1991). The possible metabolic diversions adopted by the cockroach, Periplaneta american to counteract the toxicity of fenvalerate. Biochem. Internat. 23: 259-365.
Reddy SLN, Venugopal NBRK (1990). Fuoride-induced changes in protein metabolism in the tissue of freshwater crab, *Barytelphusa guerini*. Environ. Pollut. 67:97-108.
Reitman S, Frankel S (1957). A colorimetric method for the determination of serum glutamic oxaloacetic and glutamic pyruvate transminase. Am. J. Clin. Pathol. 28:56.
Santhakumar M, Balaji M (2000). Acute toxicity of an organophours insecticide monocrotophas and its effects on behaviour of an air-breathing fish, *Anabas testudineus* (Bloch). J. Environ. Biol. 21:121-123.
Shakoori AR, Zaheer SA, Ahmed MS (1976). Effect of mALThion, dieldrin, and endrin on blood serum protein and free amino acid pool of *Channa punctatus* (bloch). Pak. J. Zool. 8:125-134.
Shivaknmar R (2005). Endosufan induced metabolic alternation in freshwater fish, *Catla catla*. Ph. D., Thesis, Karnataka University, Dharwad, Karnataka, India.
Shobha Rani AR, Sudharsan TN, Reddy, PVM, Reddy RTN (2001). Effect of arsenite on certain aspects of protein metabolism in freshwater teleost, *Tilipa mosambica* (Peters). J. Environ. Biol. 22:101-104.
Sivaramakrishna B, Radhakrishnaiah K (1998). Impact of sub lethal concentration of mercury on nitrogen metabolism of the freshwater fish, *Cyprinus carpio* (Linn). J. Environ. Biol. 19:111-117.
Sreedevi PB, Sivaramakrishna A, Suresh RK (1992). Effect of nickel on some aspects of protein metabolism in the gill and kidney of the freshwater fish, *Cyprinus carpio* (L). Environ. Pollut. 76: 355-361.
Stephenson RR (1982). Aquatic toxicology of cypermethrin. I. Acute toxicity of some feshwater fish and invertebrate in laboratory tests. Aquat. Toxicol. 2:175-185.

academicJournals

Vol. 13(10), pp. 1174-1181, 5 March, 2014
DOI: 10.5897/AJB2013.12856
ISSN 1684-5315 ©2014 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Microarray based comparative genome-wide expression profiling of major subtypes of leukemia

Harendra Modak[1], Sujayendra Kulkarni[1], Suyamindra S. Kulkarni[2], Prabhanjan Gai[3], Umesh Hallikeri[4] and Pramod B Gai[1,2]*

[1]Centre for Excellence in Molecular Haemato-oncology, Department of Applied Genetics, Karnatak University, India.
[2]Karnataka Institute for DNA Research,Dharwad, India.
[3] Institute of Tropical Medicine, University of Tübingen, Tübingen, Germany.
[4]Karnataka Cancer therapy and Research Institute, Karnataka, India.

Accepted 27 August, 2013

The uncontrolled proliferation of hematopoietic cells with no capacity to differentiate into mature blood cells leads to leukemia. Though considerable amount of work has been done in understanding the molecular basis and gene expression profiles of hematologic malignancies viz., chronic lymphocytic leukemia (CLL), chronic myelogenous leukemia (CML), acute lymphocytic leukemia (ALL) and acute myelogenous leukemia (AML), the role of various underlying genes and mechanisms predisposing the disease are poorly understood. To develop the early diagnosis, preventive and therapeutic strategies, identification of population specific novel mutations and candidate genes are required. Micro array based gene expression profiling was performed for total of 18 samples (4 from each subtype of leukemia that is, CLL, CML, ALL, AML and 2 controls) from Indian population using single color hybridization. The expression of all genes presented in terms of fold variation was subjected to F-test. The microarray data of genes showing differential regulation with respect to the control samples have been obtained from total 50, 238 probes covering 14,992 genes on Agilent's Human 8X60K Array. The experiment was conducted with expectation to have similar patterns of result in terms of gene expression but it demonstrates statistically significant relationship only among CML and ALL which are of myeloid and lymphoid origin, respectively, in contrast to other combinations. Gene expression profiles of four subtypes of leukemia were compared to each other to ascertain the overall association and significance of genes for occurrence of different types of leukemiawhich would guide in the development of common probable biomarkers for leukemias followed by effective diagnosis, prognosis and treatment. Based on their geomean fold values, the highly upregulated genes found in this study are listed.

Keywords: Leukemia, microarray, gene expression profiling, fold variation, lymphoid, myeloid, geomean fold.

INTRODUCTION

Leukemia is an uncontrolled proliferation of hemato-poietic cells having no capacity to differentiate normally to mature blood cells. It is generally classified into myeloid and lymphocytic categories based on affected cell lineages (Sawyers et al., 1991).

Several external agents like chemical exposures, treatment with chemotherapeutic agents, radiation or intrinsic factor like heredity have been entailed for the development of leukemia (Smith and Zhang, 1998). Human T-cell leukemia/-lymphotropic virus type I (HTLV-1) is also a well-empathised cause of adult T-cell leukemia (Franchini, 1995). Microarray (MA) based gene expression analyses has proved to be an important aspect of clinical and biomedical research and helps in

furnishing vital information regarding pathogenesis, diagnosis and prognosis of leukemias by increasing the knowledge on deregulated pathways in leukemia. The incisively positioned DNA probes of microarrays are projected to specifically supervise the gene expression level in parallel processing (Dunphy, 2006). Analyses of differences in gene expression at a large scale for cancer investigations can be performed by DNA microarray technology (Majeti et al., 2009).

There are several reports of gene expression profiles of chronic myelogenous leukemia (CML) (Nowicki et al., 2003; Cohen et al., 2001) and acute myelogenous leukemia (AML) (Bullinger et al., 2004; Valk et al., 2004) in bulk, whereas in few studies individual types of leukemia have been directly compared to normal hematopoietic cells (Stirewalt et al., 2008). For the first time we are reporting from Indian population about comparative gene expression profiles between four major subtypes of leukemia viz. CML, chronic lymphocytic leukemia (CLL), AML and acute lymphocytic leukemia (ALL) along with controls.

In case of leukemia, which are normally associated with a single gene abnormality viz. a single genemutation like C/EBPα, NPMc, FLT3-ITD mutation or a fusion gene due to a chromosomal translocation (for example, AML1-ETO, BCR-ABL) the use of global gene expression analysis techniques reserves for a cryptic understanding of the cellular consequences and the disease as a whole. These techniques have also been used extensively to identify prognostic determinants in leukemia patients, as well as to better understand the molecular basis of response to therapeutic agents in AML (Goswami et al., 2009).

Leukemic thymocytes disclosed typical gene expression patterns being strongly consociated with specific oncogenic transcription factors after being gone through microarray studies. Closely related signatures were also found in several samples which lacked activation of known T-ALL oncogenes. It leads to predict alternative oncogenic transcription factors able to initiate gene expression showing similar patterns (Ferrando et al., 2002). Supervised and unsupervised approaches of microarray analysis showed a distinctive pattern of gene machinery expression of the CLL clone in regression (Haslinger et al., 2004).

Wang et al. (2004) and Zent et al. (2003) reported that genes like FGR, PTPN12, IL4R, FCER2 (CD23), TMEM1, TNFRSF1B, CHS1, CCR7 and FMOD among others were differentially expressed in a consistent manner in CLL when compared with tonsillar B lymphocytes and plasma cells. The comparative gene expression profiling using cDNA micro array analysis of 5315 genes of CML and of normal donors revealed at least a 4-fold difference in the mean expression of 263 genes in which 148 up-regulated and 115 down-regulated were observed in the CMLs compared with the normal specimens (Nowicki et al., 2003).

Today, genome-wide gene expression profiling based on DNA microarrays represents one of the most powerful tools in the area of genomics (Liotta and Petricoin, 2000; Ramaswamy and Golub, 2002) since it has become economically feasible and widely accessible, thereby contributing significantly to our understanding of different types of cancers (Care et al., 2003; Kiyoi et al., 1999).

To gain discernments into the molecular alterations that cause different types of leukemia, we accomplished genome-wide comparative gene expression profiling of sixteen cases representative of four different forms of leukemia (four each) and two cases of normal blood samples as controls. The comparative analysis of leukemia genomes helps in remoulding our knowledge and depth in hemato-malignancies that could have major implications for clinical translation (Hudson et al., 2010) which fulfils the founding concept of The International Cancer Genome Consortium. Our study of genomes of sixteen leukemia patients emphasizes this evolutionary potential, nevertheless profound studies will be required to interpret these outcomes to the healthcare domain.

MATERIALS AND METHODS

Selection of patients

Clinically diagnosed blood samples of four major subtypes of leukemia viz. CML, AML, ALL, CLL were collected after ethical clearance and with the informed consent of the patients through approved hospitals followed by specific protocols. A total of 18 blood samples consisting of all the above four major types of leukemia, 4 each, with 2 controls were selected for the analysis (Table 1). The leukemia blood samples and controls are not age and sex matched.

Sample collection

Peripheral blood samples (PBC) of 2.5 ml were collected in PAXgeneBlood RNA tubes (Cat no. 762165, Qiagen) to prevent the intracellular RNA from degradation and stored at - 80°C for further experiment.

RNA extraction and target labeling

Total RNA was extracted from all the blood samples using the PAXgene Blood RNA kit (Qiagen Cat.No.762174), according to the procedure provided by the manufacturer. The RNA integrity was measured using RNA 6000 Nano Lab chip on the 2100

*Corresponding author. E-mail: pramodbgai@gmail.com.

Abbreviations: HTLV-1, Human T-cell leukemia/lymphotropic virus type I; **MA,** microarray; **CML,** chronic myelogenous leukemia; **AML,** acute myelogenous leukemia; **CLL,** chronic lymphocytic leukemia; **ALL,** acute lymphocytic leukemia; **RIN,** RNA integrity number; **DTT,** dithiothreitol.

Table 1. Clinical details of 16 patients with 2 controls.

Leukemia type	Age/ sex	Status and symptoms	Type of diagnosis	Lymphoblast count & other blood features	Chemotherapy/drugs used
AML	38/M	Fever, anaemia, thrombocytopenia, skin petechiae	Peripheral blood smear study and bone marrow study	>60-80% Blast cells in TLC*, immature cells of WBCs and platelet counts are reduced, TLC >15,000/ cm mm	Combination of daunorubicin(45 mg/m^2/ IV**/3 days) and cytarabine (100 mg/m^2/ IV/*bis-a-day*/7 days)
AML	70/M				
AML	54/F				
AML	45/F				
ALL	18/M	Fever, anaemia, enlarge lymph glands in children		Child having >50-60% blast cells in TLC, TLC >20,000/cm mm	Vincristine (1.5 mg/m^2), doxorubicin (25 mg/m^2), prednisone (40 mg/m^2), L-asparaginase (10,000 units iv/ alternate day), methotrexate (12 mg/m^{2}intrathecal)
ALL	6/M				
ALL	11/F				
ALL	10/F				
CML	40/M	Splenomegaly		>10-30% Blast cells in TLC, basophils more and platelet count mostly adequate, TLC >15,000/ cm mm	Imatinib (400 mg), hydrea (2-3 g), dasatinib (50 mg), nilotinib (100 mg)
CML	57/M				
CML	34/F				
CML	38/F				
CLL	58/M	Anaemia, enlarge lymph glands in adult patients		Elderly patients having >60-70% blast cells in TLC, Pro-lymphoblast and smudge cells are present, TLC > 20,000 /cm mm	Chlorambucil (5-10 mg/5 days/month), fludarabine (50 mg tablets/day), CHOP regimen consisting of Cyclophosphamide (750 mg/m^2/3weeks), doxorubicin (50 mg/m^2), Vincristine (1.5 mg/m^2) and prednisone (100mg/m^2 /day for 5 days)
CLL	65/F				
CLL	67/F				
CLL	60/M				
Control 1	23/M	-		Normal report	-
Control 2	24/F	-		Normal report	-

*TLC, Total Leukocyte count;**IV, intra veinous

Bioanalyser following manufacturer's protocol. Eppendorf UV-VIS Biophotometer was used to assess total RNA purity. For micro array based gene expression experiments, total RNA showing OD 260/OD280>1.8 and OD260/OD270>1.3 was used. The RNA was evaluated to be of good quality when the rRNA 285/185 ratios are greater than or equal to 1.5 along with the rRNA contribution being 30% or more. Additionally, RNA integrity number (RIN) should be >7.0.

Agilent's Quick-Amp labeling Kit (p/n5190-0442) was used for 1st labeling. Briefly, both first and second strand cDNA was synthesized by incubating 500 ng of total RNA with 1.2 µl of oligo dT-T7 promoter primer in nuclease free water at 65°C for 10 min followed by incubation with 4.0 µl of 5x first strand buffer, 2 µl of 0.1 M dithiothreitol (DTT), 1 µl of 10 mM dNTP mix 1 µl of 200 U/µl MMLV-RT and 0.5 µl of 40 U/µl RNase OUT, at 40°C for 2 h. Immediately following cDNA synthesis, the reaction mixture was incubated with 2.4 µl of 10 mM Cyanine 3-CTP (Perkin-Elmer, Boston MA) 20 µl of 4X Transcription buffer, 8 µl of NTP mixture, 6 µl of 0.1 M DTT, 0.5 µl of RNase OUT, 0.6 µl of Inorganic pyrophosphatase, 0.8 µl of TT RNA polymerase and 15.3 µl of nuclease free water at 40°C for 2 h. Qiagen RNeasy mini spin columns were used for hybridization. 825 ng of Cyanine 3 labelled cDNA in a volume of 41.8 µl was combined with 1.1 µl of 10X blocking reagent and 2.2 µl of 25X fragmentation buffer and incubated at 60°C for 30 min in the dark.

