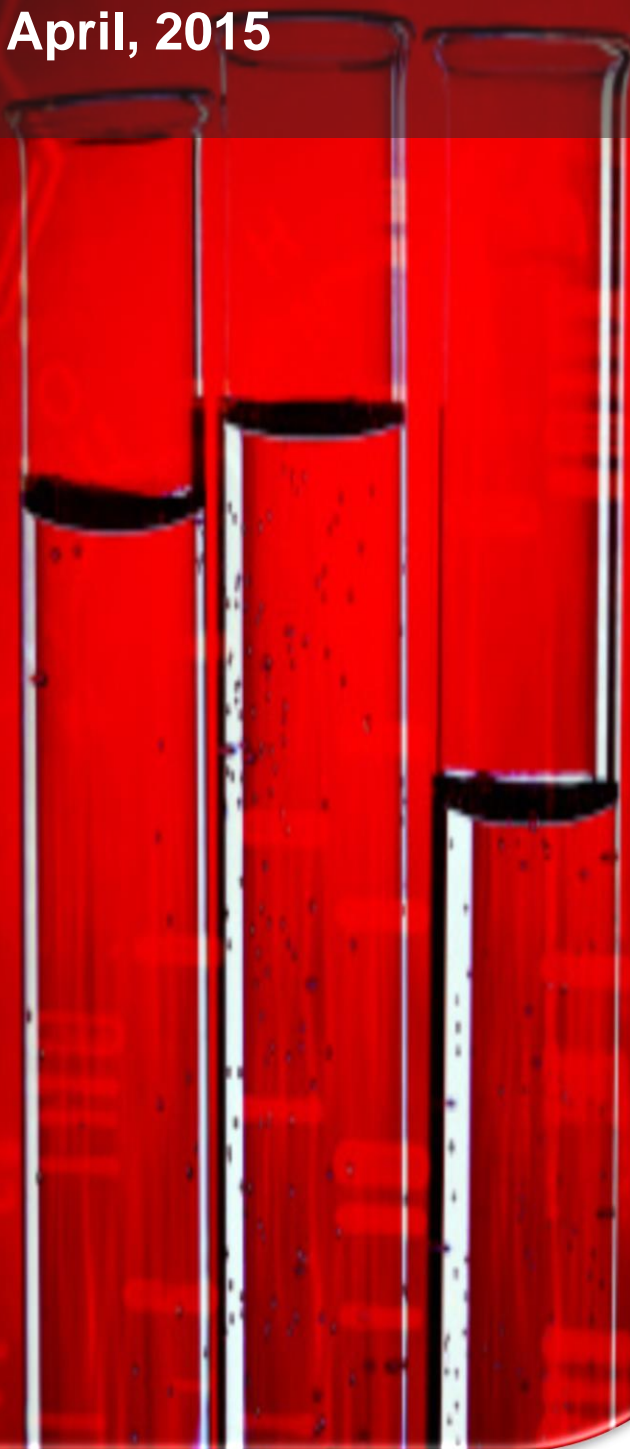


African Journal of Biotechnology

Volume 14 Number 17, 29 April, 2015

ISSN 1684-5315



*Academic
Journals*

ABOUT AJB

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

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

Submission of Manuscript

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

[Click here to Submit manuscripts online](#)

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

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

Editor-In-Chief

George Nkem Ude, Ph.D

*Plant Breeder & Molecular Biologist
Department of Natural Sciences
Crawford Building, Rm 003A
Bowie State University
14000 Jericho Park Road
Bowie, MD 20715, USA*

Editor

N. John Tonukari, Ph.D

*Department of Biochemistry
Delta State University
PMB 1
Abraka, Nigeria*

Associate Editors

Prof. Dr. AE Aboulata

*Plant Path. Res. Inst., ARC, POBox 12619, Giza, Egypt
30 D, El-Karama St., Alf Maskan, P.O. Box 1567,
Ain Shams, Cairo,
Egypt*

Dr. S.K Das

*Department of Applied Chemistry
and Biotechnology, University of Fukui,
Japan*

Prof. Okoh, A. I.

*Applied and Environmental Microbiology Research
Group (AEMREG),
Department of Biochemistry and Microbiology,
University of Fort Hare.
P/Bag X1314 Alice 5700,
South Africa*

Dr. Ismail TURKOGLU

*Department of Biology Education,
Education Faculty, Firat University,
Elaziğ,
Turkey*

Prof T. K. Raja, PhD FRSC (UK)

*Department of Biotechnology
PSG COLLEGE OF TECHNOLOGY (Autonomous)
(Affiliated to Anna University)
Coimbatore-641004, Tamilnadu,
INDIA.*

Dr. George Edward Mamati

*Horticulture Department,
Jomo Kenyatta University of Agriculture
and Technology,
P. O. Box 62000-00200,
Nairobi, Kenya.*

Dr. Gitonga

*Kenya Agricultural Research Institute,
National Horticultural Research Center,
P.O Box 220,
Thika, Kenya.*

Editorial Board

Prof. Sagadevan G. Mundree

*Department of Molecular and Cell Biology
University of Cape Town
Private Bag Rondebosch 7701
South Africa*

Dr. Martin Fregene

*Centro Internacional de Agricultura Tropical (CIAT)
Km 17 Cali-Palmira Recta
AA6713, Cali, Colombia*

Prof. O. A. Ogunseitan

*Laboratory for Molecular Ecology
Department of Environmental Analysis and Design
University of California,
Irvine, CA 92697-7070. USA*

Dr. Ibrahima Ndoye

*UCAD, Faculte des Sciences et Techniques
Departement de Biologie Vegetale
BP 5005, Dakar, Senegal.
Laboratoire Commun de Microbiologie
IRD/ISRA/UCAD
BP 1386, Dakar*

Dr. Bamidele A. Iwalokun

*Biochemistry Department
Lagos State University
P.M.B. 1087. Apapa – Lagos, Nigeria*

Dr. Jacob Hodeba Mignouna

*Associate Professor, Biotechnology
Virginia State University
Agricultural Research Station Box 9061
Petersburg, VA 23806, USA*

Dr. Bright Ogheneovo Agindotan

*Plant, Soil and Entomological Sciences Dept
University of Idaho, Moscow
ID 83843, USA*

Dr. A.P. Njukeng

*Département de Biologie Végétale
Faculté des Sciences
B.P. 67 Dschang
Université de Dschang
Rep. du CAMEROUN*

Dr. E. Olatunde Farombi

*Drug Metabolism and Toxicology Unit
Department of Biochemistry
University of Ibadan, Ibadan, Nigeria*

Dr. Stephen Bakiamoh

*Michigan Biotechnology Institute International
3900 Collins Road
Lansing, MI 48909, USA*

Dr. N. A. Amusa

*Institute of Agricultural Research and Training
Obafemi Awolowo University
Moor Plantation, P.M.B 5029, Ibadan, Nigeria*

Dr. Desouky Abd-El-Haleem

*Environmental Biotechnology Department &
Bioprocess Development Department,
Genetic Engineering and Biotechnology Research
Institute (GEBRI),
Mubarak City for Scientific Research and Technology
Applications,
New Burg-Elarab City, Alexandria, Egypt.*

Dr. Simeon Oloni Kotchoni

*Department of Plant Molecular Biology
Institute of Botany, Kirschallee 1,
University of Bonn, D-53115 Germany.*

Dr. Eriola Betiku

*German Research Centre for Biotechnology,
Biochemical Engineering Division,
Mascheroder Weg 1, D-38124,
Braunschweig, Germany*

Dr. Daniel Masiga

*International Centre of Insect Physiology and
Ecology,
Nairobi,
Kenya*

Dr. Essam A. Zaki

*Genetic Engineering and Biotechnology Research
Institute, GEBRI,
Research Area,
Borg El Arab, Post Code 21934, Alexandria
Egypt*

Dr. Alfred Dixon

*International Institute of Tropical Agriculture (IITA)
PMB 5320, Ibadan
Oyo State, Nigeria*

Dr. Sankale Shompole

*Dept. of Microbiology, Molecular Biology and
Biochemistry,
University of Idaho, Moscow,
ID 83844, USA.*

Dr. Mathew M. Abang

*Germplasm Program
International Center for Agricultural Research in the
Dry Areas
(ICARDA)
P.O. Box 5466, Aleppo, SYRIA.*

Dr. Solomon Olawale Odemuyiwa

*Pulmonary Research Group
Department of Medicine
550 Heritage Medical Research Centre
University of Alberta
Edmonton
Canada T6G 2S2*

Prof. Anna-Maria Botha-Oberholster

*Plant Molecular Genetics
Department of Genetics
Forestry and Agricultural Biotechnology Institute
Faculty of Agricultural and Natural Sciences
University of Pretoria
ZA-0002 Pretoria, South Africa*

Dr. O. U. Ezeronye

*Department of Biological Science
Michael Okpara University of Agriculture
Umudike, Abia State, Nigeria.*

Dr. Joseph Hounhouigan

*Maître de Conférence
Sciences et technologies des aliments
Faculté des Sciences Agronomiques
Université d'Abomey-Calavi
01 BP 526 Cotonou
République du Bénin*

Prof. Christine Rey

*Dept. of Molecular and Cell Biology,
University of the Witwatersand,
Private Bag 3, WITS 2050, Johannesburg, South
Africa*

Dr. Kamel Ahmed Abd-Elsalam

*Molecular Markers Lab. (MML)
Plant Pathology Research Institute (PPathRI)
Agricultural Research Center, 9-Gamma St., Orman,
12619,
Giza, Egypt*

Dr. Jones Lemchi

*International Institute of Tropical Agriculture (IITA)
Onne, Nigeria*

Prof. Greg Blatch

*Head of Biochemistry & Senior Wellcome Trust
Fellow
Department of Biochemistry, Microbiology &
Biotechnology
Rhodes University
Grahamstown 6140
South Africa*

Dr. Beatrice Kilel

*P.O Box 1413
Manassas, VA 20108
USA*

Dr. Jackie Hughes

*Research-for-Development
International Institute of Tropical Agriculture (IITA)
Ibadan, Nigeria*

Dr. Robert L. Brown

*Southern Regional Research Center,
U.S. Department of Agriculture,
Agricultural Research Service,
New Orleans, LA 70179.*

Dr. Deborah Rayfield

*Physiology and Anatomy
Bowie State University
Department of Natural Sciences
Crawford Building, Room 003C
Bowie MD 20715, USA*

Dr. Marlene Shehata

*University of Ottawa Heart Institute
Genetics of Cardiovascular Diseases
40 Ruskin Street
K1Y-4W7, Ottawa, ON, CANADA*

Dr. Hany Sayed Hafez

*The American University in Cairo,
Egypt*

Dr. Clement O. Adebooye

*Department of Plant Science
Obafemi Awolowo University, Ile-Ife
Nigeria*

Dr. Ali Demir Sezer

*Marmara Üniversitesi Eczacılık Fakültesi,
Tıbbiye cad. No: 49, 34668, Haydarpaşa, İstanbul,
Turkey*

Dr. Ali Gazanchain

*P.O. Box: 91735-1148, Mashhad,
Iran.*

Dr. Anant B. Patel

*Centre for Cellular and Molecular Biology
Uppal Road, Hyderabad 500007
India*

Prof. Arne Elofsson

*Department of Biophysics and Biochemistry
Bioinformatics at Stockholm University,
Sweden*

Prof. Bahram Goliaei

*Departments of Biophysics and Bioinformatics
Laboratory of Biophysics and Molecular Biology
University of Tehran, Institute of Biochemistry
and Biophysics
Iran*

Dr. Nora Babudri

*Dipartimento di Biologia cellulare e ambientale
Università di Perugia
Via Pascoli
Italy*

Dr. S. Adesola Ajayi

*Seed Science Laboratory
Department of Plant Science
Faculty of Agriculture
Obafemi Awolowo University
Ile-Ife 220005, Nigeria*

Dr. Yee-Joo TAN

*Department of Microbiology
Yong Loo Lin School of Medicine,
National University Health System (NUHS),
National University of Singapore
MD4, 5 Science Drive 2,
Singapore 117597
Singapore*

Prof. Hidetaka Hori

*Laboratories of Food and Life Science,
Graduate School of Science and Technology,
Niigata University.
Niigata 950-2181,
Japan*

Prof. Thomas R. DeGregori

*University of Houston,
Texas 77204 5019,
USA*

Dr. Wolfgang Ernst Bernhard Jelkmann

*Medical Faculty, University of Lübeck,
Germany*

Dr. Moktar Hamdi

*Department of Biochemical Engineering,
Laboratory of Ecology and Microbial Technology
National Institute of Applied Sciences and
Technology.
BP: 676. 1080,
Tunisia*

Dr. Salvador Ventura

*Department de Bioquímica i Biologia Molecular
Institut de Biotecnologia i de Biomedicina
Universitat Autònoma de Barcelona
Bellaterra-08193
Spain*

Dr. Claudio A. Hetz

*Faculty of Medicine, University of Chile
Independencia 1027
Santiago, Chile*

Prof. Felix Dapare Dakora

*Research Development and Technology Promotion
Cape Peninsula University of Technology,
Room 2.8 Admin. Bldg. Keizersgracht, P.O. 652,
Cape Town 8000,
South Africa*

Dr. Geremew Bultosa

*Department of Food Science and Post harvest
Technology
Haramaya University
Personal Box 22, Haramaya University Campus
Dire Dawa,
Ethiopia*

Dr. José Eduardo Garcia

*Londrina State University
Brazil*

Prof. Nirbhay Kumar

*Malaria Research Institute
Department of Molecular Microbiology and
Immunology
Johns Hopkins Bloomberg School of Public Health
E5144, 615 N. Wolfe Street
Baltimore, MD 21205*

Prof. M. A. Awal

*Department of Anatomy and Histology,
Bangladesh Agricultural University,
Mymensingh-2202,
Bangladesh*

Prof. Christian Zwieb

*Department of Molecular Biology
University of Texas Health Science Center at Tyler
11937 US Highway 271
Tyler, Texas 75708-3154
USA*

Prof. Danilo López-Hernández

*Instituto de Zoología Tropical, Facultad de
Ciencias,
Universidad Central de Venezuela.
Institute of Research for the Development (IRD),
Montpellier,
France*

Prof. Donald Arthur Cowan

*Department of Biotechnology,
University of the Western Cape Bellville 7535
Cape Town,
South Africa*

Dr. Ekhaise Osaro Frederick

*University Of Benin, Faculty of Life Science
Department of Microbiology
P. M. B. 1154, Benin City, Edo State,
Nigeria.*

Dr. Luísa Maria de Sousa Mesquita Pereira

*IPATIMUP R. Dr. Roberto Frias, s/n 4200-465 Porto
Portugal*

Dr. Min Lin

*Animal Diseases Research Institute
Canadian Food Inspection Agency
Ottawa, Ontario,
Canada K2H 8P9*

Prof. Nobuyoshi Shimizu

*Department of Molecular Biology,
Center for Genomic Medicine
Keio University School of Medicine,
35 Shinanomachi, Shinjuku-ku
Tokyo 160-8582,
Japan*

Dr. Adewunmi Babatunde Idowu

*Department of Biological Sciences
University of Agriculture Abia
Abia State,
Nigeria*

Dr. Yifan Dai

*Associate Director of Research
Revivacor Inc.
100 Technology Drive, Suite 414
Pittsburgh, PA 15219
USA*

Dr. Zhongming Zhao

*Department of Psychiatry, PO Box 980126,
Virginia Commonwealth University School of
Medicine,
Richmond, VA 23298-0126,
USA*

Prof. Giuseppe Novelli

*Human Genetics,
Department of Biopathology,
Tor Vergata University, Rome,
Italy*

Dr. Moji Mohammadi

*402-28 Upper Canada Drive
Toronto, ON, M2P 1R9 (416) 512-7795
Canada*

Prof. Jean-Marc Sabatier

*Directeur de Recherche Laboratoire ERT-62
Ingénierie des Peptides à Visée Thérapeutique,
Université de la Méditerranée-Ambrilia
Biopharma inc.,
Faculté de Médecine Nord, Bd Pierre Dramard,
13916,
Marseille cédex 20.
France*

Dr. Fabian Hoti

*PneumoCarr Project
Department of Vaccines
National Public Health Institute
Finland*

Prof. Irina-Draga Caruntu

*Department of Histology
Gr. T. Popa University of Medicine and Pharmacy
16, Universitatii Street, Iasi,
Romania*

Dr. Dieudonné Nwaga

*Soil Microbiology Laboratory,
Biotechnology Center. PO Box 812,
Plant Biology Department,
University of Yaoundé I, Yaoundé,
Cameroon*

Dr. Gerardo Armando Aguado-Santacruz

*Biotechnology CINVESTAV-Unidad Irapuato
Departamento Biotecnología
Km 9.6 Libramiento norte Carretera Irapuato-
León Irapuato,
Guanajuato 36500
Mexico*

Dr. Abdolkaim H. Chehregani

*Department of Biology
Faculty of Science
Bu-Ali Sina University
Hamedan,
Iran*

Dr. Abir Adel Saad

*Molecular oncology
Department of Biotechnology
Institute of graduate Studies and Research
Alexandria University,
Egypt*

Dr. Azizul Baten

*Department of Statistics
Shah Jalal University of Science and Technology
Sylhet-3114,
Bangladesh*

Dr. Bayden R. Wood

*Australian Synchrotron Program
Research Fellow and Monash Synchrotron
Research Fellow Centre for Biospectroscopy
School of Chemistry Monash University Wellington
Rd. Clayton,
3800 Victoria,
Australia*

Dr. G. Reza Balali

*Molecular Mycology and Plant Pathology
Department of Biology
University of Isfahan
Isfahan
Iran*

Dr. Beatrice Kilel

*P.O Box 1413
Manassas, VA 20108
USA*

Prof. H. Sunny Sun

*Institute of Molecular Medicine
National Cheng Kung University Medical College
1 University road Tainan 70101,
Taiwan*

Prof. Ima Nirwana Soelaiman

*Department of Pharmacology
Faculty of Medicine
Universiti Kebangsaan Malaysia
Jalan Raja Muda Abdul Aziz
50300 Kuala Lumpur,
Malaysia*

Prof. Tunde Ogunsanwo

*Faculty of Science,
Olabisi Onabanjo University,
Ago-Iwoye.
Nigeria*

Dr. Evans C. Egwim

*Federal Polytechnic,
Bida Science Laboratory Technology Department,
PMB 55, Bida, Niger State,
Nigeria*

Prof. George N. Goulielmos

*Medical School,
University of Crete
Voutes, 715 00 Heraklion, Crete,
Greece*

Dr. Uttam Krishna

*Cadila Pharmaceuticals limited ,
India 1389, Tarsad Road,
Dholka, Dist: Ahmedabad, Gujarat,
India*

Prof. Mohamed Attia El-Tayeb Ibrahim

*Botany Department, Faculty of Science at Qena,
South Valley University, Qena 83523,
Egypt*

Dr. Nelson K. Ojijo Olang'o

*Department of Food Science & Technology,
JKUAT P. O. Box 62000, 00200, Nairobi,
Kenya*

Dr. Pablo Marco Veras Peixoto

*University of New York NYU College of Dentistry
345 E. 24th Street, New York, NY 10010
USA*

Prof. T E Cloete

*University of Pretoria Department of
Microbiology and Plant Pathology,
University of Pretoria,
Pretoria,
South Africa*

Prof. Djamel Saidi

*Laboratoire de Physiologie de la Nutrition et de
Sécurité
Alimentaire Département de Biologie,
Faculté des Sciences,
Université d'Oran, 31000 - Algérie
Algeria*

Dr. Tomohide Uno

*Department of Biofunctional chemistry,
Faculty of Agriculture Nada-ku,
Kobe., Hyogo, 657-8501,
Japan*

Dr. Ulises Urzúa

*Faculty of Medicine,
University of Chile Independencia 1027, Santiago,
Chile*

Dr. Aritua Valentine

*National Agricultural Biotechnology Center,
Kawanda
Agricultural Research Institute (KARI)
P.O. Box, 7065, Kampala,
Uganda*

Prof. Yee-Joo Tan

*Institute of Molecular and Cell Biology 61 Biopolis
Drive,
Proteos, Singapore 138673
Singapore*

Prof. Viroj Wiwanitkit

*Department of Laboratory Medicine,
Faculty of Medicine, Chulalongkorn University,
Bangkok
Thailand*

Dr. Thomas Silou

*Universit of Brazzaville BP 389
Congo*

Prof. Burtram Clinton Fielding

*University of the Western Cape
Western Cape,
South Africa*

Dr. Brnčić (Brncic) Mladen

*Faculty of Food Technology and Biotechnology,
Pierottijeva 6,
10000 Zagreb,
Croatia.*

Dr. Meltem Sesli

*College of Tobacco Expertise,
Turkish Republic, Celal Bayar University 45210,
Akhisar, Manisa,
Turkey.*

Dr. Idress Hamad Attitalla

*Omar El-Mukhtar University,
Faculty of Science,
Botany Department,
El-Beida, Libya.*

Dr. Linga R. Gutha

*Washington State University at Prosser,
24106 N Bunn Road,
Prosser WA 99350-8694.*

Dr Helal Ragab Moussa

*Bahnay, Al-bagour, Menoufia,
Egypt.*

Dr VIPUL GOHEL

*DuPont Industrial Biosciences
Danisco (India) Pvt Ltd
5th Floor, Block 4B,
DLF Corporate Park
DLF Phase III
Gurgaon 122 002
Haryana (INDIA)*

Dr. Sang-Han Lee

*Department of Food Science & Biotechnology,
Kyungpook National University
Daegu 702-701,
Korea.*

Dr. Bhaskar Dutta

*DoD Biotechnology High Performance Computing
Software Applications
Institute (BHSAI)
U.S. Army Medical Research and Materiel
Command
2405 Whittier Drive
Frederick, MD 21702*

Dr. Muhammad Akram

*Faculty of Eastern Medicine and Surgery,
Hamdard Al-Majeed College of Eastern Medicine,
Hamdard University,
Karachi.*

Dr. M. Muruganandam

*Department of Biotechnology
St. Michael College of Engineering & Technology,
Kalayarkoil,
India.*

Dr. Gökhan Aydin

*Suleyman Demirel University,
Atabey Vocational School,
Isparta-Türkiye,*

Dr. Rajib Roychowdhury

*Centre for Biotechnology (CBT),
Visva Bharati,
West-Bengal,
India.*

Dr Takuji Ohyama

Faculty of Agriculture, Niigata University

Dr Mehdi Vasfi Marandi

University of Tehran

Dr Fügen DURLU-ÖZKAYA

*Gazi University, Tourism Faculty, Dept. of
Gastronomy and Culinary Art*

Dr. Reza Yari

Islamic Azad University, Boroujerd Branch

Dr Zahra Tahmasebi Fard

Roudehen branche, Islamic Azad University

Dr Albert Magrí

Giro Technological Centre

Dr Ping ZHENG

Zhejiang University, Hangzhou, China

Dr. Kgomotso P. Sibeko

University of Pretoria

Dr Greg Spear

Rush University Medical Center

Prof. Pilar Morata

University of Malaga

Dr Jian Wu

Harbin Medical University, China

Dr Hsiu-Chi Cheng

National Cheng Kung University and Hospital.

Prof. Pavel Kalac

University of South Bohemia, Czech Republic

Dr Kürsat Korkmaz

*Ordu University, Faculty of Agriculture,
Department of Soil Science and Plant Nutrition*

Dr. Shuyang Yu

*Department of Microbiology, University of Iowa
Address: 51 newton road, 3-730B BSB bldg. Iowa
City, IA, 52246, USA*

Dr. Binxing Li

Dr. Mousavi Khaneghah

*College of Applied Science and Technology-
Applied Food Science, Tehran, Iran.*

Dr. Qing Zhou

*Department of Biochemistry and Molecular
Biology,
Oregon Health and Sciences University Portland.*

Dr Legesse Adane Bahiru

*Department of Chemistry,
Jimma University,
Ethiopia.*

Dr James John

*School Of Life Sciences,
Pondicherry University,
Kalapet, Pondicherry*

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

Copyright: © 2015, Academic Journals.

All rights Reserved. In accessing this journal, you agree that you will access the contents for your own personal use but not for any commercial use. Any use and or copies of this Journal in whole or in part must include the customary bibliographic citation, including author attribution, date and article title.

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.

Disclaimer of Warranties

In no event shall Academic Journals be liable for any special, incidental, indirect, or consequential damages of any kind arising out of or in connection with the use of the articles or other material derived from the AJB, whether or not advised of the possibility of damage, and on any theory of liability.

This publication is provided "as is" without warranty of any kind, either expressed or implied, including, but not limited to, the implied warranties of merchantability, fitness for a particular purpose, or non-infringement. Descriptions of, or references to, products or publications does not imply endorsement of that product or publication. While every effort is made by Academic Journals to see that no inaccurate or misleading data, opinion or statements appear in this publication, they wish to make it clear that the data and opinions appearing in the articles and advertisements herein are the responsibility of the contributor or advertiser concerned. Academic Journals makes no warranty of any kind, either express or implied, regarding the quality, accuracy, availability, or validity of the data or information in this publication or of any other publication to which it may be linked.

ARTICLES

Genetic diversity analysis of mustard (*Brassica* spp.) germplasm using molecular marker for selection of short duration genotypes

Md. Shahin Iqbal, Islam Hamim, Md. Shamiul Haque and Ujjal Kumar Nath

Isolation and identification of native lower fungi for polyunsaturated fatty acid (PUFA) production in Thailand, and the effect of carbon and nitrogen sources on growth and production

Supavadee Deelai, Satinee Suetrong, Somchit Damrianant, Panida Unagul and Niramol Sakkayawong

Molecular genetic diversity study of *Lepidium sativum* population from Ethiopia as revealed by inter simple sequence repeat (ISSR) markers

Said Mohammed and Kassahun Tesfaye

Effects of selected culture media on mycellial growth of oyster mushroom (*Pleurotus ostreatus*)

Chuku, S. B., Nwachukwu, E. O. and Stanley, H. O.

The population structure of wild sorghum species in agro-ecological zones of Western Kenya

Titus O. Magomere, Eliud K. Ngugi, Silas D. Obukosia, Eunice Mutitu and Solomon I. Shibairo

Efficacy of fish compost on management of chilli fruit rot caused by *Colletotrichum capsici*

H. Ann Suji and T. Suthin Raj

Activity of the fungus *Pleurotus ostreatus* and of its proteases on *Panagrellus* sp. larvae

Hugo Leonardo André Genier, Filippe Elias de Freitas Soares, José Humberto de Queiroz, Angélica de Souza Gouveia, Jackson Victor Araújo, Fabio Ribeiro Braga, Iara Rebouças Pinheiro and Maria Catarina Megumi Kasuya

Table of Contents: Volume 14 Number 17, 29 April, 2015

Enhanced recombinant human blood coagulation factor VIII expression in the milk of goats using replicationdefective adenovirus

Zengsheng Han, Lin Wang, Kun Li, Jian Li, Hongwei Zhao, Zhiwei Liu, Wenguang Cao and Qingwang Li

Insights on predominant edible bamboo shoot proteins

Sayanika Devi Waikhom, Louis Bengyella, Pranab Roy and Narayan Chandra Talukdar

Effect of the somatic cell count on physicochemical components of milk from crossbred cows

Bruna Ariel Dias Guariglia, Priscila Alonso dos Santos, Liomar de Souza Araújo, Cristiane Isabo Giovannini, Rodrigo Balduino Soares Neves, Edmar Soares Nicolau and Marco Antônio Pereira da Silva

Full Length Research Paper

Genetic diversity analysis of mustard (*Brassica* spp.) germplasm using molecular marker for selection of short duration genotypes

Md. Shahin Iqbal^{1*}, Islam Hamim², Md. Shamiul Haque³ and Ujjal Kumar Nath⁴

¹Scientific Officer, Plant Breeding Division, Pulses Research Centre, Bangladesh Agricultural Research Institute, Ishwardi, Pabna, Bangladesh.

²Assistant Professor, Department of Plant Pathology, Bangladesh Agricultural University, Mymensingh, Bangladesh.

³Scientific Officer, Substation, Bangladesh Institute of Nuclear Agriculture, Comilla, Bangladesh.

⁴Professor, Department of Genetics and Plant Breeding, Bangladesh Agricultural University, Mymensingh, Bangladesh.

Received 21 January, 2015; Accepted 22 April, 2015

Molecular characterization of 16 mustard (*Brassica* spp.) genotypes by using 12 RAPD markers revealed that three primers GLA-11, OPB-04 and OPD-02 showed good technical resolution and sufficient variations among different genotypes. A total of 40 RAPD bands were scored of which 38 (94.87%) polymorphic amplification products were obtained. Besides, the primer OPD-02 amplified maximum number of polymorphic bands (100.00%) while the primer GLA-11 and OPB-04 generated the least (92.31%) polymorphic bands, which were minimal in number. The present study produced 13.33 scorable bands per primer and 12.67 polymorphic bands per primer. Frequencies of maximum number of polymorphic loci were found to be high with the exception of GLA-11(0.750), OPB-04 (0.875) and OPD-02 (0.750). The estimate of Nei's genetic diversity for the entire genotypes of mustard was 0.3596 and Shannon's information index was 0.535. There was a high level of genetic variation among the mustard genotypes studied from the proportion of polymorphic loci point of view. The values of pair-wise comparison of Nei's genetic distance between genotypes were computed from combined data for the three primers; ranged from 0.1054 to 0.9862. BINA Sarisha-3 and BINA Sarisha-4 showed the lowest genetic distance of 0.1054 where Tori-7 and NAP-0758-2 showed highest genetic distance of 0.9862. The 16 mustard genotypes were differentiated into three main clusters: BARI Sarisha-14, BARI Sarisha-9, BARI Sarisha-15, BINA Sarisha-4, BINA Sarisha-3, BARI Sarisha-8, Sampad and Tori-7 in cluster A, NAP-0763, NAP-0721-1, BARI Sarisha-4, BARI Sarisha-6, NAP-0762-2 and NAP-0848-2 in cluster B and NAP-0838 and NAP-0758-2 were grouped into cluster C by making dendrogram based on Nei's genetic distance using unweighted pair group method of arithmetic means (UPGMA).

Key words: Mustard, diversity analysis, RAPD marker, genetic distance, cluster analysis.

INTRODUCTION

Oilseed *Brassic*as occupy an important position in the rainfed agriculture throughout the world. In most of the regions of the world, its cultivation has increased

drastically during the last decades and, by now; it is the third largest contributor of the world supply of vegetable oil. Rapeseed is one of the most important oil and protein

rich annual crops in the world. Seed provides oil both for industrial and culinary purposes. The oils extracted from mustard contain high protein (37%), and feed concentration which is highly palatable to livestock. In Bangladesh, the seed yield of mustard/rapeseed is about 1000 kg/ha, which is very low in comparison to other developed countries. On the other hand, the area of cultivation of mustard in Bangladesh is lower due to rice based cropping system which is difficult to change. Most of the released mustard cultivars are long day duration (105 to 115 days) and thus, did not fit well for cultivation between *Aman* and *Boro* rice cropping system. Where a long duration mustard crop is grown after *Aman* and prior to the *Boro*, however, the transplanting of *Boro* may be pushed into February, resulting in a later harvest and greater exposure to early flood risk. So, if we can develop the short duration (75 to 80 days) lines, which would be successfully cultivated between *Aman* and *Boro* rice rotation without affecting this popular cropping pattern. So, it is urgent to analyze the genetic diversity and its response for the selection of short duration mustard genotypes for increasing our cropping intensity.

Diversity at marker loci is currently the most feasible strategy for characterizing diversity in mustard. Molecular markers provide the best estimate of genetic diversity, since these are independent of the confounding effects of environmental factors. In mustard, various marker systems have been used for assessing the genetic diversity. There is increasing number of reports where molecular markers like restriction fragment length polymorphism (RFLP) (Diers and Osborn, 1994; Hallden et al., 1994), random amplified polymorphic DNAs, (RAPDs) (Ghosh et al., 2009; Yildirim et al., 2010; Khan et al., 2011), amplified fragment length polymorphism (AFLP) (Sun et al., 2001; Zhao et al., 2005) and microsatellites or simple sequence repeats (SSRs) (Abbas et al., 2009; Wang et al., 2009) have been used to study genome organization, varietal differences and diversity analysis in *Brassicaceae*. Among molecular marker RAPDs are increasingly being employed in genetic research owing to their speed and simplicity (Williams et al., 1990; Welsh and McClelland, 1990). In mustard, RAPD method can be used as fast and effective approaches for genetic variation and relationship (Ananga et al., 2008), detecting polymorphism at the DNA level, genetic diversity analysis (Chen et al., 2000), measurement of genetic distance. It is important particularly for variety selection for breeding purpose, hybridization evaluation and conservation of their diverse

gene pool.

Iqbal et al. (2014) conducted an experiment entitled genetic diversity analysis of mustard germplasm based on phenotypic traits for selection of short duration genotypes in the same laboratory with same genotypes. In the present experiment, a comparative discussion was done between the diversity among the genotypes based on phenotypic traits and diversity obtained from molecular level. Keeping in mind the present study was undertaken to determine the genetic variability and diversity among different mustard genotypes.

MATERIALS AND METHODS

The experiment was conducted at the Molecular and Biotechnology Laboratory, Department of Genetics and Plant Breeding, Bangladesh Agricultural University, Mymensingh, to characterize 16 genotypes of mustard (Table 1) at molecular level with markers. DNA from 16 mustard genotypes were isolated from actively growing fresh leaves of 20 to 25 days old seedlings using Cetyl Trimethyl Ammonium Bromide (CTAB) method as described by Murray and Thompson (1980), and later modified by Doyle and Doyle (1990). DNA was precipitated with 800 μ l of absolute ethanol where DNA became visible as white strands by flicking the tube several times with fingers. DNA was pelleted by centrifugation and re-precipitation of the DNA solution was done by adding 400 μ l of 70% ethanol. The pellets were then air dried and dissolved in 50 μ l of TE buffer (10 mM Tris. HC1, 1 mM EDTA, pH = 8.0). DNA quality was checked by electrophoresis in 0.8% agarose gel and quantified using a spectrophotometer at 260 nm wave length (Spectronic GENESISTM). The DNA samples were evaluated both quantitatively and qualitatively using spectrophotometer and agarose gel electrophoresis, respectively. The primers used in RAPD analysis were found to discriminate different genotypes (two randomly chosen individuals from two different genotypes to evaluate their suitability for amplification of the DNA sequences) effectively. Among the 12 primers (Table 2) initially tested, 3 primers (GLA-11, OPB-04 and OPD-02) yielded comparatively maximum number of amplification products with high intensity and minimal smearing, good resolution and also clear bands which were selected for further analysis.

DNA amplification by PCR and electrophoresis

The amplification conditions originally recommended by Williams et al. (1990) were applied with slight modifications. The following components were used to prepare PCR cocktail (Table 3). The total volume of PCR cocktail was 8 μ l per sample. 2 μ l genomic DNA was added with 8 μ l PCR cocktail and finally, total volume was 10 μ l. DNA amplification was performed in an oil-free thermal cycler. The PCR tubes were set on the wells of the thermocycler plate. Then, the machine was run according to the following setup: Initial denaturation at 94°C for 3 min; denaturation at 94°C for 1 min;

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

Abbreviations: UPGMA, Unweighted pair group method of arithmetic means; RFLP, restriction fragment length polymorphism; RAPDs, random amplified polymorphic DNAs; AFLP, amplified fragment length polymorphism; SSRs, simple sequence repeats; CTAB, cetyl trimethyl ammonium bromide.

Author(s) agree that this article remains permanently open access under the terms of the [Creative Commons Attribution License 4.0 International License](https://creativecommons.org/licenses/by/4.0/)

Table 1. List of the mustard genotypes used in the experiment.

Name of the genotypes	Name of the species	Sources of the genotypes
BARI Sarisha-4	<i>Brassica campestris</i> L.	BARI
BARI Sarisha-6	<i>Brassica campestris</i> L.	BARI
BARI Sarisha-8	<i>Brassica napus</i> L.	BARI
BARI Sarisha-9	<i>Brassica campestris</i> L.	BARI
BARI Sarisha-14	<i>Brassica campestris</i> L.	BARI
BARI Sarisha-15	<i>Brassica campestris</i> L.	BARI
Tori-7	<i>Brassica campestris</i> L.	BARI
Sampad	<i>Brassica campestris</i> L.	BAU
BINA Sarisha-3	<i>Brassica campestris</i> L.	BINA
BINA Sarisha-4	<i>Brassica campestris</i> L.	BINA
NAP-0763	<i>Brassica napus</i> L.	RARS, Jamalpur
NAP-0762-2	<i>Brassica napus</i> L.	RARS, Jamalpur
NAP-0838	<i>Brassica napus</i> L.	RARS, Jamalpur
NAP-0721-1	<i>Brassica napus</i> L.	RARS, Jamalpur
NAP-0758-2	<i>Brassica napus</i> L.	RARS, Jamalpur
NAP-0842-2	<i>Brassica napus</i> L.	RARS, Jamalpur

The genotypes of BARI and BINA are the varieties and the genotypes of RARS, Jamalpur are the advanced lines.

Table 2. Parameters of the random primers used in the present study for screening

Primer code	Sequence (5'- 3')
OPA-01	CAGGCCCTTC
OPA-02	TGCCGAGCTG
OPA-03	AGTCAGCCAC
GLA-5	AGGGGTCTTG
GLA-9	GGGTAACGCC
GLA-11*	CAATCGCCGT
OPB-02	TGATCCCTGG
OPB-04*	GGACTGGAGT
OPC-01	TTCGAGCCAG
OPC-02	GTGAGGCGTC
OPG-5	GTGATCGCAG
OPD-02*	GGACCCAACC

*Selected for RAPD analysis of all samples of the 16 mustard genotypes.