The fragmented cDNA was mixed with 5.5 µl of 2X hybridization buffer. About 110 µl of the resulting mixture was applied to Human 8x15K Array covering 14,992 genes, (AMADID: 035928) Gene expressions Micro Array (Agilent Technologies, USA) and hybridized at 65°C for 17 h in an Agilent Microarray Hybridization Chamber with hybridization oven. After hybridization, the slides were washed with Agilent gene expression wash buffer I for 1 min at room temperature followed by 1 min wash with Agilent gene expression wash buffer II at 37°C. Slides were finally rinsed with acetonitrite for cleaning up and drying.

Hybridization, scanning, and feature extraction

Scanning of hybridized arrays was performed at a

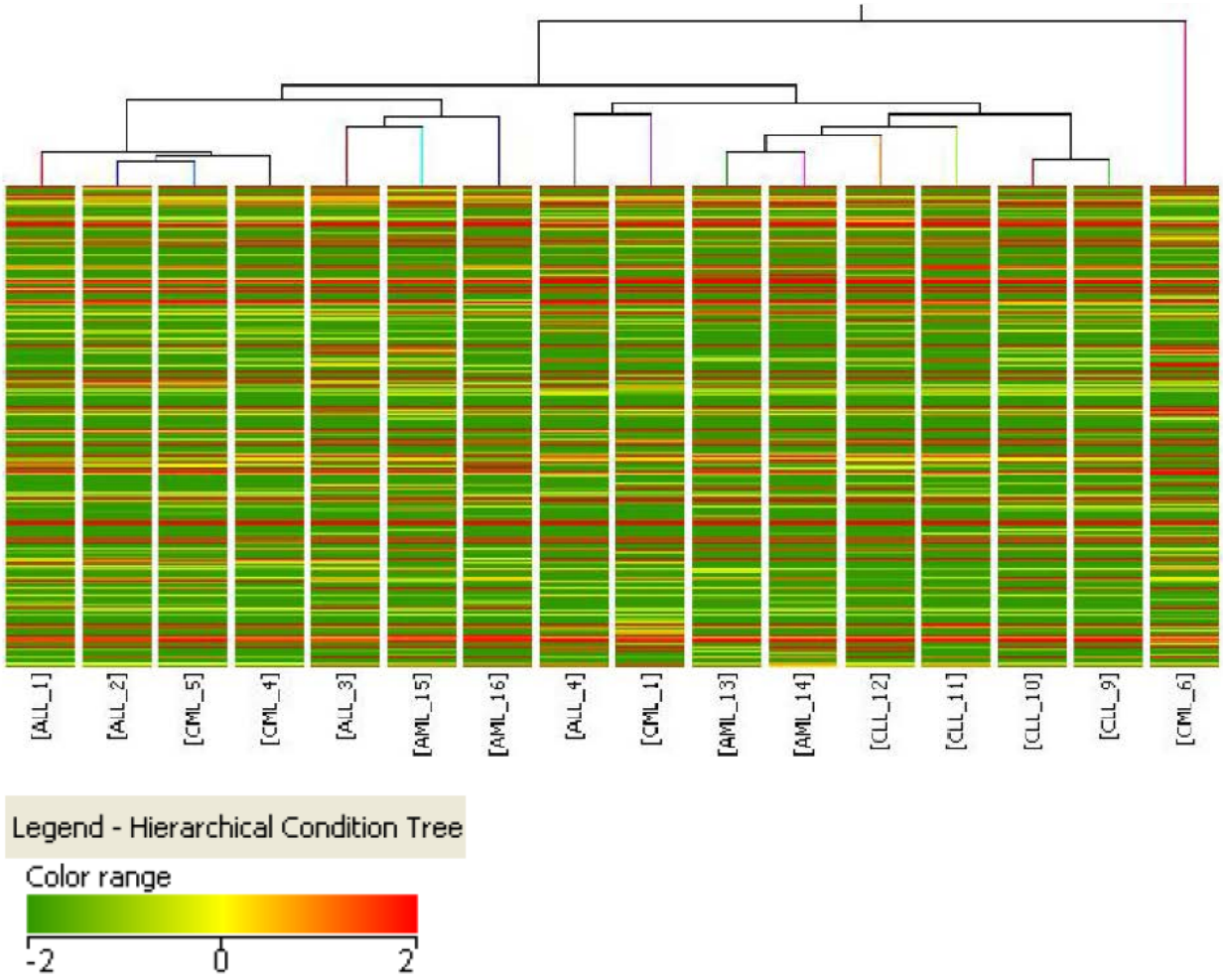


Figure 1. Clusters for intra array quality control. GeneSpring GX 11.5 software was used for normalization. [Normalization being used for QC: 75th percentile shift normalization. Percentile shift normalization was considered as a global normalization in which in an array the locations of all the spot intensities are aligned. Each column of the experiment was taken independently in this normalization, which further computes the percentile of the expression values for this array, throughout all spots (n has a range from 0-100 ; n=50 is the median). Here this value was substracted from the expression value of each entity]. ALL, Acute lymphocytic leukemia; CML, chronic myeloid leukemia; AML, acute myeloid leukemia; CLL, chronic lymphocytic leukemia.

resolution of 2 µm on an Agilent DNA Microarray scanner.Agilent feature extraction software was used for Data extraction from images.

cDNA microarray data analysis

Feature extracted data were analyzed using GeneSpring GX Version 11.5 software from Agilent. Normalization of the data was done using per spot per chip intensity dependent lowest normalization. Further quality control of normalized data was done using correlation based condition tree to eliminate bad experiments. One fold and above differentially regulated genes were filtered from the data. Differentially regulated genes were clustered by using gene tree to identify significant gene expression patterns (Figure 1).

Analysis of variance

Comparing the normal blood gene expression profiles with that of leukemia gene expression, the data were grouped. The data from all types of leukemia blood samples were subjected to F-Test in Microsoft Excel 2007.

RESULTS AND DISCUSSION

The whole genome sequence consisting of 50238 probes were used for comparative gene expression profiling of four major subtypes of leukemia viz. CML, AML, CLL and ALL by using Human 8X 60 K Array for 16 patient samples (4 for each type of leukemia) and 2 normal peripheral blood samples. The results were analysed and presented in Figure 1 and Table 2. The cluster analysis of differentially regulated genes using gene tree is to identify significant gene expression patterns (Figure 1). The clusters and sub clusters consist of different leukemia

Table 2. F-test results of all the probes for all types of leukemia.

Properties / variable	CML vs. AML		CML vs. CLL		CML vs. ALL		AML vs. ALL		AML vs. CLL		ALL vs. CLL	
	Variable1	Variabl2	Variable1	Variable2	Variable1	Variable2	Variable1	Variable2	Variable1	Variable2	Variable1	Variable2
Mean	0.3825	0.17029	0.3825	-0.17838	0.3825	0.20059	0.20059	0.17029	0.17029	-0.17838	0.20059	-0.17838
Variance	1.28342	2.08036	1.28342	2.352692	1.28342	0.874957	0.87495	2.08036	2.08036	2.35269	0.87495	2.35269
Observations	50238	50238	50238	50238	50238	50238	50238	50238	50238	50238	50238	50238
df	50237	50237	50237	50237	50237	50237	50237	50237	50237	50237	50237	50237
F	0.61692		0.54551		1.46684		0.42057		0.88425		0.37189	
P(F<=f) one-tail	0		0		0		0		0		0	
F Critical one-tail	0.98542		0.54551		1.014785		0.985429		0.985429		0.985429	

F-Test was carried out with 95% confidence and 5% α error.

samples in a mix pattern. The cluster analysis of normal samples has been performed separately. Fold variation of all the probes were obtained in terms of log in base 2.

The fold variation in terms of gene expression of all the probes were subjected to F-test two samples for variance in two groups where each type of leukemia is compared to other type and produces six different combinations. The analysis of variance was carried out with 95% confidence and 5% α error (Table 2). It was found that the critical F values in case of CML vs. AML, CML vs. CLL, AML vs. ALL, AML vs. CLL and ALL vs. CLL were greater than the calculated F-value whereas in case of CML vs. ALL the critical F-value of 1.014786 is smaller than the calculated F value of 1.466842. The F-test results revealed that there is no significant variation among AML and other types in terms of whole genome expression profiling when all the detected probes were compared. Similarly, CLL does not exhibit any significant variation in expression profiling with other types of leukemia. CML and ALL show no significant relationship with AML and CLL but have a strong relationship between them as when F-test of two samples for variance was calculated, that critical F value was found to be smaller than the calculated F value.

The results suggest that there is no significant variation between acute myeloid and chronic myeloid leukemia which are reported to be originated from myeloid line of blood cells. The same result has been found for acute lymphocytic and chronic lymphocytic leukemia where their origin is known to be lymphocytes. Similarly, Lymphoid and myeloid line of blood cells were compared to each other which render contrastive results showing AML vs. CLL to have no significant genome wide gene expression variation whereas CML vs. ALL are found to furnish statistically significant relationship. The experiment was designed with expectation to have similar patterns of result but it shows strikingly different relationships among each other. The study can be further advanced by targeting few significantly involved genes in different types of leukemia to find out any possible association among them. The highest upregulated gene found in our study was ENST00000376881 that is, ZFP57 (zinc finger protein) [Source: HGNC Symbol; Acc: 18791] in CML, LOC390413 (predicted to be similar to 60S ribosomal protein L7) in CLL, THC2585201in AML and FOXC1 in ALL (Table 3). Any gene(s) found significantly pathologically active for more than one type of leukemia could be used to design common biomarker for early diagnosis. Leukemia and lymphoma society facts (2011-2012) has cited that, approximately 31 percent of more males are living with leukemia than females but this result is not ecumenical as one of our previous report (Modak et al., 2011) has reported overall male female ratio to be 1.8:1 in leukemia cases which shows the number of male leukemia patients are almost double than that of female patients. In this particular study, the ratio between male and female of 1:1.3 has been taken for microarray gene expression analysis.

Conclusions

This study is first of its kind as per our exhaustive literature survey where the four major subtypes of leukemia were matched for their individual genetic expression to each other as well as to determine the overall association among different types of leukemia. This would lead to the development of

Table 3. Few significantly differentially upregulated genes in four leukemia subtypes with respect to clinically tested control blood samples.

Leukemia type	S/N	Probe set ID	Gene symbol	Uni Gene	Systematic name	Cyto band	p value (≤ 0.05)	Gene function	Geo mean fold
AML (geo mean value ≥ 7)	1	GT_44k_24_P153324	LOC390413	Hs.646625	XR_018341	13q22.3	0.02160		7.22
	2	GT_44k_24_P652786	THC2533996	Hs.412370	THC2533996	4p14	0.01856		7.34
	3	GT_44k_24_P76120	THC2474831	-	THC2474831	8p21.3	0.00409		7.71
	4	GT_44k_24_P922101	THC2585201	-	THC2585201	10q22.1	0.00621		10.44
	5	GT_44k_24_P926058	Y10152	Hs.546246	Y10152	7p15.1	0.00019		7.06
	6	GT_44k_24_P930276	NFE2L1	Hs.514284	L24123	17q21.32	0.00027	Protein dimerization activity	7.06
	7	GT_44k_24_P930551	THC2585049	-	THC2585049	16p11.2	0.00145		9.71
	8	GT_HS_44k_1542	SEPP1	Hs.275775	NM_001085486	5p12	0.11471	Selenium binding	7.46
ALL (geo mean value ≥ 4.5)	1	GT_44k_23_P201179	PHTF1	Hs.655824	NM_006608	1p13.2	0.00548	DNA binding activity	4.57
	2	GT_44k_23_P69652	GPR78	Hs.350588	NM_080819	4p16.1	0.00049	Rhodopsin-like receptor activity	4.61
	3	GT_44k_24_P144666	LOC401975	Hs.647716	XR_017247	1q24.1	0.04224		4.65
	4	GT_44k_32_P205110	FOXC1	Hs.348883	NM_001453	6p25.3	0.03218	Transcription factor binding	4.71
	5	GT_HsapiAK055033_00003099	HsapiAK055033		HsapiAK055033		0.01460		4.54
CML (geo mean value ≥ 4.5)	1	GT_44k_23_P6066	CPXM1	Hs.659346	NM_019609	20p13	0.02156	Carboxypeptidase A activity;	4.60
	2	GT_44k_23_P69652	GPR78	Hs.350588	NM_080819	4p16.1	0.00346	Rhodopsin-like receptor activity	4.74
	3	GT_44k_24_P144666	LOC401975	Hs.647716	XR_017247	1q24.1	0.01208		4.87
	4	GT_44k_24_P376451	GDNF	Hs.271721	ENST00000381826	5p13.2	0.03209	Growth factor activity	4.56
	5	GT_44k_24_P827491	PA2G4	Hs.524498	NM_006191	12q13.2	0.00249	RNA binding; protein binding; metalloexopeptidase activity	4.54
	6	GT_44k_32_P80245	ENST00000376881	Hs.156326	ENST00000376881	6p22.1	0.01115	Nucleic acid binding; zinc ion binding	5.44
CLL (geo mean value ≥ 7)	1	GT_44k_23_P255827	FKSG2	Hs.651853	NM_021631	8p12	0.00008		7.01
	2	GT_44k_23_P26854	KIAA0672	Hs.499758	NM_014859	17p12	0.02551	Gtpase activator activity	7.27
	3	GT_44k_24_P144666	LOC401975	Hs.647716	XR_017247	1q24.1	0.00018		8.28
	4	GT_44k_24_P153324	LOC390413	Hs.646625	XR_018341	13q22.3	0.00059		8.96
	5	GT_44k_24_P161914	LOC130728	Hs.549947	XR_019248	2p16.3	0.00058		7.43
	6	GT_44k_24_P652786	THC2533996	Hs.412370	THC2533996	4p14	0.00003		8.83
	7	GT_44k_24_P76120	THC2474831		THC2474831	8p21.3	0.00005		7.91
	8	GT_44k_24_P922101	THC2585201		THC2585201	10q22.1	0.00021		7.32
	9	GT_44k_24_P926058	Y10152	Hs.546246	Y10152	7p15.1	0.00015		7.69
	10	GT_44k_24_P930551	THC2585049		THC2585049	16p11.2	0.00009		7.01
	11	GT_44k_32_P334340	LOC653483	Hs.667982	AB016898	6q27	0.00072	Molecular function	7.27

common probable biomarkers for proper management of different types of leukemia. The significant association between CML and ALL could be clinically very useful for the trial and administration of drugs. In our study, the origin of cells viz. myeloid and lymphoid does not seem to be useful to consider as a parameter for treatment of leukemia as it varies among different combinations of CML vs. CLL, CML vs. AML, CML vs. ALL, AML vs. ALL, AML vs. CLL and ALL vs. CLL. Nevertheless, with this possibility, we are cognizant that the present study of microarray based gene expression profiling conducted by us potentially entertains several drawbacks.

Firstly, differences shown in the expression levels of probes of patients among different types of leukemia may be affected from different treatments used based on their origin or malignancy because a number of literature data suggest for the effect of drugs on the expression level of most of the distinguished probes or genes (Heuser et al., 2005; Chiaretti et al., 2004; McWeeney et al., 2010). The patients once diagnosed to any type of leukemia cannot be left untreated and in this condition, drug-induced changes may create problem in investigations into the genetic cause of disease transformation using the blood sample of such type of patient. Nevertheless, there is no such type of scientific data found to prove this assumption.

Additionally, genetic mechanism and upshots responsible for the transformation of the disease keep taking place during the patients undergoing medication. Secondly, we used peripheral blood samples from patients of different age and gender. This may give us an impression for patient-specific alterations in genetic expression. The systematic microarray study of large number of samples would probably be able to overcome these shortcomings.

ACKNOWLEDGEMENTS

We express our gratitude to the physicians, nurses, data managers, and patients from all concerned hospitals especially Karnataka Cancer therapy and Research Institute (KCTRI) for their cooperation in providing clinical specimens and information for the investigations. We express our special thanks to the Vision Group on Science and Technology (VGST), Department of Science and Technology, Government of Karnataka for providing the financial assistance for the project. We also thank the Department of Medical and Higher Education, Government of Karnataka for providing the infrastructure in Karnataka Institute for DNA Research, Dharwad, India.

REFERENCES

Bullinger L, Döhner K, Bair E, Fröhling S, Schlenk RF, Tibshirani R, Döhner H, Pollack JR (2004). Use of gene-expression profiling to identify prognostic subclasses in adult acute myeloid leukemia. N. Engl. J. Med. 350(16):1605-1616.

Care RS, Valk PJ, Goodeve AC, Abu-Duhier FM, Geertsma-Kleinekoort WM, Wilson GA, Gari MA, Peake IR, Lowenberg B, Reilly JT (2003). Incidence and prognosis of c-KIT and FLT3 mutations in core binding factor (CBF) acute myeloid leukemias. Br. J. Haematol.121(5):775-777.

Chiaretti S, Li X, Gentleman R, Vitale A, Vignetti M, Mandelli F, Ritz J, Foa R (2004). Gene expression profile of adult T-cell acute lymphocytic leukemia identifies distinct subsets of patients with different response to therapy and survival. Blood 103(7):2771-2778.

Cohen N, Rozenfeld-Granot G, Hardan I, Brok-Simoni F, Amariglio N, Rechavi G, Trakhtenbrot L (2001).Subgroup of patients with Philadelphia-positive chronic myelogenous leukemia characterized by a deletion of 9q proximal to ABL gene: expression profiling, resistance to interferon therapy, and poor prognosis. Cancer Genet. Cytogenet. 128(2):114-119.

Dunphy CH (2006). Gene Expression Profiling Data in Lymphoma and Leukemia. Arch. Pathol. Lab. Med. 130(4):483-520.

Ferrando AA, Neuberg DS, Staunton J, Loh ML, Huard C, Raimondi SC, Behm FG, Pui CH, Downing JR, Gilliland DG, Lander ES, Golub TR, Look AT (2002). Gene expression signatures define novel oncogenic pathways in T cell acute lymphoblastic leukemia. Cancer Cell 1(1):75-87.

Franchini G (1995). Molecular mechanisms of human T-cell leukemia/lymphotropic virus type I infection. Blood 86(10):3619-3639.

Goswami RS, Sukhai MA, Thomas M, Reiss PP, Kamal-Reid S(2009). Applications of Microarray Technology to Acute Myelogenous Leukemia. Cancer Inform. 7:13-28.

Haslinger C, Schweifer N, Stilgenbauer S, Döhner H, Lichter P, Kraut N, Stratowa C, Abseher R (2004). Microarray Gene Expression Profiling of B-Cell Chronic Lymphocytic Leukemia Subgroups Defined by Genomic Aberrations and VH Mutation Status. J. Clin.Oncol. 22(19) :3937-3949.

Heuser M, Wingen LU, Steinemann D, Cario G, von Neuhoff N, Tauscher M, Bullinger L, Krauter J, Heil G, Döhner H, Schlegelberger B, Ganser A (2005). Gene-expression profiles and their association with drug resistance in adult acute myeloid leukemia. Haematologica 90(11):1484-1492.

Hudson TJ et al (2010). International network of cancer genome projects. Nature 464(7291): 993-998.

Kiyoi H, Naoe T, Nakano Y, Yokota S, Minami S, Miyawaki S, Asou N, Kuriyama K, Jinnai I, Shimazaki C, Akiyama H, Saito K, Oh H, Motoji T, Omoto E, Saito H, Ohno R, Ueda R (1999). Prognostic implication of FLT3 and N-RAS gene mutations in acute myeloid leukemia. Blood 93(9):3074-3080.

Leukemia and Lymphoma Society (2011- 2012). USA (2012), Facts. 1-29.

Liotta L, Petricoin E (2000). Molecular profiling of human cancer. Nat. Rev. Genet. 1(1):48-56.

Majeti R, Becker MW, Tian Q, Lee TL, Yan X, Liu R, Chiang JH, Hood L, Clarke MF, Weissman IL (2009).Dysregulated gene expression networks in human acute myelogenous leukemia stem cells. Proc. Natl. Acad. Sci. 106(9):3396-3401.

McWeeney SK, Pemberton LC, Loriaux MM, Vartanian K, Willis SG, Yochum G, Wilmot B, Turpaz Y, Pillai R, Druker BJ, Snead JL, MacPartlin M, O'Brien SG, Melo JV, Lange T, Harrington CA, and Deininger MW (2010). A gene expression signature of $CD34^{+}$ cells to predict major cytogenetic response in chronic-phase chronic myeloid leukemia patients treated with imatinib. Blood 115(2):315-325.

Modak H, Kulkarni SS, Kadakol GS, Hiremath SV, Patil BR, Hallikeri U, Gai PB (2011). Prevalence and Risk of Leukemia in the Multi-ethnic Population of North Karnataka. Asian Pac. J. Cancer Prev. 12(3):671-675.

Nowicki MO, Pawlowski P, Fischer T, Hess G, Pawlowski T, Skorski T (2003).Chronic myelogenous leukemia molecular signature. Oncogene 22(25):3952-3963.

Ramaswamy S, Golub TR (2002). DNA microarrays in clinical oncology. J. Clin. Oncol. 20(7):1932- 1941.

Sawyers CL, Denny CT, Witte ON (1991). Leukemia and the disruption of normal hematopoiesis. Cell 64(2):337-350.

Smith MT, Zhang L (1998). Biomarkers of Leukemia Risk: Benzene as a Model. Environ. Health Perspect. 106(4):937-946.

Stirewalt DL, Meshinchi S, Kopecky KJ, Fan W, Pogosova-Agadjanyan EL, Engel JH, Cronk MR, Dorcy KS, McQuary AR, Hockenbery D,

Wood B, Heimfeld S, Radich JP (2008). Identification of genes with abnormal expression changes in acute myeloid leukemia. Genes Chromosomes Cancer 47(1):8-20.

Valk PJ, Verhaak RG, Beijen MA, Erpelinck CA, Barjesteh WD, Boer JM, Beverloo HB, Moorhouse MJ, Spek PJ, Löwenberg B, Delwel R (2004). Prognostically useful gene-expression profiles in acute myeloid leukemia. N. Engl. J. Med. 350(16):1617-1628.

Wang J, Coombes KR, Highsmith WE, Keating MJ, Abruzzo LV (2004). Differences in gene expression between B-cell chronic lymphocytic leukemia and normal B cells: a meta-analysis of three microarray studies. Bioinformatics 20(17):3166- 3178.

Zent CS, Zhan F, Schichman SA, Bumm KH, Lin P, Chen JB, Shaughnessy JD (2003). The distinct gene expression profiles of chronic lymphocytic leukemia and multiple myeloma suggest different antiapoptotic mechanisms but predict only some differences in phenotype. Leuk. Res. 27(9):765-774.

academicJournals

Vol. 13(10), pp. 1182-1187, 5 March, 2014
DOI: 10.5897/AJB2013.13326
ISSN 1684-5315 ©2014 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Fumaric acid production by *Rhizopus oryzae* and its facilitated extraction via organic liquid membrane

Yadvaindera Sood, Jayati Dhawan and Anupreet Kaur*

University Institute of Engineering and Technology, Panjab University, Chandigarh, India.

Accepted 29 January, 2014

Microbial fermentations are efficient alternatives to different manufacturing practices being followed for industrially important compounds like organic acids, food and pharmaceutically active products. However, the attractions offered by microbial processes are overshadowed by the costs associated with downstream processing involved in the recovery of pure products from such systems. Reduction of the cost in terms of financial and energy related inputs have been the goal of recent ongoing research in the field of bio-processing. This study has been focused towards development of an efficient method for simultaneous extraction of products from microbial fermentation. Through the study, the domain of liquid membranes and the possibilities of energy efficient extraction of microbial products from ongoing processes have been investigated. Liquid membrane system involves a liquid which is immiscible with the source and receiving solutions serves as a semi permeable barrier between these two liquid phases. The study uses a fumaric acid produced through fermentation of *Rhizopus oryzae* on Potato Dextrose Broth (PDB) under aerobic conditions as a model sample for extraction analysis. The culture media, that is presumably rich in fumaric acid and some other acidic products, has been subjected to extraction through liquid membrane based process. The reduction in concentration of sample used as source is measured titrimetrically. Thin layered chromatography has been deployed at certain instances for qualitative verification of the concentrations. The set up consisted of a modified layered 'liquid membrane' setup for a 'fumaric acid' source, with toluene as organic membrane and sodium hydroxide as strip phase. The liquid membrane contained a carrier for assisted transfer and was agitated. Maximum extraction takes place during the first 20 to 30 min of the run as fumaric acid concentration falls to almost 40% of its initial concentration.

Key words: Fumaric acid, carrier, trioctylamine (TOA), liquid membrane, facilitated extraction.

INTRODUCTION

Polymeric and inorganic membranes are used commercially for many applications including gas separations (Tabe-Mohammadi, 1999; Irabien et al., 2013), water purification (Zhang et al., 2008), food and diary-(Decloux et al., 2001), petrochemical (Karguri et al., 2009), pharmaceutical- (Lindner et al., 2001; Ceynowa and Koter, 2003) and biotechnology industry (Zydney and Reis, 2001; Charcosset, 2006; Escudero et al., 2013). The international nomenclature and definition of the membrane and membrane separation has been well described (Strathmann, 2006; Ulbricht, 2006). If membranes are viewed as semi permeable phase separators, then the traditional concept of membranes as polymer films can be extended to include liquids. They are defined as Liquid Membranes (LMs). Liquid membrane system involves a liquid which is immiscible with the source(feed) and receiving (strip) solutions that serves as a semi permeable barrier between these two

*Corresponding author. E-mail: anupreetz@yahoo.co.in.

liquid and gas phases. The term liquid membrane transport includes processes incorporating liquid-liquid extraction and membrane separation in one continuously operating device. It utilizes an extracting reagent solution, immiscible with water, stagnant or flowing between two aqueous solutions (or gases), the source or feed and receiving or strip phases. In most cases, the source and receiving phases are aqueous and the membrane organic, but the reverse configuration can also be used (Katalin and Petra). Liquid membranes possess higher selectivity values than solid membranes and further increase in mass flux and selectivity have been reported by incorporating some carrier, which reacts reversibly and selectively with a specific permeate in the liquid membrane (Kocherginsky et al., 2007; Ravanchi et al., 2010; Kaur and Vohra, 2010).