Table 3. Components of PCR cocktail (for 8 reactions).

Reagent	Amount per sample (µl)	Total (µl)
Ampli <i>Taq</i> polymerase buffer	1.0	8
Primer	2.5	20
dNTPs	1.0	8
Ampli <i>Taq</i> DNA polymerase	0.2	1.6
Sterile deionized water	3.3	26.4

annealing at 35°C for 1 min; elongation or extension at 72°C for 2 min; Cycle to step 2 for 40 more time; incubation at 72°C for 7 min;

completion of cycling program (45 cycles), reactions were held at 4°C. The amplified products were separated electrophoretically on

Table 4. RAPD primers with corresponding bands score and their size range together with polymorphic bands observed in 16 mustard genotypes.

Primer code	Sequences (5'-3')	Total number of bands scored	Band size ranges (bp)	Number of polymorphic bands	Proportion of polymorphic loci (%)
GLA-11	CAATCGCCGT	13	220-950	12	92.31
OPB-04	GGA CTGGAGT	13	250-980	12	92.31
OPD-02	GGACCCAACC	14	150-1000	14	100.00
Total		40		38	284.62
Average		13.33		12.67	94.87

1.5% agarose gel containing ethidium bromide. Molecular weight marker of 100 bp DNA ladder was electrophoresed alongside with the PCR products. DNA bands were observed on UV-transilluminator in the dark chamber of the Image Documentation System (uvitec, UK) and the image was viewed on the monitor, focused, acquired, saved and printed on thermal paper.

RAPD data analysis

All distinct bands or fragments (RAPD markers) were thereby given identification numbers according to their position on gel and scored visually on the basis of their presence (1) or absence (0), separately for each individual and each primer. The scores obtained using all primers in the RAPD analysis were then pooled to create a single data matrix. This was used to estimate polymorphic loci, Nei's (1973), gene diversity, population differentiation, (G_{st}), gene flow (N_m), genetic distance (D) and to construct a UPGMA dendrogram among populations using a computer program, POPGENE (Version 1.31) (Yeh et al., 1999).

RESULTS AND DISCUSSION

Twelve (12) primers were initially employed on 16 mustard genotypes for their ability to produce polymorphic bands and 3 primers (GLA-11, OPB-04 and OPD-02) which gave reproducible and distinct polymer-ic amplified products were selected. DNA amplification from all the primers tested in this study was not consistently reproducible and is a very common feature of RAPD technique. The present findings agree with those of Hadrys et al. (1992) and Williams et al. (1993). Technical problems from amplification of the RAPD technique in the field of genetic population research have also been reported by many authors Schierwater and Ender, 1993; Lynch and Milligan, 1994). A total of 40 RAPD bands were scored of which 38 (94.87%) polymorphic amplification products were obtained by using these arbitrary primers (Table 4). The size of the amplification products ranged from 100 to 1000 bp. Ghosh et al. (2009) reported 59 reproducible DNA bands generated by four arbitrary selected primers of which 58 (98.03%) bands were proved to be polymorphic and these bands ranged from 212 to 30686 bp in size. The selected 3 primers produced comparatively maximum number of high intensity band with minimal smearing, good technical resolution and sufficient variation among

different variants. The dissimilar numbers of bands were generated by primer GLA-11, OPB-04 and OPD-02 (Table 4). Besides, the primer OPD-02 amplified maximum number of polymorphic bands (100.00%) while the primer GLA-11 and OPB-04 generated the least (92.31%) polymorphic bands which were minimal in number. The banding patterns of 16 mustard genotypes using primers GLA-11, OPB-04 and OPD-02 are shown in Figures 1, 2 and 3 respectively. Number of RAPD markers scored for each individual 16 mustard genotypes for each primer are presented in Table 4.

A total of 254 clear and repeatable bands were amplified from three RAPD primers (Table 5). The primers GLA-11, OPB-04 and OPD-02 produced 74, 85 and 95, respectively polymorphic bands in 16 mustard genotypes. This proportion of polymorphism is higher compared to some previous molecular analysis in mustard genotypes in primers are confirmed in higher percentage of polymorphism. The present experiment produced 13.33 scorable bands per primer and 12.67 polymorphic bands per primer. The reasons of the considerable number of average scorable and polymorphic bands consist of 60 to 70% GC content. Fukuoka et al. (1992) observed an increase in the number of bands with increasing GC content of the primer. The explanation for this correlation between the GC content of the primer and the number of bands is that the stability of base complementation is higher when G is pairing with C by three hydrogen bonds than that of the complementation of A with T by two hydrogen bonds. The DNA polymorphisms were detected according to the presence and absence of band. Absence of band may be caused by failure of primer to anneal a site in some individuals due to nucleotide, sequences difference or by insertions or deletions between primer sites Clark and Lanigan, 1993. Frequencies of maximum number of polymorphic loci were found to be high with the exception of GLA-11(0.750), OPB-04(0.875) and OPD -02 (0.750) (Table 6). The values of Nei's (1973) genetic diversity and Shannon's information index for different accessions of 16 mustard genotypes across all loci are shown in Table 7. The estimate of Nei's genetic diversity for entire genotypes of mustard was 0.3596 and Shannon's information index was 0.535. There was a high level of genetic variation among the studied mustard genotypes

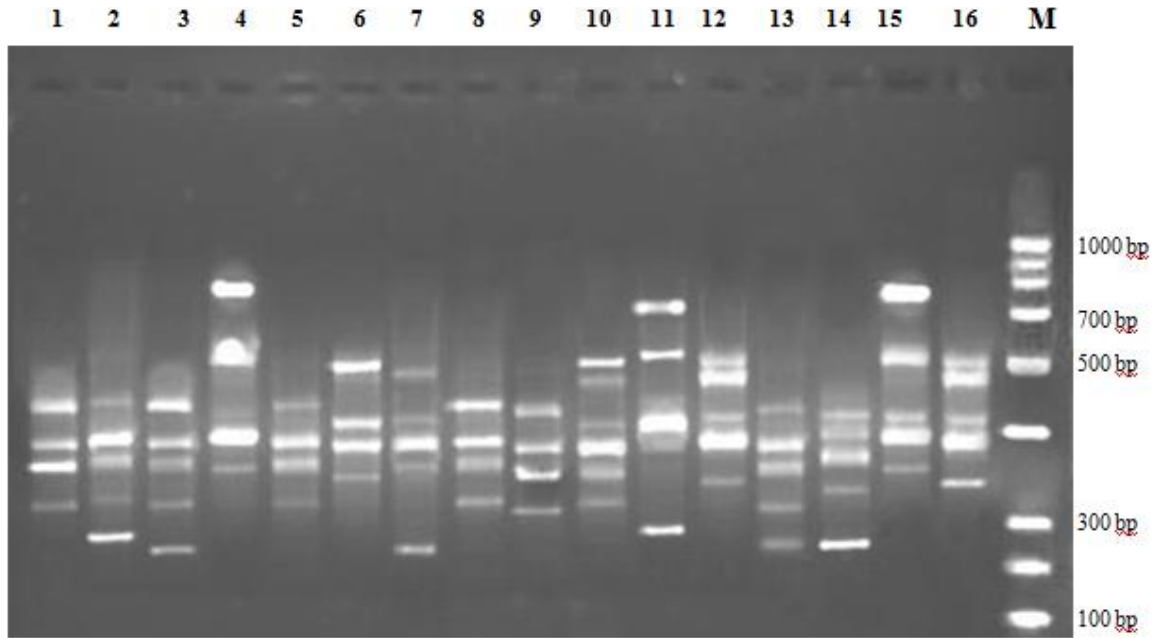


Figure 1. RAPD profile of 16 mustard genotypes using primer GLA-11. M, Molecular weight marker (100 bp DNA ladder in size). 1, BARI Sarisha-14; 2, Tori-7; 3, BARI Sarisha-8; 4, NAP-0763; 5, BINA Sarisha-4; 6, NAP-0721-1; 7, BARI Sarisha-4; 8, BINA Sarisha-3; 9, BARI Sarisha-15; 10, NAP-0838; 11, NAP-0762-2; 12, NAP-0848-2; 13, BARI Sarisha-9; 14, Sampad; 15, BARI Sarisha-6; 16, NAP-0758-2.

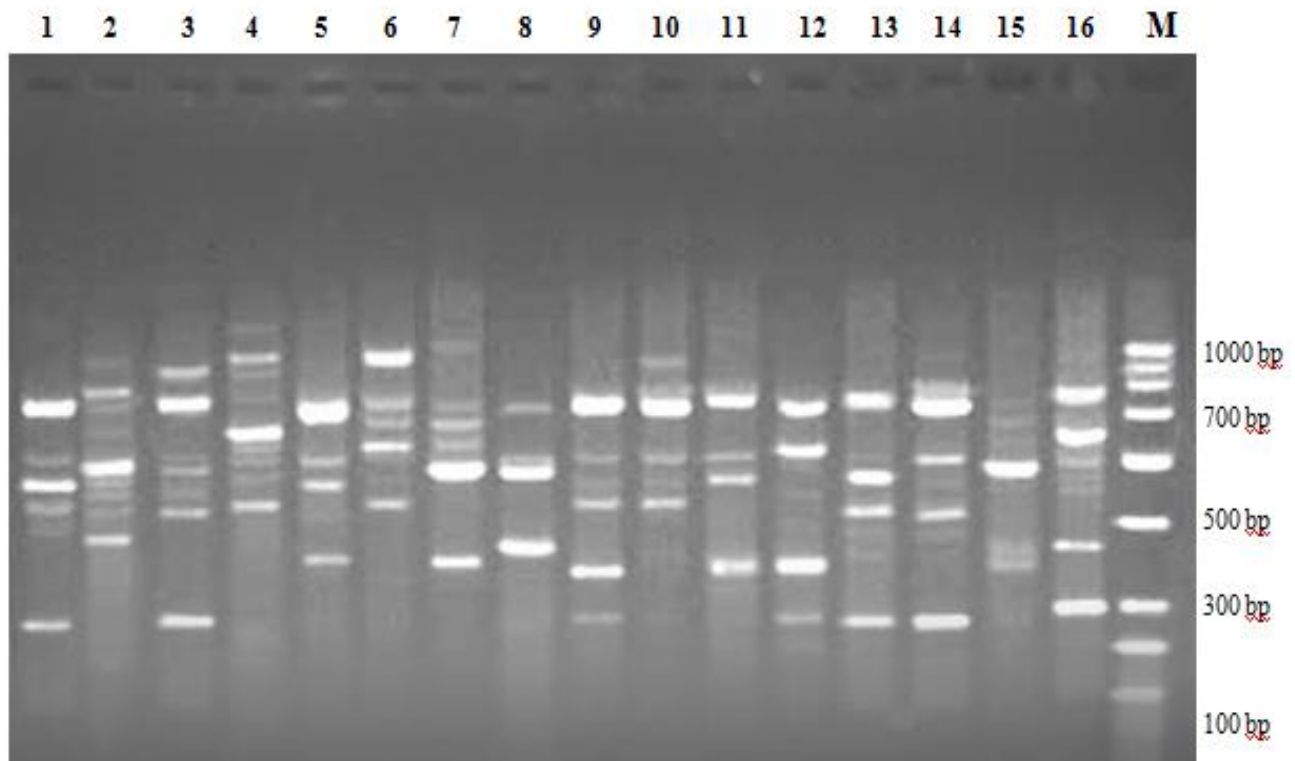


Figure 2. RAPD profiles of 16 mustard genotypes using primer OPB-04. M, Molecular weight marker (100 bp DNA ladder in size). 1, BARI Sarisha-14; 2, Tori-7; 3, BARI Sarisha-8; 4, NAP-0763; 5, BINA Sarisha-4; 6, NAP-0721-1; 7, BARI Sarisha-4; 8, BINA Sarisha-3; 9, BARI Sarisha-15; 10, NAP-0838; 11, NAP-0762-2; 12, NAP-0848-2; 13, BARI Sarisha-9; 14, Sampad; 15, BARI Sarisha-6; 16, NAP-0758-2.

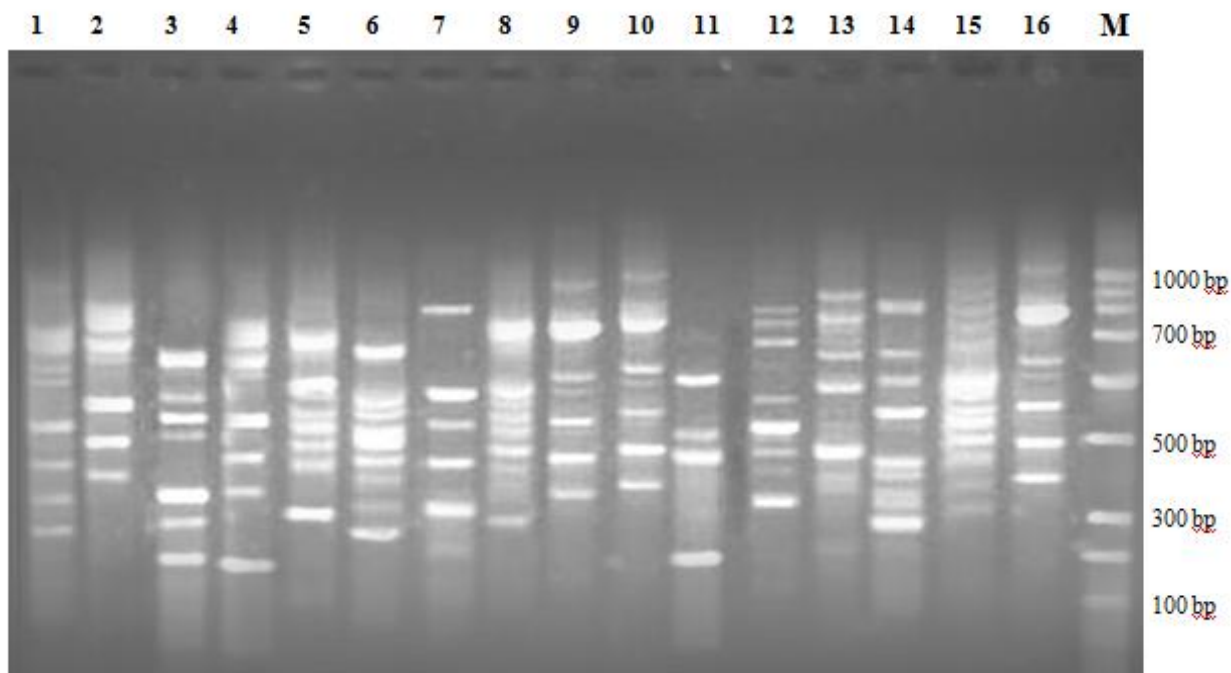


Figure 3 RAPD profiles of 16 mustard genotypes using primer OPD-02. M, Molecular weight marker (100 bp DNA ladder in size). 1, BARI Sarisha-14; 2, Tori-7; 3, BARI Sarisha-8; 4, NAP-0763; 5, BINA Sarisha-4; 6, NAP-0721-1; 7, BARI Sarisha-4; 8, BINA Sarisha-3; 9, BARI Sarisha-15; 10, NAP-0838; 11, NAP-0762-2; 12, NAP-0848-2; 13, BARI Sarisha-9; 14, Sampad; 15, BARI Sarisha-6; 16, NAP-0758-2.

Table 5. Number of polymorphic bands observed in 16 mustard genotypes after PCR amplification on with RAPD primers GLA-11, OPB-04 and OPD-02.

Genotype	GLA-11	OPB-04	OPD-02	Total bands
BARI Sarisha-14	4	6	6	16
Tori-7	4	8	5	17
BARI Sarisha-8	5	7	7	19
NAP-0763	4	8	6	18
BINA Sarisha-4	3	4	7	14
NAP-0721-1	4	5	6	15
BARI Sarisha-4	5	6	5	16
BINA Sarisha-3	4	4	7	15
BARI Sarisha-15	4	6	7	17
NAP-0838	6	6	5	17
NAP-0762-2	6	4	4	14
NAP-0848-2	5	4	7	16
BARI Sarisha-9	5	5	6	16
Sampad	5	5	8	18
BARI Sarisha-6	5	2	4	11
NAP-0758-2	5	5	5	15
Total bands	74	85	95	254

from the proportion of polymorphic loci point of view.

Considering the genetic distance values, the result

indicate that some mustard genotypes were genetically different from each other and some were tend to be

Table 6. Frequencies of polymorphic RAPD markers in mustard genotypes.

Loci	Gene frequency	Loci	Gene frequency
GLA11-1	0.1250	OPB04-8	0.8750
GLA11-2	0.0625	OPB04-9	0.3125
GLA11-3	0.5000	OPB04-10	0.5625
GLA11-4	0.2500	OPB04-11	0.2500
GLA11-5	0.4375	OPB04-12	0.4375
GLA11-6	0.5000	OPB04-13	0.4375
GLA11-7	0.0000	OPD02-1	0.1875
GLA11-8	0.7500	OPD02-2	0.1875
GLA11-9	0.2500	OPD02-3	0.4375
GLA11-10	0.1875	OPD02-4	0.5000
GLA11-11	0.5000	OPD02-5	0.1875
GLA11-12	0.1250	OPD02-6	0.5000
GLA11-13	0.2500	OPD02-7	0.1250
OPB04-1	0.0625	OPD02-8	0.7500
OPB04-2	0.1875	OPD02-9	0.4375
OPB04-3	0.2500	OPD02-10	0.7500
OPB04-4	0.1250	OPD02-11	0.5000
OPB04-5	0.8125	OPD02-12	0.5000
OPB04-6	0.1875	OPD02-13	0.1875
OPB04-7	0.3125	OPD02-14	0.1875

Table 7. Summary of genetic diversity and Shanon information index statistics for all loci.

Loci	Observed number of alleles	Effective number of alleles	Gene diversity (h)	Shanon information index (i)
GLA11-1	2.0000	1.2800	0.2188	0.3768
GLA11-2	2.0000	1.1327	0.1172	0.2338
GLA11-3	2.0000	2.0000	0.5000	0.6931
GLA11-4	2.0000	1.6000	0.3750	0.5623
GLA11-5	2.0000	1.9692	0.4922	0.6853
GLA11-6	2.0000	2.0000	0.5000	0.6931
GLA11-7	1.0000	1.0000	0.0000	0.0000
GLA11-8	2.0000	1.6000	0.3750	0.5623
GLA11-9	2.0000	1.6000	0.3750	0.5623
GLA11-10	2.0000	1.4382	0.3047	0.4826
GLA11-11	2.0000	2.0000	0.5000	0.6931
GLA11-12	2.0000	1.2800	0.2188	0.3768
GLA11-13	2.0000	1.6000	0.3750	0.5623
OPB04-1	2.0000	1.1327	0.1172	0.2338
OPB04-2	2.0000	1.4382	0.3047	0.4826
OPB04-3	2.0000	1.6000	0.3750	0.5623
OPB04-4	2.0000	1.2800	0.2188	0.3768
OPB04-5	2.0000	1.4382	0.3047	0.4826
OPB04-6	2.0000	1.4382	0.3047	0.4826
OPB04-7	2.0000	1.7534	0.4297	0.6211
OPB04-8	2.0000	1.2800	0.2188	0.3768
OPB04-9	2.0000	1.7534	0.4297	0.6211
OPB04-10	2.0000	1.9692	0.4922	0.6853
OPB04-11	2.0000	1.6000	0.3750	0.5623

Table 7. Contd.

Loci	Observed number of alleles	Effective number of alleles	Gene diversity (h)	Shanon information index (i)
OPB04-12	2.0000	1.9692	0.4922	0.6853
OPB04-13	2.0000	1.9692	0.4922	0.6853
OPD02-1	2.0000	1.4382	0.3047	0.4826
OPD02-2	2.0000	1.4382	0.3047	0.4826
OPD02-3	2.0000	1.9692	0.4922	0.6853
OPD02-4	2.0000	2.0000	0.5000	0.6931
OPD02-5	2.0000	1.4382	0.3047	0.4826
OPD02-6	2.0000	2.0000	0.5000	0.6931
OPD02-7	2.0000	1.2800	0.2188	0.3768
OPD02-8	2.0000	1.6000	0.3750	0.5623
OPD02-9	2.0000	1.9692	0.4922	0.6853
OPD02-10	2.0000	1.6000	0.3750	0.5623
OPD02-11	2.0000	2.0000	0.5000	0.6931
OPD02-12	2.0000	2.0000	0.5000	0.6931
OPD02-13	2.0000	1.4382	0.3047	0.4826
OPD02-14	2.0000	1.4382	0.3047	0.4826
Mean	1.9750	1.6183	0.3596	0.5350
Standard deviation	0.1581	0.3003	0.1269	0.1560

similar. The values of pair-wise comparison of Nei's genetic distance between genotypes were computed from combined data for the three primers, ranged from 0.1054 to 0.9862 which was near similar to previous study. The smaller number of pair-wise differences (high genetic similarity) among some genotypes was likely due to their genetical relatedness. On the other hand, large number of pair-wise differences (low genetic similarity) was observed among those genotypes developed from genetically distant parental lines. Comparatively higher genetic distance (0.9862) was found among Tori-7 vs. NAP-0758-2 genotypes pair than other genotypes combination. The lowest genetic distance (0.1054) was revealed among BINA Sarisha-3 vs. BINA Sarisha-4 as the genotypes were morphologically very similar. Considering the genetic distance values, the results indicated that the genotypes were genetically different from each other which could be used in breeding programme to have potential genetic gains.

Sixteen (16) mustard genotypes of the experiment were used to make dendrogram based on Nei's genetic distance using UPGMA. In this study, 16 mustard genotypes had been differentiated into three main clusters: BARI Sarisha-14, BARI Sarisha-9, BARI Sarisha-15, BINA Sarisha-4, BINA Sarisha-3, BARI Sarisha-8, Sampad and Tori-7 in Cluster A, NAP-0763, NAP-0721-1, BARI Sarisha-4, BARI Sarisha-6, NAP-0762-2 and NAP-0848-2 in cluster B and NAP-0838 and NAP-0758-2 were grouped in cluster C (Figure 4). Genotypes included in cluster A were totally different from the genotypes included in cluster B and cluster C. So, genetic relationship was not present between cluster A genotypes with cluster B and cluster C. The cluster A

was divided into two sub-clusters. In sub-cluster II of cluster A, there was Tori-7 alone whereas, sub-cluster I was divided in two sub-sub clusters. In sub-sub cluster II, there was BARI Sarisha-8 and Sampad whereas sub-sub cluster I was divided in two sub-sub-sub clusters. In sub-sub-sub cluster II, there was BINA Sarisha-3 and BINA Sarisha-4 whereas sub-sub-sub cluster I was divided in two sub-sub-sub-sub clusters. Sub-sub-sub-sub cluster I included BARI Sarisha-14 and BARI Sarisha-14 and Sub-sub-sub-sub cluster II included only BARI Sarisha-15. The cluster B was divided into two sub-clusters. In sub-cluster I of cluster B, there was NAP-0763 and NAP-0721-1 whereas, sub-cluster II was divided in two sub-sub clusters. Sub-sub cluster I included BARI Sarisha-4 and BARI Sarisha-6 and Sub-sub cluster II included NAP-0762-2 and NAP-0838-2. Finally, the cluster C included NAP-0838 and NAP-0758-2. According to Ghosh et al. (2009), the 9 accessions were capable of being classified into 2 major groups. One group consists of BARI Sarisha-12, Agrani, Sampad, Daulot, Rai-5, Alboglabra and another group contained BINA Sarisha-4, BINA Sarisha-5, and BARI Sarisha-13. Genotypic variations based on molecular characterization indicated that genotypes belonging to different clusters depend on their genetic components itself, but not at geographical origin at all. Therefore, it could be concluded that for further research program, especially for hybridization, genotype could be selected from different clusters, to achieve maximum heterosis regarding yield.

Iqbal et al. (2014) conducted an experiment entitled genetic diversity analysis of mustard germplasm based on phenotypic traits for selection of short duration genotypes in the same laboratory with same genotypes.

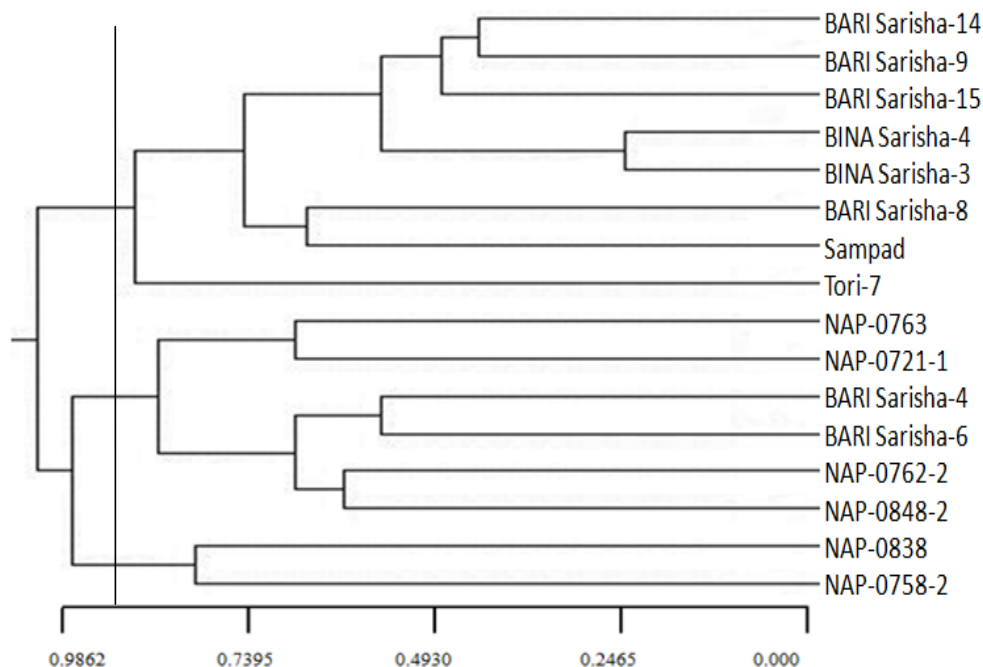


Figure 4. Unweighted pair group method of arithmetic mean (UPGMA) dendrogram based on Nei's genetic distance; summarizing data on differentiation in 16 mustard genotypes according to RAPD analysis.

They suggested that, on basis of lowest days to mature groups included Tori-7, BARI Sarisha-14, BARI Sarisha-9, BARI Sarisha-15 can be crossed with higher seed yield group which included NAP-0838, NAP-0758-2, NAP-0762-2, NAP-0848-2 and BARI Sarisha-4 for developing short duration high yielding varieties.

Now after the comparison between the results of phenotypic traits and molecular markers, it can be revealed that genotypes that belongs to lowest days to mature groups represents the genotypes of cluster I of molecular markers and genotypes that belongs to clusters III and II of molecular markers represents most of the genotypes of higher seed yield group. Therefore, it could be concluded that the next crossing combination might be between the genotypes: Tori-7, BARI Sarisha-14, BARI Sarisha-9, BARI Sarisha-15 and Sampad with the genotypes: NAP-0838, NAP-0758-2, NAP-0762-2, and NAP-0848-2 for developing short duration high yielded variety which are genotypically and phenotypically significantly differentiated.

Conflict of interests

The authors did not declare any conflict of interest.

ACKNOWLEDGEMENT

Ministry of Science and Information and Communication

Technology (MOSICT), Govt. of the People Republic of Bangladesh for awarding him National Science and Technology research fellowship (NST), 2012-2013.

REFERENCES

- Abbas SJ, Ullah F, Marwat KB, Khan IA, Munir I (2009). Molecular analysis of genetic diversity in *Brassica* species. Pak. J. Bot. 41(1): 167-176.
- Anthony OA, Ernst C, Khairy S, Ramesh K, Koffi K, Joel WO (2008). Phylogenetic relationships within and among *Brassica* species from RAPD loci associated with blackleg resistance. Afr. J. Biotechnol. 7(9):1287-1293.
- Chen YP, Cao JS, Miao Y, Zhi YW (2000). Analysis of genetics polymorphisms in vegetables crops of *Brassica campestris* by RAPD markers. J. Zhejiang Univ. Agric. Life Sci. 26: 131-136.
- Clark AG, Lanigan CMS (1993). Prospects for estimating nucleotide divergence with RAPDS. Mol. Biol. Evol. 10: 1069-1111.
- Diers BW, Osborn TC (1994). Genetic diversity of oilseed *Brassica napus* germplasm based on Restriction Fragment Length Polymorphisms. Theor. Appl. Genet. 88:662-668.
- Fukuoka S, Hosaka K, Kamijima O (1992). Use of random amplified polymorphic DNAs (RAPDs) for identification of rice accessions. Jpn. J. Genet. 67: 243-252.
- Ghosh KK, Haque ME, Parvin MS, Akhter F, Rahim MM (2009). Genetic diversity analysis in *Brassica* varieties through RAPD markers. Bangladesh J. Agril. Res. 34(3): 493-503.
- Hadrys H, Balick M, Schierwater, B (1992). Application of Random Amplified Polymorphic DNA (RAPD) in molecular ecology. Mol. Ecol. 1: 55-63.
- Hallden C, Nilsson NO, Rading IM, Sall T (1994). Evaluation of RFLP and RAPD markers in a comparison of *Brassica napus* breeding lines. Theor. Appl. Genet. 88:123-128.
- Iqbal MS, Haque MS, Nath UK, Hamim I (2014). Genetic diversity analysis of mustard germplasm based on phenotypic traits for selection

- of short duration genotypes. *Int. J. Agric. Sci. Res.* 3(8):141-156.
- Khan W M, Munir I, Farhatullah , Arif M, Iqbal A, Ali I, Ahmad D, Ahmad M, Mian A, Bakht J, Inamullah, Swati ZA (2011). Assessment of genetic diversity of *Brassica juncea* germplasm using Randomly Amplified Polymorphic DNA (RAPD) markers. *Afr. J. Biotechnol.* 10:3654-3658.
- Lynch M, Milligan BG (1994). Analysis of population genetic structure with RAPD markers. *Mol. Ecol.* 3:91-99
- Nei M (1973). Analysis of gene diversity in subdivided populations. *Proc. Natl. Acad. Sci.* 70:3321-3323.
- Schierwater B, Ender A (1993). Different thermostable DNA polymerasemay amplify different RAPD products. *Nucleic Acid Res.* 21:4647-4648.
- Sun D L, Zhao QC, Song WQ, Chen RY (2001). Relationships analysis of Chinese cabbage species by AFLP. *Acta Horticulture Sinica* 28:331-335.
- Wang J, Kaur S, Cogan NOI, Dobrowolski MP, Salisbury PA, Burton WA, Baillie R, Hand M, Hopkins C, Forster JW, Smith KF, Spangenberg G (2009). Assessment of genetic diversity in Australian canola (*Brassica napus* L.) cultivars using SSR markers. *Crop Pasture Sci.* 60:1193-1201.
- Welsh J, McClelland M (1990). Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Res.* 18:7213-7218.
- Williams JGK, Hanafey MK, Rafalski JA, Tingey SV (1993). Genetic analysis using random amplified polymorphic DNA markers. *Methods Enzymol.* 218:705-740.
- Williams JGK, Kubelik AE, Levak KJ, Rafalski JA, Tingey SC (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* 28:6531-6535.
- Yildirim E, Yildirim N, Ercisli S, Agar G, Karlidag H (2010). Genetic relationships among turnip (*Brassica rapa* var. *rapa*) genotypes. *Genet. Mol. Res.* 9:987-993.
- Zhao JJ, Wang XW, Deng B, Lou P, Wu L, Sun RF, Xu ZY, Vromans J, Koornneef M, Bonnema G (2005). Genetic relationships within *Brassica rapa* as inferred from AFLP fingerprints. *Theor. Appl. Genet.* 110:1301-1314.

Full Length Research Paper

Isolation and identification of native lower fungi for polyunsaturated fatty acid (PUFA) production in Thailand, and the effect of carbon and nitrogen sources on growth and production

Supavadee Deelai¹, Satinee Suetrong², Somchit Damrianant¹, Panida Unagul² and Niramol Sakkayawong^{1*}

¹Department of Biotechnology, Faculty of Science and Technology, Thammasat University, Ransit Center, Klong Luang, Pathum Thani, 12120, Thailand.

²BIOTEC, National Science and Technology Development Agency, Klong Luang, Pathum Thani, 12120, Thailand.

Received 22 May, 2014; Accepted 7 April, 2015

This research focuses on isolation and identification of polyunsaturated fatty acid (PUFA) producing fungi from natural sources of Thailand, followed by experimental designs for carbon and nitrogen utilizations of the potential strains. The results show that 8 and 16 isolates of lower fungi from freshwater and wet-fallen leaves, respectively, could produce PUFAs. Among these isolates, the NR06 that was isolated from wet-fallen leaves at Tad Ta Phu waterfall, Nakhon Ratchasrima province. They showed the highest level productions of both biomass ($15.49 \pm 0.24 \text{ g} \cdot \text{l}^{-1}$) and total fatty acids ($16.44 \pm 0.30\%$). The major essential fatty acid composition was found to be arachidonic acid (ARA; C20:4n6) ($32.24 \pm 0.35\%$). The minors were linoleic acid (C18:2n6) ($8.26 \pm 0.59\%$) and γ -linoleic acid (GLA; C18:3n6) ($5.48 \pm 0.08\%$). Besides the morphological characterization, taxonomic identification by the 636 bp-ITS region sequencing and phylogenetic analysis were performed. It was demonstrated that the fungal isolate NR06 was classified in the closest species of *Mortierella elongata* with 99% similarity (GenBank accession no. KF181625). Statistically based experimental designs (Design Expert 7.1 software) were applied to the optimization of biomass and fatty acid production in the NR06 culture. Using the glucose as a carbon source, there was a significant effect on both biomass and ARA production (p -values < 0.0001), whereas using yeast extract as a nitrogen source affected only ARA production (p -value = 0.02). It was suggested that the NR06 isolate could be further optimized for an improvement as a potential ARA producing strain.

Key words: Polyunsaturated fatty acids (PUFAs), arachidonic acid (ARA), lower fungi, *Mortierella elongata*.

INTRODUCTION

Polyunsaturated fatty acids (PUFAs) are long-chain fatty acids containing more than one double bond in their backbone. Particularly, long chain PUFAs that are classified as ω -3 and ω -6 essential fatty acids (EFA), play important

roles in both biomedical and nutraceutical area (Certik and Shimizu, 1999). Their biological functions are conducted via two principles. Firstly, as a part of lipid subunits in membrane of cells and organelles, PUFAs

modulate membrane dynamics, fluidity, flexibility and selective permeability. They also regulate membrane-associated processes, including membranes transport system, intercellular communication, interactions with extracellular components, cell-stimulus responses and control of some gene expressions (Gill and Valivety, 1997). Secondly, as the precursors of eicosanoids (prostaglandins, prostacyclins, thromboxanes and leukotrienes), these metabolites are essential for maintaining and regulating many diverse metabolic processes. These processes reduce inflammation and pain, control cholesterol, triglyceride and lipoprotein levels in plasma, affect platelets composition and functions, and especially regulate cognitive and visual development of fetuses and infants (Ward and Singh, 2005). As a whole, PUFAs have an impact on various cellular biochemical activities, and they are implicated in both physiological and pathological conditions including carcinogenesis and cardiovascular diseases. Since the essential fatty acids could not be synthesized *de novo* in mammals as lack of the key enzyme desaturases, the principal polyunsaturated fatty acids, that is, arachidonic acid (C20:4; ARA), eicosapentaenoic acid (C20:5; EPA) and docosahexaenoic acid (C22:6; DHA) are nowadays extensively used as dietary supplements for health and infant formulas.

The major source of PUFAs is marine fish. Their global stocks are gradually limited, resulting in inadequate production in a near future. Besides, the despicable tastes and odors of fish oil, shows that some fish especially salmon, sardine, tuna and hake, are often contaminated with heavy metals (cadmium, lead and mercury) and organic pollutants (dioxins, dioxin-like compounds and furans) that are toxic to human (Domingo et al., 2007). Related to health risks derived from the environment contaminant found in fish, a remarkable promotion of fish consumption as a source of omega-3 PUFAs has been reviewed scientifically for adverse and beneficial effects (Domingo, 2007). Thus, several assorted sources containing various types of PUFAs, that is, microalgae, bacteria, yeast, fungi and transgenic plants have been alternatively proposed.