Research and development activities within these disciplines involve diverse applications of liquid membrane technology, such as gas separations (Baker, 2002; Golemme et al., 2009), recovery of valued or toxic metals (Alizadeh et al., 2002; Kulkarni et al., 2002; Othman et al., 2006, 2004; Manchanda et al., 2011; Janardan et al., 2012), removal of organic compounds and recovery of fermentation products and some other biological systems (Schlosser et al., 2005; Dimitrov et al., 2005; Heerema et al., 2006; Boyadzhiev at al., 2006). In the last decade, studies have been carried out towards the application possibilities of ionic liquids in liquid membrane processes for the transport and separation of solids, liquids and gases (Endres et al., 2008; Koel, 2008; Wasserchied, 2007). A new class of liquid membrane called micro-encapsulated liquid membrane has also been investigated for the production of oxygen enriched air (Figoli et al., 2001). Microbial productions are being followed for industrially important compounds like proteins, organic acids, food and coloration agents and pharmaceutically active products. Organic acids production by filamentous fungi and their associated metabolic pathways have also been reviewed (Goldberg et al., 2006; Magnuson and Lasure, 2004). Fumaric acid is a naturally occurring four-carbon dicarboxylic acid that is finding increasing use as a food acidulant and beverage ingredient.

Fumaric acid has many potential industrial applications, ranging from the manufacture of synthetic resins and biodegradable polymers to the production of intermediates for chemical syntheses (Roa et al., 2008). Recently, three strains of fungus *Rhizopus* oryzae were screened to produce fumaric acid using untreated and treated corn distillers' dried grains (Thomas, 2008). Microbial productions have even been discussed using bacterial strain, *Lactobacillus* (Donnelly et al., 2001). However, the attractions offered by microbial processes are overshadowed by the costs associated with downstream processing involved in the extraction of pure products from such systems. As such, recovery techniques in submerged cultivation for fumaric acid have been scarcely studied in comparison to organic acids citric acid (Heinzle et al., 2006) and lactic acid (Joglekar et al., 2006).

Downstream processing such as reactive extraction and membrane electrodialysis have not been studied whereas simultaneous fermentation and adsorption have been studied for the same (Joglekar et al., 2006). Reduction of the cost in terms of financial and energy related inputs has been the goal of our investigations. This study has been focused towards development of an efficient method for simultaneous extraction of products from microbial fermentation. Currently, fumaric acid is produced from petroleum based derivative maleic anhydride and as the petroleum prices are rising quickly, the cost of fumaric acid production has also increased. The study uses a fumaric acid produced through fermentation of *R*. oryzae using potato dextrose broth (PDB) under aerobic conditions as a model sample for extraction analysis. The culture media, that is presumably rich in fumaric acid and some other acidic products, is subjected to extraction through liquid membrane based process. The non-miscible phases are arranged in a manner which is known as bulk liquid membrane (BLM) (Belafi-Bako et al., 2000; Clark et al., 2005). The reduction in concentration of sample used as source is measured titrimetrically.

Thin layered chromatography was deployed at certain instances for qualitative verification of the concentrations detected or undetectable through titrimetric analysis. Certainly, improvements are desirable for up-scaling the proposed method.

MATERIALS AND METHODS

Membrane solutions were prepared by dissolution of trioctylamine (TOA) (Fluka A.G. Switzerland 95%) as an extractant in toluene (S.D. Fine chemicals, 99.5%). Fumaric acid (LOBA Chemicals) solutions dissolved in water were used for controls. Strip phase solutions were prepared by dissolving sodium hydroxide pellets (Qualigens, Glaxo 97.7%) in water. Phenolphthalein indicator, methyl orange and hydrochloric acid (Qulaigens) were used for titrations. Chloroform and methanol (E. Merck 99%) were used for thin layer chromatography analysis. The lyophilized stocks *R. oryzae* were procured from MTCC, IMTECH Chandigarh. The strain is attributed with high levels of fumaric acid production. Potato dextrose agar (Himedia) and potato dextrose broth (Himedia) were used to maintain the culture strain. The media was inoculated with a lyophilized culture of a strain of *R. oryzae* MTCC-262 and incubated at 30°C for 7 days to allow fungal growth. After seven days, mycelia growth was observed on media's surface. Subculturing was carried out in 50 ml media using hyphal spores.

Set-up

The basic setup to perform experimental studies was created out of borosilicate glass molded as per the desired specifications. The assembly consisted of two concentric cylinders, the inner being shorter in height to accommodate for the liquid membrane that would connect the two liquids (Figure 1). The similar experimental apparatus has also been reported by us for the extraction of

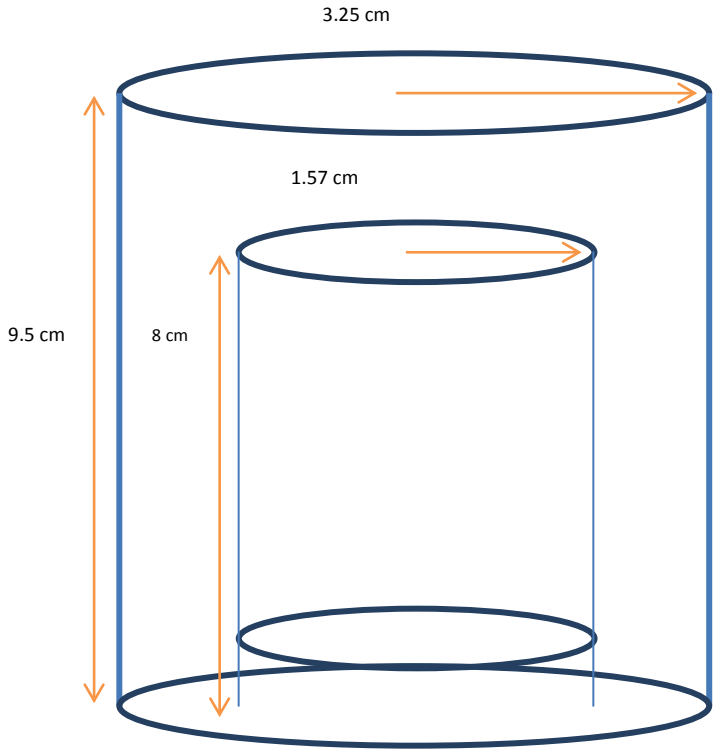


Figure 1. Specifications of the setup for holding the three liquids.

propionic and acetic acids. The facilitated extraction for the same has also been described in the same paper (Kaur and Vohra, 2010).

Source feed

The source contains components of interest that need to be recovered. In this study, samples obtained from the fermentation of *R. oryzae* were used as source feed for investigations. Pure fumaric acid solutions were used as controls in the experiments. The concentration of pure samples used was optimally kept in the ppm range to mimic actual fermentation concentrations in order to optimize the setup for such applications.

Strip phase

Under normal circumstances, strip phase for any separation process being carried out by a liquid membrane has high affinity for the product of interest yet easily dissociates from it under the effect of slight variation in conditions being provided or on the action of a chemical reagent. Keeping in mind these two characteristics, the best possible option that came up for the strip phase was an alkaline medium. Sodium hydroxide has high affinity for organic acids leading to the formation of sodium salts and water.

Liquid membrane

The liquid membrane used should be completely immiscible in the source and strip phase and should be able to maintain a distinct interface that supports high rates of diffusion across it. Given that the two other liquids selected exist in stable aqueous solutions (polar in nature) so a non polar liquid membrane was a clear choice. Toluene, with its high immiscibility in aqueous phases and easy availability, was the liquid membrane of choice.

Carrier

The carrier molecule is the essence of the whole setup – it efficiently binds to the product of interest and then releases it on coming in contact with the strip phase. The ease with which it attaches to and releases the product is of major interest and the selection of such a molecule was necessary that could fulfill both the requirements for organic acids and also be insoluble in aqueous solvents. These requirements were fulfilled to the fullest by trioctylamine (TOA) which is used as an extractant for organic acids in industrial processes and is immiscible in aqueous solutions.

Transport mechanism theory

The reaction equilibrium for the fumaric acid in the present set-up can be defined by taking into account two interfaces I and II defined as feed-phase:membrane phase interface and strip-phase:membrane phase interface, respectively. Complex formation of the extractant TOA with the fumaric acid is proposed in the mechanism at interface I.

$mFA\ (aq) + nTOA\ (org) \rightarrow FA_m.TOA_n\ (org)$

The acid-amine complex diffuses from interface I to interface II across the membrane where the extractant is released.

Table 1. Variation of fumaric acid concentration and percentage extraction during reactive extraction process in fermentation samples of different days of fermentation.

Sample time (min)	Amount of fumaric acid /100 ml							
	Day 1		Day 2		Day 5		Day 6	
	Amount of FA/100 ml	Percentage extraction	Amount of FA/100 ml	Percentage extraction	Amount of FA/100 ml	Percentage extraction	Amount of FA/100 ml	Percentage extraction
0	0.1323	-	0.1822	-	0.3017	-	0.2855	-
10	0.0943	28.73	0.1184	35.02	0.1950	35.36	0.1706	40.24
20	0.0856	35.30	0.0987	45.83	0.1695	45.82	0.1486	47.95
30	0.0885	33.10	0.0789	56.70	0.1184	60.75	0.1172	58.94
40	0.0783	40.82	0.0789	56.70	0.1137	62.32	0.1137	60.18
50	0.0783	40.82	0.0789	56.70	0.1126	62.68	0.1114	60.98
60	0.0812	38.62	0.0777	57.35	0.1126	62.68	0.1102	61.40

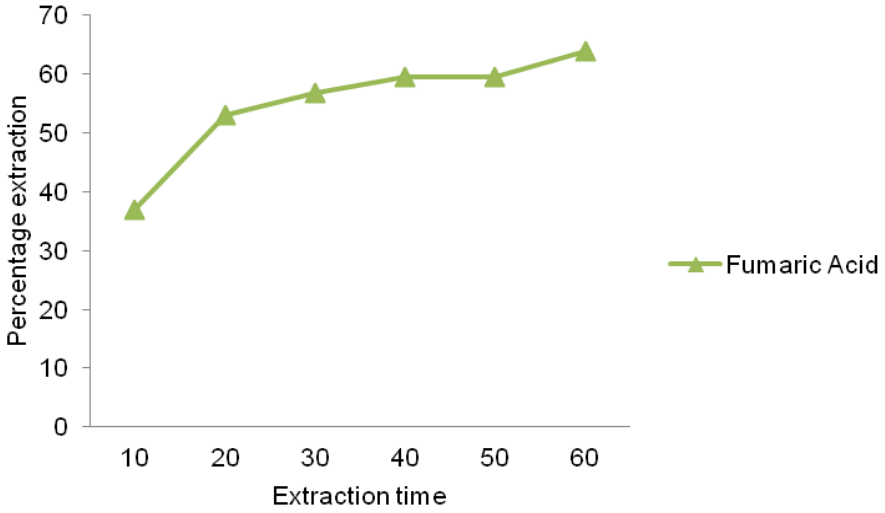


Figure 2. Trend of percentage extraction of fumaric acid (1 g/100 ml) in the proposed BLM set-up.

$FA_m.TOA_n$ (org) → FA (org) + TOA (org)

TOA diffuses back to the interface I across the membrane. The ionized acid reacts further with the strip phase comprised of sodium ions and produces a non-diffusive compound. Transfer mechanisms for the recovery of acetic acid, propionic acid and fumaric acid have been described elsewhere in a similar fashion (Kaur and Vohra, 2010; Zhang et al., 2009).

RESULTS

Experiments to investigate the extraction potential of the proposed BLM set-up was carried out firstly with pure fumaric acid (1 g/100 ml) where above 60% extraction has been achieved (Figure 2). Secondly, cell-free supernatant was then used as source feed to investigate the extraction potential of the proposed BLM for fumaric acid from a mixed solution. It was observed that fumaric acid concentration increases as fermentation proceeds. It was also observed that the concentration of fumaric acid falls with time for all the samples when subject to reactive extraction process (Table 1). The concentration of fumaric acid drops significantly during first few minutes of experiment and then becomes almost constant. Investigations were carried out with cell-free supernatant as obtained from fermentation of *R. oryzae* on different days of fermentation as a source feed along with 1 N sodium hydroxide as strip phase and 4% (v/v) TOA in toluene as liquid membrane. As initial concentration of fumaric acid increases with fermentation days, maximum extraction achieved also increased from 38 to 61% (Figure 3). It is further concluded that the maximum percentage extraction for the two cases remained close to 60%. Based on the results, it is confirmed that the proposed BLM set-up has the potential to extract the fumaric acid both pure and from a mixture of other acids

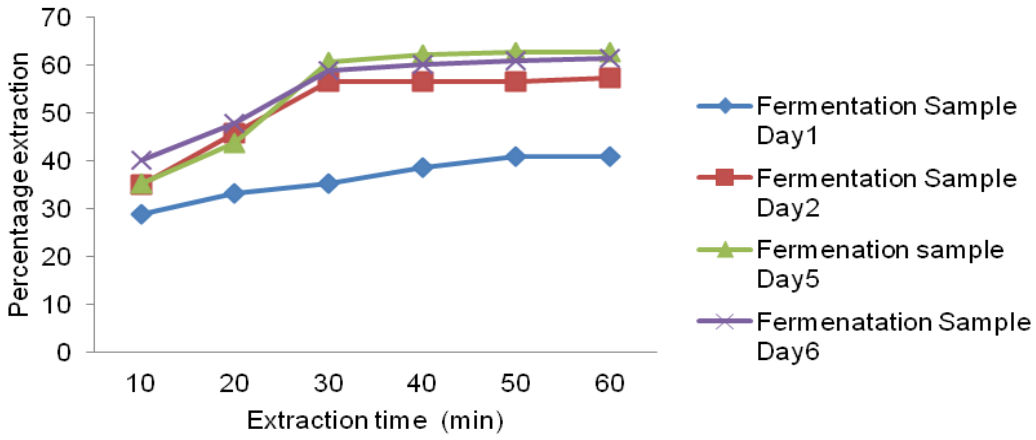


Figure 3. Variation of percentage extraction of fumaric acid in the samples obtained from fermentation of *Rhizopus oryzae*.

and that too even in dilute concentrations. Further studies are required to optimize the system and make the technique commercially viable so that it can be used for *in-situ* extraction of fumaric acid from the solution.