The green alga, *Parietochloris incina*, was reported to be a potent producer of ARA. Since more than 90% of the ARA was deposited onto storage triglyceride molecules, EFA bioavailability is important in further studies (Bigogno et al., 2002). While natural and transgenic plants are the main alternative sources of PUFAs for human consumption, oleaginous microorganisms, particularly fungi also gain prominence as potential sources. In comparison with the plants, besides the main advantages of less competition for land to grow and the independence to seasonal variations

variations, fatty acid profiles from fungi are naturally high in EFAs. They can also be induced to synthesize higher yields of targeted fatty acids by controlling environmental and nutritional conditions (Certik and Shimizu, 1999; Dyal and Narine, 2005). Since lipogenesis mechanism in certain strains was well studied, various desaturase and elongase mutants can be manipulated. Also, it is able to incorporate and transform exogenous fatty acids (Dong and Walker, 2008; Shimizu and Sakuradani, 2009). Moreover, high growth rates on wide substrates would provide utilizing cheap materials including local agricultural products and industrial by-products or wastes (Fakas et al., 2008; Gema et al., 2002). Production of fungal storage lipids as the single cell oils (SCO) have been obviously targeted to Zygomycetes especially of the genera *Cunninghamella*, *Mucor*, *Mortierella*, *Rhizopus* and *Zygorhynchus* (Yongmanitchai and Ward, 1989; Gill and Valivety, 1997; Gema et al., 2002; Shimizu and Sakuradani, 2009). However, lipid accumulation was observed when the organisms were under stress conditions, for example, a high carbon to nitrogen ratio in the growth medium (Murphy, 1991). Besides, some fungal strains have been indicated as promising sources of certain long chain fatty acids, the ability of their fermentation to compete economically with traditional sources of EFA is limited by relatively low productivities and excessively long fermentation times (Barclay and Zeller, 1996; Leman, 1997). So far, many researches have been focusing on finding potential species those produce high specific EFA with relatively inexpensive cost and effective fermentation systems for industrial scales.

The aim of this study was to isolate the native lower fungi producing PUFAs from Thailand. To the best of our knowledge, this is the first survey for the isolation of oleaginous fungi from a freshwater and wet-fallen leaves from various natural sources, for which no previous data are available. The potential isolates for PUFAs production would be further characterized and investigated. Taxonomic identification was carried out through molecular methods. The effect of carbon and nitrogen sources on growth and ARA production was also evaluated.

MATERIALS AND METHODS

Isolation of lower fungi

Freshwater from rivers, waterfalls and reservoirs, and wet-fallen leaves around there were samples for isolation of PUFA producing fungi. The samples were collected into sterile bottles, from 5 provinces of Thailand, that is, Lopburi, Rayong, Chonburi, Nokorn

*Corresponding author. E-mail: bunyason@hotmail.com. Tel: +66-2564-4444. Ext: 2835. Fax: +66-2564-4500.

Abbreviations: PUFAs, Polyunsaturated fatty acids; ARA, arachidonic acid.

Author(s) agree that this article remains permanently open access under the terms of the [Creative Commons Attribution License 4.0 International License](https://creativecommons.org/licenses/by/4.0/)

Ratchasima, Chachoengsao and Trang. For the freshwater samples, mixed vegetable seeds (basil seeds, hairy basil seeds, cucumber seeds and pumpkin seeds) were put into the vials containing 5 ml of distilled water, followed by autoclaving at 121°C for 15 min. The freshwater samples were added to the vials and incubated at 25°C for 24 h. When fungal mycelia appeared around the seeds, freshwater samples were poured out. To suppress bacterial growth, the fungal seeds were washed three times with 50 ml of the antibiotic mixture solution (0.25 mg·l⁻¹ of penicillin and 0.25 mg·l⁻¹ streptomycin). The fungal seeds were further cultivated on potato dextrose agar (PDA) at 25°C for three to five days. For the wet-fallen leaves samples, they were cut into 0.5 x 0.5 cm pieces and washed three times with 50 ml of the antibiotic mixture solution. Then they were transferred to glucose peptone yeast extract (GPY) agar (0.1% yeast extract, 0.1% peptone, 1.0% glucose, 1.6% sea salt and 1.5% agar) and incubated at ambient temperature for 24 h. The morphology of all appeared fungi from both PDA and GPY plates was characterized. The fungal mycelia from different plates were mounted on slides for general staining with lactophenol cotton blue and oleagineous staining with Sudan Black B to observe sporangiophores and fat globules under a compound microscopy, respectively. The lower fungi (no septate fungi) with fat globules were selected to cultivate on PDA for more two to three times. Isolated pure cultures were maintained on PDA slants, stored at 4°C, and subcultured every two weeks.

Cultivation and harvest

After three to five days of growth on PDA, pure mycelia with agar were cut into 1.5x1.5 cm pieces and minced. They were transferred to 50 ml of potato dextrose broth (PDB) and cultivated on rotary shaker at 200 rpm and 25°C for five days. Growth fungi were taken and analyzed for polyunsaturated fatty acids. Culture broth was filtered by Whatman No.1 filter paper and washed three times with distilled water. The supernatant was determined for glucose residual by high-performance liquid chromatography (HPLC: HPLC model Waters 2690) equipped with a SHODEX SH-1011 COL analytical column and a refractive index detector. The mobile phase was 5 mM sulfuric acid and nanopure water with a flow rate of 0.6 ml·min⁻¹, and the column temperature was 60°C. The fungal cells were freeze dried for three days, followed by determination of biomass and polyunsaturated fatty acid content.

Extraction and fatty acid analysis

The fifty milligrams of fungal cells were added to the extraction tube containing 100 µl of internal standard (1% (w/v) heptadecanoic acid (C17:0)), and 2.0 ml of 5% (v/v) H₂SO₄ in methanol. The solution was mixed well and incubated in water bath at 90°C for 1 h. After cooling at room temperature, 1 ml of hexane and 1 ml of distilled water were added. The solution was centrifuged at 1,000 rpm for 5 min. The supernatant was analyzed for PUFA composition with gas chromatography (GC-Shimadzu), equipped with Omega WaxTM 250 in a fused silica (Rtx1 fused silica) capillary column (0.25 mm ID x 30 m) and a flame ionization detector (FID). Nitrogen and hydrogen were used as carrier gases at a flow rate of 1.22 ml·min⁻¹. The column and detector temperatures were 150 and 260°C, respectively. An injection was performed in split mode 100:0 at 250°C. Lipid and fatty acid compositions were determined and paralleled with the chromatograms of standard polyunsaturated fatty acids (fatty acid methyl esters (FAME) chemical standards, Supelco, U.S.A.), i.e., palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2n6), γ-linolenic acid (GLA, C18:3n6), eicosenoic acid (C20:1n9), dihomo-linolenic acid (DGLA, C20:3n6), arachidonic acid (ARA, C20:4n6) and eicosapentaenoic acid (EPA, C20:5n3).

DNA sequencing of nuclear ribosomal internal transcribed space (ITS) region and phylogenetic analysis

DNA extraction was modified from O'Donnell et al. (1997) method. The fine powder of fungal mycelia of 50-100 mg was added to 1.5 ml-microcentrifuge tube. Lysis buffer (1 M EDTA 50 ml and 50 mM Tris HCl 10 ml in 1.16 g·l⁻¹ of NaCl), 700 µl, was added and mixed well. The solution was incubated in heat block at 65°C for 1 h. It was centrifuged at 12,000 rpm, 25°C, for 20 min, and transferred to a new microcentrifuge tube. Equal volume of phenol : chloroform : isoamylalcohol (24:25:1) was added and turned up side down slowly for 20 times. The mixture was kept at -20°C for overnight. After that, the mixture was centrifuged at 12,000 rpm, 4°C, for 10 min. The DNA pellets were washed with 75% ethanol and air dried for overnight. Finally, DNA was resuspended in 20-40 fold of elution buffer NE (Macherey-Nagel GmbH & Co. KG, Germany). PCR amplification in ITS region was performed by using a pair of both primers ITS5 (5' GGA AGT AAA AGT CGT AAC AAG G 3') and ITS4 (5' TTC TCC GCT TAT TGA TAT GC 3') was used in 50 µl PCR reactions, which contained 35.2 µl of 10x buffers, 5.0 µl of 25 mM MgCl₂, 1.5 µl of 1 mM dNTP, 4.0 µl of forward and reverse primers (10 µM), 1.5 µl of 20-100 ng DNA sample, 1.8 µl of ddH₂O and Taq DNA polymerase (1 unit/20 µl) (Fermentas, Canada). The thermal cycling for PCR protocol was initially denatured at 94°C for 2 min. Then, the 2-35 cycles were performed at 94°C for 1 min, 55°C for 1 min and 72°C for 2 min. The final extension step was managed at 72°C for 10 min, were incubated at 94°C for 1 min, 55°C for 1 min and 72°C for 2 min, and final extension step was managed at 72°C for 10 min. After analysis with 1.5% (w/v) agarose gel electrophoresis, the PCR products were purified exactly following the manufacturer's instruction of Nucleo spin extract II (Macherey-Nagel GmbH & Co. KG, Germany). The PCR products of the NR06 isolate were further directly sequenced by Marcrogen (Korea). Each sequence was checked for ambiguous bases and assembled using Bio edit V.7.0.5 (Hall, 1999). The consensus sequences for each DNA region were multiple aligned by Cluster W 1.6 along with other sequence obtained from the GenBank database. The phylogenetic analysis were performed using PAUP*4.0b 10 and similarity test of ITS region.

Effect of carbon and nitrogen sources on growth and PUFA production

This study was performed in flask scale for carbon and nitrogen assimilation. The appropriated carbon and nitrogen sources will promote growth and polyunsaturated fatty acid production. The carbon sources in this experiment included cellulose, dextrose, starch, glycerol, glucose, sucrose and xylose, in a concentration of 10 g·l⁻¹ with yeast extract 5 g·l⁻¹, MgSO₄·7H₂O 1.0 g·l⁻¹ and K₂HPO₄ 1.0 g·l⁻¹. The nitrogen sources used in this study were corn meal, corn steep solid, meat extract, NH₄NO₃, peptone, soytone, yeast extract, in a concentration of 5.0 g·l⁻¹ with glucose 10.0 g·l⁻¹, MgSO₄·7H₂O 1.0 g·l⁻¹ and KH₂PO₄ 1.0 g·l⁻¹. The samples were taken every 3 and 5 days of cultivation times. All samples were determined in triplicate for dried cell weight and polyunsaturated fatty acid production. The experimental design using statistical program (Design expert 7.1 software) was employed to analyze the analysis of variance (ANOVA) of the quadratic regression models. It was used to screen factors affecting the production of biomass and ARA by the NR06 isolate. The variable evaluations are listed in Table 4. These included seven nutrients (glucose, glycerol, sucrose, meat extract, soytone, peptone and yeast extract) and four dummies (unassigned variables). This statistic program consists of seven variables with 30 runs (N) with D1 to D4 being the dummy variables employed to evaluate the standard errors (SE) of the experiments (Saelao et al., 2011).

Statistical analyses were applied to identify the variables that had significant effects on the responses (biomass, ARA production and total fatty acid (TFA)). The effect by variable ($E_{(x_i)}$) on a response was determined by substrate on the average response of the lower level (R_i^-) from that of the high level (R_i^+) using the following standard equation:

$$E(X_i) = 2 \frac{[\sum R_i^+ - \sum R_i^-]}{N} \quad (1)$$

Where, N is total number of experiments or runs ($N=30$). The effects of the dummy variables were used to calculate SE as follows:

$$SE = \sqrt{\frac{\sum (Ed)^2}{N}} \quad (2)$$

Where, Ed is the effect of each dummy variable and n is the number of dummy variables ($n = 4$).

$$Y = \beta_0 + \sum_{i=1}^k \beta_i x_i + \sum_{i=1}^k \beta_{ii} x_i^2 + \sum_{i=1}^k \beta_{ij} x_i x_j \quad (3)$$

Where, Y is the predicted response, β_0 is the model constant, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, β_{ij} is the interaction coefficient.

RESULTS

A total of 10 freshwater samples and 75 wet-fallen leaves samples from different natural sources, that is, Bang Pakong River (Chachoengsao Province), Lopburi River (Lopburi Province), Sikao River (Trang Province), Bang Phra Reservoir (Chonburi Province), Khao Cha Mao (Rayong Province), Wang Champi Waterfall and Tad Ta Phu Waterfall (Nakorn Rachasima Province) were used for determination of fungal growth and selected for pure colonies on PDA plates. Fatty acid producing strains of lower fungi were found to be 8 and 16 isolates from freshwaters and wet-fallen leaves samples, respectively (Table 1). The results show that the biomass concentration varied from 1.20-15.49 $g \cdot l^{-1}$ for all isolates. The lowest biomass production was found in the CC04 culture and the highest biomass producer was the NR06 culture. Similarity, total lipid content (percent) production varied from 2.11±0.12 to 16.44±0.30%, with the same cultures of the lowest and the highest. Thus, the NR06 isolate was considered as a potent culture for the maximum production of biomass (15.49±0.24 $g \cdot l^{-1}$) and total lipid (16.44±0.30%) among all 24 isolates, and it was selected for further characterization and identification.

The NR06 isolate was cultivated from wet-fallen leaves near Tad Ta Phu waterfall, Nakorn Ratchasima Province. Morphological characteristics of the NR06 isolate are shown in Figure 1. After cultivation on PDA for 7-10 days, the culture was occurred as fluffed up white cottony mycelia in Petri dish (Figure 1A). Non-septated mycelia were classified as lower fungus, with many lipid globules

inside those stained by Sudan black B, and their swollen sporangiophores were observed (Figure 1B, C and D). Molecular phylogeny characterization of the NR06 isolate was carried out for taxonomic identification using ITS gene sequence. The PCR product obtained 636 bp-ITS gene which was further used to perform BLAST. The lower fungus isolate NR06 was identified to the closest species as *Mortierella elongata* with 99% similarity, with GenBank accession no. KF181625.

The fatty acid composition of all fatty acid producing strains was analyzed by GC (Table 2). Total fatty acid contents of fungal isolates were C16-C20 fatty acids, i.e. palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2n6), γ -linolenic acid (GLA, C18:3n6), eicosenoic acid (C20:1n9), dihomo-linolenic acid (DGLA, C20:3n6), arachidonic acid (ARA, C20:4n6) and eicosapentaenoic acid (EPA, C20:5n3). Although palmitic, stearic and oleic acids were commonly found in all isolates, polyunsaturated fatty acid profiles among the isolates were remarkably different. Ten (10) isolates from a total of 24 fungal isolates could produce essential unsaturated fatty acids. The NR06 isolate which showed the highest biomass and fatty acid content, could produce significantly high level of essential long chain unsaturated fatty acids ($p \leq 0.05$), especially arachidonic acid (ARA; C20:4n6) (32.24±0.35 %). Other unsaturated fatty acids, i.e., linoleic acid (C18:2n6), γ -linolenic acid (GLA; C18:3n6) and oleic acid (C18:1) were also found in lipid profiles of the NR06 culture, with the levels of 8.26±0.59, 5.48±0.08 and 23.48±0.22%, respectively (Figure 2).

The effect of various carbon sources, i.e., cellulose, dextrose, glycerol, glucose, starch, sucrose and xylose, on growth and polyunsaturated fatty acid production by the lower fungal NR06 isolate are demonstrated in Table 3. The results indicate that glycerol, dextrose and glucose gave relatively high biomass with the values of 8.37±0.72, 7.93±0.67 and 7.87±0.72 $g \cdot l^{-1}$, the ARA productions of 22.51±1.9, 22.07±1.22 and 21.00±0.01% (w/w, ARA by total fatty acid) and the total fatty acids of 12.61±0.12, 10.83±0.56, 11.41±1.58% (w/w, total fatty acid by biomass), respectively. Thus, glycerol, dextrose and glucose were chosen for further studies as carbon source variables. Various nitrogen sources, i.e., corn meal, corn steep solid, meat extract, peptone, soy tone and yeast extract, except NH_4NO_3 , were found to support growth in a range of 6.10±0.10 to 7.10±0.26 $g \cdot l^{-1}$. However, since ARA productions from media containing meat extract, peptone, soytone and yeast extract were 23.62±2.46, 23.91±9.86, 32.36±4.12 and 27.03±6.41% (w/w, ARA by total fatty acid), respectively, these nitrogen sources were selected for the variables.

All selected carbon and nitrogen sources were evaluated to be the important factors for statistical program, Design expert 7.1 software, USA. The variable factors of substrate combination between carbon and nitrogen sources were performed in Table 4. After 30 trials, the results showed

Table 1. Biomass and total fatty acid contents of fungal isolates from various natural sources.

Natural sources	Samples	Fungal isolates	DW (g·l ⁻¹)	TFA (%)
Bang Pakong river, Chachoengsao province	Leaves	CC01	4.78±0.16 ⁱ	3.65±0.34 ^{ghi}
		CC02	1.59±0.19 ^{no}	3.32±0.13 ^{ghijk}
		CC03	1.49±0.23 ^o	2.22±0.15 ^{lm}
		CC04	1.20±0.08 ^o	2.11±0.12 ⁿ
Lopburi river, Lopburi province	Freshwater	LB01	11.19±0.23 ^c	10.38±0.22 ^c
		LB02	10.33±0.08 ^d	2.50±0.21 ^{km}
		LB03	10.93±0.13 ^c	4.83±0.11 ^f
		LB04	4.98±0.19 ⁱ	4.02±0.13 ^{fgh}
Sikao river, Trang province	Freshwater	TR03	4.85±0.07 ^j	4.74±0.36 ^f
		CB01	7.25±0.13 ^g	3.05±0.23 ^{ijkl}
		CB02	8.23±0.25 ^f	3.92±0.47 ^{fghi}
		CB04	12.12±0.24 ^b	2.99±0.05 ^{ijklm}
Bang Phra reservoir, Chonburi province	Leaves	CB05	6.31±0.08 ^h	2.67±0.16 ^{ijklm}
		CB06	3.09±0.17 ⁱ	4.68±0.04 ^f
		CB07	6.11±0.18 ^h	3.54±0.17 ^{ghij}
		CB08	2.60±0.08 ^k	6.61±0.24 ^e
Khao Cha Mao waterfall, Rayong province	Leaves	RY01	7.43±0.16 ^g	10.39±0.94 ^c
		RY02	7.52±0.42 ^g	8.45±1.41 ^d
Wang Champi waterfall, Nakorn Ratchasima province	Leaves	NR01	2.33±0.11 ^{kl}	9.61±0.07 ^c
		NR02	1.89±0.15 ^{mn}	13.86±0.11 ^b
		NR03	2.08±0.18 ^m	14.11±0.24 ^b
		NR04	9.34±0.16 ^e	4.10±0.25 ^{fg}
	Freshwater	NR05	1.20±0.07 ^o	3.12±0.18 ^{hijkl}
Tad Ta Phu waterfall, Nakorn Ratchasima province	Leaves	NR06	15.49±0.24 ^a	16.44±0.3 ^a

Data expressed as means±SD of three replicates. Different superscript letters are significantly different ($p \leq 0.05$). *DW, weight of dry biomass; TFA, total fatty acid.

wide variation of responses for biomass, total fatty acid content and ARA production. The amounts of biomass production varied from 7.06 to 26.46 g·l⁻¹. The obtained total fatty acids were in a wide range of 5.06 to 56.3% (w/w) with ARA productions varying from 12.06 to 56.3% (w/w) (Table 5). Variables of selected carbon sources (glucose, sucrose and glycerol) and selected nitrogen sources (meat extract and yeast extract) had a significant effect on growth with confidence levels >95.0%. The statistical experimental design revealed that biomass and ARA production were significantly affected by growth of cell cultures of the NR06 isolate with coefficients of determination (R^2) of 0.9956 and p -values <0.05.

The combination of carbon and nitrogen sources using the experimental design displayed the best results from the mixtures of substrates as in the equations. The ANOVA of the quadratic regression models for growth and ARA production indicated the 'Prob>F' <0.0015 and

0.0011, respectively. The models implicit that both statistical designs maintain the significance of experimental data. The statistically experimental design coefficients estimated by regression analysis for each factor are also shown in Table 6. The significance of each coefficient was determined by p -values. The smaller the p -values, the higher the significance of the corresponding coefficients. The results showed that glucose as the carbon source affected both biomass and ARA production (p -values of <0.0001), while yeast extract as the nitrogen source affected only ARA production ($p = 0.02$). The mathematical model, incorporating the different interactions of low and high levels of varied factors, is proposed with effect on growth (Equation 4) and ARA production (Equation 5).

$$Y_{\text{ARA}} (\% \text{ of TFA}) = -16.0 A + 0.63 B + 1.42 C - 2.57 D - 1.47 E + 0.11 F - 2.49 G + 1.62 H + 0.92 J - 0.045 K + 0.086 L - 0.082 A B - 1.59 A C + 1.92 A D + 1.10 A E - 0.46 A H + 1.52 A G - 0.26 A H - 0.63 A J - 0.021 A K - 0.40 A L - 0.66 B C \quad (4)$$

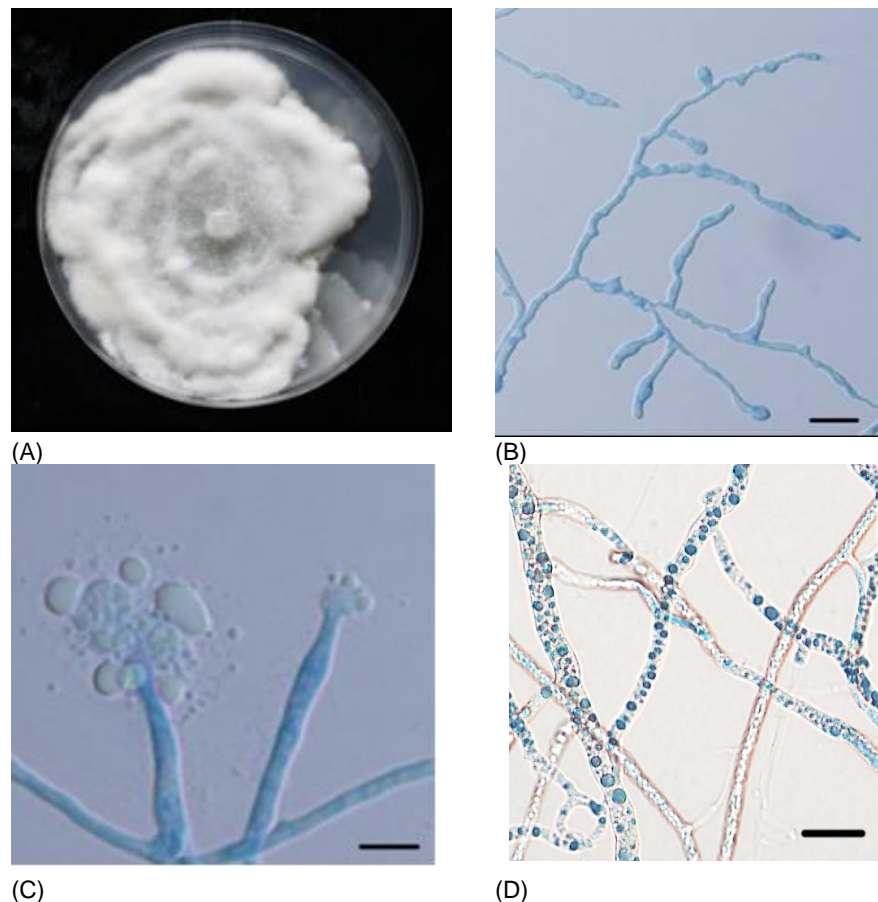


Figure 1. Morphological characteristics of the NR06 isolate. (A) Growth on PDA plate (B) The spores, 20x. (C) The sporangiophores, 40x. (D) The mycelia with many lipid globules, 40x. Horizontal bars indicate 1 μm (B, C and D).

$$Y_{(\text{biomass g}\cdot\text{L}^{-1})} = +3.99A - 0.45B + 0.56C + 0.25D - 0.28E + 0.39F + 0.33G + 0.39H - 0.82J - 0.17K + 0.28L - 0.43A + 0.86A + 0.049AD + 0.012A - 0.30A + 0.85A + 1.17A - 0.057AJ - 0.40AK - 0.64AL - 1.48BC \quad (5)$$

DISCUSSION

A number of the lower fungi, especially *Mucor* spp., *Mortierella* spp., *Rhizopus* spp. and *Cunninghamella* spp., have been exploited for the production of PUFA as they have the ability to synthesize intracellular lipids from carbohydrates and store them in the mycelium as triacylglycerols. However, the fungal economic yield is still limited. Thus, attempts have been continued to search for either the best new producer or the same strains with improved cultivation conditions for specific fatty acid productions. Since the degree of unsaturated fatty acids increases with decreased temperature, the production of EPA and ARA is stimulated by lower temperatures (Hansson and Dostálek, 1988). Botha et al. (1999) developed an isolation procedure for ARA

producing species at low temperature (5°C). Chen et al. (1997) screened for low temperature (10°C) ARA producer fungal strains and obtained the Wuji-H4 isolate with high content of ARA (42.4%) in lipids. Shimizu et al. (1988) investigated the effect of temperature on the fatty acid composition of *Mortierella* fungi. It was observed that the optimum temperature for ARA production in *Mortierella alpina* was 28°C while that of EPA production was stimulated at lower temperatures. In the present study, screening for the production PUFA was practically performed at 25°C. Fatty acid producing strains of native lower fungi could be isolated from wet-fallen leaves (16 isolates), more than those from freshwater (8 isolates). Aki et al. (2001) demonstrated a filamentous fungus isolated from freshwater pond samples, assigned to the species *Mortierella alliacea* YN-15 that accumulated high level of ARA in its mycelia. More native lower fungi, *Mortierella* sp. (Botha et al., 1999) and *Mucor rouxii* (Mamatha et al., 2010) were isolated from soil samples. Kamlangdee and Fan (2003) isolated DHA (docosahexaenoic acid, 22:6n3) producing strains of *Schizochytrium* sp. from fallen, senescent leaves from mangrove tree

Table 2. Fatty acid composition of 24 fungal isolates.

Isolates	Fatty acid composition (percentage of total fatty acid)									
	C16:0 (Palmitic acid)	C16:1 (Palmitoleic acid)	C18:0 (Stearic acid)	C18:1 (Oleic acid)	C18:2n6 (Linoleic acid)	C18:3n6 (GLA*)	C20:1n9 (Eicosenoic acid)	C20:3n6 (DGLA*)	C20:4n6 (ARA*)	C20:5n3 (EPA*)
CC01	27.48±0.52 ^c	1.56±0.28 ^e	2.92±0.15 ^{ijk}	30.10±0.49 ⁱ	14.23±0.30 ^j	ND	ND	ND	7.52±0.37 ^c	12.08±0.13 ^g
CC02	29.74±0.16 ^b	1.30±0.08 ^f	2.86±0.23 ^{ijk}	35.10±0.20 ^f	12.60±0.34 ^k	ND	ND	ND	ND	9.91±0.16 ⁱ
CC03	31.83±0.25 ^a	2.41±0.23 ^d	3.29±0.38 ^{hij}	30.28±0.18 ⁱ	14.25±0.02 ^j	ND	ND	ND	6.76±0.51 ^d	11.45±0.34 ^h
CC04	22.32±0.28 ^{ef}	1.59±0.49 ^e	2.00±0.05 ^{kl}	31.16±0.29 ^h	19.30±0.49 ^g	ND	ND	ND	10.19±0.09 ^b	13.97±0.07 ^d
LB01	17.36±0.15 ^{ik}	0.34±0.16 ^{hi}	11.07±0.25 ^{bc}	32.21±0.24 ^g	36.39±0.28 ^e	ND	0.59±0.25 ^b	ND	ND	ND
LB02	18.55±0.30 ^j	0.59±0.34 ^{hi}	3.07±0.25 ^{ij}	16.36±0.16 ⁿ	57.82±0.28 ^b	ND	ND	ND	ND	ND
LB03	18.77±0.45 ⁱ	0.84±0.16 ^{gh}	2.03±0.17 ^{kl}	11.25±0.41 ^p	63.54±0.63 ^a	ND	ND	ND	ND	ND
LB04	12.65±0.44 ⁿ	34.27±0.44 ^a	4.52±0.42 ^g	41.62±0.58 ^c	ND	ND	ND	ND	ND	ND
TR03	22.11±0.22 ^f	1.07±0.25 ^{efg}	3.82±0.23 ^{ghi}	23.58±0.18 ^l	49.26±0.01 ^c	ND	ND	ND	ND	ND
CB01	21.16±0.28 ^g	ND	1.43±0.52 ^l	10.81±0.30 ^{pq}	19.54±0.87 ^g	0.97±0.08 ^{hi}	ND	ND	7.45±0.31 ⁱ	17.03±0.20 ^a
CB02	18.17±0.60 ^{ij}	ND	1.09±0.09 ^l	10.27±0.07 ^f	18.25±0.42 ^h	1.11±0.20 ^{hi}	ND	ND	6.20±0.07 ^c	12.61±0.40 ^f
CB04	23.18±0.28 ^d	ND	1.19±0.05 ^l	12.97±0.11 ^o	23.34±0.17 ^f	22.52±0.52 ^c	ND	ND	1.79±0.19 ^h	2.37±0.21 ^k
CB05	16.67±0.15 ^j	ND	3.83±0.17 ^l	28.88±0.30 ^j	10.98±0.18 ^l	33.74±0.21 ^a	ND	ND	ND	ND
CB06	17.52±0.33 ^j	ND	2.75±0.28 ^{ghi}	10.67±0.16 ^{pq}	16.05±0.23 ⁱ	1.03±0.06 ^{hi}	ND	ND	6.32±0.16 ^e	16.17±0.31 ^b
CB07	18.14±0.12 ^{ij}	ND	1.12±0.04 ^l	11.45±0.14 ^p	16.24±0.16 ⁱ	0.63±0.10 ⁱ	ND	ND	5.78±0.19 ^f	13.45±0.30 ^e
CB08	10.90±0.18 ^o	ND	6.53±0.32 ^f	45.08±0.22 ^b	13.12±0.23 ^k	0.39±0.17 ⁱ	ND	ND	4.40±0.28 ^g	8.44±0.17 ^j
RY01	20.62±0.52 ^{gh}	5.89±0.89 ^b	12.03±1.39 ^a	7.47±1.04 ^s	22.82±0.72 ^f	20.17±0.45 ^d	ND	ND	ND	ND
RY02	20.12±0.72 ^h	6.06±0.66 ^b	4.05±0.69 ^{gh}	21.50±0.88 ^m	23.46±0.62 ^f	23.93±2.02 ^b	ND	ND	ND	ND
NR01	20.29±0.20 ^h	4.10±0.18 ^c	8.57±0.40 ^{de}	48.11±0.34 ^a	8.88±0.10 ^m	6.67±0.31 ^e	ND	ND	ND	ND
NR02	22.45±0.13 ^{def}	ND	11.84±0.26 ^{ab}	37.34±0.39 ^d	5.10±0.15 ⁿ	2.10±0.11 ^{gh}	0.77±0.06 ^a	ND	15.68±0.42 ⁱ	0.56±0.20 ^l
NR03	23.04±0.22 ^{de}	ND	10.54±0.58 ^c	36.08±0.14 ^e	6.07±0.3 ⁿ	3.16±0.19 ^g	0.60±0.20 ^b	2.53±0.24 ^b	15.18±0.28 ⁱ	0.54±0.25 ^l
NR04	13.97±0.49 ^m	0.21±0.02 ^{hi}	5.66±0.33 ^f	26.11±0.37 ^k	8.96±0.71 ^m	2.28±1.20 ^g	ND	19.92±0.57 ^a	ND	15.01±0.32 ^c
NR05	19.98±0.21 ^h	ND	7.85±0.19 ^e	25.58±0.38 ^k	46.59±0.48 ^d	ND	ND	ND	ND	ND
NR06	15.09±0.74 ⁱ	ND	9.38±0.26 ^d	23.07±0.22 ^l	8.26±0.59 ^m	5.48±0.08 ^f	ND	ND	32.34±0.35 ^a	ND

Data expressed as means±SD of three replicates. Different superscript letters are significantly different ($p \leq 0.05$). ND = Not detectable. GLA, γ -linolenic acid; DGLA, dihomo-linolenic acid; ARA, arachidonic acid; EPA, eicosapentaenoic acid.

(*Kandelia candel*). Since the NR06 was isolated from wet-fallen leaves at the waterfall, unfortunately there was no record on a type of the collected leaves. Thus, interestingly, it was suggested to study further with an original tree of the leaves.

The NR06 isolate, which was subsequently identified as *M. elongata*, produced the highest level of total fatty acids contents, which mainly contained ARA (more than 30%). Similar results were also found in *M. alpina* and *M. alliacea*

(Shinmen et al., 1989). As the PUFAs are intracellular products, high biomass concentration is required for commercially high productivity. Since the effects of several environmental factors, that is, temperature, pH, the chemical composition

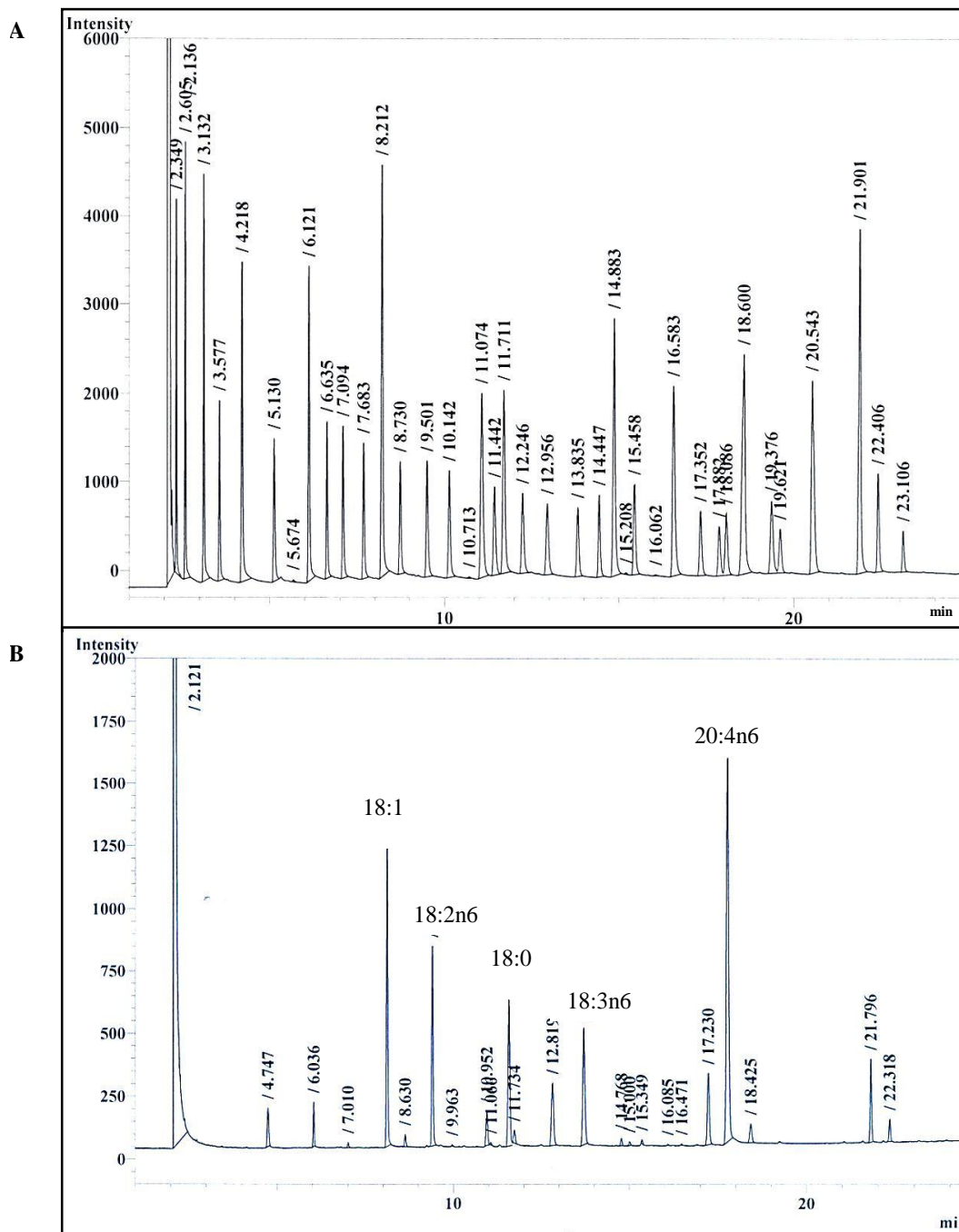


Figure 2. Chromatogram of fatty acid methyl esters (FAME) chemical standards (Supelco, U.S.A.) (A) and chromatogram of the NR06 isolate (B).

of the nutrient media and culture age, have been studied extensively, it was shown that the fungal strains could utilize a wide variety of carbon sources. In the cultures of *Mortierella* for ARA production, glucose is the most frequently used carbon source. Aki et al. (2001) investigated various carbon sources for the strains of *M. alpina* and *M. alliacea*, and found that glucose was suitable for ARA production. In the case of *M. alliacea*,

which was isolated from rice grains, usage of starch as a carbon source was also practical. Totani et al. (2002) reported the effect of glucose concentration on ARA production. They pointed out that more than 20% glucose inhibited the growth of *M. alpina* and high glucose concentration also induced the formation of filamentous morphology. Various studies have also demonstrated that vegetable oil addition was beneficial to enhance ARA

Table 3. Growth, fatty acid content and productions of ARA and EPA of the NR06 isolate in the media containing various carbon and nitrogen sources.