In a similar set of studies, recovery of fumaric acid from waste solutions have been reported using a different carrier triakylamine by hollow-fibre supported liquid membrane (Zhang et al., 2009). The study has investigated several different classes of solvents along with cosolvents. Combination of the reverse osmosis and complex extraction and stripping has also been reported for the treatment of industrial wastewater containing fumaric acid (Zhang et al., 2008). Therefore, the current study confirms that the proposed set-up has potential to recover fumaric acid from wastewater and fermentation broth and could emerge as a fast, simpler and cheaper alternate for the same.

Conclusion

Based on the literature survey, *R. oryzae* appeared to be the microorganism with the highest productivity of fumaric acid. The fumaric acid production can be improved by exploring the field of genetic engineering with the help of acid-resistant strain. Another alternative is to replace petroleum based maleic acid and to use fermentatively produced fumaric acid. This production can be enhanced by overcoming product inhibition by applying *in-situ* removal of fumaric acid during fermentation.

The latter has been achieved partly with the help of current studies. Further optimizations in terms of carrier, carrier concentration and strip phase concentration are thus desirable to make the technique commercially viable. The validation of our findings and improvements thereafter promise considerable reduction in the costs associated with processes deployed for production of different industrially important microbial metabolites. Apart from reduction of overall costs, the application of these principles can also enhance productivity in cases of self-inhibited processes through simultaneous removal of product.

REFERENCES

Alizadeh N, Salimi S, Jabbari A (2002). Transport study of palladium through a bulk liquid membrane using hexadecylpyridinium as carrier. Sep. Purif. Technol. 28:173-180.

Baker WR (2002). Future directions of membrane gas separation technology. Indus. Engg. Chem. Res. 41(6):1393-14112.

Belafi-Bako K, Gubicza L, Mulder M (2000). Integration of membrane processes into Bioconversions, KluwerAcademic Publishers ISBN: 978-3064-6437-9, New York.

Boyadzhiev L, Dimitrov K, Metcheva D (2006). Integration of solvent extraction and liquid membrane separation: an efficient tool for recovery of bio-active substances from botanicals. Chem. Eng. Sci. 61:4126-4128.

Ceynowa J, Koter I (2003). Kinetic resolution of chiral alcohols in bifunctional membrane exhibiting enzyme activity and enantioselective permeation. J. Mol. Catal. B: Enzymatic 24-25:17-26.

Charcosset C (2006). Membrane processes in biotechnology: an overview. Biotechnol. Adv. 24:482-492.

Clark JD, Han B, Bhown AS, Wickramasinghe SR (2005). Amino acid resolution using supported liquid membranes, Sep. Purif. Technol. 42(3):201-211, 13835866.

Decloux M, Daufin G, Escudier JP, Carrere H, Berot S, Fillaudeau L (2001). Recent and emerging applications of membrane processes in the food and dairy industry. Trans Institution of Chemical Engineers 79 (c). 89-102.

Dimitrov K, Metcheva D, Boyadzhiev L (2005). Integrated processes of extraction and liquid membrane isolation of atropine from *Atropa belladonna* roots. Sep. Purif. Technol. 46:41-45.

Donnelly M, Millard CS, Stols L (2001). Mutant E.coli strain with increased succinic acid production. USRE37393.

Endres F, Abbott AP, MacFarlane DR (2008). Electrodeposition from Ionic liquids. Wiley-VCH Verlag GmbH & Co. KGaA. ISBN: 978-3-527-31565-9, Weinheim, Germany.

Escudero I, Geanta RM, Ruiz MO (2013). Micellar-enhanced ultrafilteration for the recovery of lactic acid and citric acid from beet molasses with sodium dodecyl sulphate. J. Memb. Sc. 430: 11-23.

Figoli A, Sager WFC, Mulder MHV (2001). Facilitated oxygen transport in liquid membranes: review and new concepts. J. Memb. Sci. 181,

97-110.
Goldberg I, Rokem JS, Pines O (2006). Organic acids: old metabolites, new themes. J. Chem. Techol. Biotechnol. 81:1601-1611.
Golemme G, Bernardo P, Driolo E (2009). Membrane gas separation: A review/state of the art. Indus. Engg. Chem. Res. 48(10):4638-4663.
Heerema L, Roelands M, Hanemaaijer JH, Bont J de, Verdoes D (2006). In-situ phenol removal from fermentation broth by pertraction. Desalination 200:485-487.
Heinzle E, Biwer AP, Cooney CL (2006). Development of sustainable bioprocess-modelling and assessment. Wiley, Chichester.
Irabien A, santos E, Albo J, Daniel CI, Portugal CAM, Crespo JG (2013). Permeability modulation of supported magnetic ionic liquid membranes (SMILMs) by an external magnetic field. J. Memb. Sc. 430: 56-61.
Janardan P, Panja S, Mohapatra PK, Tripathi SC, Gandhi PM (2012). Supported liquid membrane transport studies on Am (III), Pu(IV), U(VI) and Sr(II) using irradiated TODGA. J Hazard Mater. 237-38:339-346.
Joglekar HG, Rahman I, Babu S, Kulkarni BD, Joshi A (2006). Comparative assessment of downstream processing options for lactic acid, Sep. Purif. Technol. 52, 1-17.
Karguri A, Ravanchi MT, Kaghazchi T (2009). Application of membrane separation processes in petrochemical industry: a review. Desalination 235:199-244.
Katalin BB, Petra C. Application of ionic liquids in membrane separation processes. www.intechopen.com.
Kaur A, Vohra DK (2010). Study of bulk liquid membrane as a separation technique to recover acetic and propionic acids from dilute solutions. Indian J. Chem. Tech. 17:133-138.
Kocherginsky NM, Yang Q, Seelam L. (2007). Recent advances in supported liquid membrane technology. Sep and Purif Technol. 53(2):171-177.
Koel M (2008). Ionic liquids in chemical analysis. CRC Press Taylor & Francis Group. ISBN: 978-1-4200-4646-5, USA.
Kulkarni PS, Mukhopadhyay S, Bellary MP, Ghosh SK (2002). Studies on membrane stability and recovery of uranium(VI) from aqueous solutions using a liquid emulsion membrane process. Hydrometallurgy 64:49-58.
Lindner W, Maier NM, Franco P (2001). Separation of enantiomers: needs, challenges, perspectives. J. Chromat. A 906:3-33.
Magnuson JK, Lasure LL (2004). Organic acid production by filamentous fungi. In: Tracz JS, Lange L(eds) Advances in fungal biotechnology for industry, agriculture and medicine. Kluwer/Plenum, New York, USA, 307-340.
Manchanda VK, Bhattacharyya A, Mohapatra PK, Gadly T, Raut DR, Ghosh SK (2011). Liquid-liquid extraction and flat sheet supported liquid membrane studies on Am(III) and Eu(III) separation using 2,6-bis(5,6-dipropyl-1,2,4-triazin-3-yl)pyridine as the extractant. J. Hazard Mater. 195:238-244.
Othman N, Mat H, Goto M (2006). Separation of silver from photographic wastes by emulsion liquid membrane system. J. Memb Sci. 282, 171-177.
Othman N, Mat H, Goto M (2004). Liquid membrane technology for precious metals recovery from industrial waste. http://eprints.utm.my/1064/1/NorasikinOthman2004_LiquidMembrane TechnologyFor Precious.pdf
Ravanchi MT, Kaghazchi T, Kargari A (2010). Supported liquid membrane separation of propylene-propane mixtures using a metal-ion carrier. Desalination 250(1):130-135.
Roa Engel CA, Straathof AJ, Zijlmans TW, van Gulik WM, van der Wielen LA (2008). Fumaric acid production by fermentation. Appl. Microbiol. Biotechnol. 78(3), 379-389.
Schlosser S, Kertesz R, Martak J (2005). Recovery and separation of organic acids by membrane-based solvent extraction and pertraction: an overview with a case study on recovery of MCPA. Sep. Purif. Technol. 41:237-266.
Strathmann H (2006). Application of membrane processes for the separation of molecular mixtures. European J. Lipid Sci. Technol. 82(4):152-166.
Tabe-Mohammadi A (1999). A review of the applications of membrane separation technology in natural gas treatment. Sep. Sci. Technol. 34(10):2095-2111.
Thomas PW (2008). Fumaric acid production by Rhizopus oryzae on corn Distillers' Grains with solubles. Res. J. Microbiol. 3(1), 35-40.
Ulbricht M (2006). "Advanced functional polymer membranes" (Invited Feature Article), Polymer 47, 2217-2262.
Wasserchied P, Welton T (2007). Ionic liquids in synthesis. VCH-Wiley ISBN:978-3-527-31239-9, Weinheim, Germany.
Zhang L, Li S, Zhang J, Zhi T, Chen H (2008). Combination of complex extraction with reverse osmosis for the treatment of fumaric acid industrial waste water. Desalination 234:362-369.
Zhang L, Li SJ, Chen HL (2009). Recovery of fumaric acid by hollow-fiber supporting liquid membrane with strip dispersion using trialkylamine carrier. Sep. Purif. Technol. 66:25-34.
Zydney A, Reis RY (2001). Membrane separations in biotechnology. Curr. Opinion Biotechnol. 12:208-211.

academicJournals

Vol. 13(10), pp. 1188-1193, 5 March, 2014
DOI: 10.5897/AJB2013.12335
ISSN 1684-5315 ©2014 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Identification and chemical studies of pelagic masses of *Sargassum natans* (Linnaeus) Gaillon and *S. fluitans* (Borgessen) Borgesen (brown algae), found offshore in Ondo State, Nigeria

O. O. Oyesiku[1]* and A. Egunyomi[2]

[1]Department of Plant Science and Applied Zoology, Olabisi Onabanjo University Ago-Iwoye, Nigeria.
[2]Department of Pure and Applied Biology, Ladoke Akintola University of Technology, Ogbomosho Nigeria.

Accepted 29 January, 2014

The pelagic seaweed found offshore and negatively impacting fishing activity in Ondo State Nigeria, has been identified to be a mixture of *Sargassum natans* and *Sargassum fluitans* which presumably floated from the Sagasso Sea of North Atlantic. In a bid to harness the potential uses of the seaweed biomass, the mixed *Sargassum* species were analyzed for the proximate composition, some minerals and phytochemical constituents using standard methods. The mixed *Sargassum* species contained 154 mg/100 g% protein, 86.5 mg/100 g ash content, 25.5 mg/100 g fat, 71.5 mg/100 g fibre and 573 mg/100 g carbohydrate. Thus it could be consumed by humans if cleaned. Owing to the small concentration of Nitrogen (6.3 mg/100 g), phosphorus (96.5 mg/100 g) potassium (28 mg/100 g), the percentage ratio of N-P-K (1-10-3) of *Sargassum* spp. was recommended as fertilizer. The presence of flavonoids, tannins, terpenoids and saponins show that the species can be harnessed for their medicinal potentials.

Key words: *Sargassum natans, Sargassum fluitans,* brown algae, proximate analysis, phytochemical, fertilizer, Nigeria.

INTRODUCTION

In July 2012, the Nigerian media reported the occurrence of an unknown seaweed floating massively off the coast of Ajegunle-Erun-Ama (Lat. 06° 19′ 32″, Long. 04° 30′ 32″E, alt 5 m) in Ondo State, Southwestern Nigeria. The fishermen in the area observed that between April and July during the wet season, their nets were often filled with the seaweed mass instead of fishes. Consequently, their fishing occupation was adversely affected. Additionally, handling the seaweed biomass resulted in skin and eye irritation. Owing to these reports, two field trips were made to the locality with a view to collecting the seaweed and getting any useful information from the fishermen. The seaweed was instantly identified by the second author as *Sargassum*, a brown alga.

On a global scale, *Sargassum* (*Sargassum natans* and *Sargassum fluitans*) has broad distribution offshore of the North Atlantic (Schneider and Searles, 1991) and outside the Sargasso Sea there are more *Sargassum* floating in the Gulf of Mexico than anywhere else in the world (Conver and Sieburth, 1964; Gower and King, 2008). *S. fluitans* has recently been added to the Global database of Invasive Species Specialist Group (ISSG) (2011). The floating *S. natans* and *S. fluitans* are taxonomically valid speciesthat dominate the algal communities of the

*Corresponding author. E-mail: busik1000@gmail.com.

Figure 1. Sargassum mass (*Sargassum natans* and *S. fluitans*) found at Ajegunle Erun-Ama beach, Ondo State, Nigeria. Part of the lateral branches (A), gas bladders (B).

Sargasso Sea and both occur in the western Atlantic Ocean (Guiry and Guiry 2011; Wynne 2011). Based on six years record of satellites imagery tracking of movement and distribution of floating *Sargassum* spp. in the Gulf of Mexico and West Atlantic, Gower and King (2008) reports that floating *Sargassum* species originate from Gulf of Mexico and have a life span nearly always one year.

Five *Sargassum* species, *Sargassum acinarium*, *Sargassum cyniaun Sargassum filipendula, Sargassum ramifolum* and *Sargassum vulgare* were described and reportedly found in some West African countries (John et al., 2003). Of all these species, only *S. vulgare* and its variety (var. *foliosissimum* are distributed in 12 West African countries including Nigeria. It was thought desirable to ascertain whether the features of *Sargassum* from Ondo State, Nigeria, resemble any of the species recorded for West Africa.

Seaweeds are of economic importance. *Sargassum* species are used as fodder and fertilizer in China and many parts of Asia (Round, 1973). *Sargassum* forms about 10% of the average diet in Japan where tender parts of the plant are eaten raw as salad or cooked with coconut milk. Also in Bermuda, indigenes spread out salt free *Sargassum* clumps as fertilizer around the base of banana. Algin, a carbohydrate found in *Sargassum* is extracted for use in textile, paper and pharmaceutical industries (Chennubhotla et al., 1981). *Sargassum* bio-biomass is used as a potential renewable energy resource such as biogas (Wang et al., 2009, Yokoyama et al., 2007). Despite the annual invasion of *Sargassum* biomass and its attendant negative impact on the traditional fisheries in Ondo State, the plant material caught in the nets could be useful to humans. In view of this, the present study was undertaken to identify the invading *Sargassum* species and carry out chemical studies using the biomass. This is with a view to harnessing the potential uses of the seaweed.

MATERIALS AND METHODS

Collection and Identification of Plant materials

Plant materials were collected into glass jar containing sea water during the two visits made to Ajegunle-Erun-Ama beach, Ondo State Nigeria Figure 1. Detailed identification of the plant material was done in the laboratory with the aid of dissecting microscope and verified with illustrations by Széchy et al. (2012), Dawes and Mathieson (2008), John et al. (2003) Littler and Littler (2000) and Schneider and Searles (1991).

Proximate analysis

The powdered *Sargassum* sample was analyzed for crude protein, crude fat, fibre, moisture, ash using the methods described in AOAC (2005). The carbohydrate content was calculated by using the formula below. The analyses were duplicated.

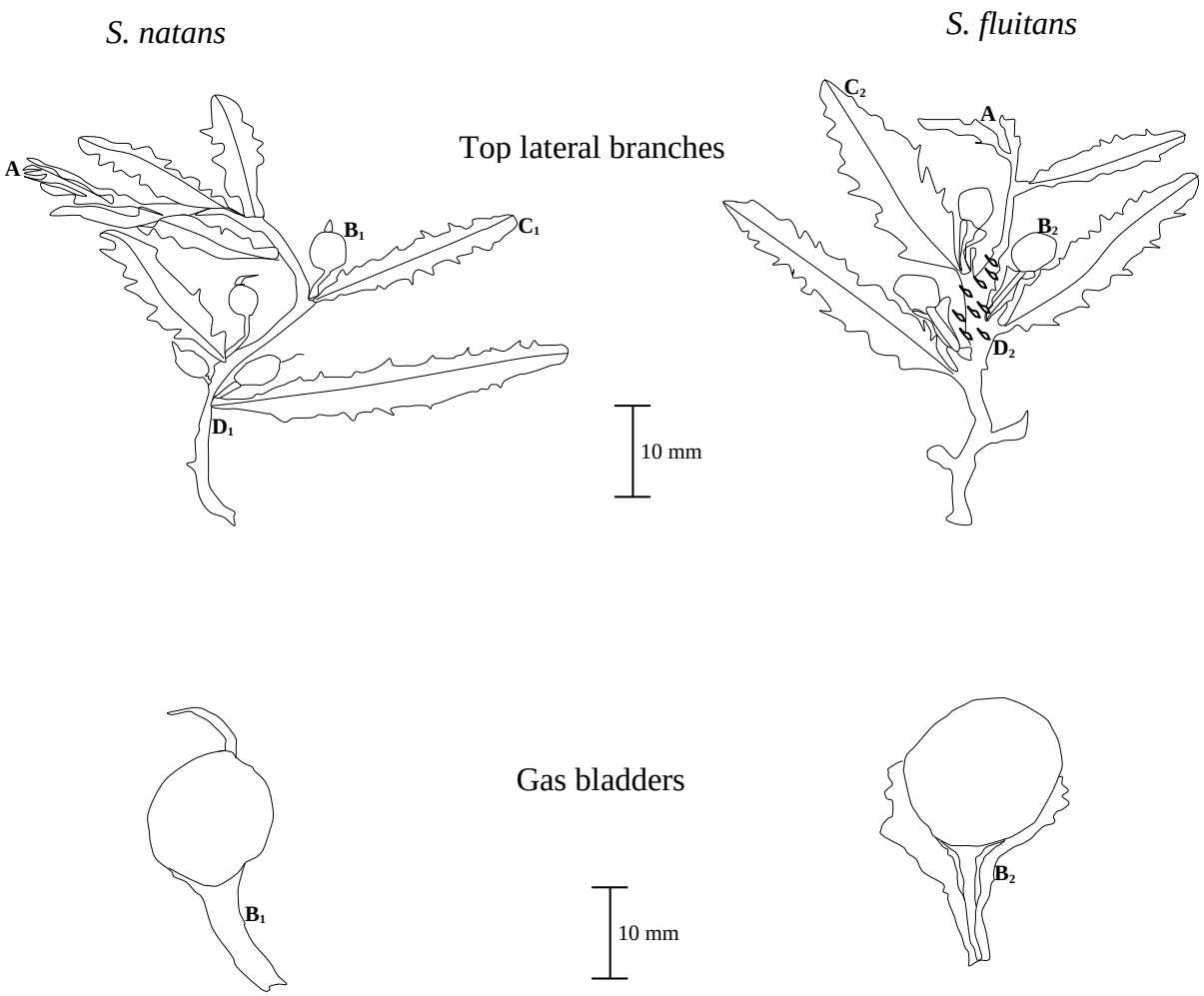


Figure 2. *Sargassum* (*S. natans* and *S. fluitans*. Receptacle (A), gas bladder with spine, stalk without wing (B_1), gas bladder without spine, stalk with wing (B_2), linear leaf with seriated margin (C_1), lanceolate leaf with seriated margin(C_2), lateral branch without spines (D_1), lateral branch with spines (D_2).

$$\%CHO = 100 - \%\text{moisture} + \%\text{ash} + \%\text{fat} + \%\text{protein} + \%\text{fibre}$$

Mineral analysis

The method of Walsh (1971) was used for the digestion of dried *Sargassum* material. After digestion, percentage of potassium, magnesium, zinc, iron, iodine were analyzed using atomic absorption spectrophotometer (FC 210/211, VGP Bausch Scientific AAS). Nitrogen content was determined by Kjeldahl method. Phosphorus was determined using Vanadomolybdate yellow method (AOAC, 2005). Percentage transmittance was determined at 400nm using Spectronic 20 (Bausch and Lomb) Colorimeter. Analyses were duplicated.

Phytochemical analysis

Phytochemical qualitative analysis of the plant sample was carried out using standard procedures described by Sofowora (1993) and Evans (2002).

RESULTS AND DISCUSSION

The *Sargassum* mass is shown in Figure 1. Based on light microscopical studies, the *Sargassum* 'wracks' was identified as a mixture of *S. natans* (Linnaeus), Gaillon and *S. fluitans* (Borgesen) Borgesen (Figure 2). The two species were differentiated as outlined in Table 1. Of all the morphological differences, two are particularly remarkable - the usual occurrence of spines on the air bladder of *S. natans* and their absence in *S. fluitans.* The other distinguishing character is that while *S. natans* had long stalk bladder, the *S. fluitans* had short stalk with wing tissue around it (Figure 2).

Several workers (Schnieder and Searles, 1991; Littler and Littler, 2000; Dawes and Mathieson, 2008; Conti, 2008; Shapiro, 2004) observed that *S. fluitans* is separated from *S. natans* by the lack of spines on the gas (H_2S) bladder (pneumatocysts) and the presence of

Table 1. Morphological comparison of *Sargassum natans* and *S. fluitans* collected from Ajegunle - Erun-Ama beach in Ondo State, Nigeria.

Species	Stem	Leaf shape	Leaf size (mm)	Leaf apex	Leaf margin	Vesicle	Vesicle stalk
S. natans	Flat on one side and slender tapering on other side, branchlet smooth	Narrow, linear-oblong	1-3[a] x 15-20[b]	obtuse	shallowly toothed	Pea-shaped with spine tip (ca. 1-2[a] x 0.5-3 [b] mm)	Long and slender (ca.1-3 mm
S. fluitans	Flat on one side and slender tapering on other side, branchlet spiny	Broad, lanceolate-elliptical	3-5[a] x 15-21[b]	acute	deeply toothed	Pea-shaped without spine tip (ca. 1-3[a] x 1-3 [b] mm)	Long and winged (ca.1-3 mm)

A, Width range; b, length range.

Table 2. Proximate analysis of mixture of *Sargassum natans* and *S, fluitans* powder.

[a]Content	Percentage (%)
Carbohydrates (by difference)	57.3 ± 0.21
Protein	15.4 ± 0.0
Moisture content	9.0 ± 0.14
Ash	8.65 ± 0.07
Crude Fibre	7.15 ± 0.21
Ether Extract (Fat)	2.5 ± 0.07

a, Mean of two readings ± SD.

winged tissue around the bladder stalk. However the two species are difficult to differentiate from one another once they have reached the shoreline (Shapiro, 2004). The present study also confirms this observation. That both *S. natans* and *S. fluitans* were mixed together in the present study, is highly suggestive of the fact that the invading seaweed must have originated from the Sargasso sea in the North Atlantic Ocean where according to Stoner and Greening (1984), both species co-exist. *S. natans* and *S. fluitans* are two most similar and common holo-pelagic brown algae (Conover and Sieburth, 1964), reproducing vegetatively by fragmentation (Awasthi, 2005; Rogers, 2011). They remain completely pelagic during their lifecycle and are never attached to the sea floor during their life cycle (Hemphill, 2005; Round, 1981). However, strong currents occasioned by several storms in May or June bump into the edges of the Sargasso Sea breaking off segments of the ecosystem (Grower and King, 2008). In the process, the sea currents and winds carry the *Sargassum* fragments along, eventually sweeping it into West African coastal regions of countries like Nigeria and Gabon (Colombini and Chelazzi, 2003). *Sargassum,* being highly tolerant of variations in environmental parameters like desiccation, sunlight, salinity and temperature occupies a broad range of habitats (Abbott and Dawson, 1978, Fritsch, 1965).

The proximate analysis of mixed *Sargassum* sample is shown in Table 2. Crude protein (154 mg/100 g) was relatively higher when compared with the crude protein of two commonly consumed Nigerian vegetables. Idris (2011) reported that *Telfairia occidentale* had 87.3 mg/100 g protein and Adeboye (2004) obtained 116-123 mg/100 g protein for two varieties of *Senecio biafrae.* Proteins are required for growth and body building. In this study, ash content in *Sargassum* sample was 86.5 mg/100 g but Gbadamosi et al. (2012) reported 87.2 mg/100 g for *S. biafrae*. The crude fat (25.5 mg/100 g) of the seaweed is lower than that of *T. occidentale* (35.7 mg/100 g) obtained by Gbadamosi et al. (2012). All these show that *S. natans* and *S. fluitans* have nutritional potentials.

Seaweeds and their products are used in coastal Asia to enhance growth and productivity, however, horticultural uses of *Sargassum* are limited (Eyras et al., 2008; Klock-Moore, 2000; Win and Saing, 2008). Although using *Sargassum* biomass as fertilizer is another way of harnessing its potentials, *Sargassum* is natural fertilizer for *Panicum amarum*, a saline habitat grass (Williams, 2008). Ocean algae consistently have lower nitrogen content when compared to compost from traditional feed stocks (Eyras et al., 1998; Maze et al., 1993). As shown in Table 3, the percentage ratio of N-P-

Table 3. Mineral analysis of mixture of *Sargassum natans* and *S. fluitans* powder.

Mineral	[b]mg/100 g
Phosphorus	96.5 ± 2.12
Magnesium	42.75 ± 0.35
Potassium	28.0 ± 0.74
Iron	8.7 ± 0.28
[a]Nitrogen (Kjeldahl)	6.360. ± 0.2
Zinc	0.05 ± 0.0
Iodine	0.04 ± 0.0

a, Converted from % to mg/100 g by multiplying

Table 4. Quantitative phytochemical analysis of mixture of *Sargassum natans and S. fluitans* powder.

[a]Bioactive compound	mg/100 g
Flavonoids	775.0 ± 7.07
Saponins	525.01 ± 0.0
Tannins	122.5 ± 3.53
Phenolics	80.0 ± 0.0
Alkaloids	77.5 ± 3.50
Terpenoids	66.5 ± 2.12
Cardiac glycosides	16.5 ± 2.12

a, Mean of two readings ± SD.

K (1-10-3) was relatively small. However, high N-P-K percentage does not necessarily mean a better fertilizer. Besides, N-P-K percentage ratios of organic fertilizers are typically smaller compared to inorganic ones. Organic fertilizers are not water soluble and thus release nutrients slowly over time, especially when enhanced by a soil pH 6.5-6.8, thus increasing soil fertility capacity (Granstedt and Kjellenberg, 1997). Since nitrogen generally promotes foliage growth, phosphorus promotes root growth and fruit production and potassium promotes overall health of the plant, the percentage ratio (1-10-3) of *Sargassum* in the present study is recommended as fertilizer for a new lawn and for seedling establishment. The low concentration of iodine (0.04 mg/100 g) in this study agrees with the view of Liu et al. (2012) that the contribution of iodine in *Sargassum* for treating thyroid and related diseases seems to have been over estimated.