Media	Biomass (g·l ⁻¹)	Total Fatty acid (%w/w)	PUFA in TFA (%W/W)	
			ARA	EPA
Carbon sources (1%)				
Cellulose	5.13±0.83	4.83±0.15	27.24±5.04	0.66±0.06
Dextrose	7.93±0.67	10.83±0.56	22.07±1.22	0.37±0.06
Glycerol	8.37±0.72	12.61±0.12	22.51±1.91	0.29±0.24
Glucose	7.87±0.72	11.41±1.58	21.00±0.01	0.45±0.00
Starch	7.13±0.72	7.89±2.01	20.07±1.26	0.55±0.11
Sucrose	6.00±0.46	4.45±1.10	21.77±3.05	1.09±0.54
xylose	3.83±0.12	5.48±0.13	27.42±1.98	0.79±0.34
Nitrogen sources (0.5%)				
Corn meal	7.03±0.91	14.15±0.03	9.27±0.04	0.10±0.01
Corn steep solid	7.03±0.40	21.62±1.07	11.45±1.75	0.24±0.01
Meat extract	7.10±0.26	10.52±0.40	23.62±2.46	0.41±0.00
NH ₄ NO ₃	4.90±0.89	8.17±1.27	12.2±1.44	ND
Peptone	6.43±0.35	10.34±6.41	23.91±9.86	0.41±0.21
Soytone	6.10±0.10	6.15±0.71	32.36±4.12	0.41±0.01
Yeast extract	6.77±0.59	7.55±1.21	27.03±6.41	0.57±0.09

Data expressed as means±SD of three replicates. ND = Not detectable. ARA, arachidonic acid; EPA, eicosapentaenoic acid; PUFA, polyunsaturated fatty acid; TFA, total fatty acid.

Table 4. Selected variables were chosen for studies of biomass, total fatty acid and ARA productions by cell cultures of the NR06 isolate, using the statistical program, Design Expert 7.1 software.

Code	Variables	Low level (+)	High level (-)
		(g·l ⁻¹)	(g·l ⁻¹)
A ₁	Glucose	10	60
B ₂	Glycerol	10	60
C ₃	Sucrose	10	60
D ₄	Meat extract	5	15
E ₅	Soytone	5	15
F ₆	Peptone	5	15
G ₇	Yeast extract	5	15
H	Dummy 1 (-)	-	-
I	Dummy 2 (-)	-	-
J	Dummy 3 (-)	-	-
K	Dummy 4 (-)	-	-

yield (Dong and Walker, 2008).

The ratio of carbon and nitrogen (C/N) in the growth media is considered as the most important factor to achieve biomass and lipid accumulation (Fakas et al., 2008). Complex organic nitrogen sources such as yeast extract and corn steep liquor are generally practicable for growth. Yokochi et al. (1998) investigated several alternative carbon and nitrogen sources for the algal culture of

Schizochytrium limacinum SR 21. It was found that glucose, fructose, glycerol and oleic acid led to high levels of cell growth over 10 g·l⁻¹ and DHA production over 0.5 g·l⁻¹, while saccharose, lactose, maltose, starch and linseed oil decreased levels of DHA production. Among various nitrogen sources tested, corn steep liquor or yeast extract had the highest DHA production levels (over 0.5 g·l⁻¹). The fungal strain *M. alpine*, could produce

Table 5. Statistical program, Design Expert 7.1 software, design matrix to evaluate variables affecting biomass, total fatty acid and ARA production by the NR06 isolate.

Run	A	B	C	D	E	F	G	H	I	J	K	ARA production (%, w/w)	TFA production (%, w/w)	Biomass production (g·l ⁻¹)
1	+	+	-	+	+	+	-	-	-	+	-	14.37	18.80	14.70
2	-	+	+	-	+	+	+	-	-	-	+	46.12	9.31	8.40
3	+	-	+	+	-	+	+	+	-	-	-	14.58	17.51	26.46
4	-	+	-	+	+	-	+	+	+	-	-	36.56	7.57	7.26
5	C	C	+	-	+	+	-	+	+	+	-	59.28	6.35	9.20
6	C	C	C	+	-	+	+	-	+	+	+	37.27	8.50	10.16
7	+	C	C	-	+	-	+	+	-	+	+	14.08	21.34	17.50
8	+	+	C	-	-	+	-	+	+	-	+	19.22	16.34	16.44
9	+	+	+	-	-	-	+	-	+	+	-	14.06	18.13	14.94
10	+	+	+	+	-	-	-	+	-	+	+	52.25	9.46	9.14
11	+	+	+	+	+	-	-	-	+	-	+	13.70	19.53	17.82
12	-	-	-	-	-	-	-	-	-	-	-	49.10	7.83	8.64
13	C	C	C	C	C	C	C	-	-	-	-	27.09	14.29	9.92
14	C	C	C	C	C	C	C	-	-	-	-	16.90	16.53	10.70
15	C	C	C	C	C	C	C	-	-	-	-	20.41	8.82	11.78
16	-	-	+	-	-	-	+	+	+	-	+	56.31	5.76	7.52
17	+	-	-	+	-	-	-	+	+	+	-	16.75	19.18	14.02
18	-	+	-	-	+	-	-	-	+	+	+	50.79	12.06	10.56
19	+	-	+	-	-	+	-	-	-	+	+	14.38	18.26	17.34
20	+	+	-	+	-	-	+	-	-	-	+	13.40	19.98	16.64
21	+	+	+	-	+	-	-	+	-	-	-	17.59	17.65	16.86
22	-	+	+	+	-	+	-	-	+	-	-	51.86	13.19	7.06
23	-	-	+	+	+	-	+	-	-	+	-	34.32	9.14	9.06
24	-	-	-	+	+	+	-	+	-	-	+	40.92	6.49	8.90
25	+	-	-	-	+	+	+	-	+	-	-	12.06	23.07	13.48
26	-	+	-	-	-	+	+	+	-	+	-	48.73	9.11	9.62
27	+	+	+	+	+	+	+	+	+	+	+	13.47	13.17	16.38
28	C	C	C	C	C	C	C	0	0	0	0	23.49	15.29	9.10
29	C	C	C	C	C	C	C	0	0	0	0	22.97	15.71	9.38
30	C	C	C	C	C	C	C	0	0	0	0	25.68	16.19	10.20

The four variables (D1-D4) are designed as “dummy variables”. +, high level; -, low level; ARA, arachidonic acid; TFA, total fatty acid; C, central values.

ARA 5.3 g·l⁻¹, where glucose is a carbon source and yeast extract a nitrogen source (Jang et al., 2005). Zhu et al. (2003) described a method for ARA production by *M. alpina* on glucose/defatted soybean meal and sodium nitrate with the yield of 1.87 g·l⁻¹ ARA (17.3% of total lipids) from 31.2 g biomass in 7 days. Lan et al. (2002) showed that glutamate enhanced ARA production, while other PUFAs were retained. For 7 days of growth, the yields of biomass and ARA were 25 and 1.4 g·l⁻¹, respectively. Eroshin et al. (1996) developed a medium containing aspirin to select ARA producing strain of *Mortierella* sp., and obtained three isolates producing up to 40% of ARA in total lipid content. Production of ARA in photoautotrophic algae, such as *Porphyridium cruentum* and *Parietochloris incise*, appeared to be optimal under

conditions of slow growth in nitrogen free or nitrogen-starved conditions (Ward and Singh, 2005). Kyle (1994) described a method for production of ARA by the heterotrophic alga, *Pythium insidiosum*. Relatively high rate of growth (15 g in 50 h) were achieved in glucose-yeast extract medium. Biomass oil content was 5-6% (0.75-0.9 g·l⁻¹, of which 30-35% was ARA). However, ARA yields and productivities were only 0.3 and 0.15 g·l⁻¹·h⁻¹, respectively.

ARA productivity of the NR06 strain was evaluated by cultivation in liquid media containing different carbon sources and nitrogen sources. Optimization of growth and production of total intracellular fatty acids including ARA was accomplished by a combination of glucose and yeast extract, which was evaluated by the Design

Table 6. Regression coefficients of the variables in response to biomass, total fatty acid and ARA productions.

Variable	Biomass production						ARA production					
	Coefficient estimate	SE	SS	df	F value	p value (Prob > F)	Coefficient estimate	SE	SS	df	F value	p value (Prob > F)
Model	10.18	0.32	543.43	1	37.76	0.0015	30.86	0.74	6908.04	1	24	0.0011
A	3.99	0.16	372	1	620.78	<0.0001	-16.05	0.74	6145.22	1	469.60	<0.0001
B	-0.45	0.16	4.78	1	7.97	0.0476	0.63	0.74	9.41	1	0.72	0.4351
C	0.56	0.16	7.39	1	12.32	0.0247	1.42	0.74	48.08	1	3.67	0.1134
D	0.26	0.35	0.34	1	0.57	0.4910	-2.57	0.74	157.51	1	12.04	0.0179
E	-0.29	0.35	0.43	1	0.72	0.4437	-1.47	0.74	51.42	1	3.93	0.1043
F	0.41	0.39	0.67	1	1.12	0.3493	0.11	0.74	0.32	1	0.024	0.8827
G	0.32	0.35	0.5	1	0.83	0.4135	-2.49	0.74	148.26	1	11.33	0.02
H	0.37	0.39	0.54	1	0.91	0.3953	1.62	0.74	62.65	1	4.79	0.0803
I	-0.84	0.39	2.8	1	4.67	0.0968	0.92	0.74	20.19	1	1.54	0.2693
J	-0.19	0.39	0.14	1	0.24	0.6521	-0.045	0.74	0.048	1	3.68 ^{E-003}	0.9540
K	0.3	0.39	036	1	0.59	0.4840	0.086	0.74	0.18	1	0.013	0.9123

The four variables (H, I, J and K) are designed as “dummy variables”. A, glucose; B, glycerol; C, sucrose; D, meat extract; E, soytone; F, peptone; G, yeast extract; SE, standard error; SS, sum of square; df, degree of freedom; ARA, arachidonic acid.

expert 7.1 software. Moreover, to enhance the production of polyunsaturated fatty acids, the combination of carbon and nitrogen sources using the statistically experimental design revealed the best results obtained from the mixtures of substrates as in the equations. These results agree with those of Ghobadi et al. (2011) who used the Plackett-Burman screening design providing the effect of combination of substrates for enhancement of biomass and ARA yield of *M. alpina* CBS754.68. Saelao et al. (2011) also optimized biomass and ARA production by a novel marine gliding bacterium, *Aureispira maritima* using response surface methodology. They reported that tryptone and culture temperature affected biomass, whereas pH and agitation rate had a significant effect on ARA production. Subsequent statistical optimization of these four factors was verified to increase both biomass and ARA yield.

The isolated native NR06 fungal strain from the present study was classified as *M. elongata*, with the high biomass of $15.49 \pm 0.24 \text{ g} \cdot \text{l}^{-1}$, total fatty acids of $16.44 \pm 0.30\%$ and the major essential fatty acid of arachidonic acid (ARA; C20:4n6) ($32.24 \pm 0.35\%$). It has been recommended that further optimizations are required for improvement to be a potential ARA producing strain.

Conflict of interests

The authors did not declare any conflict of interest.

ACKNOWLEDGEMENTS

This work was supported by the Higher Education Research Promotion and National Research

University Project of Thailand, Office of the Higher Education Commission. The authors are thankful to BIOTEC, National Science and Technology Development Agency for instrument support.

REFERENCES

- Aki T, Nagahata T, Ishihara K, Tanaka Y, Morinaga T, Higashiyama K, Akimoto K, Fujikawa S, Kawamoto S, Shigeta S, Ono K, Suzuki O (2001). Production of arachidonic acid by filamentous fungus, *Mortierella alliaeae* strain YN-15. *J. Am. Oil Chem. Soc.* 78:599-604.
- Barclay WR, Zeller S (1996). Nutritional enhancement of n-3 and n-6 fatty acids in rotifers and *Artemia napuli* by feeding spray dried *Schizochytrium* sp. *J. World Aquac. Soc.* 27: 314-322.
- Bigogno C, Khozin-Goldberg I, Anderstein D, Cohen Z (2002). Biosynthesis of arachidonic acid in the oleaginous microalga *Parietochloris incisa* (Chlorophyceae): radiolabeling studies. *Lipids* 37:209-216.
- Botha A, Paul I, Roux C, Kock JLF, Coetzee DJ, Strauss T, Maree C (1999). An isolation procedure for arachidonic acid producing *Mortierella* species. *Antonie Van Leeuwenhoek*

- 75:253-256.
- Certik M, Shimizu S (1999). Biosynthesis and regulation of microbial polyunsaturated fatty acid production. *J. Biosci. Bioeng.* 87:1-14.
- Chen CX, Chen HC, Chang CC (1997). Optimization of Arachidonic acid production by *Mortierella alpina* Wuji-H4 isolation. *J. Am. Oil Chem. Soc.* 74:569-578.
- Domingo JL (2007). Omega-3 fatty acids and the benefits of fish consumption: Is all that glitters gold. *Environ. Int.* 33: 993-998.
- Domingo JL, Bocio A, Falco G, Llobet JM (2007). Benefits and risks of fish consumption Part I. A quantitative analysis of the intake of omega-3 fatty acids and chemical contaminants. *Toxicology* 230: 219-226.
- Dong M, Walker T (2008). Addition of polyunsaturated fatty acids to canola oil by fungal conversion. *Enzyme Microb. Technol.* 42:514-520.
- Dyal SD, Narine SS (2005). Implication of the use of *Mortierella* fungi in the industrial production of essential fatty acids. *Food Res. Int.* 38: 445-467.
- Eroshin VK, Dedyukhina EG, Chistyakova TI, Zhelifonova, VP, Kurtzman CP, Bothast RJ (1996). Arachidonic acid production by species of *Mortierella*. *World J. Microbiol. Biotechnol.* 12:91-96.
- Fakas S, Papannikolaou S, Galoitou-Panayotou M, Komitis M, Aggelis G (2008). Organic nitrogen of tomato waste hydrolysate enhances glucose uptake and lipid accumulation in *Cunninghamella echinulata*. *J. Appl. Microbiol.* 105:1062-1070.
- Gema H, Kavadia A, Dimou D, Tsagou V (2002). Production of γ -linoleic acid by *Cunninghamella echinulata* cultivated on glucose and orange peel. *Appl. Microbiol. Biotechnol.* 58:303-307.
- Ghobadi Z, Hamidi-Esfahani Z, Azizi MH (2011). Determination of effective variables on arachidonic acid production by *Mortierella alpina* CBS754.68 in solid state fermentation using Plackett-Burman screening design. *World Acad. Sci. Eng. Technol.* 57:678-680.
- Gill I, Valivety R (1997). Polyunsaturated fatty acids, part 1: Occurrence, biological activities and applications. *Trends Biotechnol.* 15: 401-409.
- Hall TA (1999). Bio Edit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acid Symp. Ser.* 41:95-98.
- Hansson L, Dostálek M (1988). Effect of culture conditions on mycelia growth and production of γ -linoleic acid by the fungus *Mortierella ramanniana*. *Appl. Microbiol. Biotechnol.* 28:240-246.
- Jang HD, Lin YY, Yang SS (2005). Effect of culture media and conditions on polyunsaturated fatty acids production by *Mortierella alpina*. *Bioresour. Technol.* 96:1633-1644.
- Kamlangdee N, Fan KW (2003). Polyunsaturated fatty acids production by *Schizochytrium* sp. *Songklanakarin J. Sci. Technol.* 25: 643-650.
- Kyle D (1994). Microbial oil mixtures and uses thereof. United States Patent 5,374,657.
- Lan W, Qin W, Yu L (2002). Effect of glutamate on arachidonic acid production from *Mortierella alpina*. *Let. Appl. Microbiol.* 35: 357-60.
- Leman J (1997). Oleaginous microorganisms: An assessment of the potential. *Adv. Appl. Microbiol.* 43:195-243.
- Mamatha SS, Halami PM, Venkateswaran G (2010). Identification and characterization of the n-6 fatty acid-producing *Mucor rouxii* native isolate CFR-G15. *Eur. J. Lipid Sci. Technol.* 112: 380-389.
- Murphy D (1991). Storage lipid bodies in plants and other organisms. *Prog. Lipid Res.* 29:299-324.
- O'Donnell K, Cigelnik E, Weber NS, Trappe JM (1997). Phylogenetic relationships among ascomycetous truffles and the true and false morels inferred from 18S and 28S ribosomal DNA sequence analysis. *Mycologia* 89:48-65.
- Saelao S, Kanjana A, Kaewsuan S (2011). Optimization of biomass and arachidonic production by *Aureispira maritime* using response surface methodology. *J. Am. Oil Chem. Soc.* 88:619-629.
- Shimizu S, Kawashima H, Shinmen Y, Akimoto K, Yamada H (1988). Production of eicosapentaenoic acid by *Mortierella* fungi. *J. Am. Oil Chem. Soc.* 65:1455-1459.
- Shimizu S, Sakuradani Y (2009). Single cell oil production by *Mortierella alpina*. *J. Biotechnol.* 144:31-36.
- Shinmen Y, Shimizu S, Akimoto K, Kawashima H, Yamada H (1989). Production of arachidonic acid by *Mortierella* fungi. *Appl. Microbiol. Biotechnol.* 31:11-16.
- Totani N, Yamaguchi A, Yawata M, Ueda T (2002). The role of morphology during growth of *Mortierella alpina* in arachidonic acid production. *J. Oleo Sci.* 51:531-538.
- Ward OP, Singh A (2005). Omega-3/6 fatty acids: Alternative sources of production. *Process Biochem.* 40:3627-3652.
- Yokochi T, Honda D, Higashihara T, Nakahara T (1998). Optimization of docosahexaenoic acid production by *Schizochytrium limacinum* SR 21. *Appl. Microbiol. Biotechnol.* 49:72-76.
- Yongmanitchai W, Ward P (1989). Omega-3 fatty acids: alternative source of production. *Process Biochem.* 24:117-125.
- Zhu M, Yu LJ, Wu YX (2003). An inexpensive medium for production of arachidonic acid by *Mortierella alpina*. *J. Ind. Microbiol.* 30:75-79.

Full Length Research Paper

Molecular genetic diversity study of *Lepidium sativum* population from Ethiopia as revealed by inter simple sequence repeat (ISSR) markers

Said Mohammed^{1*} and Kassahun Tesfaye²¹Biology Department, College of Natural and Computational Sciences, Debre Berhan University, Ethiopia.²Institute of Biotechnology, Addis Ababa University, Ethiopia.

Received 28 February, 2015; Accepted 13 April, 2015

Lepidium sativum L. (family Brassicaceae), is an underutilized medicinal plant with worldwide distribution. In Ethiopia, *L. sativum* occurs in all regions and agro-ecologies at different altitudinal ranges. The study was conducted to assess the genetic diversity of *L. sativum* population from Ethiopia using inter simple sequence repeat (ISSR) marker. Molecular data generated from ISSR bands recorded was used for computing gene diversity, percent polymorphism and Shannon diversity index and AMOVA. Moreover, the ISSR data was used to construct unweighted pair group method with arithmetic mean (UPGMA) and principal coordinated analysis (PCO) plot using Jaccard's coefficient. Tigray and Amhara population showed higher gene diversity (0.24) and Shannon information index (0.35). All UPGMA, neighbor-joining (NJ) and PCO analysis showed very weak grouping among individuals collected from the same regions. Generally, Tigray and Amhara regions showed moderate to high diversity in ISSR analysis. Different geographical regions of Ethiopia, showed different level of variation; thus conservation priority should be given for those regions that have high genetic diversity. This result also indicates the presence of genetic diversity that can be exploited to improve the productivity of *L. sativum* in Ethiopia.

Key words: Genetic diversity, ISSR, *Lepidium sativum*.

INTRODUCTION

The genus *Lepidium* L. comprises about 150 species distributed worldwide. In tropical Africa, only nine species are found. The genus *Lepidium* belongs to the family Brassicaceae. The garden cress, *Lepidium sativum* L., a fast growing annual herb is native to Egypt and West Asia (Zhan et al., 2009).

L. sativum is a fast growing (30-60 cm) annual herb. Leaves are entire (upper-sessile and lower- petiolate);

flowers- white, small and long racemes, fruits- small pods, obviate, two seeds per pod; seeds- brownish red and slimy when soaked in water, seed shape-elliptic (Zhan et al., 2009). This species reproduces sexually pollen; it is both self and cross pollinating plant. Insects are well known for cross pollination (Quirós and Cárdenas, 1998).

The exact origin of *L. sativum* L. is not known. However,

*Corresponding author. E-mail: seya1muhe@gmail.com. Tel: + +251 (0) 913 13 98 55. Fax: +251116812065.

it is believed to have originated in the highland of Ethiopia and central and Southwest Asia, and then spread to the rest part of the world. This species is commonly cultivated in SW Asia (perhaps Persia) from where it spread many centuries ago, to West Europe, as shown by the philosophical trace of its names in different Indo-European languages (Muhammad and Hussain, 2010).

Medicinal plants are excellent sources of unknown chemical substances for therapeutic effects (Rao, 2004). *L. sativum* seeds contain flavonoids, coumarins, sulphur, glycosides, triterpenes, sterols and various imidazole alkaloids (Radwan et al., 2007; Agarwal and Verma, 2011; Datta et al., 2011). Ethno-medicinal uses of *L. sativum* leaves include its use as salad, cooked with vegetables, curries and also used as fodder for cattle (Moser et al., 2009; Patel et al., 2009; Rehman et al., 2010). The leaves are stimulant, diuretic, used in scorbutic disease and hepatic complaints (Raval and Pandya, 2009).

In Ethiopia, *L. sativum* occurs in all regions and agro-ecology at different altitudinal range. It is not cultivated widely; instead it is cultivated with teff field and available in all local markets. It is not cultivated in large amount as other crops. The main purpose of its cultivation in Ethiopia is to use it as a medicinal plant. It is used for human abdominal ache and diarrhea. Moreover, *L. sativum* is also used to treat skin diseases and other internal problems in livestock.

Despite of its medicinal use, there was no genetic diversity study on Ethiopian *L. sativum*, particularly using molecular markers. Very few studies have been carried out using morphological markers outside Ethiopia. Hence, this study is proposed to investigate the genetic diversity and population structure of *L. sativum* population collected from Ethiopia. Variation was studied using ISSR molecular marker. This gave the overall genetic variability, patterns of distribution and population structure which was very critical to design sustainable conservation and use strategy.

MATERIALS AND METHODS

Tissue harvest and DNA extraction

The experiment was designed to characterize accessions using inter simple sequence repeat (ISSR) markers. Young leaves were collected separately from five randomly selected individual plants per accession after four weeks of planting and dried in silica gel. Approximately equal amount of the dried leaf samples were bulked for each accession and ground with pestle and mortar. Total genomic DNA was isolated from about 0.4 g of the pulverized leaf sample using modified triple cetyl trimethyl ammonium bromide (CTAB) extraction technique as described by Borsch et al. (2003) (Table 1).

Primer selection and optimization

The ISSR marker assay was conducted at Genetics Laboratory of the Microbial, Cellular and Molecular Biology Program Unit, College

of Natural Sciences, Addis Ababa University, Addis Ababa. A total of 10 primers were obtained from the Genetic Research Laboratory (Primer kit UBC 900) and primers used by Kim et al. (2002) were used for the initial testing of primers variability and reproducibility. One individual was selected from each population to screen the primers with 1:5 dilutions. A total of four polymorphic and reproducible ISSR primers (812, 834, 873 and 880) were selected after testing and screening. Table 2 shows the list of primers used and tested, their annealing temperature with respective sequences and other properties.

PCR and gel electrophoresis

The polymerase chain reaction was conducted in Biometra 2003 T3 Thermo cycler. PCR amplification was carried out in a 25 µl reaction mixture containing 1 µl template DNA, 13.45 µl H₂O, 5.60 µl dNTP (1.25 mM), 2.6 µl Taq buffer (10X buffer S), 1.25 µl MgCl₂ (50 mM), 0.6 µl primer (20 pmol/µl) and 0.5 µl Taq Polymerase (3 u/µl). The amplification program was 4 min preheating and initial denaturation at 94°C, then 40 x 15 s at 94°C, 1 min primer annealing at 45°C/ 48°C based on primers used, 1.30 min extension at 72°C and the final extension for 7 min at 72°C. The PCR reactions were stored at 4°C until loading on gel for electrophoresis. The amplification products were differentiated by electrophoresis using an agarose gel (1.67% agarose with 100 ml 1xTBE) and 8 µl amplification product of each sample with 2 µl loading dye (six times concentrated) was loaded on gel. DNA marker 100 bp was used to estimate molecular weight and size of the fragments. The electrophoresis was done for 3 h at constant voltage of 100 V. The DNA was stained with 10 mg/ml ethidium bromide which were mixed with 250 ml distilled water for 30 min and washed with distilled water for 30 min.

Statistical analysis

The bands were recorded as discrete characters, presence '1' or absence '0' and '?' for missing data. Based on recorded bands, different software's were used for analysis. POPGENE version 1.32 software (Yeh et al., 1999) was used to calculate genetic diversity for each population as number of polymorphic loci, percent polymorphism, Gene diversity (H) and Shannon diversity index (I). Analysis of molecular variance (AMOVA) was used to calculate variation among and within population using Arlequin version 3.01 (Excoffier et al., 2006). NTSYS- pc version 2.02 (Rohlf, 2000) and Free Tree 0.9.1.50 (Pavlicek et al., 1999) software's were used to calculate Jaccard's similarity coefficient.

The unweighted pair group method with arithmetic mean (UPGMA) (Sneath and Sokal, 1973) was used to analyze and compare the population and generate phenogram using NTSYS- pc version 2.02 (Rohlf, 2000). To further examine the patterns of variation among individual samples on 3D, a principal coordinated analysis (PCO) was performed based on Jaccard's coefficient (Jaccard, 1908). The calculation of Jaccard's coefficient was made with PAST software version 1.18 (Hammer et al., 2001). The first three axes were used to plot the three dimensional PCO with STATISTICA version 6.0 software (Hammer et al., 2001; Statistica soft, Inc.2001).

RESULTS

Genetic diversity as revealed by percent polymorphism, shannon and gene diversity values

Of the total 53 loci scored, 81.13% (43) were observed to be polymorphic. From all the population studied, Amhara

Table 1. List of *L. sativum* accessions, altitude and regions of collection used in the study and their respective symbol used in molecular analysis result

Number	Accession number	Altitude	Region	Region	Accession symbol
1	90018	2500	Amhara	Wello	A1
2	208030	*Uk	Amhara	Gonder	A2
3	90004	2400	Amhara	Gonder	A3
4	205162	*Uk	Amhara	Gonder	A4
5	229799	2550	Amhara	Gojam	A5
6	215713	2580	Amhara	Wello	A6
7	229205	2690	Amhara	Shewa	A7
8	241777	2110	Amhara	Wello	A8
9	214243	*Uk	Amhara	Gonder	A9
10	215714	2200	Amhara	Wello	A10
11	212628	1500	Amhara	Wello	A11
12	235892	2270	Amhara	Gonder	A12
13	229200	2150	Amhara	Shewa	A13
14	207542	*Uk	Amhara	Gonder	A14
15	229203	2620	Amhara	Shewa	A15
16	229201	2310	Amhara	Shewa	A16
17	229798	2250	Amhara	Gojam	A17
18	229204	2580	Amhara	Shewa	A18
19	229202	2600	Amhara	Shewa	A19
20	205162	2820	Amhara	Gonder	A20
21	90020	2000	Amhara	Shewa	A21
22	229199	2220	Amhara	Shewa	A22
23	208769	1900	Oromia	Wollega	O1
24	208667	1900	Oromia	Harerrge	O2
25	212852	*Uk	Oromia	Balle	O3
26	90002	*Uk	Oromia	Harerrge	O4
27	215808	1950	Oromia	Wollega	O5
28	212853	*Uk	Oromia	Balle	O6
29	215807	1720	Oromia	Wollega	O7
30	208666	*Uk	Oromia	Harerrge	O8
31	208693	*Uk	Oromia	Harerrge	O9
32	230830	2450	Oromia	Harerrge	O10
33	230831	2050	Oromia	Harerrge	O11
34	237991	2150	Oromia	Balle	O12
35	230524	*Uk	Oromia	Harerrge	O13
36	90023	*Uk	Oromia	Keffa	O14
37	90021	2620	Oromia	Shewa	O15
38	90005	*Uk	Oromia	Harrerge	O16
39	216885	1570	Oromia	Arsi	O17
40	208669	2180	Oromia	Harerrge	O18
41	90022	1480	Oromia	Wollega	O19
42	234828	*Uk	Oromia	Wollega	O20
43	216886	1570	Oromia	Arsi	O21
44	216816	*Uk	Oromia	Harerrge	O22
45	219961	*Uk	Tigray	Tigray	T1
46	219960	1940	Tigray	Tigray	T2
47	233984	1860	Tigray	Tigray	T3
48	219959	*Uk	Tigray	Tigray	T4
49	233981	2140	Tigray	Tigray	T5

Table 1. Contd

50	233986	2080	Tigray	Tigray	T6
51	207910	*Uk	Tigray	Tigray	T7
52	237512	1900	Tigray	Tigray	T8
53	242609	*Uk	Tigray	Tigray	T9
54	234355	2090	Tigray	Tigray	T10
55	207991	*Uk	Tigray	Tigray	T11
56	233983	1950	Tigray	Tigray	T12
57	233982	2210	Tigray	Tigray	T13
58	219958	2130	Tigray	Tigray	T14
59	233370	*Uk	Tigray	Tigray	T15
60	233679	*Uk	Tigray	Tigray	T16
61	240396	1950	SNNPR	Keffa	SN1
62	242916	2200	SNNPR	Keffa	SN2
63	214660	*Uk	SNNPR	Sidamo	SN3
64	202116	*Uk	SNNPR	Keffa	SN4
65	225799	2650	SNNPR	Gammo goffa	SN5
66	205141	1900	SNNPR	Shewa	SN6
67	240578	*Uk	SNNPR	Gammo goffa	SN7
68	240397	*Uk	SNNPR	Keffa	SN8
69	90016	*Uk	SNNPR	Gammo goffa	SN9
70	225725	*Uk	SNNPR	Gammo goffa	SN10
71	240808	1950	SNNPR	Gammo goffa	SN11
72	90009	*Uk	Unknown	Unknown	UN1
73	90014	*Uk	Unknown	Unknown	UN2
74	90012	*Uk	Unknown	Unknown	UN3
75	90017	*Uk	Unknown	Unknown	UN4
76	90010	*Uk	Unknown	Unknown	UN5
77	240579	*Uk	Unknown	Unknown	UN6
78	90008	*Uk	Unknown	Unknown	UN7
79	80001	*Uk	Unknown	Unknown	UN8
80	80002	*Uk	Unknown	Unknown	UN9
81	216815	*Uk	Somali	Somali	SO1
82	230829	*Uk	Somali	Somali	SO2
83	231210	1910	Somali	Somali	SO3
84	230523	*Uk	Somali	Somali	SO4
85	90007	*Uk	Somali	Somali	SO5

Source: IBC database. *Uk= Unknown.

and Tigray were 6.04%, Oromia 50.94%, SNNPR 47.17%, and Somali 45.28% polymorphic. Amhara and Tigray showed more percent polymorphism; while the least polymorphism was detected in population from Somali region. No unique bands were observed for either the accessions or the populations (Table 3).

Among the *L. sativum* accessions evaluated using ISSR marker, samples from Tigray and Amhara exhibited the highest gene diversity ($H = 0.24$), whereas samples from Oromia had ($H = 0.17$), from SNNPR ($H = 0.18$) and Somali ($H = 0.18$) gene diversity values. The average gene diversity for the total population (H_T) was 0.27 (Table 3, Figure 1).

Primer 873 showed highest gene and Shannon diversity (0.36 and 0.53, respectively) and primer 812 was the least (0.20 and 0.31, gene and Shannon diversity, respectively) (Table 4).

Analysis of molecular variance

Analysis of molecular variance was carried out on the overall ISSR data score of *L. sativum* accessions without grouping by region or geographic location. AMOVA revealed high percentage of variation (94%) that was attributed to within population variation while the remaining

Table 2. List of primers, annealing temperature, primer sequence, amplification quality and repeat motives used for optimization.

Primer	Annealing temperature (°C)	Primer sequence	Amplification quality	Repeat motive
810	45	GAGAGAGAGAGAGAT	Monomorphic	Dinucleotide
812	45	GAGAGAGAGAGAGAA	Polymorphic, reproducible	Dinucleotide
818	48	CACACACACACACAAG	Monomorphic	Dinucleotide
824	48	TCTCTCTCTCTCTCG	Monomorphic	Dinucleotide
834	45	AGAGAGAGAGAGAGYT	Polymorphic, reproducible	Dinucleotide
844	45	GAGAGAGAGAGAGAYT	No banding	Dinucleotide
872	38	GATAGATAGATAGATA	No banding	Tetra- nucleotide
873	45	GACAGACAGACAGACA	Polymorphic, reproducible	Tetra- nucleotide
878	45	GGATGGATGGATGGAT	No banding	Tetra- nucleotide
880	45	GGAGAGGAGAGGAGA	Polymorphic, reproducible	Penta- nucleotide

Source: Primer kit 900 (UBC 900); Single-letter abbreviations for mixed base positions: R = (A, G); Y = (C, T).

Table 3. Banding patterns generated using the four selected primers, their repeat motifs, amplification patterns and number of scored bands.

Primer	Repeat motif	Amplification quality	Number of scored bands
812	(GA)8A	Excellent	14
834	(AG)8YT	Excellent	11
873	(GACA)4	Excellent	16
880	(GGAGA)3	Excellent	12
Total			53

Single-letter abbreviations for mixed base positions: R = (A, G) Y = (C, T).

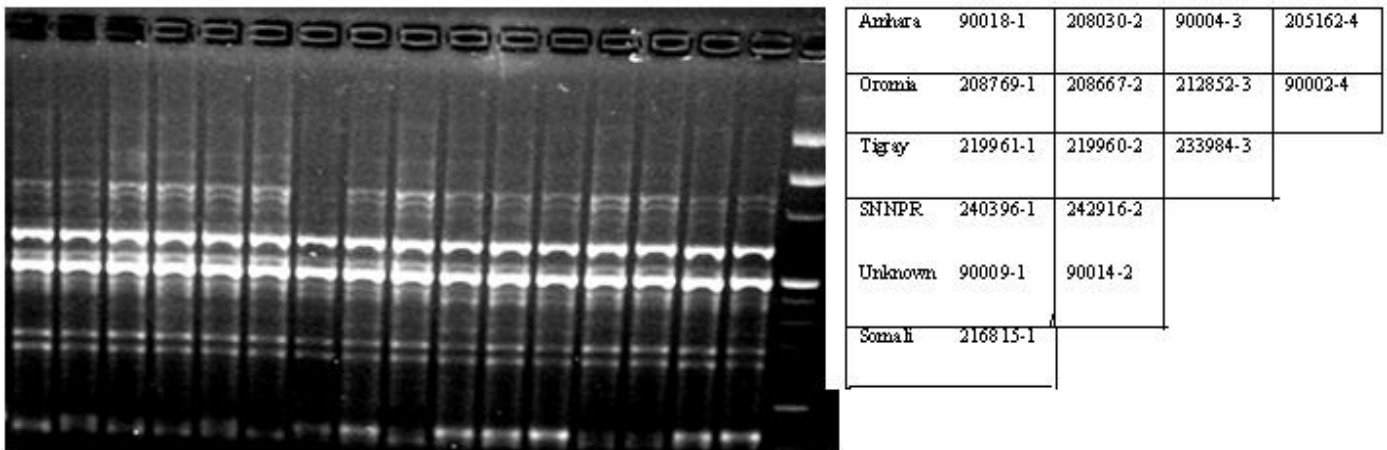


Figure 1. ISSR fingerprint generated from 16 individual accessions using primer 873.

variation was due to population variation (6%). The highest polymorphic loci (35) and percent polymorphism (66.04) were observed in Amhara and Tigray regions (Table 5). Similarly, the highest genetic diversity (0.24) and Shannon information index (0.35) were recorded in Amhara and Tigray regions (Table 5).

The variation was found to be highly significant at P = 0.00. The result shows that there was high gene flow or

seed flow among population in different region; this resulted in low genetic variation and differentiation among population (Table 6).

Clustering analysis

UPGMA and neighbor joining tree construction methods

Table 4. Number of scorable bands (NSB), number of polymorphic loci (NPL), percent polymorphism (PP), genetic diversity (H), Shannon index information (I) of 85 *L. sativum* accessions based on all primers used.

Primer	NSB	NPL	PP	H±SD	I±SD
812	14	9	64.29	0.20±0.20	0.31±0.28
834	11	9	81.82	0.24±0.15	0.38±0.22
873	16	15	93.75	0.36±0.13	0.53±0.18
880	12	10	83.33	0.25±0.15	0.39±0.22
Over all	53	43	81.13	0.27±0.17	0.41±0.24

Table 5. The number of polymorphic loci (NPL), percent polymorphism (PP), genetic diversity (H) and Shannon information index (I) among the five regions of Ethiopia.

Population	NPL	PP	H±SD	I±SD
Amhara	35	66.04	0.24±0.19	0.35±0.28
Oromia	27	50.94	0.17±0.19	0.26±0.28
Tigray	35	66.04	0.24±0.19	0.35±0.27
SNNPR	25	47.17	0.18±0.21	0.27±0.30
Somali	24	45.28	0.18±0.21	0.26±0.30
Total	182	343.39	1.27±1.2	1.87±1.72

Table 6. Analysis of molecular variance (AMOVA) of *L. sativum* accessions in Ethiopia without grouping.

Source of variation	Sum of squares	Variance components	Percentage of variation	Fixation	P
Among populations	4.122	0.02834	6.00	0.06	0.00
Within population	34.765	0.44387	94.00		
Total	38.888	0.47221	100		

were used to construct dendrogram for six population and 85 individuals based on 53 PCR bands amplified by two di-nucleotides (812 and 834), one penta nucleotide (880) and one tetra nucleotide (873). The dendrogram derived from neighbor-joining analysis of the whole ISSR data with 85 *L. sativum* accessions showed four distinct clusters and two sub-clusters within each major cluster. Most of the individual accessions collected from the same region tend to spread all over the tree without forming their own grouping. The wider distribution of *L. sativum* accession all over the tree showed the low divergence among population from different localities. UPGMA analysis based on regions of collection of *L. sativum* revealed three major groups. The first cluster contained Oromia, Amhara and Tigray; while the second cluster contains SNNPR and individual from unknown origins. The final major cluster contained the Somali group (Figures 2 and 3).