The quantitative phytochemical analysis of the mixed *Sargassum* species is shown in Table 4. *Sargassum* seaweeds have been used in more than 226 prescriptions to treat various diseases in China (Liu et al., 2012). The uses in traditional Chinese medicine presumably depend on the phytochemical compounds in the seaweed. Although Liu et al., (2012) listed 80 *Sargassum* species that have been analyzed for their phytochemical constituents, *S. natans* and *S. fluitans* are not included. Liu et al. (2009) isolated two flavonoids from *Sargassum pallidum,* but in low concentrations. The occurrence of relatively high flavonoid constituents (750 mg/100 g) in the mixed *Sargassum* (*S. natans* and *S. fluitans*) shows that the biomass is a rich source of flavonoids hence useful medicinally. Flavonoids are known to have antiviral, antineoplastic, antithrombotic and vasodilatory activities (Alan and Miller, 1996). The occurrence of tannins in appreciable amount (122.5 mg/100 g) is significant. According to Okwu and Okwu (2004), tannins hasten the healing of wounds and inflamed mucous membrane. Phlorotannin fractions from mixed *Sargassum* species showed anticoagulant and antioxidant activities (Wei et al., 2007).

The mixed *Sargassum* sample had 66.5 mg/100 g of terpenoids. Terpenoids have antimicrobial and antiamoebic activities (Amaral et al., 1998). The present study supports the report of Liu et al. (2012) that meroterpenoids (class of compounds containing terpenoid elements) are particularly abundant in *Sargassum*. Meroterpenoids are antioxidant, anticancer, antimalarial, and antiviral and neuroprotective agents (Tsang and Kamei, 2004). Saponin constituent (525 mg/100 g) was relatively high in the mixed *Sargassum* sample. Saponins are used in manufacturing insecticides, shampoos and in the synthesis of steroid hormones (Okwu, 2003). That alkaloid (77.5 mg/100 g) occur in the *Sargassum* sample may explain the usefulness of the seaweed in the management of high blood pressure. Harborne (2000) reported that alkaloids such as rynchosphylline help in improving cardiac conditions by reducing blood pressure, increasing circulation and inhibiting deposition of arteriosclerosis plaque and blood clots.

The Nigerian traditional fishermen reported that the invading *Sargassum* mass caused skin and eye irritation when handled. This effect might be due to the production of some chemical substances as the seaweed decomposed. As observed by the present study, *Sargassum* was harmless when dry but decomposed very fast in wet conditions on the beaches, emitting foul odor (poisonous hydrogen sulphide). The inhalation of the gas could trigger irritation of the eyes and the respiratory system.

Conclusion

It was evident that the features of *Sargassum* from Ondo State, Nigeria, did not resemble any of the species recorded for West Africa. In consideration of seasonal appearance on coastal beaches, *Sargassum* constitutes a nuisance to both the fishermen and the beach lovers. Since biological, chemical and mechanical removal approaches may not be effective, attention should be focused on how the pelagic masses can be economically useful.

ACKNOWLEDGEMENTS

The authors sincerely thank Mr. Methuselah Aligaji, the leader of the fishermen at Ajegunle-Erun-Ama Community for the valuable information. To Prof AE Adegbite, Mr David Adegbite, Mr Shadrach Olowoparija, Mr Kunle Akinyemi, Mr Omolade Toyo and Mr Bayo Ojeleke for their participation in the field visits and comments.

REFERENCES

Abbott IA, Dawson EY (1978). How to know the seaweeds. (2nd ed.). Dubuque, Iowa: Wm. C. Brown Co. Publishers.

Adeboye OC (2004). Senecio biafrae (Oliv & Hiern) C. Jeffrey. Edited by Grubben GJH, Denton OA. PROTA, Wageningen, Netherlands.

Alan L, Miller ND (1996). Antioxidant flavonoidflavonoids structure, function and clinical usage. Alt. Med. Rev. 1: 103-111.

Amaral JA, Ekins A, Richards SR, Knowles R (1998). Effect of selected monoterpenes on methane oxidation, dentrification and aerobic metabolism by bacteria in pure culture. Appl. Environ. Microbiol. 34: 520-525.

AOAC (2005). Official Methods of Analysis, 18th Edition, Association of Official Analytical Chemists, Washington, DC, USA.

Awasthi DK (2005).Cryptogams: Algae, Bryophyta and Pteridophyta. NewDehli: Krishna Prakashan Media.

Chennubhotla VSK, Kaliaperumal N, Kalimuthu S (1981). Seaweed recipes and other practical uses of seaweeds. Seafood Exp. J. 13: 9-16.

Colombini I, Chelazzi L (2003). Influence of marine allochthonous input on sand beach communities. Oceanog. Marine. Biol. 41: 115-159.

Conover JT, Sieburth JM (1964). Effect of *Sargassum* distribution on its epibiota and antibacterial activity. Botanica Marina. 6: 147-157.

Conti R (2008). *Sargassum* beach maintenance in Nueces County, 2007, effects on beach morphology. Symposium conducted at the meeting of the *Sargassum* Symposium, Corpus Christi, TX.

Dawes CJ, Mathieson AC (2008). The seaweeds of Florida, Gaires ville, University Press of Florida, USA.

Evans WC, (2002). Trease and Evan's Pharmacognosy 15th Ed, B. Sanders Co. Ltd. Singapore.

Eyras MC, Defosse GE, Dellatore F (2008). Seaweed compost as an amendment for horticultural soils in Patagonia, Argentina. Compost. Sci. Utiliz. 16: 119-124.

Eyras MC, Rostagno CM, Defosse GE (1998). Biological evaluation of seaweed composing. Compost Sci. Utiliz. 6: 74-81.

Fritsch FE (1965). The structure and the reproduction of algae, II. Cambridge: University Press.

Gbadamosi IT, Alia AE, Okolosi O (2012). In-vitro antimicrobial activities and nutritional assessment of roots of ten vegetables. New York Sci. J. 5(12): 234-240.

Gower J, King S (2008). Satellite images show the movement of floating *Sargassum* in the Gulf of Mexico and Atlantic Ocean. Available from Nature Proceedings.

Granstedt A, Kjellenberg L (1997). Long-term field experiment in Sweden: Effects of organic and inorganic fertilizers on soil fertility and crop quality. In: Proceedings of an International conference in Boston, Tufts University, Agricultural Production and nutrition, Massachusetts. pp. 19-21.

Guiry MD, Guiry GM (2011). AlgaeBase: world-wide electronic publication. Galway: National University of Ireland.

Harborne JB, (2000). Arsenal for Survival: Secondary plant products. Taxon. 49 (3): 435-449.

Hemphill AH (2005). Conservation on the high sea-drift algae habitat as an open ocean cornerstone. High sea marine protected areas. Park 15(3): 48-56.

Idris S (2011). Compositional Studies of *Telfairia occidentalis* Leaves. A. J. Chem. 1(2): 56-59.

Invasive Species Specialist Group (ISSG) (2011). Global invasive species database.

John DM, Lawson GW, Ameka GK (2003). The Marine Macroalgae of the Tropical West Africa sub-region. Nova Hedw. 125: 217.

Klock-Moore KA (2000). Comparison of *Salvia* growth in seaweed compost and biosolid compost. Compost Sci. Utiliz. 6: 24-28.

Littler DS, Littler MM (2000). Caribbean Reef Plants. Washington: Offshore Graphics, Inc. United State of America.

Liu L, Heinrich N, Myers S, Dworjanyn SA (2012). Towards a better understanding of medicinal uses of the brown seaweed *Sargassum* in Traditional Chinese Medicine: A phytochemical and pharmacological review. J. Ethnopharmacol. 142: 591-619.

Liu X, Wang CY, Shao CL, Wei Y X, Wang BC, Sun LL, Zheng CJ, Guan HS (2009). Chemical constituents from *Sargassum pallidum* (Turn), C. Agardh. Biochem. Syst. Ecol. 37: 127-129.

Maze J, Morand P, Potoky P (1993). Stabilization of "green tide" *Ulva* by a method of composting with a view to pollution limitation. J. Appl. Phycol. 5: 183-190.

Okwu DE (2003). The potentials of *Ocimum gratissimum, Pergularia extensa* and *Tetrapleura teraptera as spice and flavouring agents*. Nig. Agric. J. 34: 143-148.

Okwu DE, Okwu ME (2004). Chemical composition of *Spondias mombin* Linn. Plant parts. J. Sust. Agric. Environ. 6: 140-147.

Rogers (Ed.) K (2011). Fungi, algae and protists. New York, NY: Britannica Educational Publishing.

Round FE (1973). The Biology of the Algae, 2nd Ed. Edward Arnold (Publishers Ltd.) London.

Round FE (1981). The ecology of algae. New York, NY: Cambridge University Press.

Schneider CW, Searles RB (1991). Seaweeds of the southeastern United States: Cape Hatteras to Cape Canaveral Durham: Duke University Press.

Shapiro L (2004). Sargassum natans (Linnaeus) Gaillon. EOL Special Rapid Response.

Sofowora A (1993). Medicinal Plants and Traditional Medicine in Africa. 2nd Edition Ibadan, Nigeria: Spectrum Books Ltd.

Stoner AW, Greening HS (1984). Geographic variation in the macrofaunal associates of pelagic *Sargassum* and some biogeographic implications. Marine Ecol. Prog. Series. 20: 185-192.

Széchy MTM, Guedes PM, Baeta-Neves MH, Oliveira EN (2012). Verification of Sargassum natans [Linnaeus] Gaillon [Heterokontophyta: Phaeophyceae] from the Sargasso Sea off the coast of Brazil, western Atlantic Ocean. Checklist 8 (4): 638-641.

Tsang CK, Kamei Y (2004). Sargaquinonic acid supports the survival of neuronal PCIZD cells in a nerve growth factor - independent manner. Eur. J. Pharmacol. 488: 11-18.

Walsh LM (1971). Instrumental methods for analysis of soils and plant tissue. Madison. Wis. U.S.A: Soil Science Society of America Inc.

Wang S, Jiang XM, Han XX Liu JG (2009). Combustion characteristics of seaweeds biomass. 1. Combustion characteristics of *Enteromorpha clathrata* and *Sargassum natans*. Energy Fuels. 23: 5173-5178.

Wei Y, Li J, Wang J, Qi H (2007). Screening of different fractions of phlorotannins from *Sargassum thumbergii* Kuntze for anticoagulant activity. Zhongguo Shenghua Yaowu Zazhi. 28: 227-229.

Williams A (2008). *Sargassum* as a fertilizer for *Panicum amarum*. Symposium conducted at the meeting of the *Sargassum* Symposium, Corpus Christi, TX.

Win LL, Saing KM (2008). Effectiveness of Myanmar brown seaweed (*Sargassum* spp.) extract as organic fertilizer in pot trial of rice. GMSARN International Conference on Sustainable Development: Issues and Prospects for the GMS: pp.1-4.

Wynne MJ (2011). A checklist of benthic marine algae of the tropical and subtropical western Atlantic: third revision. Nova Hedwigia. 140: 1-166

Yokoyama S, Jonouchi K, Imou K (2007). Energy production from marine biomass: fuel cell power generation driven by methane produced from seaweed. World Aca. Sci. Eng. Tech. 28: 320-323.

academicJournals

Vol. 13(10), pp. 1194-1197, 5 March, 2014
DOI: 10.5897/AJB2013.13001
ISSN 1684-5315 ©2014 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Short Communication

Allelotoxicity of *Oudneya africana* R. Br. aqueous leachate on germination efficiency of *Bromus tectorum* L. and *Triticum aestivum* L.

Salhi Nasrine[1]*, Salama M. El-Darier[2] and Halilat M. El-Taher[1]

[1]Université KasdiMerbah Ouargla Laboratoire de Bio-ressources sahariennes : préservation et valorisation, Faculté des Sciences de la Nature et de la Vie et des Sciences de la Terre et de l'Univers Ouargla 30 000 Algérie.
[2]Departments of Botany and Microbiology, Faculty of Science, University of Alexandria, Alexandria, Egypt.

Accepted 28 October, 2013

This present study was conducted to investigate the possible allelopathic effect of *Oudneya africana* (donor species) on *Bromus tectorum* (weed species) and *Triticum aestivum* (cv. Sahel1; crop species) through germination bioassay experiment. *B. tectorum* is a winter annual grass that grows in winter wheat and other crops and in both disturbed and undisturbed grasslands. The effect of *O. africana* aqueous leachate (OAAL) on germination and seedling growth of *B. tectorum* and *T. aestivum* was investigated. Higher concentrations of the aqueous leachate significantly reduced the germination rate of *B. tectorum*. Similarly, coleoptile (CL) and radicle (RL) lengths of *B. tectorum* seeds was significantly inhibited. To go through with this, seedling growth of the weed species was also affected. On the other hand, the germination parameters of wheat seeds were slightly affected with applying different OAAL concentration levels compared to *B. tectorum*. In conclusion, allelochemicals extracted from the donor species caused a significant reduction in germination and growth parameters of *B. tectorum* > *T. aestivum.*

Key words: Aqueous leachate, *Oudneya africana*, *Bromus tectorum,* germination rate.

INTRODUCTION

Allelopathy was defined as the direct or indirect harmful or beneficial effects of one plant or another through the production of chemical compounds that escape into the environment (Rice, 1984). Hence, Plants or organisms that release these compounds are called "donor species", while those that are influenced in their growth and development are called "target or recipient species" (Torres et al., 1996; Inderjit and Keating, 1999). Allelochemicals are plant secondary metabolites mainly produced from medicinal and aromatic plants (Delabys et al., 1998); have been identified, including the phenolic acids, coumarins, terpenoids, flavoniods, alkaloids, glycosides and glucosinolates. These chemical substances (phytotoxic) are known to be exuded by plants to suppress emergence or growth of the other plants; allelopathic effects of these compounds are often observed to occur early in the life cycle, causing inhibition of seed germination and seedling growth. These compounds exhibit a wide range of mechanisms of action and interpretations of mechanisms of action are complicated by the fact that individual compounds can have multiple phytotoxic effects (Einhellig, 2002).