PCO analysis

All the data obtained using the four ISSR primers were used in PCO analysis using Jaccard's coefficient of

similarity. The first three coordinates of the PCO having Eigen values of 4.83, 4.55 and 1.63 with variance of 18.28, 17.26 and 6.20%, respectively were used to show the grouping of individuals using two and three coordinates. In 3D most of the individual accessions that represent different populations spread all over the plot. Using two coordinates (Figures 4 and 5) almost similar result was observed like that of three coordinates. Overall, no clear grouping was observed among individuals collected from different locality.

DISCUSSION

Molecular diversity and its implications for improvement and conservation

In the present study, ISSR was used for the first time to assess genetic variation of *L. sativum* population from Ethiopia. This method provides an alternative choice to other system for obtaining highly reproducible marker without any necessity for prior sequence information for various genetic analyses. Due to the abundant and rapidly

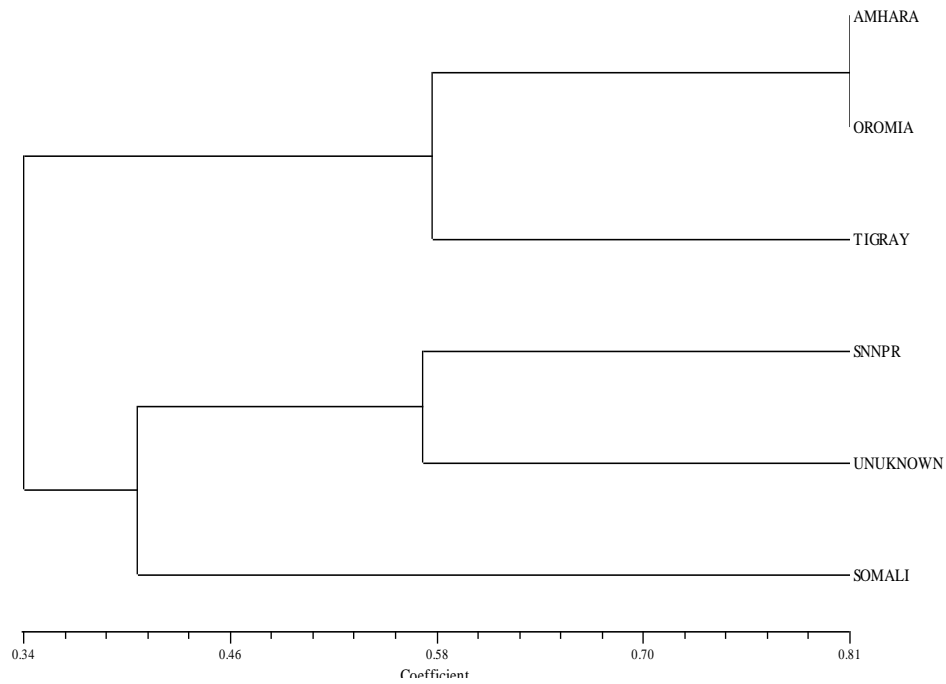


Figure 1. UPGMA based dendrogram for 6 *L. sativum* population using 4 ISSR (2 di, 1 penta and 1 tetra nucleotide) primers.



Figure 3. Neighbor-joining analysis of 85 individuals based on 53 PCR bands amplified by two dinucleotide (812 and 834); one tetranucleotide (873); and one pentanucleotide (880) primers. The neighbor joining algorithm is based on Jaccard's coefficient.

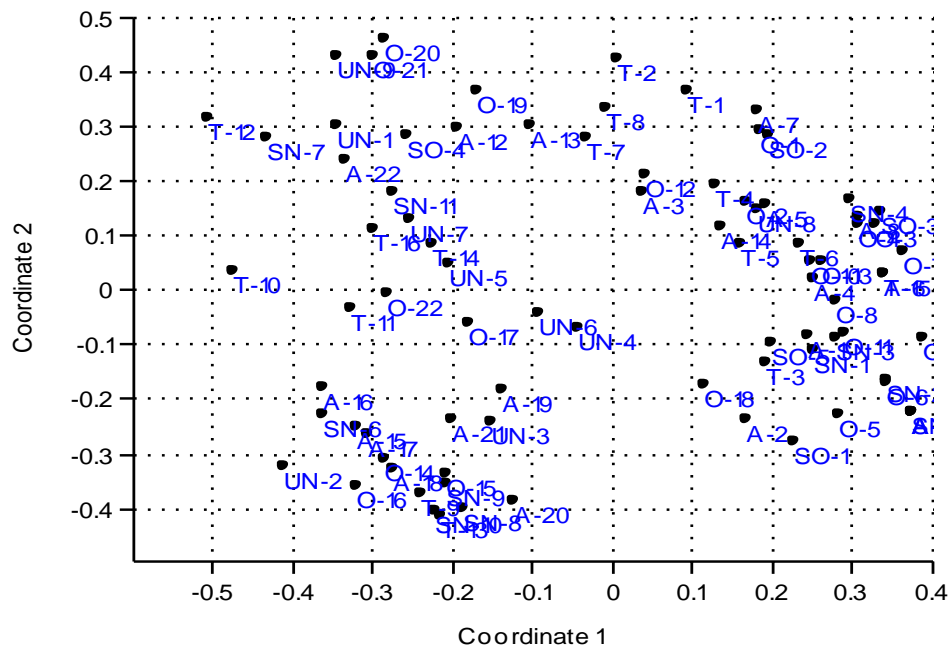


Figure 4. Two dimensional representation of principal coordinate analysis of genetic relationships among 85 accessions of *L. sativum* accessions using ISSR data.

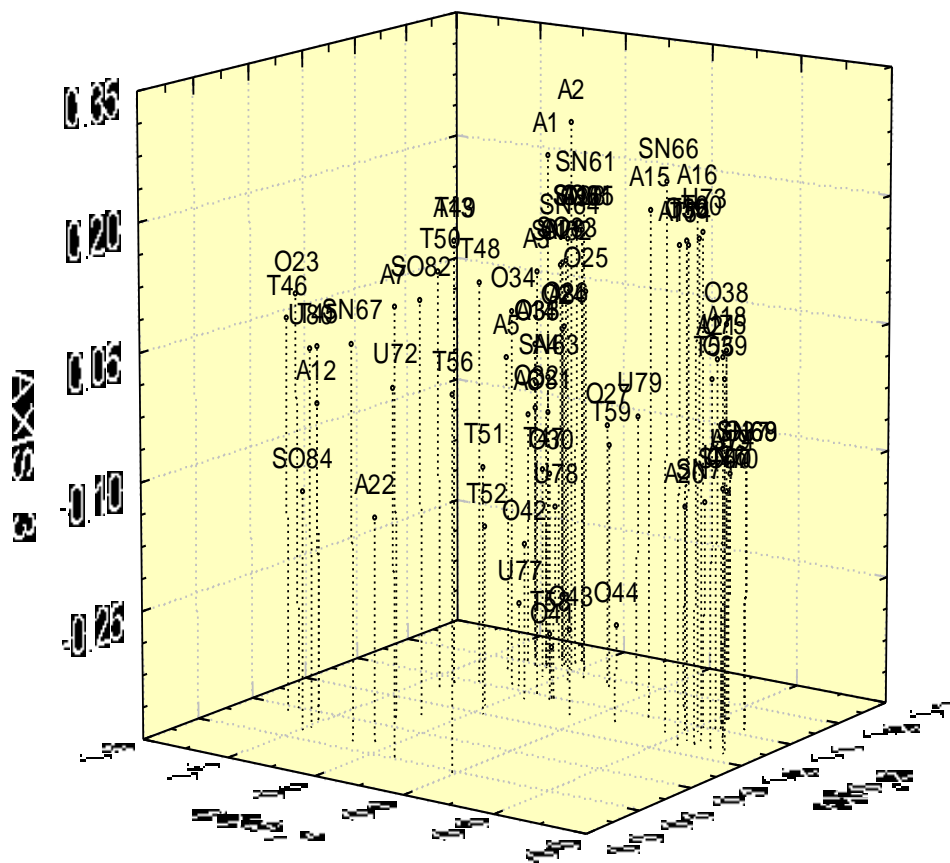


Figure 5. Three dimensional representation of principal coordinate analysis of genetic relationships among 85 accessions of *L. sativum* accessions.

evolving SSR regions, ISSR amplification has the potential of illuminating much larger number of polymorphic fragments per primer than any other marker system used such as RFLP or microsatellites. ISSRs are regions that recline within the microsatellite repeats and offer great potential to determine intra-genomic and inter-genomic diversity compared to other arbitrary primers, since they reveal variation within unique regions of the genome at several loci simultaneously. Several properties of microsatellite such as high variability among taxa, ubiquitous occurrence and high copy number in eukaryotic genome make ISSRs extremely useful marker for variability analysis (Morgante et al., 2002).

In this study, bulk sampling approach was chosen, since it permits representation of the vast accession by optimum number of plants. Yang and Quiros (1993) reported that bulked samples with 10, 20, 30, 40 and 50 individuals had resulted in the same RAPD profiles as that of the individual plant constituting the bulk sample. Gilbert et al. (1999) also reported that pooling of DNA from individuals within accessions is the most appropriate strategy for assessing large quantities of plant material and concluded that 2-3 pools of five genotypes is sufficient to represent the genetic variability within and between accessions in the lupin and similar collections. Edossa et al. (2010) used bulked samples for diversity assessment in lentil collected from Ethiopia. The technique revealed higher genetic diversity, and, therefore, validated the usefulness of bulk sample analyses. Dagmawi (2011) also used bulked sample in germplasm diversity study of sesame populations, and found moderate genetic diversity of both Ethiopian and exotic population.

The present study showed that out of 53 loci generated by four primers two di, one penta and one tetra; 43 of them were polymorphic with 81.13% polymorphism. In regions based analysis, Amhara and Tigray showed higher percent polymorphism (66.04%); while, SNNPR and Somali showed least polymorphism with 47.17 and 45.28%, respectively. The same patterns of diversity were observed with gene diversity and Shannon index. Generally, *L. sativum* populations from Amhara and Tigray showed higher diversity than the other regions.

Edossa et al. (2010) studied the morphological and molecular diversity of Ethiopian lentil (*Lens culinaris Medikus*) using four ISSR primers and found 59.57% polymorphism with higher percent variation attributed within population (56.28%). Gezahegn et al. (2009) studied wild and cultivated rice species of Ethiopia using six ISSR primers and reported 38.3 and 28.3% polymorphism of wild and cultivar rice species, respectively. Moreover, higher proportion of genetic diversity was observed within populations of rice (Gezahegn et al., 2009). Hence, the present study shows higher percent polymorphism and higher proportion of diversity within population of *L. sativum* comparable with that of Edossa et al. (2010) and Gezahegn et al. (2009).

In general Amhara and Tigray had good genetic diversity than Oromia, SNNPR and Somali.

AMOVA analysis resulted in high genetic diversity within population (94%) and very low genetic diversity among population (6%). This could be due to high seed exchange among different regions and markets which could lead to intermix of population between regions. Unlike other landraces of cultivated plants, *L. sativum* in Ethiopia is not restricted to a given area rather it is widely exchanged among local community and markets. This showed that there is very high gene flow between population and regions. Jiang et al. (2012) who studied on the genetic diversity of *Chimonanthus grammatus* population by using ISSR marker showed that there was 73.6% within population variation whereas the rest 26.4% was due to among population variation. As compared to the present study, there was less gene flow. Jiang et al. (2012) recommended that gene flow, genetic drift and evolutionary history might have important influence on genetic structure and diversity of a given population.

L. sativum is both self and cross pollinated plant (Quirós and Cárdenas, 1998). Hence, the proportion of genetic variation is dependent on the type of pollination that the species undergoes. If the species has large proportion of cross pollination, then we expect high genetic variation within population and less divergence among population. In addition to pollination, behavior of insects; market exchange could facilitate gene flow among regions which could result in higher percent variation within population and less genetic structure. This is also supported with the spread of individual accessions on UPGMA and PCO graphs.

Dendrogram of the present study by using UPGMA of Jaccard's coefficient of similarity showed that Amhara and Oromia population of *L. sativum* were closely related. Based on this study, the samples with unknown origins could probably have been collected from Southern part of Ethiopia since they closely clustered with the SNNPR population. The Somali population had its own lineage far from the other population and diverted as an out lies. Genetic distance is a measure of the allelic substitutions per locus that have occurred during the separate evolution of two population or species.

Smaller genetic distances indicate a close genetic relationship, whereas large genetic distances indicate a more distant genetic relationship. Crosses between distantly related individuals are expected to give better offspring than those between closely related genotypes. Therefore, prior knowledge of the genetic distance between genotypes or accessions is important in designing breeding program.

Genetic diversity of plant population is largely influenced by factors such as reproduction system, genetic drift, evolutionary history and life history (Loveless and Hamrick, 1984). In broad-spectrum, out crossing species have higher levels of genetic diversity than selfing and clonal plants (Rossetto et al., 1995).

Conclusion

Analysis of molecular variance for the accessions studied showed that the highest proportion of genetic variation was attributed to within population than among population. It is also highly significant. This confirms that there was a high level of gene flow and low level of genetic differentiation. Based on the UPGMA data, the Amhara, Tigray and Oromia accessions were clustered into one group, whereas the SNNPR and the unknowns to the other cluster. Samples from Somali formed a distinct cluster and showed that it is distantly related to accessions from the entire regions.

Competing interest

The authors declare that they have no competing interests.

ACKNOWLEDGEMENTS

Our sincere thanks to the Ethiopian Institute of Biodiversity (EIB) for providing germplasms with full passport data of *L. sativum* assembled from Ethiopia. We are also grateful to Professor Klaus mummenhoff for his help during laboratory work.

REFERENCES

- Agarwal J, Verma D L (2011). Antioxidative activity and flavonoid composition from *Lepidium Sativum*. *Nat. Sci.* 9: 21-25.
- Borsch T, Hilu KW, Quandt D, Wilde V, Neinhuis C, Barthlott W (2003). Non coding plastid trnT-trnF sequences reveal a well resolved phylogeny of basal angiosperms. *J. Evol. Biol.* 16: 558-576.
- Dagmawi T (2011). Germplasm Study of Ethiopian and Exotic Sesame Populations. M. Sc. Thesis. Addis Ababa University, Addis Ababa.
- Datta P K, Diwakar BT, Viswanatha S, Murthy KN, Naidu K A (2011). Safety evaluation studies on garden cress (*Lepidium sativum* L.) seeds in Wistar rats. *Int. J. Appl. Res. Nat. Prod.* 4:37-43.
- Edossa Fikiru, Kassahun Tesfaye, Endashaw Bekele (2010). A comparative study of morphological and molecular diversity in Ethiopian lentil (*Lens culinaris Medikus*) landraces. *Afr. J. Plant Sci.* 4:242-254.
- Gezahegn G, Kassahun T, Endashaw B (2010). Inter simple sequence repeat (ISSR) analysis of wild and cultivated rice species from Ethiopia. *Afr. J. Biotechnol.* 9: 5048-5059.
- Gilbert JE, Lewis RV, Wilkinson MJ, Caligari PDS (1999). Developing an appropriate strategy to assess genetic variability in plant germplasm collections. *Theor. Appl. Genet.* 98: 1125-1131.
- Hammer O, Harper D, Ryan P (2001). PAST: Paleontological statistics software for education and data analysis. *Paleontología Electrónica* 4:1-9.
- Jiang Y, Li Y, Lu S, Liu Y, Peng J, Zhu D (2012). Genetic diversity of *Chimonanthus grammatus* populations determined with inter-simple sequence repeats (ISSR) analysis: Implications for conservation. *J. Med. Plants Res.* 6:1272-1278.
- Kim DH, Zur G, Danin-Poleg Y, Lee S W, Shim K B, Kang C W, Kashi Y (2002). Genetic relationships of sesame germplasm collection as revealed by inter-simple sequence repeats. *Plant Breed.* 121:259-262.
- Loveless M D, Hamrick JL (1984). Ecological determinants of genetic structure in plant populations. *Annu. Rev. Ecol. Syst.* 15:65-69.
- Morgante M, Hanafey H, Powell W (2002). Microsatellites are preferentially associated with non repetitive DNA in plant genome. *Nat. Genet.* 30:194-200.
- Moser B R, Shah SN, Winkler-Moser J K, Vaughn S F, Evangelisa R L (2009). Composition and Physical Properties of Cress (*Lepidium sativum* L.) and Field Pennycress (*Thlaspi arvense* L.) oils. University St., Peoria, USA.
- Muhammad Z, Hussain F (2010). Vegetative growth performance of five medical plants under NaCl salt stress. *Pak. J. Bot.* 42: 303-316.
- Patel U, Kulkarni M, Undda U, Bhosale A (2009). Evaluation of diuretic activity of aqueous and methanol extracts of *Lepidium sativum*, garden cress (Cruciferaceae) in rats. *Trop. J. Pharm. Res.* 8:215-219.
- Pavlicek A, Hrdá S, Flegr J (1999). Free-tree-soft ware program for construction of phylogenetic trees on the basis of distance data and bootstrap/jackknife analysis of the tree robustness. Application in the RAPD analysis of genus *Frenkelia*. *Folia Biol. (Praha)* 45: 97-99.
- Quiros C F, Cárdenas R A (1998). MACA (*Lepidium meyenii* Walp.). University of California, USA.
- Radwan HM, El-Missiry MM, Al-Said WM, Ismail AS, Abdel SH, Seif-Elnasr MM (2007). Investigation of the glucosinolate of *Lepidium sativum* growing in Egypt and their biological activity. *Res. J. Med. Med. Sci.* 2: 127-132.
- Rao NK (2004). Plant genetic resources. Advancing conservation and use through biotechnology. *Afr. J. Biotechnol.* 3:136- 145.
- Raval ND, Pandya JN (2009). Clinical trait of *Lepidium sativum* in management of sandhivata. *AYU* 30:153-157.
- Rohlf FJ (2000). NTSYS-pc ver 2.11T. Exter Software, Setauket, New York.
- Rossetto M, Weaver PK, Dixon KW (1995). Use of RAPD analysis in devising conservation strategies for the rare and endangered *Grevillea scapigera* (Proteaceae). *Mol. Ecol.* 4:321-329.
- Sneath PHA, Sokal RR (1973). Numerical taxonomy. Freeman, San Francisco.
- Statistica soft Inc. (2001). Statistica data analysis system, Statistica software.
- Yang X, Quiros C (1993). Identification and classification of celery cultivars with RAPD markers. *Theor. Appl. Genet.* 83: 2-3.
- Yeh FC, Yang RC, Boyle TJ B, Ye Z, Mao JX (1999). POPGENE, the user Friendly Shareware for Population Genetics Analysis, Version 1.3.1. Molecular Biotechnology center, University of Alberta, Canada.
- Zhan LJ, Fontane E, Tibaldi G, Nicola S (2009). Qualitative and physiological response of minimally processed garden cress (*Lepidium sativum* L.) to harvest handling and storage conditions. *J. Food Agric. Environ.* 7:43-50.

Short Communication

Effects of selected culture media on mycelial growth of oyster mushroom (*Pleurotus ostreatus*)

Chuku, S. B.¹, Nwachukwu, E. O.¹ and Stanley, H. O.²

¹Department of Plant Science and Biotechnology, University of Port Harcourt, Nigeria.

²Department of Microbiology, University of Port Harcourt, Nigeria.

Received 12 November, 2014; Accepted 13 April, 2015

The effect of selected culture media on mycelial growth of *Pleurotus ostreatus* was studied on six different culture media: three synthetic [malt extract agar (MEA), potato dextrose agar (PDA) and Sabouraud dextrose agar (SDA)] and three formulated from locally available agricultural wastes [corn cob (CCEA), palm waste (PWEA) and cassava peels (CPEA)]. The tissue culture of the mushroom was done on MEA which was used as the stock culture. The mycelia were subcultured severally to get a pure culture. Pure culture of the mycelia was subcultured on the different media types and incubated at room temperature for five days. The experiment was set up in three replicates. The mycelial growth, density and growth rate were obtained. The values obtained were subjected to statistical analysis. The results for maximum mycelia growth, mycelial density and growth rate were as follows: MEA (9.0 cm, 5+ and 1.8 cm/day), PDA (6.4 cm, 3+ and 1.3 cm/day), SDA (7.8 cm, 5+ and 1.6 cm/day), CPEA (9.0 cm, 4+ and 1.8 cm/day), PWEA (9.0 cm, 3+ and 1.8 cm/day) and CCEA (2.4 cm, 2+ and 0.5 cm/day). Generally, all the media types used supported the growth of the mycelia. The result indicates that MEA, SDA and CPEA gave abundant growth of *P. ostreatus* mycelia while CCEA recorded scanty mycelial growth.

Key words: *Pleurotus ostreatus*, malt extract agar, potato dextrose agar, Sabouraud dextrose agar, mushroom, mycelia growth.

INTRODUCTION

The fungus (*Pleurotus ostreatus*) is an edible species of the white rot fungi belonging to the class Basidiomycetes and family Pleurotaceae. *P. ostreatus* (Jacq: Fr.) Kummer, tree oyster mushroom, is the most cultivated species among oyster mushrooms and the type species

of the genus *Pleurotus*. The *Pleurotus* fungi are found in both tropical and temperate climates throughout the world (Change and Miles, 2004). In nature, the fungus is found to grow on a wide range of dead plant species rich in lignin and cellulose. The white rot fungi belong to a

*Corresponding author. E-mail: talk2soso@yahoo.co.uk.

Abbreviations: MEA, Malt extract agar; PDA, potato dextrose agar; SDA, saubroud dextrose agar; CCEA, corncob extract agar; PWEA, palm waste extract agar; CPEA, cassava peels extract agar; PPEA, plantain peel extract agar; YPDA, yeast peptone dextrose agar.

Author(s) agree that this article remains permanently open access under the terms of the [Creative Commons Attribution License 4.0 International License](https://creativecommons.org/licenses/by/4.0/)

physiological grouping that comprises those fungi that are capable of extensively degrading Lignin, within Ligno cellulosic substrates (Pointings, 2001). The degrading fungi excrete lignolytic enzymes extracellularly, triggering oxidation of substrates in the extracellular environment (Mester and Tien, 2000). Due to the importance of the mushroom (Oyster mushroom) as a source of food for humans and feed for animals and it is fast growing demand for other medicinal and industrial uses (mycoremediation) in recent times, the need to cultivate the mushroom is in high demand without only depending on its natural occurrence in the wild. The mycelium is the vegetative part of the mushroom which increases the surface area and facilitates absorption and digestion of nutrients and develops into the actual fruiting body.

The purpose of this study was to determine media types that support fast multiplication of the mycelia. *P. ostreatus*, has been studied by Nasim et al. (2001) comparing the effect of three culture media [malt extract agar (MEA), potato dextrose agar (PDA) and Murashige and Skoog's (MS) Medium] on the mycelial growth. MEA was shown to have the highest growth while Potato Dextrose Agar (PDA) ended up with the least growth. Similar work was also reported by Stanley and Nyenke (2011) where they studied mycelial growth on different media using MEA, PDA, Sabouraud dextrose agar (SDA), corn cob extract agar (CCEA), cassava peel extract agar (CPEA) and plantain peel extract agar (PPEA). They discovered that MEA, CCEA and CPEA showed an excellent growth rate and density of *Pleurotus pulmonarius* while PDA and PPEA were observed to support poor mycelia growth. A similar research carried out by Uddin et al. (2012) showed that PDA provided slower mycelia growth rate of *P. ostreatus* than MEA. Dey et al. (2007) worked on mycelial growth of *P. ostreatus* on PDA, MEA and Yeast Peptone Dextrose Agar (YPDA) and found out that YPDA was superior to PDA and MEA for mycelial colony proliferation. Similar work has also been reported by Mansur et al. (2012) on *P. ostreatus* using PDA, Yeast Mannitol Agar (YMA) and MS medium and it was reported that PDA recorded the best growth than YMA and MS medium. In this study, mycelial growth of *P. ostreatus* was studied on six (6) different media MEA, SDA, PDA, CCEA, CPEA and PWEA.

MATERIALS AND METHODS

The fungus (*P. ostreatus*) used was obtained from the demonstration farm, Faculty of Agriculture, University of Port Harcourt, Choba, Port Harcourt. All the instruments used were properly sterilized; all glassware (Petri dishes, test tubes and McCartney bottles) were sterilized by autoclaving at 121°C for 30 min. The other instruments used like forceps, scapel and inoculating loop were cleaned with 70% ethanol, flamed to red hot and allowed to cool close to fire area and the work surface was properly scrubbed with 90% ethanol and hypochlorite. Six (6) selected media types were used, 3 ready to use and 3 formulated from agricultural wastes. The media preparations were done as

follows:

Malt extract agar (MEA)

To prepare 1 L, 50 g of MEA powder was added to 1 L of distilled water and autoclaved at 121°C for 20 min. When cooled to about 45°C, 2% lactic acid was added.

Potato dextrose agar (PDA)

Thirty nine grams of PDA powder was added to 1 L of distilled water

Sabouraud dextrose agar (SDA)

Sixty five grams of SDA powder was added to 1 L of distilled water.

Corn cob extract agar (CCEA)

200 g of sliced corncob waste was placed in 1 L of distilled water and boiled for 30 min. The extract was strained through a fine sieve. 20 g of Agar was added to the filtrate and distilled water was added to make up to 1 L. The mixture was then autoclaved at 121°C for 20 min. When cooled to about 45°C, 2% lactic acid was added.

Cassava peel extract agar (CPEA) and palm waste extract agar (PWEA)

CPEA and PWEA were prepared with the same method above. The tissue culture was done using freshly picked and tender mushrooms were gently cleaned with 70% ethanol, small piece of deep mushroom tissues were sectioned using an already sterilized scalpel (flamed to red hot and cooled around the flame area) and placed on MEA medium already poured and solidified in Petri dishes. The inoculated Petri dishes were immediately sealed with masking tape to prevent contamination and then incubated without light for 14 days at room temperature of about 28°C±2. Then several subcultures were done to get a pure culture. The pure culture was subcultured to the different media types under study, in each case the inoculum was placed at the centre of the Petri dishes. The mycelia growth was determined using a transparent ruler to measure the colony diameter. The mycelia density was graded by visual observation as described by Kadiri (1998). The growth rate was calculated as described by Stanley and Nyenke (2011) using the formula.

$$\text{Growth rate} = \frac{\text{Colony diameter on the last day (cm)}}{\text{Number of days measurement was taken after inoculations}}$$

RESULTS AND DISCUSSION

The result in (Figure 1) on the 5th day shows that *P. ostreatus* had the highest mycelial colony diameter of 9.0 cm and growth rate of 1.8 cm/day on CPEA, MEA and PWEA but they differed in their mycelial density which were 4+, 5+ and 3⁺, respectively, for CPEA, MEA and PWEA. On SDA medium, the highest colony diameter was 7.8 cm, mycelial density of 5+ and growth rate of 1.6 cm/day. Furthermore, the growth on PDA was poor with 6.4 cm as highest mycelial colony diameter, 3+ mycelial

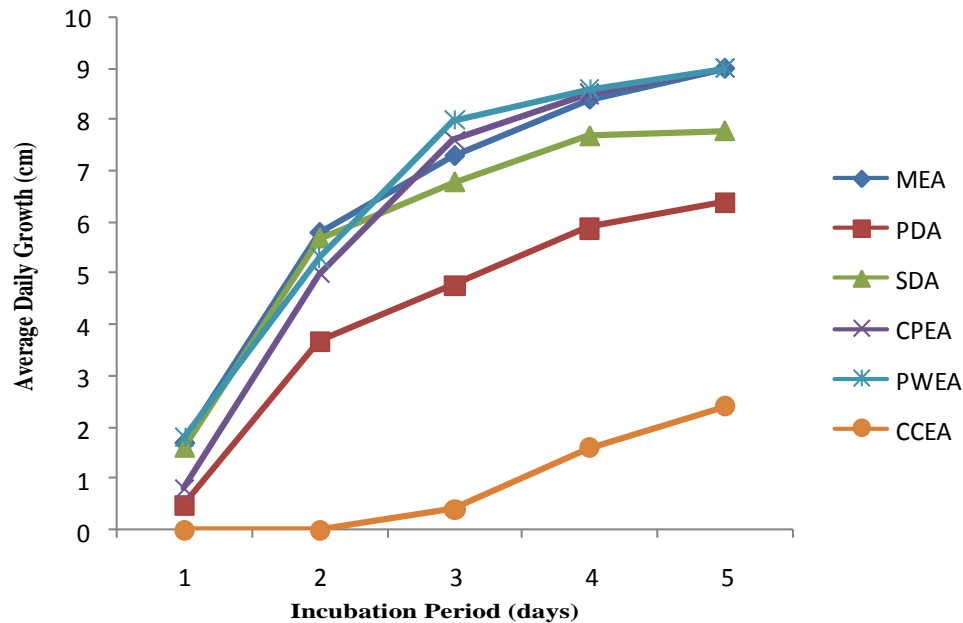


Figure 1. Composite graph of growth of *Pleurotus ostreatus* all media types used.

density and 1.3 cm/day as growth rate. The least growth was recorded on CCEA with 2.4 cm as highest mycelial colony diameter, 2+ as mycelial density and 0.5 cm/day as growth rate.

The results obtained during the experiments showed that all media types used supported the growth of the mycelium of *P. ostreatus*. All the culture media [composed from agricultural (organic) wastes and synthetic] stimulated and sustained high mycelial growth except the CCEA. The results revealed that mushroom mycelial growth was high for MEA, CPEA, PWEA and SDA as compared to PDA and CCEA media which supported moderate and scanty mycelial growth, respectively. The differences observed in mycelia growth is due to varying composition of nutrients in the substrates used. These mushroom mycelia require lignocellulosic substrates that are good sources of carbon and nitrogen to sustain the growth. This study is similar to those of Suharban and Anthony (1996) who worked on the stability of different tuber crops on mycelia growth of *P. ostreatus* variety sajor caju in liquid and solid media.

The study shows that the mycelia of oyster mushroom took five (5) days for complete colonization in the media plates with MEA, PWEA and CPEA but in the case of the other media types (PDA, SDA and CCEA), the media plates were not fully ramified even after the 5th day (Plate 1a to 1e). The findings are in line with those of Nasim et al. (2001) who found that MEA provided fastest *P. ostreatus* mycelia growth rate than that of Murashige and Skoog's (MS) medium and PDA. They also found that slowest growth was observed on PDA medium. The result of the study is also in line with those of Uddin et al. (2012) which showed that PDA provided slower mycelial

growth rate of *P. ostreatus* than MEA.

In contrast, Dey et al. (2007) found out that YPDA media was superior to PDA and MEA for mycelia colony proliferation while Munsur et al. (2012) found out that PDA recorded the best mycelia than YMA and MS media. The other parameters used to compare the growth of mushroom mycelia on different culture media were mycelial density and growth rate. Mycelial density was very abundant on MEA and SDA, abundant on CPEA while PDA and PWEA was moderate and scanty for CCEA. This is similar to the findings of Stanley and Nyenke (2011) in the cultural studies on mycelia of *P. pulmonarius* (Oyster mushroom) in selected culture media, where they found that mycelia density was very abundant (5⁺) on MEA, SDA, CCEA and CPEA then on PDA it was abundant (4⁺) whereas on PPEA, it was moderate. The growth rate was high on MEA, CCEA and CPEA in slightly descending order respectively, but was lowest on PPEA. From the study, growth rate was found to be highest in MEA, CPEA and PWEA then least in CCEA. The reason for the different growth performances on the media types used is due to their different nutritional compositions.

Conclusion

The method for production of excellent quantity and quality of active mycelia should be encouraged as it can be said to be the powerhouse for the development of the mushroom industry all over the world (Stanley and Nyenke, 2011). The use of organic waste materials for the production of mycelia is also a good development that

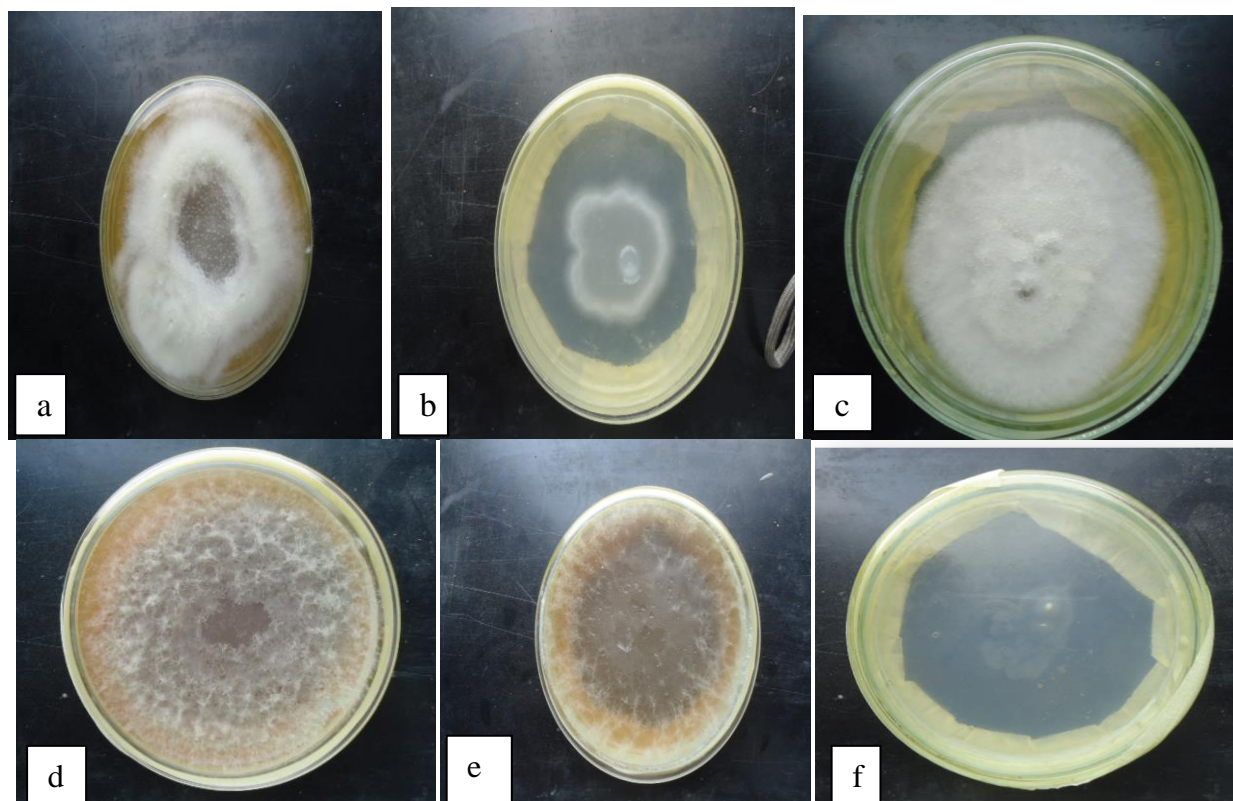


Plate 1. Mycelia growth on MEA (a), PDA (b), SDA (c), CPEA (d), PWEA (e) and CCEA (f), respectively.

will help in the utilization of these wastes to produce useful biomass and help manage these wastes that are readily available and detrimental to life. It is recommended that MEA, SDA and CPEA should be used for fast multiplication of the mycelia of *P. ostreatus* for mushroom production and other industrial uses (mycoremediation).

Conflict of interests

The authors did not declare any conflict of interest.

REFERENCES

- Change ST, Miles PG (2004). *Pleurotus*- A Mushroom of Broad Adaptability. *Mushrooms: Cultivation, Nutritional value, Medicinal Effect, and Environmental Impact* (2nd ed.) CRC Press. pp. 315-325.
- Dey RC, Nasiruddin KM, Al Munsur MAZ (2007). Effect of hormone, media and variety on mycelial growth of mushroom. *J. Bangladesh Agril Univ.* 5(2): 181-187.
- Kadiri M (1998). Spawn and Fruit body Production of *Pleurotus sajou caju* in Abeokwuta, Nigeria. *Niger. J. Bot.* 11:125-131
- Mester T, Tien M (2000). Oxidation Mechanism of Ligninolytic Enzymes Involved in the degradation of environmental pollutants. *Int. Biodeterior. Biodegrad.* 46:51-59.
- Munsur MA, Miah A, Rahman MH, Rahman MM, Yahia ASM (2012). Effects of varieties and media on mycelia growth and substrate on spawn production of Oyster mushroom. *Bangladesh Res. Publications J.* 7(4):361-366
- Nasim G, Malik SH, Bajwa R, Afzal M, Mianm SW (2001). Effect of three Different Culture Media on Mycelia Growth of Oyster and Chinesae Mushrooms. *J. Biol. Sci.* 1 (12):1130-1133.
- Pointings S (2001). Feasibility of Bioremediation by white-rot. *J. Fungi Appl. Microbiol. Biotechnol.* 57:20-33.
- Stanley HO, Nyenke CU (2011). Cultural Studies on Mycelia of *Pleurotus pulmonarius* (Oyster Mushroom) in selected Culture Media. *Int. J. Sci. Nat.* 2 (2):183-185.
- Suharban M, Anthony A (1996). Suitability of Different Tuber Crops on the Mycelia Growth of *Pleurotus sajou caju*. Fr. Singer, the Oyster Mushroom. *Tropical Tuber Crops: Problems, Prospects and Future Strategies.* pp. 513-515.
- Uddin MJ, Nasiruddin KM, Haque ME, Biswas AK, Islam MS (2012). Influence of Different Media Variety and Growth Regulator on Myelial colony proliferation of mushroom. *J. Environ. Sci. Nat. Resour.* 5(1):223-227.

Full Length Research Paper

The population structure of wild sorghum species in agro-ecological zones of Western Kenya

Titus O. Magomere^{1,4*}, Eliud K. Ngugi¹, Silas D. Obukosia², Eunice Mutitu¹ and Solomon I. Shibairo³

¹Plant Science and Crop Protection Department University of Nairobi, P.O. Box 29053-00625, Nairobi, Kenya.

²Africa Harvest Biotechnology Foundation International, P.O. Box 642, 00621-Village Market, Nairobi, Kenya.

³Kibabii University College, Masinde Muliro University of Science and Technology, P.O Box 1699-50200 Bungoma, Kenya.

⁴Department of Biochemistry and Biotechnology, School of Pure and Applied Sciences, Kenyatta University, P.O. Box 43844, Nairobi, Kenya.

Received 18 February, 2015; Accepted 22 April, 2015

There is need to understand the genetic structure of wild sorghums that grow alongside cultivated traditional sorghum varieties in order to assess the potential effect of crop genes in wild populations. In this study, 175 wild sorghum samples were collected from 13 agroecological zones (AEZs) from three counties in Western Kenya and genotyped using microsatellite markers. Crop alleles were observed in wild sorghum populations. The range of allelic frequencies varied from low (<0.4), to moderate (0.4-0.7) and to high (0.7) in the AEZs. Wild sorghum populations had moderate to high expected heterozygosity (H_E) values of between 0.453 in LM₁ to 0.715 in LM₂. Differences in the magnitude of diversity was significant in the counties (Busia $H_E = 0.59 - 0.71$; Homabay $H_E = 0.58-0.68$ and Siaya $H_E = 0.45-0.59$) but not distinct among the AEZs. Whole population F_{IS} , F_{ST} and F_{IT} values were low at 0.15, 0.16 and 0.29, respectively indicating low level of inbreeding, low genetic differentiation of the population and low to moderate deviation from Hardy-Weinberg (HW) equilibrium respectively. The deviation from HW equilibrium was significant in some wild populations from Siaya and Busia. Intra-population diversity (H_S) was larger than inter-population diversity (D_{ST}) in 13 populations from the sampled AEZs, indicating the importance of gene flow between populations of wild sorghums. Heterozygosity values under mutation drift equilibrium (H_{EQ}) varied under infinite allele model (IAM), two-phase model (TPM) and the step wise mutation model (SMM). However, significant population bottlenecks were absent in the wild sorghums. Presence of significant geographic county clusters and lack of significance on AEZ clusters indicate that human activities have had more influence on the distribution and diversity of wild sorghums than the prevailing climatic conditions. Efforts towards physical and genetic containment of crops genes need to be enhanced for successful ecologically sensitive confined field trials and future adoption of transgenics in cropping systems.

Key words: Diversity, *Sorghum bicolor*, *Sorghum halepense*, *Sorghum sudanense*, microsatellite loci.

INTRODUCTION

The sympatric nature of the members of the sorghum genus over time may have contributed to the spon-

taneous occurrence of *Sorghum bicolor* alleles in *Sorghum halepense* and *Sorghum sudanense* populations

(Ejeta and Grenier, 2005; Mutegi et al., 2009). *S. sudanense*, a cultivated form of *S. bicolor* ssp. *drummondii*, has shown natural outcrossing ranging from 0-100% on individual plants with averages of 39 and 57% when most tillers were at anthesis (Pedersen et al., 1998). Morrell et al. (2005) found *S. bicolor* alleles in *S. halepense* weeds suggesting persistent natural and spontaneous out-crossing in the species. The potential for crop to wild hybridization in sorghum populations in Ethiopia (a center of origin) and in Niger was found to be widespread (Tesso et al., 2008; Adugna et al., 2013). Crop and wild sorghums have been found to be intermixed in farming communities where they were interfertile and had synchronized flowering with several putative crop wild hybrids being observed (Tesso et al., 2008). Ancient and recent cross hybridization events after speciation in the sorghum genus have maintained several crop genes in the wild sorghum species (Morrell et al., 2005). In some cases the spontaneous hybrids formed could have obtained features from both parents, whose heterosis and unique features may have resulted in formation of a bridge species within the sorghum genus. Crop alleles might have an impact on the fitness of the crop x weed hybrids and may enhance or diminish their weedy potential (Hokanson et al., 2010).

Diversification within the sorghum genus heavily draws from disruptive selection (Dogget and Majisu, 1968). This phenomenon favors extreme phenotypes to the intermediates leading to wide variability within the species on specific traits (Rueffler et al., 2006). This has led to the wide variability on the morphological features of sorghum and on important food and forage associated traits as a result of artificial and natural selection for more than one level of a particular character within populations of crop and weed sorghums (Doggett, 1988). This diverse variability is also observed on important microsatellite locus in the genus (Yonemaru et al., 2009). The polymorphism on the microsatellite loci shows maintenance of extreme mutations showing losses or addition of several repeats on mononucleotide, di-nucleotide, tri-nucleotide and tetra-nucleotide SSR motifs. Individual species within the genus also show similar variability at the genotypic and phenotypic levels (Yonemaru et al., 2009).

Analysis of the population structure of crop and wild sorghum species is important in Africa and Ethiopia, which is a probable "center of origin" of the genus.

Therefore, wide genetic diversity is expected to be naturally maintained in East and Central Africa regions and other secondary centers of diversity where sorghum plays an important role in human diets. Recent studies have shown that there is heterozygosity excess and diversity in crop and wild sorghum populations in some parts of Kenya (Mutegi et al., 2011) Ethiopia (Adugna et al., 2013) and Africa (Barnaud et al., 2007; Sagnard et al., 2011) with the wild sorghums contributing higher magnitudes of diversity than the crops or landraces. North-West and South-East wild sorghum populations from Kenya had H_{EQ} of 0.34 and 0.56 each indicating no apparent loss of genetic variability from the expected diversity (Muraya et al., 2010). Mutation models are commonly used for the analysis of population bottlenecks in natural populations. The step wise mutation model (SMM) considers the size of microsatellite repeats, thus it may be subject to problems of homoplasy in its interpretation. The infinite allele model (IAM) assumes that each mutation can create any new allele randomly and considers any point mutation along a stretch of DNA within a locus to constitute a new allele (Goodman, 1998; Schlotterer, 2000). The two-phase model (TPM) is an intermediate model that provides better analysis of how DNA sequences evolve (Di Rienzo et al., 1994). Excess of H_{EQ} over the observed heterozygosity on loci evolving under the IAM, SMM, or TPM models would suggest environmental evolutionary pressures had minimal effect on reducing the population size and thus the allelic diversity (Cornuet and Luikart, 1996). Therefore, indicating absence of significant population bottlenecks in the recent past. This situation is sustained despite the pressures that the wild populations would have experienced as a result of agricultural practices like weeding and changes in biotic and abiotic conditions. If such pressures were significant, mutation and genetic drift would have been important in the populations thus an excess of observed heterozygosity over H_{EQ} would have been apparent. In some members of Poaceae spontaneous occurrence of crop alleles and crop transgenes in wild and landrace populations have been observed (Gepts and Papa 2003). In maize (*Zea mays*) distinct transgenes have been shown to exist in landraces in Mexico showing evidence of genetic contamination of the centers of origin (Piñeyro-Nelson et al., 2009). Hybridization between maize and teosinte (*Zea mays* ssp. *parviglumis*) (wild) has been shown to

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

Abbreviations: AEZ, Agro-ecological zone; DST, inter-population gene diversity; FIS, fixation index; FIT, index of deviation from HW equilibrium; FST, degree of population differentiation; GST, proportion of inter-population gene diversity; HE, expected heterozygosity; HEQ, heterozygosity values under mutation drift equilibrium; HO, observed heterozygosity; HT, total gene diversity; IAM, infinite allele model; SMM, stepwise mutation model; TPM, two-phase model; LM, lower Midlands; UM, upper midlands; HB, Homabay; SY, Siaya; BU, Busia counties; SSR, simple sequence repeats.

Author(s) agree that this article remains permanently open access under the terms of the [Creative Commons Attribution License 4.0 International License](https://creativecommons.org/licenses/by/4.0/)

exist naturally despite the distinct morphological differences that exist between the two (Doebley, 2004). In rice, natural hybridization between *O. rufipogon* and *O. sativa* has been reported in important rice cultivation regions globally (Song et al., 2002).

With the recent development and introduction of transgenic crops (James et al., 2010), it is vital to isolate and characterize the role of crop alleles within local wild populations of sorghum and study their spatial and temporal significance (Hokanson et al., 2010). Intra-population and inter-population statistics provide a means to elucidate the diversity of wild sorghum populations in the agro-ecological zones (AEZs) from three counties in Western Kenya. This study evaluated the effect of spontaneous hybridization in conspecific sorghum species on the spatial and temporal allelic composition of wild sorghum populations. The study also evaluated allelic differentiation among wild sorghum species across AEZs in Homabay, Siaya and Busia counties. The local population structure of wild sorghum species in AEZs from the three counties of Kenya was examined. In addition heterozygosity deficiency under the infinite allele model (IAM), under the two-phase model (TPM) and step wise mutation model (SMM) was evaluated.

MATERIALS AND METHODS

Three counties (Homabay, Siaya and Busia) were sampled to represent Western Kenya sorghum producing regions around Lake Victoria (Figure 1). Sampling units or farms that had wild sorghum forms within the counties were clustered based on the mapped AEZs. Sorghum farms were randomly sampled and 175 samples were collected from 13 AEZs from three counties in July 2011 (Table 1). Sampling was done after seed set in farms that had both wild and crop sorghums. Some wild sorghums growing on road reserves and uncultivated land in the clusters were also sampled. The seeds and first two leaves next to the panicle were collected labelled and kept on ice. Sampled AEZs in the three counties represented the diversity of wild sorghums in varying ecological conditions (Table 2).

DNA extraction and electrophoresis analysis

Sampled leaves and seeds were maintained on ice during transportation to the laboratory located at the College of Agriculture and Veterinary Sciences (CAVS) (-1° 14' 59.72", +36° 44' 30.79") of the University of Nairobi. The leaves were stored at -20°C in the laboratory freezers while the seeds were dried in the greenhouse. The seeds were germinated and young leaves collected and maintained on ice for DNA extraction. Sampled leaves and young leaves were washed with detergent in running water to remove dust particles and other debris. Leaves were weighed and 0.3 g kept in clean and labelled polyethylene bags on ice until grinding. The seeds from sampled panicles were threshed and kept in labelled plastic bags. Two procedures were applied to obtain genomic DNA from young leaves (two to five weeks old plants), from old leaves (five weeks old to flowering plants) and from dried seeds. Genomic DNA was extracted from young leaves by using a modified CTAB extraction procedure (Doyle and Doyle, 1990; Barnaud et al., 2008). Total nucleic acid extraction from seed was done by a modified CTAB-based seed extraction protocol (Delobel et al., 2007).

Agarose gel electrophoresis for genomic DNA extracted from young leaf, old leaf and seed tissues was done before running multiplex PCR. The multiplex PCR products were separated and analysed using a 4% UltraPure™ Agarose from Invitrogen™.

Simple sequence repeat (SSR) marker assay

Primers were designed based on the polymorphic SSR sequences from crop sorghum physical map genomic clones on five linkage groups chromosomes found in Phytozome databases. To ensure the specificity of the primers prior to synthesis, e-PCR was performed for all primer combinations. Annealing sites were also confirmed by using BLASTn procedure against the NCBI GenBank database. The primers were selected on the basis of their polymorphism and ability to distinguish several wild sorghum accessions (Table 3). Three out of the seven primers analysed had high polymorphic index.

PCR was done in 0.5 ml reaction tubes with a hybaid® thermocycler. Reaction volumes of 11 µl were used in all experiments. The reaction conditions were set in conventional PCR format using Invitrogen™ PCR reagent system, Taq DNA polymerase with (W-1) Invitrogen™ and 10mM dNTP Mix, PCR grade Invitrogen™. The PCR components in the reaction mix included; nuclease free water, 1X Taq DNA polymerase reaction buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl], 1.5 mM magnesium chloride, 0.2 mM of each of the four dNTPs, 0.05% W1 detergent, 0.5mM of each of the two primers, 0.5-1 U Taq DNA polymerase enzyme and 50-100 ng template DNA depending on the PCR product size and number of alleles. Mineral oil overlay was added to control evaporation of the reaction. A master mix for 50 samples was prepared and placed on ice and the appropriate volumes aliquoted into each reaction tube before adding the DNA template.

The PCR products were separated and analysed using a 4% UltraPure™ Agarose, Invitrogen™. PCR products were evaluated on 3-4% UltraPure™ agarose gels depending on fragment size separation requirement. UltraPure™ Agarose, Invitrogen™ (3-4) was added in 300 ml of 1X TBE buffer (recommended rate with modification in run time and voltage) at room temperature and stirred to remove clumps. The agarose was melted by heating in the microwave oven till boiling. Ethidium bromide (15 µl) (10 mg/ml) was added after cooling to approximately 60 to 70°C. This was poured in gel casting equipment with 50 well combs and left to solidify, air bubbles were removed manually. Mineral oil was removed by pipetting from PCR products and 1 µl of loading dye (BlueJuice™ gel loading buffer) Invitrogen™ was added to 11 µl of the PCR products including a 100bp DNA ladder Invitrogen™ 1 µg/µl. These were loaded in the wells while the gel was submerged in electrophoresis buffer. Electrophoresis was done at 120 v for 2-4 h to ensure maximum separation of fragments with varying molecular weight. The gel was placed on a UV transilluminator and a photograph taken.

Data analysis

Genotypic and allelic data was recorded after analysis of PCR agarose gels. The data was loaded into analysis software POPGENE version 32 (http://www.ualberta.ca/~fyeh/popgene_download.html) to evaluate intra-population and inter-population diversity on study loci. Analysis of molecular variance and test for HW equilibrium in the populations was done in ARLEQUIN version 3.5 (<http://cmpg.unibe.ch/software/arlequin3>). Dissimilarity among populations were analysed in DARwin version 5.0.158 (<http://darwin.cirad.fr/darwin>). Population bottlenecks among populations of wild sorghum growing in three counties in Western

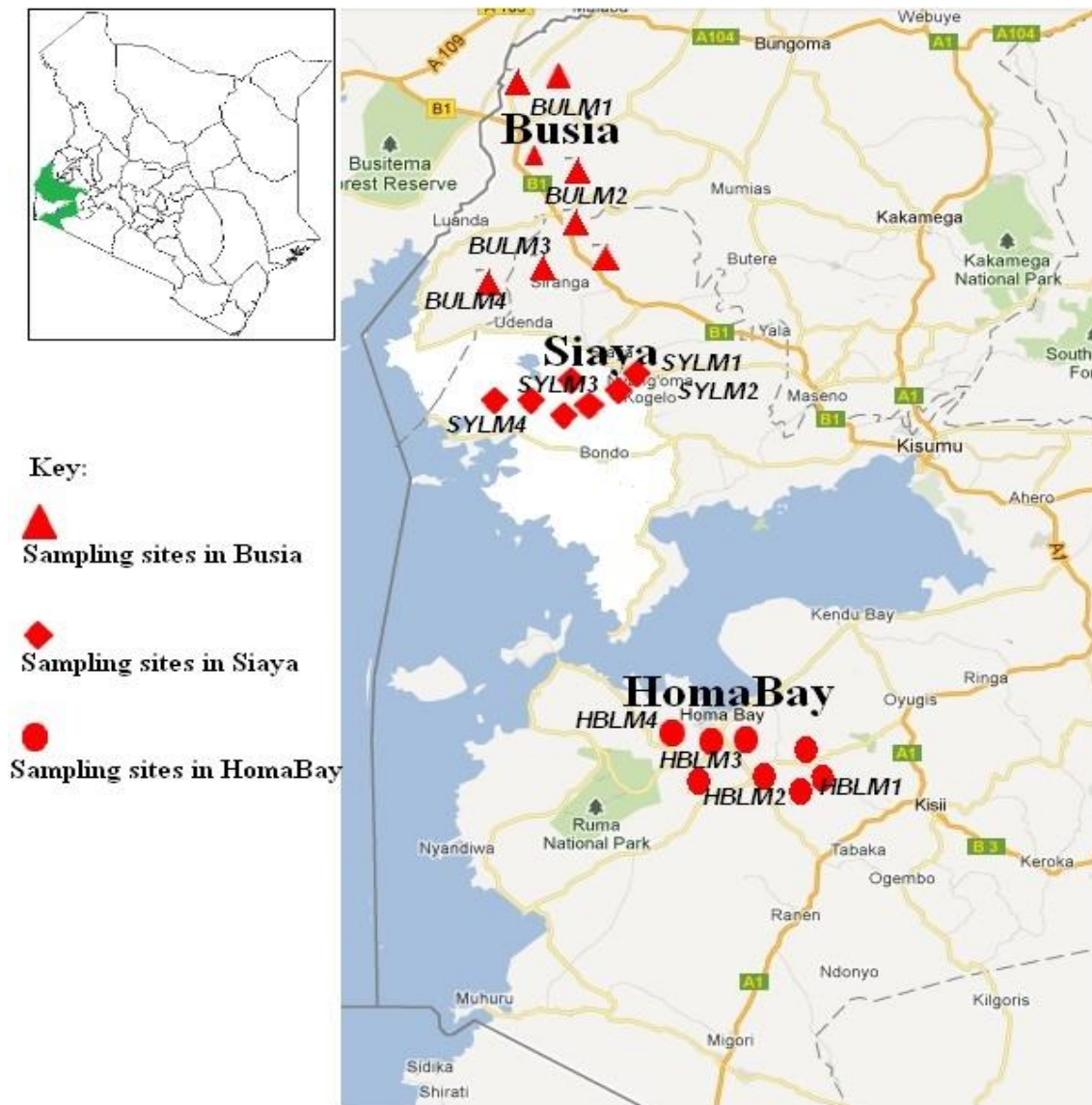


Figure 1. Sampling of wild sorghums in agro-ecological zone characteristics in survey sites in Homabay, Siaya and Busia counties of Kenya.

Kenya were analysed in BOTTLENECK version 1.2.02 (www.montpellier.inra.fr/URLB/bottleneck/bottleneck.html).

Total gene diversity (H_T) was partitioned into inter-population and intra-population components (Nei, 1973) to derive intra-population gene diversity (H_S) and inter-population gene diversity ($D_{ST} = H_T - H_S$) values. Furthermore, the coefficient of gene differentiation (G_{ST}), which represents the relative magnitude of gene differentiation among subpopulations, was calculated as the proportion of inter-population gene diversity to total gene diversity as ($G_{ST} = D_{ST}/H_T$). Expected heterozygosity and diversity (Mondini et al., 2009) indices were estimated using the ARLEQUIN version 3.5 software (Excoffier et al., 2005). Analysis of molecular variance (AMOVA) was done using ARLEQUIN software to estimate the significance of the covariance components allocated for within individuals, within populations, within groups of populations and among groups using non-parametric permutation procedures (Excoffier et al., 1992). The Ewens-watterson neutrality tests were

performed with ARLEQUIN software to test for the selective neutrality based on Ewens sampling theory in a population at equilibrium (Ewens, 1972).

Allelic variations on all loci, Shannon index on the polymorphic information on each locus and the overall allele frequency were calculated. F-statistics (fixation index $-F_{IS}$, Index of deviation from HW equilibrium $-F_{IT}$ and the degree of population differentiation F_{ST}) were estimated for multiple populations (Hartl and Clark, 1989; Weir, 1990; Mohammadi and Prasanna, 2003). Test of Hardy Weinburg (HW) equilibrium was done by computing expected genotypic frequencies under random mating using the algorithm by Levene (1949), and performing chi-square (X^2) tests. Probability values were used to determine the populations under HW equilibrium. Nei's unbiased genetic distance and genetic identity was estimated with the POPGENE software version 32 (Yeh and Boyle, 1997; Yang and Yeh, 1993).

The classical linkage disequilibrium coefficient measuring

Table 1. Number of wild sorghums collected from various agro-ecological zone sites in Homabay, Siaya and Busia counties of Kenya.

County	AEZs	Number of samples
Homabay	LM 1	9
	LM 2	8
	LM 3	12
	LM 4	21
	UM 1	11
Siaya	LM 1	11
	LM 2	12
	LM 3	11
	LM 4	19
Busia	LM 1	18
	LM 2	13
	LM 3	16
	LM 4	14
Total		175

Table 2. Agro-ecological zone characteristics in Homabay, Siaya and Busia counties of Kenya where wild sorghums were collected.

Agro-ecological zone	Altitude (m)	Annual mean temperature (°C)	Annual mean rainfall (mm)
Busia			
(+0° 28' 45.07", +34° 7' 11.39")			
Lower midlands (LM)			
LM1	1200-1440	22.2-21.0	1650-2000
LM2	1200-1350	22.3-21.4	1420-1800
LM3	1140-1500	22.7-21.0	1100-1450
LM4	1135-1200	22.7-22.3	900-1100
Homabay			
(-0° 36' 15.25", +34° 29' 57.86")			
Upper midlands (UM)			
UM1	1500-1570	21.0-20.5	1600-1900
Lower Midlands (LM)			
LM1	1300-1500	21.8-20.9	1500-1900
LM2	1200-1350	22.3-21.5	1400-1600
LM3	1140-1250	22.7-22.0	1020-1390
LM4	1135-1200	22.7-22.3	890-1020
LM5	1135-1180	22.7-22.4	-
Siaya			
(+0° 2' 18.43", +34° 12' 19.88")			
Upper midlands (UM)			
UM1	1500-1620	20.5-19.9	1600-1800
UM2-3	1500-1800	20.5-19.3	1450-1600
UM3-4	1500-1700	20.5-19.3	1200-1450

Table 2. Contd.

Agro-ecological zone	Altitude (m)	Annual mean temperature (°C)	Annual mean rainfall (mm)
Lower midlands (LM)			
LM1	1300-1550	21.7-20.4	1600-1800
LM2	1300-1550	21.7-20.4	1300-1600
LM3	1140-1550	22.7-20.4	900-1500
LM4	1140-1450	22.7-20.9	800-1300
LM5	1135-1300	22.7-21.7	700-900

Farm management hand book of Kenya (2005, 2009).

Table 3. SSR loci used to analyze polymorphism in the wild sorghum populations.

Locus	Chromosome	Forward primer (5' -> 3')	Reverse primer (5' -> 3')
SB1764	3	CTTGTGCTTGCTTGCACCATATTC	GTCGATGAGGAGCTTCATGCTCAG
SB3420	6	GAGCCAGCATGCATGATAATTGTT	CACAAAGGCATGACAGTCAATCAA
SB4688	9	CTGTAAGCATGATGAAGGTCGTGG	AAGAAGGTGATGACAGGGATGGAG
SB5058	9	GAGAATTGGAAGAAAGCCTCGTT	CAGAGCTCCTAAACGGTCCTCAAA
SBI000X	10	CTAGAGGATTGCTGGAAGCG	CTGCTCTGCTTGTCTGTTGAG
SB858	1	TTTCGTCTTCTCCACATGACCAC	AGAAGATGGCGACAATCAGAAAGG
SB5293	10	TGAATAATGCACGCAGTAGCGTCT	TATCCACGGCTCGCTAGCTACT

deviation from random association between alleles at different loci (Lewontin and Kojima, 1960) D and its standardization by the maximum value it can take (D_{max}), given the allele frequencies (Lewontin, 1964) were estimated in POPGENE. Dendrogram for populations from AEZs were based on Nei's genetic distances using UPGMA, which is an adoption of the program NEIGHBOR of PHYLIP version 3.5c.

The Wilcoxon test was applied in the Bottleneck software to test for heterozygosity deficiency under the infinite allele model (IAM), the two-phase model (TPM) and the step wise mutation model (SMM). Estimates of H_{EQ} from different mutation models (IAM, SMM and TPM) and analysis for presence of population bottlenecks were performed in BOTTLENECK software. Dissimilarity matrixes were developed in DARwin software using the DICE procedure for presence or absence data below:

$$d_{ij} = \frac{b+c}{2a+(b+c)} \quad (1)$$

Where, d_{ij} is the dissimilarity between units i and j ; a is the number of variables where $x_i = \text{presence}$ and $x_j = \text{presence}$; b is the number of variables where $x_i = \text{presence}$ and $x_j = \text{absence}$; c is the number of variables where $x_i = \text{absence}$ and $x_j = \text{presence}$

Axial and radial phylogenetic trees were developed using the weighted neighbourhood joining Saitou and Nei (1987) in DARwin below.

$$\partial_{(S,K)} = \frac{(C_i + C_j) \partial_{(i,K)} + (C_j + C_k) \partial_{(i,K)} - C_k \partial_{(i,i)}}{C_i + C_j + C_k} \quad (2)$$

Where, i, j and k are elements units or groups of units; C_i, C_j and C_k are the unit numbers of these elements; $\partial_{(S,K)}$, weighted average of dissimilarities between k and elements i and j ; i, j and k - elements units or groups of units.

RESULTS

Allelic variation for microsatellite loci and allele frequency in populations of wild species

Microsatellite loci SB1764, SB3420 and SB4688 showed polymorphisms within the populations from Homabay, Siaya and Busia counties and in sub-populations clustered along AEZs (UM₁, LM₁, LM₂, LM₃, and LM₄). Locus SB1764 showed 5 alleles from which 3.27 alleles were effective. A Shannon information index of 1.325 was observed (Table 4). Five alleles were seen from the analysis of locus SB3420 in all populations, where 3.17 out of the five alleles were effective. A Shannon information index of 1.3 was observed for this locus. For locus SB4688, five alleles were observed in the populations but only 2.92 of the alleles were effective, thus a Shannon information content of 1.26 was recorded (Table 4).

PCR amplification of three loci gave bands ranging from 250bp to 520bp in the wild populations from the three Western Kenya counties (Figure 2a, 2b, 2c). Amplifications were obtained on loci SB4688 and SB3420 in wild sorghums collected from Siaya and Homabay. Some wild materials did not amplify on loci SB4688 (lanes 34 to 38, Figure 2a) and SB3420 (lanes 14, 15, 20, 21, 22, 25, 40, 41 and 42, Figure 2b), these were different from the expected alleles in *S. halepense*, *S. verticilliflorum*, *S. bicolor* and *S. sudanense* (Figure 2c). A 550bp band from locus SB3420 (Lanes 5, 9, 12, 31 and 32, Figure 2b) demonstrated a *S. verticilliflorum* origin, while 300 and 290 bp from the same locus demonstrated

Table 4. Observed number of alleles and Shannon Index of the for SSR loci assayed.

Locus	Chromosome	Sample size	na	ne	I
SB1764	3	332	5.0000	3.2686	1.3247
SB3420	6	332	5.0000	3.1734	1.2899
SB4688	9	332	5.0000	2.9202	1.2551
SB5058	9	112	3.0000		0.5553
SBI000X	10	64	2.0000		0.2338
SB858	1	124	4.0000		0.9824
SB5293	10	152	3.0000		0.8092

na = Observed number of alleles; ne = effective number of alleles [Kimura and Crow (1964)] (only for primers with above one I); I = Shannon's Information index [Lewontin (1972); Sherwin et al., 2006].

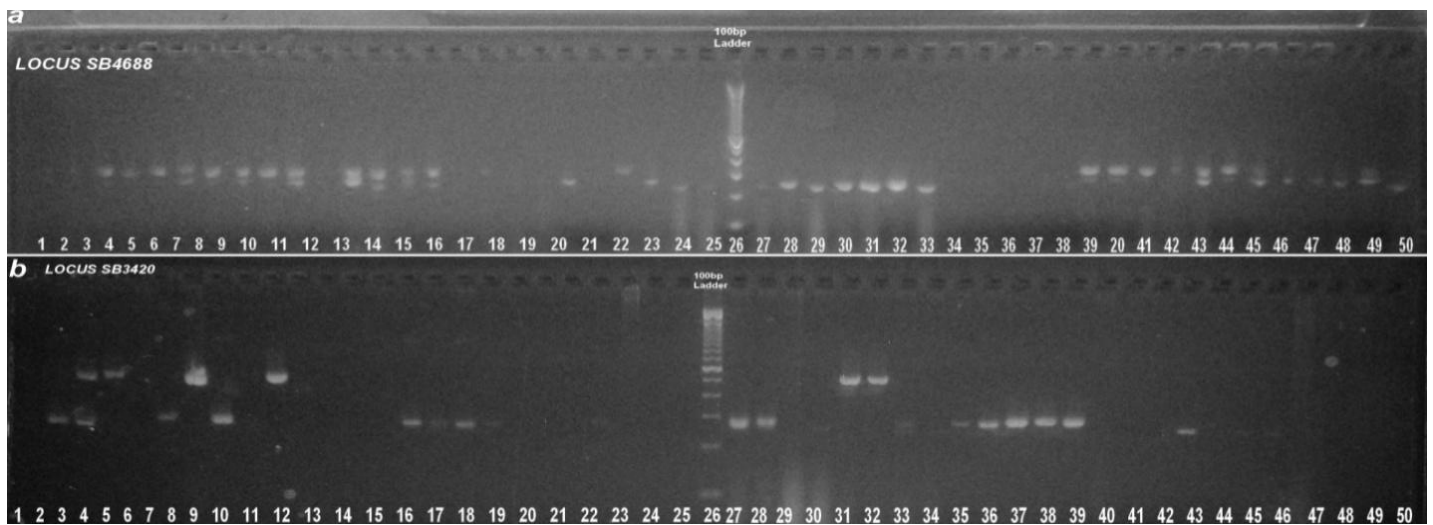


Figure 2. Agarose gel electrophoresis showing alleles 50 representative samples of wild populations from Western Kenya counties and AEZS. **(a)** Locus SB4688, **(b)** locus SB3420. *S. bicolor* is used as a positive control in lane 50 and in lane 3 (a) and (b) respectively; water was used as a negative control and loaded with the 100 bp ladder in lane 26.

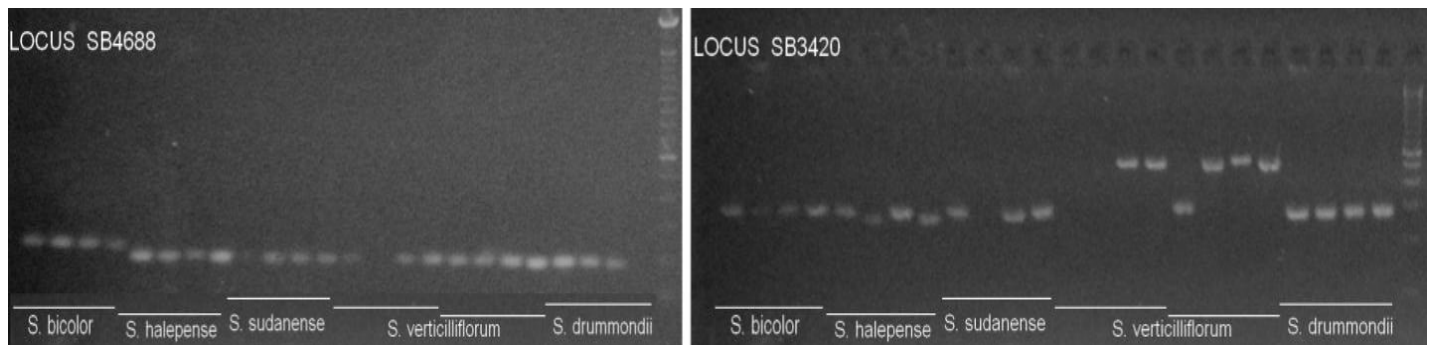


Figure 2c. Agarose gel electrophoresis showing expected alleles from sorghum species on loci SB4688 and SB3420.

a *S. bicolor* and *S. sudanense* origin (Figure 2b). Some materials had two alleles showing recent hybridization

events between *S. bicolor* and *S. verticilliflorum* on locus SB3420 (Lanes 4, Figure 2b).

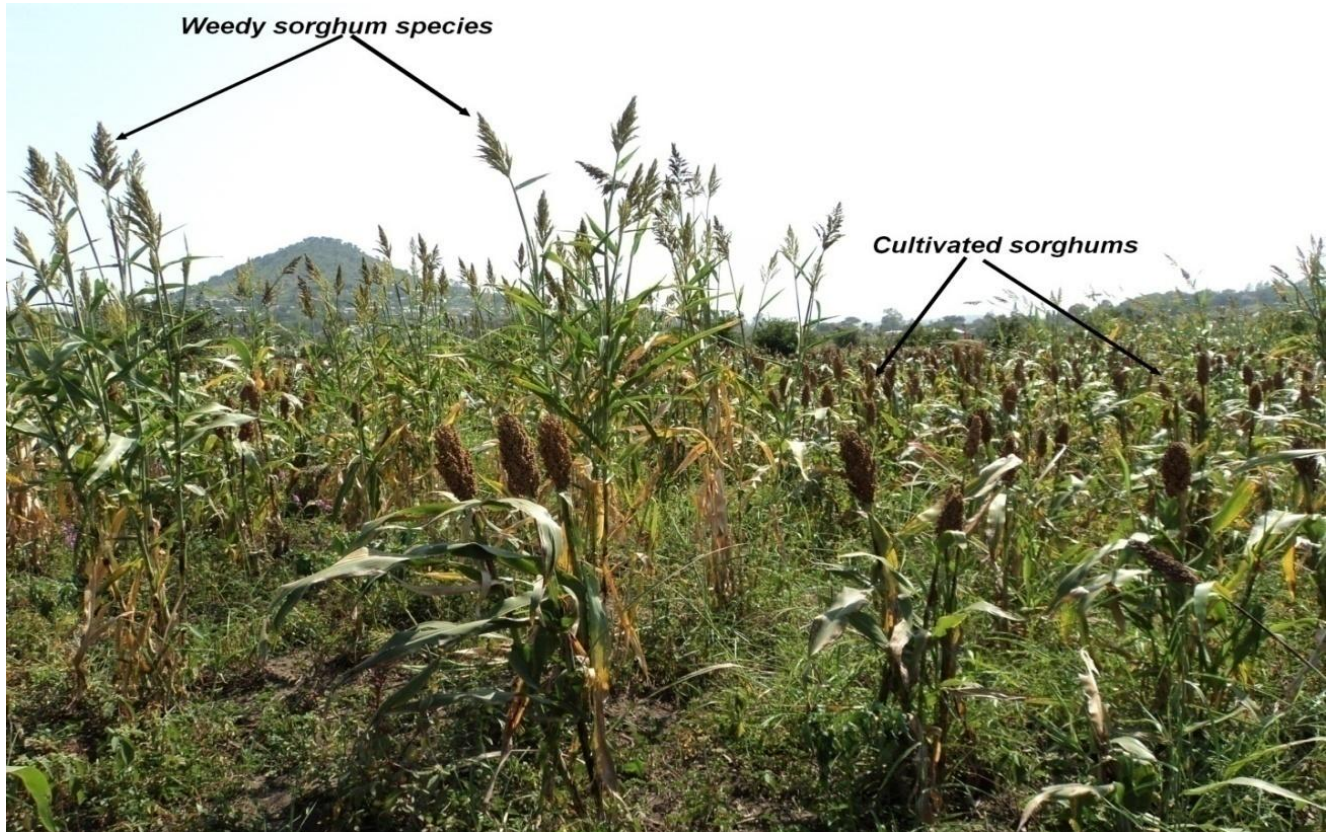


Figure 3. Wild species in crop sorghum farms in Western Kenya.

This is a result of their proximity in crop sorghum stands (Figure 3).

Locus SB1764 showed high (above 0.7) frequencies of alleles A and C in SYLM₃, SYLM₄ and BULM₄ (Table 5). Alleles A and C of SB1764 were the most frequent in the population (Table 5). Allele C had medium (0.4 to 0.7) frequencies in all AEZs except in HBLM₄, HBLM₁, SYLM₄, BULM₁ and BULM₂ (Table 5). Alleles B, D and E of locus SB1764 had low (below 0.4) frequencies in all AEZS (Table 5). Locus SB3420 alleles had medium (0.4 to 0.7) to low (below 0.4) in all populations except in SYLM₁ where allele E had a frequency of 0.7. Allele B had medium frequencies (0.4 to 0.7) in 8 AEZ populations except HBLM₃, HBLM₄, SYLM₁, SYLM₂ and BULM₄. Medium frequencies were also observed on allele D and allele E (Table 5). Locus SB44688 showed high frequencies on allele C in BULM₄ and allele E in SYLM₁, and SYLM₃. Medium frequencies on this locus were seen on alleles B, C, E, F and G. Low frequencies were observed on alleles B, C, F and G. Allele E was the most frequent in the population (Table 5).

Genetic diversity within and among wild sorghum populations from AEZS in Western Kenya

Total heterozygosity was high in AEZs from Busia (0.712),

followed by those from Homabay (0.665) and Siaya (0.564). Observed heterozygosity (H_O) was high (0.762) in BULM₁ (1200-1440 masl) wild sorghum groups. However, H_O was low towards the lower AEZs LM₂, LM₃, and LM₄ in Busia, similar trend was seen in Homabay AEZs with high H_O values of 0.62 in HBLM₁ and HBLM₂ and low values in HBLM₃ (0.444) and HBLM₄ (0.567) (Table 6). In Siaya all the zones had H_O of less than 0.4 (Table 6).