Medicinal plants have inhibitory effects (Lin et al., 2003, 2004) on selected weeds and its allelochemicals inhibiting weed growth. Therefore, it was easier to screen allelopathic plants from medicinal ones than other plants

*Corresponding author. Email: salhi.ne@univ-ouargla.dz, nesrinemed@yahoo.fr.

Table 1. The effects of *Oudneya Africana* aqueous leachate on germination and seedling growth of *Bromus tectorum* and *Triticum aestivum*.

Concentration (%)	0%	2.5%	5 %	7.5%	10%	Statistical analysis
Germination (%)						
Bromus tectorum	100.0^{a}	73.3^{b}	26.6^{c}	13.3^{d}	6.6^{e}	**
Triticum aestivum	100.0^{a}	100.0^{a}	100.0^{a}	100.0^{a}	90.0^{b}	**
Coleoptile length (mm)						
Bromus tectorum	19.66^{a}	6.33^{b}	0.66^{c}	0.00^{c}	0.00^{c}	**
Triticum aestivum	42.00^{a}	25.00^{b}	22.5^{c}	18.00^{d}	15.50^{e}	**
Radicle length (mm)						
Bromus tectorum	34.00^{a}	3.00^{b}	1.66^{bc}	0.83^{c}	0.83^{c}	**
Triticum aestivum	56.66^{a}	26.50^{b}	17.5^{c}	16.00^{c}	12.00^{d}	**

Different letters within each column indicate significance at P<0.05 one-way ANOVA; **: Significant at 0.01.

possibly because they have the ability to accumulate certain metabolic compounds curing many diseases of mankind (Qasem and Hassan, 2003). The genus *Oudneya* belongs to the Brassicaceae family and it comprises four thousands species (Quezel and Santa, 1963). They occur mainly in temperate and cold regions of the Northern Hemisphere (Brooks, 1987). *O. africana* (locally named Henat l`ibel) is an endemic plant of sahara and is used in folk medicine by local people of Ouargla (Algeria) to treat wound cicatrisation and against the scorpion's bites. The phytochemical tests of the aerial parts of *O. africana* showed the presence of saponosids, flavonoids, sterols, steroids and tannins in different quantities (Bouhadjera et al., 2005).

The present research is a part of a specific study carried out in Algeria to explore the allelopathic effects of *O. africana* (donor species) aqueous leachate on germination efficiency of *B. tectorum* (weed species) and *T. aestivum* (crop species) under laboratory conditions.

MATERIALS AND METHODS

Samples from the aerial shoots of *O. africana* were collected from the natural habitats. The samples were air- dried, ground in a Wiley Mill to fine uniform texture then stored in glass jars until use. Stock aqueous extract was obtained by soaking 50 g air-dried plant material in 500 ml of cold distilled water (10% w/v) at room temperature (20 ± 2°C) for 24 h with occasional shaking. The mixture were filtered through two layers of cheesecloth and centrifuged for 20 min at 10.000 r.p.m to remove particulate material and the purified extract. Different concentrations (2.5, 5, 7.5 and 10% of *O. africana* aqueous leachate; OAAL) were prepared from the stock solution in addition to the control (distilled water). To achieve this experiment, 10 seeds of each of the weed and crop species were arranged in 9-cm diameter Petri-dishes lined with two discs of Whatman No. 1 filter paper under normal laboratory conditions with day temperature ranging from 19 to 22°C and night temperature from 12 to 14°C. 2 ml of each level of OAAL were added daily to three replicates. Before sowing, the seeds were surface sterilized with 2% sodium hypochlorite for 2 min then rinsed four times with distilled water. The sterilized seeds were soaked in aerated distilled water for 24 h. The germination percentage (GP), coleoptile (CL) and radicle (RL) lengths were recorded after one weak at the end of the experiment.

Statistical analysis

Data of the present study were subjected to standard one-way analysis of variance (ANOVA) using the COSTAT 2.00 statistical analysis software manufactured by CoHort Software Company (Zar, 1984).

RESULTS

Germination percentage (GP)

The present data implies the significant promoting influence (P≤ 0.01) of OAAL on GP of *B. tectorum* (Table 1). At control, GP values were 100% thereafter decreased to 73.3, 26.6, 13.3 and 6.6% at 2.5, 5, 7.5 and 10% OAAL concentrations respectively. Table 1 also showed that GP of wheat seeds were apparently varied with applying different OAAL concentration levels which is supported statistically (P≤ 0.01). At control, 2.5, 5 and 7.5% OAAL concentrations, the values were 100% while it was 90% at 10% OAAL concentration.

Coleoptile (CL) and radicle (RL) lengths

Statistically, the applied concentrations of OAAL are significantly (P≤ 0.01) affecting CL of *B. tectorum* (Table 1). The values of CL were 19.66 mm at control level thereafter it reduced to 6.33 mm at 2.5% OAAL and was completely inhibited at 7.5 and 10% OAAL concentrations. Regarding *T. aestivum*, the values of CL were 42, 60 mm at control level. Afterward, it reduced to 25 and 22.5 mm at 2.5 and 5% OAAL respectively. Expectedly, the maximum

allelopathic action of 7.5 and10% OAAL concentration has reduced CL to 18 and 15.5 mm. Compared to the control, a gradual decrease in RL of *B. tectorum* was observed along gradual OAAL concentrations. RL implication was significantly affected at P≤ 0.01 (Table 1). At control, RL was 34 mm and at 2.5 and 5% concentrations, RL decreased to 3 and 1.66 mm, respectively. Constantly, it continues reduction till it attained a value of about 0.83 mm at 7.5 and 10% OAAL concentration level. With respect to *T. aestivum* RL attained a value of about 56.66 mm at control level and values of 26.5 and 17.5 mm at 2.5 and 5% of OAAL concentrations, respectively. At 7.5 and 10% OAAL concentration levels the values were about 16 and 12 mm, respectively.

DISCUSSION

The allelopathic effect of 2.5, 5, 7.5 and 10% of *O. africana* aqueous leachate (OAAL) beside the control was clearly demonstrated on germination percentage, coleoptile and radicle length of *B. tectorum* a weeds associated with crop species (*T. aestivum*). Considering the foregoing results, it seemed that there are significant phytotoxic effect of the donor species on germination and coleoptile (CL) and radicle (RL) lengths. These results correlated with the findings that allelochemicals presented in the aqueous extracts of plant species have been reported to affect different physiological processes through their effects on enzymes responsible for phytohormone synthesis and were found to associate with inhibition of nutrients and ion absorption by affecting plasma membrane permeability.

The reduction in germination could be attributed to inhibitory effect of allelopathic substances present in the extract. According to the study of Diwani et al. (2009), *O. Africana* contain substances such as phenolic compounds, which succeeded to be used as natural anti-oxidant for the protection of oils. The results appeared to be in agreement with that of Swaminathan et al. (1989) who reported that the potential compounds, which are able to induce inhibitory effect on germination, are identified as phenolic acids. The release of phenolic compounds adversely affects the germination and growth of plants through their interference in energy metabolism, cell division, mineral uptake and biosynthetic processes (Rice, 1984).

The results of Dias et al. (2005) appeared that the extracts of some plants inhibit the growth of others. With regard to allelopathic effects of plant secondary metabolites, it is now generally recognized that some terpenoids, mainly monoterpenes and sesquiterpenes present in the volatile fractions (Fischer, 1991; von Poser et al., 1996) and phenolic compounds are the principal responsible for growth inhibition of competing plants (Harborne, 1993). Polyphenol compounds such as tannins, flavonoids and phenolic acids were the most prominent components of the crude extracts investigated and could contribute to the germination and growth inhibitory activity of the *Hypericum* species extracts (Dall'Agnol et al., 2003). Many biological activities of the flavonoids including pollinator attractants, oviposition stimulants, feeding attractants and deterrents, phyto-alexins and allelopathy have been found. Several flavonoids such as quercetin, isoquercitrin, rutin, and quercetrin among many others have shown effects on plant growth (Rivera-Vargas et al., 1993; Parvez et al., 2004; Iqbal et al., 2005).

Conclusion

The study reveals OAAL affect the germination efficiency of both weed and crop species and the effect was highly prominent in the weed compared to the crop species. Based on the results of this study the species with the strongest allelopathic potential such as *O*. *Africana* must be examined for their selective action on other specific plants including weeds and crops under field conditions, their allelopathic activity will be much more detailed. Analysis of possible allelochemicals in these plants is also required. There is possibility of using these allelochemicals directly or as structural leads for the discovery and development of environment friendly herbicides to control weeds.

REFERENCES

Bouhadjera K, Kebir T, Baba-Ahmed A, Bendahou M (2005). Antimicrobial activity of the sterols and steroids extracted from the Algerian Oudneya Africana R. Br. Pakistan J. Biol. Sci. 8:834-838.

Cadiz.von Poser GL, Menut C, Toffoli ME, Verin P, Sobral M, Bessiere JM, Lamaty G, Henriques AT (1996). Essential oil composition and allelopathic effect of the Brazilian Lamiaceae*Hesperozygisringens* (Benth.) Epling and HesperozygisrhododonEpling. J. Agric. Food Chem. 44:1829-1832.

Dall'Agnol R, Ferraz A, Bernardi AP, Albring D, Nor C, Sarmento L, Lamb L, Hass M, von Poser GL, Schapoval EES (2003). Antimicrobial activity of some Hypericum species. *Phytomedicine,* 10:141-147.

Delabys A, AnCay A, Mermillod G (1998). Recherch d`espèces végétales a propriétés allopathiques .Annales de la 17e Conférence du COLUMA, 9,10,11, Dijon (souspresse).

Dias JFG, Círio GM, Miguel MD, Miguel OG (2005). ContribuiçãoaoestudoalelopáticodeMaytenusilicifoliaMart. exReiss., Celastraceae. *Revista Brasileira de Farmacognosia,* 15:220-223.

Einhellig FA (2002). The physiology of allelochemical action: clues and views. In: Allelopathy, from Molecules to Ecosystems, M.J. Reigosa and N. Pedrol, Eds. Science Publishers, Enfield, New Hampshire.

El Diwani G, El Rafie S, Hawash S (2009). Protection of Biodiesel and Oil from Degradation by Natural Antioxidants of Egyptian Jatropha. Int. J. Environ. Sci. Technol. 6(3):369-378.

Fischer NH (1991). Plant terpenoids as allelopathic agents. In: Harborne JB, Tomas-Barberan FA (ed.) Ecological chemistry and biochemistry of plant terpenoids. Oxford: Clarendon Press. pp. 377-398.

Harborne J (1993). Introduction to ecological biochemistry.London:Academic Press.

Inderjit, Keating KI (1999). Allelopathy: principles, procedures, processes, and promises for biological control. Advances in Agronomy. 67:141-231.

Iqbal Z, Golisz A, Furubayashi A, Nasir H, Fujii Y (2005). Allelopathic

potential of buckwheat.Fourth World Congress of Allelopathy.WaggaWagga, Australia.
Lin D, Tsuzuki E, Sugimoto Y, Dong Y, Matsuo M, Terao H (2004). Elementary Identification and biological activities of phenolic allelochemicals from dwarf lilyturf plant (Ophiopogonjaponicus K.) against two weeds of paddy rice field. Plant Product. Science, 7(3):260-265.
Lin D, Tsuzuki E, Sugimoto Y, Dong Y, Matsuo M, Terao H (2003). Assessment of dwarf lilyturf (Ophiopogonjaponicus K.) dried powders for weed control in transplanted rice. Crop Protection. 22 (2):431-435.
Parvez MM, Tomita-Yokotani K, Fujii Y, Konishi T, Iwashina T (2004). Effects of quercetin and its seven derivatives on the growth of Arabidopsis thaliana and Neurosporacrassa. Biochemical Systematic and Ecology. 32:631-635.
Qasem, Rand J, Hassan AA (2003). Harbicidal Properties of some Medicinal plants against *Malvasylvestris* and *Portulaca oleracea*. Agric. Sci. J. 30(1):84-99.
Quezel P, Santa S (1963). In: Nouvelle Flore de l'Algerie et des regions de sertiquesmeridionales, Vols. 1-2. CNRS, Paris. pp. 600-601.
Rice EL (1984). Allelopathy.Second edition. New York: Academic Press, Orlando, FL. p. 422.
Rivera-Vargas LI, Schmitthenner AF, Graham TL (1993).Soybean fl avonoid effects on and metabolism by Phytophthorasojae. Phytochemistry, 32:851-857.
Swaminathan C, Vinayrai RS, Suresh KK (1989). Allelopathicproctivities of *Acacia nilotica*. J. Trop. Forest Sci. 2:56-60.
Torres A, Oliva RM; Castellano D, Cross P (1996). First world congress on allelopathy. A science of the future. SAI (University of Cadiz). Spain. p. 278
Zar JH (1984) (ed). Biostatistical Analysis Prentice-Hall: Inc. *New* Jersey, p. 718.

UPCOMING CONFERENCES

5th International Conference on Biotechnology and Food Science (ICBFS 2014), Erzurum, Turkey, 24 Apr 2014

7th Annual World Congress of Industrial Biotechnology (IBIO-2014), Dalian, China, 25 Apr 2014

Conferences and Advert

April 2014

XI International Symposium on Plant Biotechnology, Jardines del Rey archipelago, Cuba, 9 Apr 2014

5th International Conference on Biotechnology and Food Science (ICBFS 2014), Erzurum, Turkey, 24 Apr 2014

7th Annual World Congress of Industrial Biotechnology (IBIO-2014), Dalian, China, 25 Apr 2014

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