High expected heterozygosity (H_E) values were observed in Lower midlands in Busia. Population from BULM₂ had H_E of 0.715 while BULM₁, BULM₃ and BULM₄ had H_E values of between 0.590 to 0.690. Expected heterozygosity in HBLMs ranged between 0.531 (HBLM₁) to 0.683 (HBLM₂). Expected heterozygosity was low in AEZs from Siaya (0.453 to 0.557). Expected heterozygosity was greater than the Inter-population gene diversity (D_{ST}) and the proportion of inter-population gene diversity (G_{ST}) in all wild sorghum populations from different AEZS. However the D_{ST} and G_{ST} of HBLM₁ (0.134 and 0.202), SYLM₁ (0.111 and 0.197) and BULM₃ (0.116 and 0.163) had higher values than other AEZS (Table 6).

The degree of inbreeding and therefore heterozygote deficiency (F_{IS}) was high in HBLM₁, HBLM₂, HBLM₃ and HBLM₄. HBLM₁ had an F_{IS} index of 0.596 which was almost similar to that observed in SYLM₁ (0.582) (Table

Table 5. Allele frequency for loci SB1764, SB3420 and SB4688 in the wild sorghum populations obtained from three sorghum growing counties around Lake Victoria in Western Kenya.

Population	HBLM ₁	HBLM ₂	HBLM ₃	HBLM ₄	HBUM ₁	SYLM1	SYLM2	SYLM3	SYLM4	BULM ₁	BULM ₂	BULM ₃	BULM ₄
Locus/Allele													
SB1764/A	0.5625	0.2500	0.1250	0.2250	0.0909	0.1667	0.1818	0.1364	0.7105	0.5357	0.4286	0.2000	0.0455
SB1764/B	0.3125	0.2500	0.2500	0.3250	0.1364	0.0000	0.2727	0.0000	0.2368	0.2500	0.0000	0.1333	0.0455
SB1764/C	0.1250	0.5000	0.6250	0.3500	0.5455	0.6667	0.5000	0.7273	0.0526	0.1071	0.3571	0.5333	0.7273
SB1764/D	0.0000	0.0000	0.0000	0.1000	0.2273	0.0833	0.0455	0.1364	0.0000	0.0000	0.1071	0.1333	0.0909
SB1764/E	0.0000	0.0000	0.0000	0.0000	0.0000	0.0833	0.0000	0.0000	0.0000	0.1071	0.1071	0.0000	0.0909
SB3420/A	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0455	0.0000	0.0526	0.1071	0.1429	0.2000	0.2273
SB3420/B	0.5625	0.4375	0.1667	0.2250	0.5455	0.2917	0.3636	0.4545	0.4474	0.4286	0.5000	0.4333	0.3182
SB3420/D	0.4375	0.3125	0.4167	0.1250	0.0909	0.0000	0.0000	0.0455	0.0000	0.1429	0.2500	0.2333	0.1818
SB3420/E	0.0000	0.2500	0.2917	0.6250	0.3636	0.7083	0.5909	0.3636	0.5000	0.3214	0.1071	0.1333	0.2273
SB3420/F	0.0000	0.0000	0.1250	0.0250	0.0000	0.0000	0.0000	0.1364	0.0000	0.0000	0.2857	0.0000	0.0455
SB4688/B	0.6875	0.4375	0.1667	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
SB4688/C	0.3125	0.2500	0.3333	0.4000	0.4091	0.2500	0.3636	0.2727	0.4211	0.2500	0.2143	0.7667	0.2273
SB4688/E	0.0000	0.3125	0.5000	0.6000	0.5909	0.7500	0.6364	0.7273	0.5263	0.2857	0.2500	0.1333	0.3636
SB4688/F	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.4286	0.2500	0.0667	0.0909
SB4688/G	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0526	0.0357	0.0000	0.0333	0.3182

HB, Homabay; SY, Siaya; BU, Busia counties. LM, lower midlands; UM, upper midlands; HBLM₁, Homabay lower midlands 1.

Table 6. Molecular diversity indices of wild sorghum populations obtained from three sorghum growing counties around lake Victoria in Western Kenya.

Region	Population	Number of individuals	H _o	S _d	H _E (H _s)	S _d	H _T	S _d	F _{IS}	D _{ST}	G _{ST}
Homabay	HBLM ₁	8	0.625	0.250	0.531	0.075	0.665	0.031	0.19318	0.134	0.202
Homabay	HBLM ₂	8	0.625	0.125	0.683	0.014	0.665	0.031	0.09091	0.019	0.028
Homabay	HBLM ₃	12	0.444	0.210	0.640	0.087	0.665	0.031	0.31518	0.025	0.037
Homabay	HBLM ₄	20	0.567	0.104	0.593	0.123	0.665	0.031	0.04508	0.072	0.108
Homabay	HBUM ₁	11	0.242	0.189	0.583	0.074	0.665	0.031	0.59596	0.082	0.123
Siaya	SYLM ₁	12	0.194	0.127	0.453	0.075	0.564	0.098	0.58152	0.111	0.197
Siaya	SYLM ₂	11	0.364	0.091	0.557	0.100	0.564	0.098	0.35829	0.007	0.013
Siaya	SYLM ₃	11	0.333	0.189	0.514	0.138	0.564	0.098	0.36232	0.050	0.089
Siaya	SYLM ₄	19	0.456	0.030	0.523	0.065	0.564	0.098	0.13011	0.042	0.074
Busia	BULM ₁	14	0.762	0.149	0.684	0.030	0.712	0.010	0.11828	0.028	0.039
Busia	BULM ₂	14	0.548	0.419	0.715	0.052	0.712	0.010	0.24112	0.003	0.004
Busia	BULM ₃	15	0.511	0.139	0.596	0.171	0.712	0.010	0.14702	0.116	0.163

Table 6. Contd.

Region	Population	Number of individuals	H _O	Sd	H _E (H _S)	Sd	H _T	Sd	F _{IS}	D _{ST}	G _{ST}
Busia	BULM ₄	11	0.606	0.292	0.670	0.174	0.712	0.010	0.09910	0.042	0.060
	Total	166*	0.483	0.178	0.595	0.090	0.648	0.045		0.056	0.087

H_O= Observed heterozygosity; sd=standard deviation; H_E (H_S); expected heterozygosity (intra-population gene diversity); H_T; total gene diversity; F_{IS}= heterozygote deficiency/ degree of inbreeding; D_{ST}= inter-population gene diversity (H_T-H_S); G_{ST}= proportion of inter-population gene diversity (D_{ST}/H_T).

Table 7. Expected heterozygosity (H_E) of wild sorghum populations obtained from three sorghum growing counties around lake Victoria in Western Kenya.

Locus#	HBLM ₁	HBLM ₂	HBLM ₃	HBLM ₄	HBUM ₁	SYLM ₁	SYLM ₂	SYLM ₃	SYLM ₄	BULM ₁	BULM ₂	BULM ₃	BULM ₄	Mean	S.D.	Tot. Het
SB1764	0.608	0.667	0.554	0.729	0.654	0.536	0.671	0.455	0.448	0.651	0.690	0.662	0.472	0.600	0.096	0.608
SB3420	0.525	0.692	0.728	0.556	0.589	0.431	0.515	0.671	0.562	0.706	0.680	0.724	0.797	0.629	0.106	0.525
SB4688	0.458	0.692	0.638	0.492	0.506	0.391	0.485	0.416	0.558	0.696	0.775	0.402	0.740	0.558	0.135	0.458
Mean	0.531	0.683	0.640	0.593	0.583	0.453	0.557	0.514	0.523	0.684	0.715	0.596	0.670	0.595	0.079	0.531
S.D.	0.075	0.014	0.087	0.123	0.074	0.075	0.100	0.138	0.065	0.030	0.052	0.171	0.174	0.090	0.049	0.075

HB, Homabay; SY, Siaya; BU, Busia counties. LM, lower midlands; UM, upper midlands; HBLM₁, Homabay lower midlands 1.

6). These F_{IS} values imply low to moderate inbreeding the two populations. Low F_{IS} values were observed in HBLM₄ (0.045), SYLM₄ (0.130) and BULM₄ (0.099) (Table 6), these populations had higher levels of outcrossing.

Expected heterozygosity on each locus varied across populations in the AEZs. Locus SB1764 gave high H_E values in HBLM₄ (0.729) (Table 7). Midway H_E values of between 0.69 and 0.455 were observed in the rest of the populations. Locus SB3420 showed higher diversity within HBLM₃, BULM₁, BULM₃ and BULM₄ (>0.7). The least H_E value on the locus was observed in SYLM₁ (0.431) (Table 7) suggesting low intra-population gene diversity. Locus SB4688 had higher intra-population gene diversity in BULM₂ and BULM₄ with H_E values of 0.775 and 0.740 respectively. The least H_E value on the locus was observed in the SYLM₁ population just as was the

case with locus SB3420 (Table 7).

Analysis of molecular variance (AMOVA) as a weighted average over the three loci SB1764, SB3420, SB4688 showed that 3.2% of the total variation was explained by among group variations, 10.5% by among populations within groups' differences and 16.3% by among individuals within populations. Large variations of 69.97% were explained by differences within individuals on the three loci (Table 8).

Population specific indices in wild sorghum populations from AEZS in Western Kenya

Wild sorghum from BULM₂ showed high degree of inbreeding (F_{IS} of 0.9) on locus SB1764, while loci SB3420 had high inbreeding in HBUM₁ (0.7) and SYLM₁ (0.81). Loci SB4688 had high inbreeding in

HBUM₁ (0.83) (Table 9). Midway heterozygosity deficiency (F_{IS}) values were observed on locus Sb1764 in HBLM₁ (0.4) HBLM₃ (0.56) SYLM₃ (0.41) and BULM₄ (0.43) (Table 9). Similar values were observed on locus SB3420 in SYLM₂ (0.48) and BULM₃ (0.46) Similar inbreeding degrees were seen on locus SB4688 in SYLM₁ (0.59) and SYLM₃ (0.57). Low values of less than 0.4 were observed on locus SB1764 in AEZs in Homabay, Siaya and Busia counties. However negative F_{IS} values were more present on loci SB3420 and SB4688 in sorghum species obtained from HBLM₁, HBLM₂, HBLM₄, BULM₂, BULM₃ and BULM₄ (Table 9). This shows differential heterozygosity deficiency coefficients on the three loci in wild sorghum population with higher affinity for fixation on loci SB1764. On a whole population basis of wild sorghums from Western Kenya, the degree of inbreeding (F_{IS}) values were generally

Table 8. AMOVA showing percentage of variation on each of the three loci assayed.

Source of variation	Locus SB1764 Percentage of variation	Locus SB3420 Percentage of variation	Locus SB4688 Percentage of variation	3 loci Percentage of variation
Among groups	-3.14523	5.81122	7.01745	3.20
Among populations within groups	15.81786	4.81671	10.98644	10.52
Among individuals within populations	28.66450	8.02441	12.18045	16.30
Within individuals	58.66288	81.34767	69.81566	69.97
	100%	100%	100%	100%

Table 9. Population specific F_{IS} (heterozygote deficiency) indices per polymorphic locus (absolute values) in wild sorghums from AEZs in Western Kenya

LOCUS	HBLM ₁	HBLM ₂	HBLM ₃	HBLM ₄	HBUM ₁	SYLM ₁	SYLM ₂	SYLM ₃	SYLM ₄	BULM ₁	BULM ₂	BULM ₃	BULM ₄
SB1764	0.400	0.263	0.560	0.389	0.315	0.389	0.333	0.412	-0.059	0.013	0.900	-0.007	0.434
SB3420	-0.750	-0.091	0.088	-0.173	0.701	0.814	0.483	0.195	0.161	-0.331	-0.273	0.456	-0.029
SB4688	-0.400	0.103	0.357	-0.226	0.828	0.585	0.259	0.574	0.250	-0.028	0.081	-0.167	0.018
Mean	0.193	0.091	0.315	0.045	0.596	0.582	0.358	0.362	0.130	0.118	0.241	0.147	0.099

HB - Homabay; SY - Siaya and BU - Busia counties. LM -Lower Midlands and UM-Upper Midlands. HBLM₁ -Homabay Lower Midlands 1.

Table 10. Whole population degree of inbreeding (F_{IS}), deviation from Hardy Weinberg (HW) equilibrium (F_{IT}), degree of population differentiation (F_{ST}) and estimated gene flow, based on loci SB1764, SB3420 and SB4688.

Locus	F statistics			
	F_{IS}	F_{IT}	F_{ST}	Nm*
SB1764	0.3015	0.4147	0.1620	1.2935
SB3420	0.0336	0.1521	0.1226	1.7887
SB4688	0.1196	0.3022	0.2074	0.9555
Mean	0.1514	0.2901	0.1634	1.2800

*Nm = Gene flow estimated from $F_{ST} = 0.25(1 - F_{ST})/F_{ST}$.

low on all loci giving a population mean of 0.1514. Locus SB1764 showed F_{IS} index of 0.3015, locus SB3420 had 0.0336 while locus SB4688 had an index of 0.1196 on all collections from Homabay, Siaya and Busia AEZs (Table 10). The whole

population leaned towards the Hardy- Weinberg (HW) equilibrium with F_{IT} index (deviation from Hardy Weinberg (HW) equilibrium ($=1-H/H_E$) of (0.2901). Attainment of the HW equilibrium was observed more on Locus SB3420 with F_{IT} of

0.1521. Locus SB4688 had an index of 0.3022, while deviation from the HW equilibrium was most visible on locus SB1764 with an F_{IT} index of 0.4147 (Table 10).

Inter-population differences were most observed

Table 11. χ^2 Test of HW equilibrium within wild sorghum populations in AEZs in Homabay, Siaya and Busia counties by estimation of expected genotypic frequencies under random mating (Levene, 1949).

Locus	HBLM ₁ χ^2	P	HBLM ₂ χ^2	P	HBLM ₃ χ^2	P	HBLM ₄ χ^2	P	HBUM ₁ χ^2	P
SB1764	15.250	0.002	15.571	0.001	10.489	0.015	43.500	0.000	8.149	0.227
SB3420	4.083	0.043	3.357	0.340	12.250	0.057	3.617	0.728	25.580	0.000
SB4688	1.273	0.259	7.714	0.052	4.049	0.256	1.012	0.314	8.205	0.004
	SYLM ₁ χ^2	P	SYLM ₂ χ^2	P	SYLM ₃ χ^2	P	SYLM ₄ χ^2	P	BULM ₁ χ^2	P
SB1764	46.650	0.000	9.380	0.153	8.658	0.034	38.872	0.000	20.114	0.003
SB3420	8.874	0.003	3.173	0.366	8.767	0.187	3.108	0.375	9.313	0.157
SB4688	4.606	0.032	0.769	0.380	4.050	0.044	4.263	0.234	6.400	0.380
	BULM ₂ χ^2	P	BULM ₃ χ^2	P	BULM ₄ χ^2	P				
SB1764	36.727	0.000	18.833	0.004	42.200	0.000				
SB3420	5.646	0.464	21.202	0.002	15.400	0.118				
SB4688	16.857	0.010	1.162	0.979	10.250	0.115				

on locus SB4688 with an F_{ST} index of 0.2074. Locus SB1764 had an F_{ST} index of (0.1620) while locus SB3420 had an index of 0.1226). All three loci gave a degree of population differentiation of 0.1634 (Table 10). Gene flow (Nm) estimated from the degree of population differentiation (F_{ST}) had a whole population mean of 1.28. Gene flow was most observed on locus SB3420, moderate on locus SB1764 (1.294) and least on locus SB4688 (0.96) (Table 10).

Population equilibrium within wild sorghum populations in agro-ecological zones in Western Kenya

Homogeneity tests of gene frequencies in wild sorghums across counties and AEZs within

counties using χ^2 tests demonstrated that the wild sorghum populations were within the HW equilibrium (Table 11). This equilibrium was true for all loci (SB1764, SB3420 and SB4688). χ^2 computation for wild sorghum from lower midlands in Homabay, Siaya and Busia showed significant adherence for the HW equilibrium. Wild sorghums from HBLM₄, SYLM₂, SYLM₄, BULM₁ and BULM₄ did not adhere to the HW equilibrium on more than two loci, as shown by the high non-significant P values (Table 11).

Population bottlenecks in wild sorghum populations in the different Agro-ecological zones in Homabay, Siaya and Busia counties

There was no evidence to suggest existence of population bottleneck on wild sorghums growing

in AEZs in Homabay, Siaya and Busia. The Wilcoxon test for heterozygosity deficiency under the infinite allele model (IAM) had a probability of 1.00, under the two-phase model (TPM) P was 1.00 and under the step wise mutation model (SMM) P was 1.00 showing no significant pattern. Heterozygosity excess was not significantly different under the IAM, TPM and SMM model ($p=0.0625$). A similar situation was seen when both heterozygosity excess and deficiency was tested ($p=0.125$) (Table 12a).

The sign test showed that the expected number of loci with heterozygosity excess under IAM was 1.68 under TPM was 1.74 and under SMM was 1.82. No loci were observed with heterozygosity deficiency but none had significant heterozygosity excess under all models (Table 12a). Despite lack of significant heterozygosity excess or deficiency

Table 12a. Sign and Wilcoxon tests for heterozygosity excess and deficiency under the IAM, SMM and TPM models.

Model	Sign test			p	Wilcoxon test		
	Expect number of loci with heterozygosity excess	loci with heterozygosity deficiency	loci with heterozygosity excess		Probability heterozygosity deficiency	Probability heterozygosity excess	Probability heterozygosity excess and deficiency
I.A.M	1.68	0	3	0.17553	1.00000	0.06250	0.12500
T.P.M	1.74	0	3	0.19409	1.00000	0.06250	0.12500
S.M.M	1.82	0	3	0.22393	1.00000	0.06250	0.12500

I.A.M, infinite allele model; T.P.M, two-phase model; S.M.M, stepwise mutation model.

Table 12b. Comparison between (Ho) Heterozygosity (He) Heterozygosity and the expected values under the IAM, SMM and TPM models.

Locus	Heterozygosity		Population bottlenecks					
	H _O	H _E	I.A.M. H _{EQ}	Probability	S.M.M. H _{EQ}	Probability	T.P.M. H _{EQ}	Probability
SB1764	0.3750	0.6083	0.432	0.0430	0.648	0.3460	0.543	0.0960
SB3420	0.8750	0.5250	0.428	0.0450	0.647	0.3880	0.538	0.1090
SB4688	0.6250	0.4583	0.434	0.1070	0.641	0.4810	0.540	0.2020
Mean	0.6250	0.5306						

H_O, Observed heterozygosity; H_E, expected heterozygosity; H_{EQ}, heterozygosity under mutation-drift equilibrium.

pattern in the models applied to explain bottlenecks in the populations there was variation in the calculated heterozygosity values. This was true when compared to the heterozygosity under mutation - drift equilibrium (H_{EQ}) values in IAM, TPM and SMM models (Table 12b). On locus SB1764 H_E was 0.6083 but H_{EQ} was computed at 0.432, 0.648 and 0.543 under IAM, SMM and TPM model (Table 12b). Locus SB3420 had H_E of 0.5250 but under IAM, SMM and TPM H_{EQ} values of 0.428, 0.647 had 0.538 respectively (Table 12b). Locus SB4688 had H_E of 0.4583 and H_{EQ} 0.434, 0.641 and 0.540 values under IAM, SMM and TPM respectively.

Genetic distance and genetic identity among wild sorghum populations from agro-ecological zones in Homabay, Siaya and Busia counties

Wild sorghum population within AEZS in three Western Kenya counties (Homabay, Siaya and Busia) showed significant levels of genetic identity and distance (Table 13). Population HBLM₁ was less identical to other population. It was most distant to SYLM₁ (1.3148) SYLM₃ (1.028), BULM₄ (0.9838), SYLM₂ (0.9017), HBLM₄ (0.8464) and HBUM₁ (0.791) (Table 13). Moderate genetic distances were observed between HBLM₁ and

HBLM₃ (0.5727), SYLM₄ (0.5228) and Busia AEZs. Similar midway genetic distance values were obtained between SYLM₁ and BULM₁ (0.4729), BULM₂ (0.4357) and BULM₃ (0.4563). Population from SYLM₄ and BULM₁ showed moderate to high genetic distances 0.6316 and 0.5314 respectively. Low genetic distances were observed between population from HBUM and SYLM₁ (0.0771), SYLM₂ (0.0325) and SYLM₃ (0.0173). Similar low values were observed between populations HBLM₄ and HBUM₁ (0.0906); HBLM₄ and SYLM₂ (0.0039); HBLM₂ and HBLM₃ (0.0701) (Table 13).

Population from Homabay, Siaya and Busia

Table 13. Nei's unbiased measures of genetic identity and genetic distance among wild sorghum populations from AEZs in Homabay, Siaya and Busia counties

Pop ID	HBLM ₁	HBLM ₂	HBLM ₃	HBLM ₄	HBUM ₁	SYLM ₁	SYLM ₂	SYLM ₃	SYLM ₄	BULM ₁	BULM ₂	BULM ₃	BULM ₄
HBLM ₁	****	0.8564	0.5640	0.4290	0.4634	0.2685	0.4059	0.3577	0.5928	0.6462	0.6509	0.5988	0.3739
HBLM ₂	0.1550	****	0.9323	0.7948	0.8505	0.7496	0.8221	0.8091	0.7024	0.7142	0.7777	0.7827	0.7883
HBLM ₃	0.5727	0.0701	****	0.8777	0.8752	0.8414	0.8771	0.8839	0.6307	0.6163	0.6915	0.7818	0.8622
HBLM ₄	0.8464	0.2296	0.1304	****	0.9134	0.9278	0.9962	0.8674	0.8571	0.7462	0.6436	0.7120	0.7349
HBUM ₁	0.7691	0.1619	0.1332	0.0906	****	0.9258	0.9680	0.9828	0.7552	0.6881	0.7706	0.8338	0.8707
SYLM ₁	1.3148	0.2882	0.1727	0.0750	0.0771	****	0.9752	0.9637	0.7305	0.6232	0.6468	0.6336	0.8285
SYLM ₂	0.9017	0.1959	0.1312	0.0039	0.0325	0.0251	****	0.9437	0.8367	0.7329	0.6848	0.7393	0.8156
SYLM ₃	1.0280	0.2118	0.1234	0.1422	0.0173	0.0369	0.0580	****	0.6907	0.6143	0.7361	0.7307	0.8957
SYLM ₄	0.5228	0.3532	0.4610	0.1542	0.2808	0.3140	0.1783	0.3701	****	0.8871	0.7398	0.6458	0.5317
BULM ₁	0.4367	0.3366	0.4840	0.2927	0.3738	0.4729	0.3108	0.4873	0.1198	****	0.8908	0.6697	0.5878
BULM ₂	0.4295	0.2514	0.3688	0.4408	0.2605	0.4357	0.3786	0.3064	0.3014	0.1156	****	0.7911	0.8361
BULM ₃	0.5128	0.2451	0.2462	0.3397	0.1817	0.4563	0.3021	0.3138	0.4373	0.4009	0.2343	****	0.8020
BULM ₄	0.9838	0.2378	0.1482	0.3080	0.1384	0.1881	0.2038	0.1102	0.6316	0.5314	0.1790	0.2206	****

Nei's genetic identity (above diagonal) and genetic distance (below diagonal).

made significant clusters in dendrogram analysis. Moderate to high bootstrap values (30 to 83) were observed on county branches (Figure 4). Populations from Siaya clustered away from Busia and Homabay, however, three sub-clusters were observed within the major cluster (Figure 4). Populations from Busia clustered away from the population from Homabay. Less minor clusters were observed in Busia than those that were seen in Homabay. Some populations from Siaya were found to cluster with those from Homabay. A similar situation was observed with populations from Busia clustering with those from Siaya and Homabay (Figure 4). Populations sampled from AEZs in the three counties showed a trend on the distribution of wild sorghum genotypes in given ecological zonation (Figure 5). Wild sorghums from LM₄ zones made clusters with wild sorghums from other counties. SYLM₄ clustered with populations from lower midlands from Busia. BULM₄ clustered with SYLMs while HBLM₄

clustered with SYLMs. Wild sorghums from each of the counties clustered together in most instances irrespective of their AEZ of origin and showed no significant pattern on the distribution (Figure 5).

DISCUSSION

Crop alleles were observed in the wild sorghum populations obtained from sorghum growing Western Kenya counties around Lake Victoria. Crop allele frequencies varied among the loci studied and in the AEZs from where the material was sampled. Low (<0.4), moderate (0.4-0.7) and high (0.7) crop allele frequencies were observed in this study. The presence of crop alleles at varying frequencies in wild forms could be directly attributed to the wild sorghum sympatric growth patterns and cultivation of crop sorghum. Localized sympatry was observed in all regions of

growth, where wild sorghums were seen growing in crop stands as weeds, on boarder rows, hedges and road side reserves. Tillering in sorghums (especially wild genotypes) extended their flowering period and increased the chances of interspecific hybridization. The climatic conditions in different geographic locations and the rainfall pattern encourage synchrony in germination and flowering and possibly interspecific hybridization. Previous studies in sorghum have shown the existence of crop alleles in populations of weed sorghums (Morrell et al., 2005). Proximity of wild sorghums to crop sorghums has been shown to result in both crop to weed and weed to crop interspecific hybridization (Warwick et al., 2009; Arriola and Ellstrand, 1996; Sahoo and Schmidt, 2010). The persistence of crop alleles in wild populations would be attributed to their weedy nature in given environments (Paterson et al., 1995).

Wild sorghum population had moderate to high

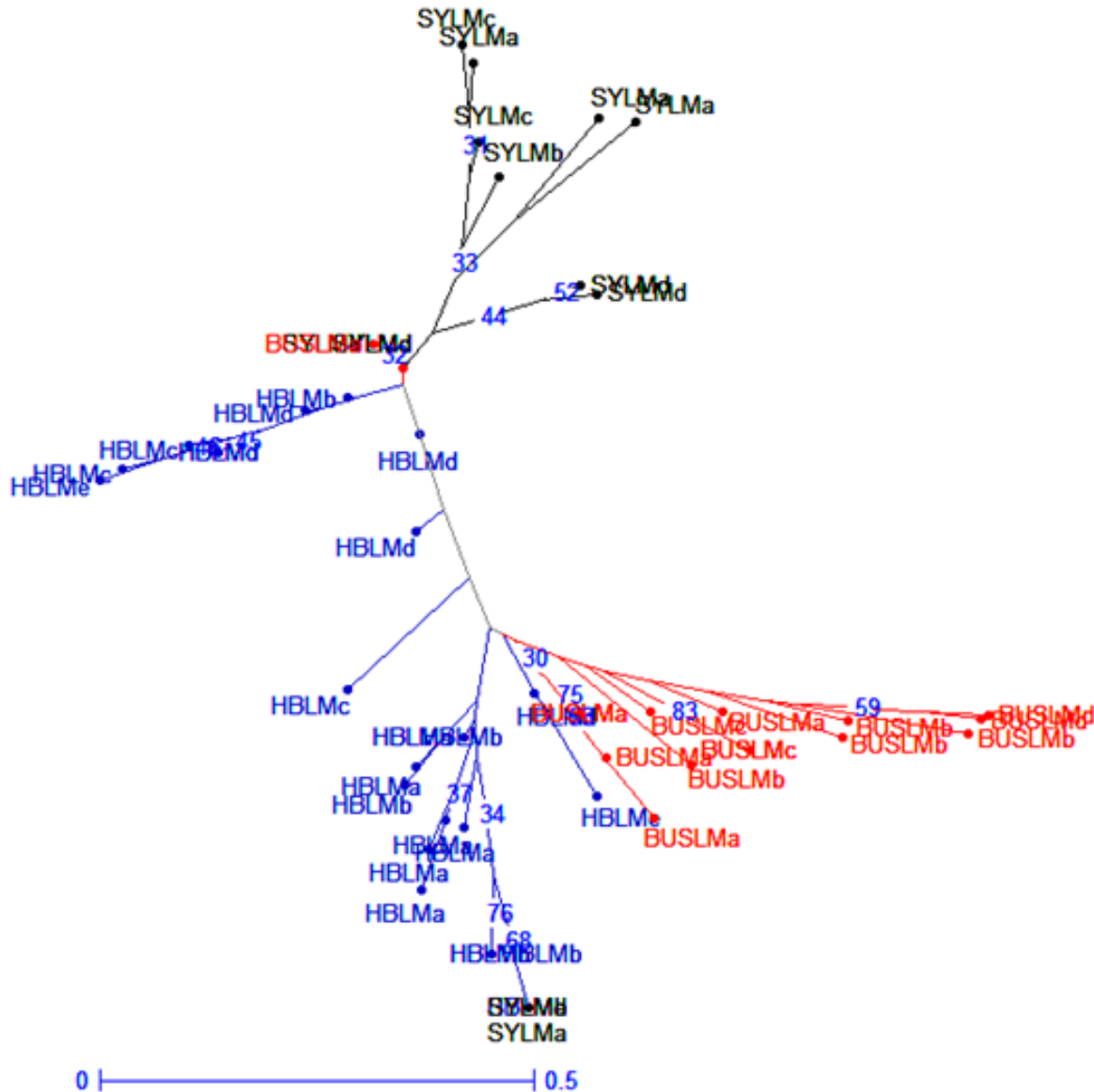


Figure 4. Dendrogram showing clusters of populations from Homabay, Siaya and Busia counties of Western Kenya.

diversity on the SSR loci assayed from crop sorghum producing AEZs in Western Kenya. H_E values of between 0.453 in LM_1 to 0.715 in LM_2 were obtained in the wild populations. No distinct patterns were observed on the magnitude of diversity of wild sorghums based on AEZs. There was minimal differences in the magnitude of diversity among the counties (Busia $H_E = 0.59 - 0.71$; Homabay $H_E = 0.58-0.68$, Siaya $H_E = 0.45-0.59$). Weed control and seed dissemination agricultural practices by local human populations may have influenced the diversity of the weeds. Such practices may have had low input on the diversity of wild sorghums in Busia and most in Siaya. High diversity in wild sorghums was also observed in other regions of Kenya $H_E = 0.69$ (Mutegei et al., 2011), in Africa $H_E = 0.59$ (Casa et al., 2005) in

Cameroon (Barnaud et al., 2007) and in Mali and Guinea (Sagnard et al., 2011).

Wild sorghum populations exhibit intraspecific and interspecific hybridization. Intraspecific hybridization results in low heterozygosity values in populations while interspecific hybridization explains gene flow within wild populations and thus the evolution and diversity of biotypes. Several intermediate types may also be observed in populations, which may play the role of “bridge species”. However, interspecific hybridization seems to increase variability of traits per species as a result of disruptive selection in wild sorghums (Doggett and Majisu, 1968).

Wild sorghums in Western Kenya were maintained at HW equilibrium due to low inbreeding and high

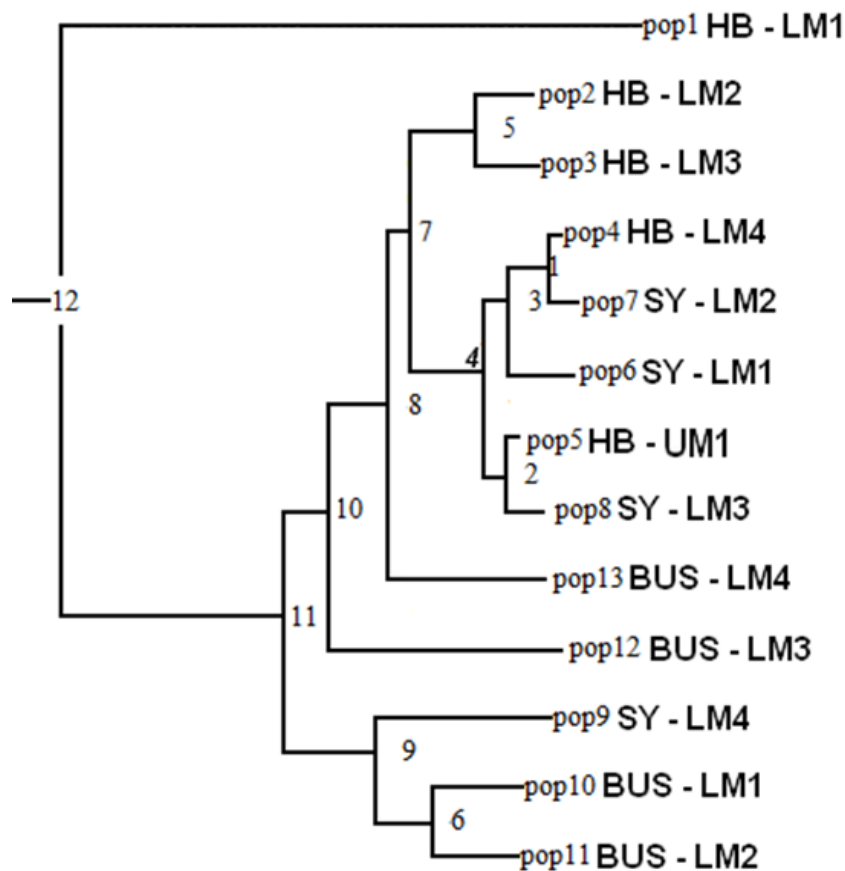


Figure 5. Cluster of populations from study AEZs. Dendrogram Based Nei's (1978) Genetic distance: Method = UPGMA, Modified from neighbor procedure of PHYLIP Version 3.5, in popgene.

heterozygosity. The degree of inbreeding (F_{IS}) was low in most AEZs except in UM_1 from Homabay and LM_1 from Siaya. These two regions also had fairly low heterozygosity (H_E). F_{IS} and the degree of genetic differentiation of the population (F_{ST}) have substantial impact on the HW equilibrium at whole population level. Low F_{IS} , F_{IT} and F_{ST} values were observed at a whole population basis, indicating that the population was at HW equilibrium. However, populations from Homabay UM_1 and Siaya LM_1 had moderate F_{IS} values of 0.59 and 0.58 respectively and did not conform to the HW equilibrium. The deviation was probably due to farmer selection practices during weeding. Farmers might have allowed some species of wild sorghums to grow to maturity in or around sorghum fields. This may have a similar effect to inbreeding in wild sorghum population. However, on a whole population basis, inbreeding was low and heterozygosity was high. Previous results show that populations of crop and wild sorghum were heterozygous with low F_{IS} in other parts of Kenya (Mutegi et al., 2011) and in other parts of Africa (Barnaud et al., 2007, Sagnard et al., 2011). Allele frequencies within populations of wild sorghums were high on the loci

assayed. This indicates the presence of gene flow among wild sorghums. Intra-population diversity ($H_S=H_E$) was moderate to high in these wild sorghum populations signifying existence of pollen or seed mediated outbreeding and geneflow among the wild sorghums. Thus, there is potential for proliferation and maintenance of exotic crop genes in these wild populations. In addition, low F_{IS} values were observed in the populations showing low inbreeding. Partitioning of variances shows that variation within individuals in populations was higher than variations among populations, confirming diminished inbreeding within individuals in populations. This also indicates that pollen and seed mediated gene flow was important in the wild sorghum populations. Furthermore, the intra-population diversity (H_S) was larger than the inter-population diversity (D_{ST}) in all populations, thus allelic differences between populations was not huge. The flow of genes from population to population could be accomplished by both pollen flow and seed distribution. This is important due to the movement towards adopting transgenics in cropping system in the near future. If this happened there would be a risk of unintentional escape of transgenes into wild populations thereby boosting their

adaptive advantage and therefore their wild character (Warwick et al., 2009).

The wild sorghum populations did not have significant patterns to explain recent population bottlenecks. Heterozygosity under mutation drift equilibrium (H_{EQ}) varied under IAM, TPM and SMM models. The H_{EQ} estimated by the mutation models show lower values probably due to selective weeding in sorghum fields and the presence of sorghum wild volunteers. Bird preference for the larger seeded weedy sorghums may have had some impact on reducing the estimated H_{EQ} . Growth of small-scale agriculture in the regions around Lake Victoria may have led to heavy weeding of some wild sorghum species to extinction and therefore contributed to the lower H_{EQ} values estimated in the mutation models. Previous studies in sorghum growing regions of Ethiopia did not show recent bottlenecks in sorghum populations (Adugna and Bekele, 2015).

Phylogenetic analysis of wild sorghums showed that counties and AEZs clustered in different ways. The dendrograms from the counties had consistent clusters in contrast to those from the various AEZs that did not show a consistent pattern. For instance in Figure 4, genetic material from one county clustered away from material from other counties. However, in Figure 5, genetic material from specific AEZs did not cluster together, indicating that wild sorghums were not clearly segregated along AEZ boundaries in Western Kenya. The presence of consistent geographic county clusters and lack of consistent AEZs clusters indicate higher influence of human population activities rather than climatic conditions on the distribution and diversity of wild sorghums. This could be attributed to practices such as seed contamination with specific weed seed during harvest and also due to selective weeding of the non crop sorghums. Furthermore, selective maintenance of certain non-crop sorghums on hedges and sharing of contaminated seed with specific wild sorghum seeds could be important in the distribution and diversity of wild sorghums. These practices enhance the populations of given wild sorghums within certain geographic locations. Selective weeding and seed distribution practices seem to differ from county to county, culminating in differences in diversity of weed types present. The influence of strong selection by man has been shown to influence sorghum phylogeographic position in Africa (de Alencar Figueiredo et al., 2008). The diversification trend of the wild types found in Western Kenya seem to follow the diversification pattern of crop sorghum which is strongly linked to geographic biotype / race classifications (Deu et al., 1995; 2006, Dje et al., 2000; Barnaud et al., 2007).

Conclusions

Crop alleles were observed in wild, sorghum populations. In addition wild sorghum population had moderate to high

diversity on SSR loci assayed. H_E values of between 0.453 in LM_1 to 0.715 in LM_2 were obtained in the wild populations. The wild populations had low inbreeding, low genetic differentiation and low to moderate deviation from HW equilibrium. Intra-populations diversity (H_S) was larger than inter-population diversity (D_{ST}) in all wild populations. This shows pollen and seed mediated gene flow is important in the wild sorghum populations. Analysis of H_{EQ} values under IAM, TPM and SMM mutation models suggest absence of recent bottlenecks in the wild populations. Human influence maybe more important than climatic conditions in explaining the distribution and diversity of wild sorghums populations. Future concerns over the persistence of robust crop alleles including transgenes in wild sorghums will need to be addressed. There is need to expand the capacity for testing and evaluation of the presence and effect of crop genes and in wild sorghums growing around crop sorghum production regions.

Conflict of interests

The authors did not declare any conflict of interest.

ACKNOWLEDGEMENTS

I wish to express my appreciation to the University of Nairobi, the Africa Harvest Biotechnology Foundation International and the Danforth Center Plant Science Center for the financial and material support they offered during the course of the research.

REFERENCES

- Adugna A, Bekele E (2015). Assessment of recent bottlenecks and estimation of effective population size in the Ethiopian wild sorghum using SSR allele diversity and mutation models. *Plant Genet. Resour.* DOI:10.1017/S1479262115000015.
- Adugna A, Snow AA, Sweeney PM, Bekele E, Mutegi E (2013) Population genetic structure of in situ wild *Sorghum bicolor* in its Ethiopian center of origin based on SSR markers. *Genet. Resour. Crop Evol.* 60: 1313-1328.
- Arriola PE, Ellstrand NC (1996). Crop-to-weed gene flow in the genus sorghum (Poaceae): Spontaneous interspecific hybridization between johnsongrass, *Sorghum halepense*, and crop sorghum, *S. bicolor*. *Am. J. Bot.* 83:1153-1160.
- Barnaud A, Deu M, Garine E, McKey D, Joly HI (2007). Local genetic diversity of sorghum in a village in northern Cameroon: structure and dynamics of landraces. *Theor. Appl. Genet.* 114(2):237-48.
- Barnaud A, Trigueros G, Garine E, McKey D, Joly HI (2008). High outcrossing rates in fields with mixed sorghum landraces: How are landraces maintained? *Heredity* 101: 445-452.
- Casa HM, Mitchell SE, Hamblin MT, Sun H, Bowers JE, Paterson AH, Aquadro CF, Kresovich S (2005). Diversity and selection in sorghum: simultaneous analyses using simple sequence repeats. *Theor. Appl. Genet.* 111(1):23-30.
- Cornuet JM, Luikart G (1996). Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data. *Genetics* 144:2001-2014.
- de Alencar Figueiredo LF, Calatayud C, Dupuits C, Billot C, Rami JF,

- Brunel D, Perrier X, Courtois B, Deu M, Glaszmann JC (2008). Phylogeographic evidence of crop neodiversity in sorghum. *Genetics* 179(2): 997–1008.
- Delobel C, Moens W, Querci M, Mazzara M, Cordeil S, Van den Eede G (2007). Maize seeds sampling and DNA extraction; report on the validation of a DNA extraction method from maize seeds and grains. European commission, Directorate General- joint research centre institute for health and consumer protection (IHCP). Biotechnology and GMOs unit. Website www.gmo-crl.jrc.ec.europa.eu/summaries/3272_DNAExtrReport.pdf Date: 10-1-2012.
- Deu M, Hamo P, Chanterreau J, Dufour P, D'Hont A, Lanaud C (1995). Mitochondrial DNA diversity in wild and cultivated sorghum. *Genome* 38:635–645.
- Di Rienzo A, Peterson AC, Garza JC, Valdes AM, Slatkin M, Freimer NB (1994). Mutational processes of simple sequence repeat loci in human populations. *Proc. Natl. Acad. Sci. USA* 91:3166–3170.
- Doebley JF (2004). The genetics of maize evolution. *Annu. Rev. Genet.* 38:37-59.
- Doggett H (1988). *Sorghum*, Ed 2. John Wiley and Sons, Inc., New York.
- Doggett H, Majisu BN (1968). Disruptive selection in crop development. *Heredity* 23:1-23.
- Doyle JF, Doyle JL (1990). Isolation of plant DNA from fresh tissue. *Focus* 12:13-15.
- Ejeta G, Grenier C (2005). Sorghum and its weedy hybrids. In: *Crop Fertility and Volunteerism*, edited by Jonathan Gressel, CRC Taylor and Francis, London.
- Ewens WJ (1972). The sampling theory of selectively neutral alleles. *Theor. Popul. Biol.* 3:87-112.
- Excoffier L, Laval G, Schneider S (2005). Arlequin ver. 3.0: An integrated software package for population genetics data analysis. *Evol. Bioinform. Online* 1:47-50.
- 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.
- Gepts P, Papa R (2003). Possible effects of (trans) gene flow from crops on the genetic diversity from landraces and wild relatives. *Environ. Biosafety Res.* 2:89-103.
- Goodman SJ (1998). Patterns of extensive genetic differentiation and variation among European harbor seals (*Phoca vitulina vitulina*) revealed using microsatellite DNA polymorphisms. *Mol. Biol. Evol.* 15(2):104-118.
- Hartl DL, Clark AG (1989). *Principles of Population Genetics*. Sinauer Associates, Sunderland, MA.
- Hokanson KE, Ellstrand NC, Ouedraogo JT, Olweny PA, Schaal BA, Raybould AF (2010). Biofortified sorghum in Africa: using problem formulation to inform risk assessment. *Nat. Biotechnol.* 28(9):900-903.
- James C (2010). Global Status of Commercialized Biotech/GM Crops: 2010. ISAAA Brief No. 42. ISAAA: Ithaca, NY.
- Levene H (1949). On a matching problem arising in genetics. *Ann. Math. Stat.* 20:91-94.
- Lewontin RC (1964). The interaction of selection and linkage. I. General considerations: Heterotic models. *Genetics* 49:49-67.
- Lewontin RC (1972). The apportionment of human diversity. *Evol. Biol.* 6:381-398.
- Lewontin RC, Kojima K (1960). The evolutionary dynamics of complex polymorphisms. *Evolution* 14:458-472.
- Mohammadi SA, Prasanna BM (2003). Review interpretation analysis of genetic diversity in crop plants salient statistical tools. *Crop Sci.* 43:1235-1248.
- Mondini L, Noorani A, Pagnotta MA (2009). Assessing Plant Genetic Diversity by Molecular Tools. *Diversity* 1(1):19-35.
- Morrell PL, Williams-coplin D, Lattu AL, Bowers EJ, Chandler JM, Paterson AH (2005). Crop-to-weed introgression has impacted allelic composition of Johnson grass populations with and without recent exposure to cultivated sorghum. *Mol. Ecol.* 14:2143-2154.
- Muraya MM, Sagnard F, Parzies HK (2010). Investigation of recent population bottlenecks in Kenyan wild sorghum populations (*Sorghum bicolor* (L.) Moench ssp. *verticilliflorum* (Steud.) De Wet) based on microsatellite diversity and genetic disequilibria. *Genet. Resour. Crop Evol.* 57(7):995-1005.
- Mutegi E, Sagnard F, Muraya M, Kanyenji B, Rono B, Mwangera C, Marangu C, Kamau J, Parzies H, de Villiers S, Semagn K, Traoré PS, Labuschagne M (2009). Ecogeographical distribution of wild, weedy and cultivated *Sorghum bicolor* (L.) Moench in Kenya: implications for conservation and crop-to-wild gene flow. *Genet. Resour. Crop Evol.* 57(2):243-253.
- Mutegi E, Sagnard F, Semagn K, Deu M, Muraya M, Kanyenji B, de Villiers S, Kiambi D, Herselman L, Labuschagne M (2011). Genetic structure and relationships within and between cultivated and wild sorghum (*Sorghum bicolor* (L.) Moench) in Kenya as revealed by microsatellite markers. *Theor. Appl. Genet.* 122(5):989-1004.
- Nei M (1973). Analysis of gene diversity in subdivided populations. *Proc. Natl. Acad. Sci. U.S.A.* 70:3321-3323.
- Nei M (1978). Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics* 89:583–590.
- Paterson AH, Schertz KF, Lin YR, Liu SC, Chang YL (1995). The weediness of wild plants: Molecular analysis of genes influencing dispersal and persistence of johnsongrass, *Sorghum halepense* (L.) Pers. *Proc. Natl. Acad. Sci. U.S.A.* 92:6127-6131.
- Pedersen JF, Toy JJ, Johnson B (1998). Natural outcrossing of sorghum and sudangrass in the central great plains. *Crop Sci.* 38:937-939.
- Piñeyro-Nelson A, van Heerwaarden J, Perales H, Serratos J, Rangel A, Hufford, M, Gepts P, Garay-Arroyo A, Rivera-Bustamante R, Alvarez-Buylla E (2009). Transgenes in Mexican maize: molecular evidence and methodological considerations for GMO detection in landrace populations. *Mol. Ecol.* 18:750-761.
- Rueffler C, Van Dooren TJM, Leimar O, Abrams PA (2006). Disruptive selection and then what? *Trends Ecol. Evol.* 21(5):238-45A.
- Sagnard F, Deu M, Dembélé D, Leblois R, Touré L, Diakité M, Calatayud C, Vaksman M, Bouchet S, Mailé Y, Togola S, Traoré PCS (2011). Genetic diversity, structure, gene flow and evolutionary relationships within the *Sorghum bicolor* wild-weedy-crop complex in a western African region. *Theor. Appl. Genet.* 123(7):1231-1246.
- Sahoo L, Schmidt J (2010). Growth and fitness components of wild x cultivated *Sorghum bicolor* (Poaceae) hybrids in Nebraska. *Am. J. Bot.* 97(10):1610-1617.
- Saitou N, Nei M (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4:406-425.
- Schlotterer C (2000). Evolutionary dynamics of microsatellite DNA. *Chromosoma* 109: 365-371.
- Sherwin, WB, Jabot F, Rush R, Rossetto M (2006). Measurement of biological information with applications from genes to landscapes. *Mol. Ecol.* 15(10): 2857–2869
- Song ZP, Lu BR, Zhu YG, Chen JK (2002). Pollen competition between cultivated and wild rice species (*Oryza sativa* and *O. rufipogon*). *New Phytol.* 153: 289–296.
- Tesso T, Kapran I, Grenier C, Snow A, Sweeney P, Pedersen J, Marx D, Bothma G, Ejeta G (2008). The potential for crop-to-wild gene flow in sorghum in Ethiopia and Niger: A geographic survey. *Crop Sci.* 48:1425-1431.
- Warwick SI, Beckie HJ, Hall LM (2009). Gene Flow, Invasiveness, and Ecological Impact of Genetically Modified Crops. *The Year in Evolutionary Biology 2009: Ann. N.Y. Acad. Sci.* 1168:72-99.
- Weir BS (1990). *Genetic data analysis*. 1st ed. Sinauer Associates, Sunderland, MA.
- Yang RC, Yeh FC (1993). Multilocus structure in *Pinus contorta* Dougl. *Theor. Appl. Genet.* 87:568–576.
- Yeh FC, Boyle TJB (1997). Population genetic analysis of codominant and dominant markers and quantitative traits. *Belg. J. Bot.* 129:157.
- Yonemaru J, Ando T, Mizubayashi T, Kasuga S, Matsumoto T, Yano M (2009). Development of Genome-wide Simple Sequence Repeat Markers Using Whole-genome Shotgun Sequences of Sorghum (*Sorghum bicolor* (L.) Moench). *DNA Res.* 16(3):187-193.

Short Communication

Efficacy of fish compost on management of chilli fruit rot caused by *Colletotrichum capsici*

H. Ann Suji^{1*} and T. Suthin Raj²¹CAS in Marine Biology, Faculty of Marine Sciences, Annamalai University, Chidambaram, India.²Department of Plant pathology, Faculty of Agriculture, Annamalai University, Chidambaram, India.

Received 5 March, 2015; Accepted 13 April, 2015

Anthracnose of chilli caused by *Colletotrichum capsici* is a serious disease affecting the yield and quality of fruits. The efficacy of fish compost against chilli fruit rot was assessed in comparison with other compost. Green house study was conducted to study the disease control by application of fish compost, farm yard manure, neem cake, mahua cake, sheep manure, paddy husk, press mud, poultry manure, coir pith compost and groundnut cake. Application of fish compost of 12.5 ton/ha recorded the maximum disease incidence and increased the fruit yield when compared to control.

Key words: *Colletotrichum capsici*, *Capsicum annuum* L., fish compost.

INTRODUCTION

Chilli (*Capsicum annuum* L.) is an important spice crop of India. It is affected by several fungal, bacterial and viral diseases, of which, chilli anthracnose causes considerable damage, inflicting severe quantitative and qualitative losses. The estimated loss due to this disease ranged from 8 to 60% in different parts of India. The fungus *Colletotrichum capsici* infects both unripe (green) and ripe (red chilli) fruits and survives on seed as acervuli and micro sclerotia (Suthin Raj and Christopher, 2009). Infection of *C. capsici* is higher at the mature fruit stage than in the early fruit stage. The fungus pathogen is both seed borne and air borne and affects seed germination and vigour to a greater extent. Several fungicides have been reported to be effective in the management of fruit rot of chilli (Gopinath et al., 2006; Shovan et al., 2008). However, the indiscriminate use of fungicides leads to

toxic residues on chilli products, development of fungicide resistance and also serves as a cause for environmental pollution (Suthin Raj et al., 2012). Therefore, under intensive chilli cultivation, there is an urgent need to develop alternative disease control measures. The present investigation screened various composts against *C. capsici* *in vitro* based on their efficacy to control fruit rot diseases.

MATERIALS AND METHODS

Site selection for composting, collection of raw materials (bulking agents) and their nutrient content analysis

Inexpensive locally available bulking agents (farm yard manure, neem cake, mahua cake, sheep manure, paddy husk, press mud,

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

Table 1. The intensity of fruit rot.

Category value	Percent fruit area diseased
0	0
1	1-5
2	6-10
3	11-25
4	26-50
5	51 and above

poultry manure, coir pith compost and groundnut cake), trash fish, fish and shell fish processing wastes were collected as raw materials for composting and their percentage contents of phosphorus, potassium, carbon, nitrogen, moisture, ash (Tandon, 2009) etc., were analysed.

Preparation of fish powder extracts

Two marine fish species (trash fish and edible fish) were processed at a local processing plant, using 3.5% sucrose and 0.15% phosphate as cryoprotectants. The frozen blocks were transported to the laboratory and stored at 18°C until drying. A 500 g block of each fish was dried using a Labconco Freeze Dry System at a temperature of 40°C until the moisture content reached 5%. The samples were milled and sieved using a 40 mm screen mesh. The resulting powder was vacuum packed and stored at 4°C. Powdered samples were soaked in chloroform (1:4 w/v) and extracted for 2 days at room temperature and the extracts were collected and concentrated (Ann Suji, 2004). Without drying and powdering, bad odor may be emitted from fish products. So, powdered fish sample was used for the preparation of compost.

Composting procedure

The compost pile were prepared and the environmental conditions such as: temperature, moisture, nitrogen, carbon, phosphorus, pH, potassium (Tandon, 2009), etc. which favour the production of nutrient enriched compost were monitored at regular intervals.

Quality assessment of mature compost

The mature compost was analysed for its microbial status, pH, phosphorus, potassium, organic matters, carbon and nitrogen ratio, mercury, arsenic, cadmium, copper, iron, molybdenum, zinc, sodium, humic acid, fulvic acid (Tandon, 2009).

Pot culture study

Fresh seed samples were collected from the Department of Agronomy, Annamalai University, Chidambaram, Tamilnadu. A pot culture experiment was conducted in unsterilized soil incorporating the different organic amendments viz., fish compost (12.5 t/ha), farm yard manure (FYM) (12.5 t/ha), neem cake (250 kg/ha), mahua cake (250 kg/ha), sheep manure (12.5 t/ha), paddy husk (3 t/ha), press mud (12.5 t/ha), poultry manure (12.5 t/ha), coir pith compost (12.5 t/ha) and groundnut cake (250 kg/ha), to assess their efficacy against chilli anthracnose. The organic amendments

at the calculated quantities were mixed with pot culture soil a week before planting. 30 days old seedlings were planted at 3 per/pot. Three replications were kept with four pots in each. The pots were maintained inside a glass house with judicious, uniform and regular watering. After 90 days, plants were inoculated with a spore suspension ($1 \times 10^6 \text{ m}^{-1}$) of *C. capsici* thoroughly over the plant canopy by pin pricking method. The anthracnose disease incidence was recorded on 120 days after planting. The intensity of fruit rot was calculated as percent disease index (PDI) as per the grade chart proposed by Ravinder Reddy (1982) using the formula proposed by McKinney (1923) (Table 1). The percent disease index (PDI) was calculated using McKinney (1923) infection index.

$$\text{PDI} = \frac{\text{Sum of numerical ratings}}{\text{Total number of fruits observed}} \times \frac{100}{\text{Maximum category value}}$$

RESULTS AND DISCUSSION

Application of organic amendments significantly reduced the fruit rot incidence and enhanced the fruit yield and dry matter production. Among them, fish compost at 12.5 tons/ha (T_1) recorded a minimum disease incidence (49.10%) and significantly increased both fruit yield (360.20 g/plant) and dry matter production (8.96 g/plant). It was followed by FYM at 12.5 tons/ha (T_2) which recorded a 51.3% disease incidence of 357.0 g/plant fruit yield and dry matter production of 8.76 g/plant and neem cake, 250 kg/ha (T_3) recorded 54.5% disease incidence, 348.7 g/plant fruit yield and dry matter production of 8.42 g/plant (Table 2).

The present investigation was undertaken to study the effect of different organic amendments against chilli fruit rot disease incidence. Fish compost at 12.5 ton/ha recorded the maximum growth and yield parameters. Stone (2003) reported that paper mill residue compost amended to sandy field soils consistently was suppressive to anthracnose and brown spot disease in snap bean. Zhang et al. (1996) reported that fish compost induced systemic acquired resistance in cucumber to *Pythium* root rot and anthracnose.

Conflict of interests

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

Table 2. Effect of fish compost against *C. capsici* on under greenhouse condition.

Treatment	Mean no. of fruits/plant	Fruit yield (g/plant)	Fruit rot incidence on 120 th day	Dry matter production (g/plant)
T ₁ – Fish compost (12.5 t/ha)	102 ^{*a}	360.20 ^a	49.10 ^a	8.96 ^a
T ₂ – FYM (12.5 t/ha)	99 ^b	357.00 ^b	51.30 ^b	8.76 ^b
T ₃ – Neem cake (250 kg/ha)	95 ^c	348.70 ^b	54.50 ^d	8.42 ^c
T ₄ – Mahua cake (250 kg/ha)	91 ^d	334.50 ^c	58.20 ^f	7.72 ^f
T ₅ – Sheep manure (12.5 t/ha)	94 ^c	346.80 ^c	55.60 ^e	8.22 ^d
T ₆ – Paddy husk (3 t/ha)	90 ^d	332.80 ^c	57.00 ^f	7.60 ^g
T ₇ – Press mud (12.5 t/ha)	97 ^b	351.60 ^b	52.60 ^c	8.63 ^b
T ₈ – Poultry manure (12.5 t/ha)	92 ^c	344.00 ^c	56.20 ^e	7.84 ^e
T ₉ – Coir pith compost (12.5 t/ha)	82 ^e	324.60 ^e	58.90 ^g	7.34 ⁱ
T ₁₀ – Groundnut cake (250 kg/ha)	88 ^e	329.00 ^d	60.40 ^h	7.49 ^h
T ₁₁ – Control	42 ^f	162.40 ^f	65.55 ⁱ	7.02 ^j

*Values in the column followed by common letters do not differ significantly by DMRT (P=0.05).

REFERENCES

- Gopinath K, Radhakrishnan NV, Jayaraj J (2006). Effect of propiconazole and difenoconazol on the control of anthracnose of chilli fruit caused by *Colletotrichum capsici*. *Crop Prot.* 25: 1024-1031.
- McKinney HH (1923). A new system of growing grading plant diseases. *J. Agric. Res.* 26:195-218.
- Ravinder Reddy M (1982). Evaluation of fungicides against major diseases of chilli. *M.Sc. (Ag.)*, Thesis, Tamil Nadu Agric. Univ., Coimbatore, India, p. 64.
- Shovan LR, Bhuiyan MKA, Begum JA, Pervez Z (2008). *In vitro* control of *Colletotrichum dematium* causing anthracnose of soybean by fungicides, plant extracts and *Trichoderma harzianum*. *Int. J. Sustain. Crop Prod.* 3:10-17.
- Stone AG (2003). Effect of organic amendments on soil borne and foliar diseases in field-grown snap bean and cucumber. *Plant Dis.* 87:1037-1042.
- Suthin Raj T, John Christopher D, Ann Suji H (2012). Morphological, cultural and genetic variabilities in fruit rot of Chilli caused by *Colletotrichum capsici* in Tropical region of South India. *Ann. Plant Prot. Sci.* 20(2):421-425.
- Suthin Raj T, John Christopher D (2009). Effect of bio-control agents and fungicides against *Colletotrichum capsici* causing fruit rot of chilli. *Ann. Plant Prot. Sci.* 17(1):143-145.
- Zhang W, Dick WA, Hoitink HAJ (1996). Compost-induced systemic acquired resistance in cucumber to Pythium root rot and anthracnose. *Phytopathology* 86:1066-1070.

Full Length Research Paper

Activity of the fungus *Pleurotus ostreatus* and of its proteases on *Panagrellus* sp. larvae

Hugo Leonardo André Genier^{1*}, Filippe Elias de Freitas Soares², José Humberto de Queiroz², Angélica de Souza Gouveia², Jackson Victor Araújo³, Fabio Ribeiro Braga^{3,4}, Iara Rebouças Pinheiro¹ and Maria Catarina Megumi Kasuya⁵

¹Departamento de Engenharia Rural, Universidade Federal do Espírito Santo, Alegre-ES. Cep: 29500-000, Alegre.

²Departamento de Bioquímica e Biologia Molecular, Universidade Federal de Viçosa, Viçosa-MG. Cep: 3657000, Alegre.

³Departamento de Veterinária, Universidade Federal de Viçosa, Viçosa-MG, Alegre.

⁴Universidade Vila-Velha, Vila Velha, Espírito Santo. Cep: 29102206, Alegre.

⁵Departamento de Microbiologia, Universidade Federal de Viçosa, Viçosa-MG, Alegre.

Received 25 January, 2015; Accepted 22 April, 2015

Biological control has been shown to be one of the possible biotechnological applications of fungi and their proteases. The objective of this study was to evaluate the nematocidal activity of the fungus *Pleurotus ostreatus* and its proteases on *Panagrellus* sp. larvae. Proteolytic activity of *P. ostreatus* (PLO 06) was measured and characterized at different pHs, temperatures and in the presence of an inhibitor (PMSF). Daily samples of culture medium were collected in order to determine the period of maximum enzyme production. A zymogram showed the profile of several proteases. Predatory activity of the fungus *P. ostreatus* (PLO 06) was evaluated on *Panagrellus* sp. larvae (assay A) as well as the nematocidal activity of PLO 06 proteases on the same larvae (assay B). At pH 9 and 60°C, the activity of the proteases reached the maximum. In the presence of inhibitor, there was no proteolytic activity. A sample collected on the fifth day of incubation showed the highest enzyme activity. *P. ostreatus* demonstrated capture activity on larvae *Panagrellus* sp. The values of the reduction of the larvae (Assay A) were: day 1 (65.6%); day 2 (77.4%); day 3 (95.2%). The reduction of the larvae (Assay B) was 42%. *P. ostreatus* (PLO 06) and its proteases were very effective against *Panagrellus* sp. larvae, demonstrating great potential for use in integrated biological control.

Key words: *Pleurotus*, protease, *Panagrellus* sp., biological control, nematocidal.

INTRODUCTION

Biological control assumes increasing importance in integrated management programs of agricultural pests

(Parra et al., 2002) and thus is a "strategic component" in the fight against gastrointestinal nematode parasites and

*Corresponding author. E-mail: hugogenier@hotmail.com. Fax: +55 (28) 3552-8603.

Abbreviation: PMSF, Phenylmethylsulfonyl fluoride.

Author(s) agree that this article remain permanently open access under the terms of the [Creative Commons Attribution License 4.0 International License](http://creativecommons.org/licenses/by/4.0/)

phytonematodes (Hibbett and Thorn, 1994; Grønvold et al., 1996; Eira, 2004). Living organisms that act as biological control agents constitute the group of natural enemies, which is formed by parasitoids, predators and pathogens. Accordingly, a wide variety of fungi have been studied for this purpose: nematophagous fungi (Larsen, 1999; Lopez-Llorca et al., 2008) and specifically here, fungi of the genus *Pleurotus* (Okorie et al., 2011). The genus *Pleurotus* (class of Basidiomycetes) hosts several species with important biological characteristics. Among these we can highlight: (1) ability to colonize and degrade a wide variety of lignocellulosic residues, (2) high value medicinal and gastronomical properties, (3) require shorter time of cultivation when compared to other edible genera (Cohen et al., 2002; Bonatti et al., 2004; Satou et al., 2008; Okorie et al., 2011) and (4) nematicidal activity against phytonematodes. In relation to this fact, in general, fungi of this genus exhibit a unique method of capturing nematode, which can be described as the production of toxic metabolites that cause the decrease of helminth head and subsequent paralysis (Thorn and Barron, 1984; Hibbett and Thorn, 1994). This toxin has been identified as trans-2-decenedioic acid (Kwok et al., 1992). The species *P. ostreatus* are known to prey on nematodes. However, in relation to predatory activity, this has not been fully elucidated, thus requiring more studies from biological models under laboratory conditions.

Extracellular proteases produced by nematophagous fungi are directly involved in the stages of the infection. They act in the digestion of nematode's protection barrier, rich in protein (Braga et al., 2010). However, there is a lack of studies aimed at elucidating the molecular mechanism of interaction of the proteases from fungi of the genus *Pleurotus*. Thus, the objective of this study was to evaluate the nematicidal activity of the fungus *Pleurotus ostreatus* and its proteases.

MATERIALS AND METHODS

Fungi

One fungal isolate was used; *P. ostreatus* (PLO 06, GenBank accession number KC782771). This fungi is derived from the soil of Viçosa, Brazil, and belong to the Nucleus Collection of the Department of Microbiology/UFV. These organisms have been maintained by means of continuous transfer to solid medium containing 2% water-agar (2% WA) in the Department of Molecular Biology and Biochemistry, Federal University of Viçosa, Brazil. Subsequently, the fungus was again replicated in Petri dishes for removal of the inoculum in the form of disks of 2 cm and has grown on 28°C, in the dark. Free-living nematodes of the genus *Panagrellus* were used in the experiment. These organisms have been kept in Petri dishes in moistened and kneaded oat flakes medium, at the Laboratory of Parasitology, Department of Veterinary, Federal University of Viçosa, Brazil. For the test trial, these nematodes were extracted with a Baermann apparatus and collected in hemolysis tubes after 6 h of decanting.

Proteases production

Fungal mycelia of PLO 06 were transferred to flasks (250 ml) previously autoclaved containing 50 ml of liquid medium according to the methodology described by Soares et al. (2013). The liquid medium was composed of glucose 10 g /l; yeast extract, 10 g/l; K₂HPO₄, 5 g/l; and MgSO₄, 0.10 g/l. The fungal inoculum was grown in the flasks under agitation at 120 rpm at 28°C for seven days. Then, the fermented medium was filtered using filter paper Whatman No. 1 at 4°C and then centrifuged for 10 min, at 10,000 g, 4°C, to obtain the protease.

Activity assay

The proteolytic activity of *P. ostreatus* (PLO 06) was measured according to the methodology described by Soares et al. (2013). One protease unit was defined as the amount of enzyme required to release 1.0 µg of tyrosine per minute under the conditions used in the tests.

Proteases characterization

All characterization experiments were performed with three replications.

Effects of pH and temperature

The proteolytic activity was characterized at different pH values. Thus, the 50 mM phosphate-citrate buffer was used for pH 3, 4, 5, 6, 7, 8 and 9. The assay temperature was 40°C. Evaluation of the effect of temperature on the proteolytic activity was performed at the pH with the maximal activity obtained in the above assay at different temperature values (30, 40, 50, 60, 70 and 80°C).

Effect of Inhibitors

Proteases were incubated with the inhibitor phenylmethylsulfonyl fluoride (PMSF), at the pH and temperature that showed the highest activity in the previous assays.

Proteolytic activity during the days of incubation

Samples of culture medium were collected every day in order to measure the proteolytic activity over time in order to obtain the time of highest enzyme production.

Zymogram

A zymography was performed using casein as the substrate (casein-SDS-PAGE) (Hummel et al., 1996) to obtain the profile of the proteases produced by *P. ostreatus* (PLO 06). Samples were subjected to gel electrophoresis on 10% polyacrylamide containing 1% casein and mixed with gel-native sample buffer (glycerol 30% (v/v), bromophenol blue 1% (p/v), Tris- HCl 0.25 M). After the electrophoresis procedure, the gel was incubated in a Triton X-100 2.5% solution for 30 min for removal of SDS. Then, the gel was washed three times with water. Subsequently, it was incubated in Tris-HCl 50 mM (pH 9.0) for 1 h at 60°C. In the developing procedure, the gel was stained with Coomassie Brilliant Blue R-250 and then was immediately destained with decolorizing solution (10% acetic acid and 50% alcohol aqueous solution).

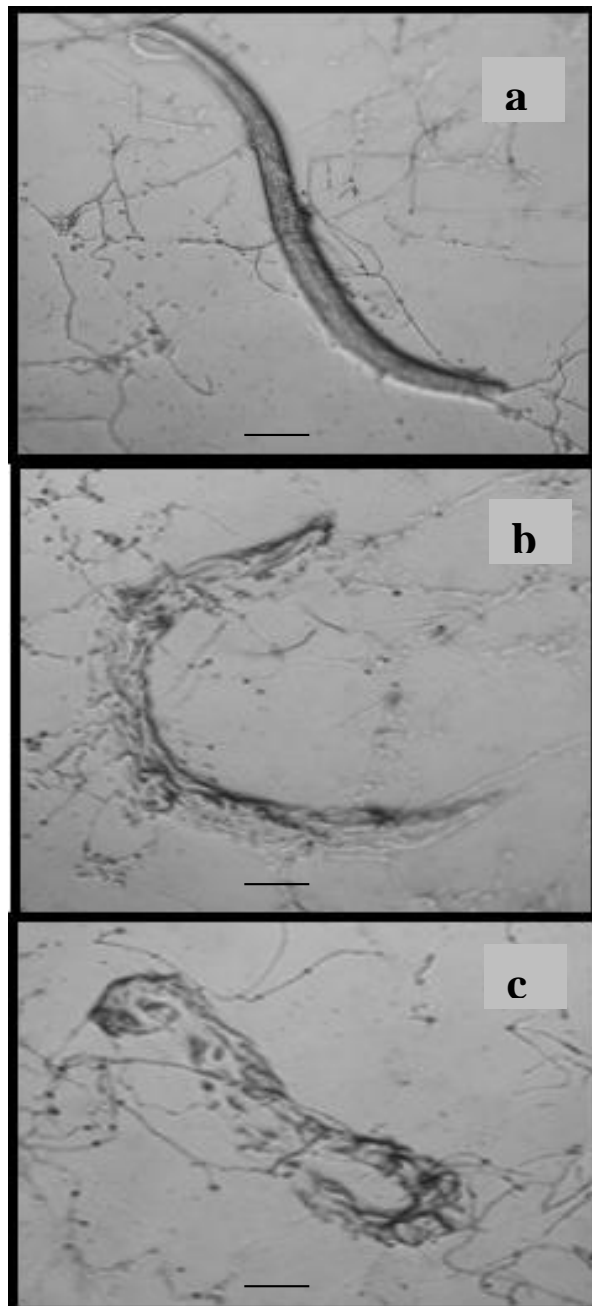


Figure 1. a. Initial action of hyphae of the fungus *Pleurotus ostreatus* on larvae *Panagrellus* sp. b. Destruction of larvae and colonization by hyphae of *Pleurotus ostreatus*. c. Destruction and/or digestion of *Panagrellus* sp. larvae by *Pleurotus ostreatus*. Bars: a-7,000 μm ; b-4,500 μm and c-6,000 μm .

Experimental assays

Two assays (A and B) were performed. In the assay A, predatory activity of the fungus *P. ostreatus* (PLO 06) was evaluated on *Panagrellus* sp. larvae. In the assay B, the nematicidal activity of *P. ostreatus* (PLO 06) proteases was evaluated on larvae of *Panagrellus* sp.

Assay A

Two groups were formed on Petri dishes 4.5 cm containing 10 ml of 2% WA; one treated group and one control group, with six replicates for each group. The Petri dishes were previously marked in fields of 4 mm in diameter. In the treated group, each Petri dish contained 1000 *Panagrellus* sp. larvae and the fungus. The control group (without fungi) contained only 1000 *Panagrellus* sp. larvae. Both groups of plates were incubated in a BOD incubator chamber in the dark at 28°C. For three days, every 24 h, 10 random fields of 4 mm in diameter in each plate of the treated and control groups were observed under an optical microscope at 10x objective by counting the number of non-destroyed larvae in each, according to the methodology adapted from Braga et al. (2010). Photomicrographs were taken for proof of capture activity and subsequent destruction of nematodes.

Destruction efficiency of *Panagrellus* sp. larvae compared to the control was assessed by the Tukey test at 1% probability. The data were interpreted by analysis of variance at significance levels of 1 and 5% probability (Ayres et al., 2003). Subsequently, the average reduction percentage of the larvae was calculated according to the following formula:

$$\text{Reduction (\%)} = \frac{\text{Average larvae recovered from control} - \text{Average larvae recovered from treatment}}{\text{Average larvae recovered from control}} \times 100$$

Assay B

Two groups were formed in microtubes. One treated group in which about 50 *Panagrellus* sp. larvae were incubated with the proteases and one control group, in which about 50 larvae *Panagrellus* sp. were incubated with denatured proteases (boiled for 1 h). Six replicates were performed for each group. Both groups were incubated at 28°C for 24 h. After this period, the total number of larvae was counted (Soares et al., 2013). The data were interpreted by analysis of variance in significance levels of 1 and 5% probability. The efficiency of the destruction of the larvae in the control was evaluated by the Tukey test at 1% probability (Ayres et al., 2003). The average reduction percentage of the larvae was calculated according to the following equation:

$$\text{Reduction (\%)} = \frac{\text{Average larvae recovered from control} - \text{Average larvae recovered from treatment}}{\text{Average larvae recovered from control}} \times 100$$

RESULTS AND DISCUSSION

The fungus *P. ostreatus* (PLO 06) showed high *in vitro* capture activity on larvae *Panagrellus* sp. After 24 h, an interaction of hyphae of the fungus with nematodes was observed, as shown in Figure 1a. Figure 1b shows the destruction of the larvae, as well as colonization by fungal hyphae. Figure 1c shows the complete destruction and or digestion of the larvae by the tested fungi. Figure 2b illustrates the production of toxic droplets by the fungus *P. ostreatus*. The tested fungi were effective in destroying *Panagrellus* sp. larvae, as shown in Table 1. On the first day of the experiment, the percentage reductions



Figure 2. a. *Panagrellus* sp. larvae (white arrow). b. Toxic droplet production by the fungi *Pleurotus ostreatus* (black arrow / detail). Bar: a-15,000 μ m.

Table 1. Daily average, standard deviation and percentage reduction of *Panagrellus* sp. larvae in water-agar (2% WA) during the period of three days in the treatments with the fungus *Pleurotus ostreatus* and the control group without fungi.

Days	<i>Pleurotus ostreatus</i>	Control	Reduction (%)
1	10.2 ^a \pm 4.3	29.1 ^b \pm 8.92	65.6
2	6.2 ^a \pm 3.4	15.7 ^b \pm 10.6	77.4
3	1.4 ^a \pm 0.8	29.4 ^b \pm 11.15	95.2

Averages followed by the same lowercase letter in the lines do not differ statistically of ($p > 0.01$).

observed were higher than 50%, reaching values higher than 90% on the third day. The results of this study have demonstrated the nematicidal activity of fungi of the genus *Pleurotus* on the free-living nematode *Panagrellus* sp. (Table 1). Fungal species tested have activity against phytonematodes, however, these isolates have never been tested on the real possibility of biological control. In this sense, it can be argued that among the various species of fungi there are variations between the action of different isolates, which must be constantly tested (Mendoza-de Gives, 1999). This information is also in line with those of Okorie et al. (2011) who tested the

nematicidal activity of two species of fungi of the genus *Pleurotus* and demonstrated no distinction in their activities. Hibbett and Thorn (1994) mention that the basic mechanism of the activity of predatory fungi of the genus *Pleurotus* is the production of small droplets of anti-nematode toxin derived from linoleic acid. It is further described that the paralyzed nematodes are subsequently invaded by hyphae. These results are in agreement with the present work's results, where PLO 06 showed nematicidal activity and production of these toxic droplets against *Panagrellus* sp. larvae (Figure 2a, b). Some works that targeted *in vitro* control of plant parasitic

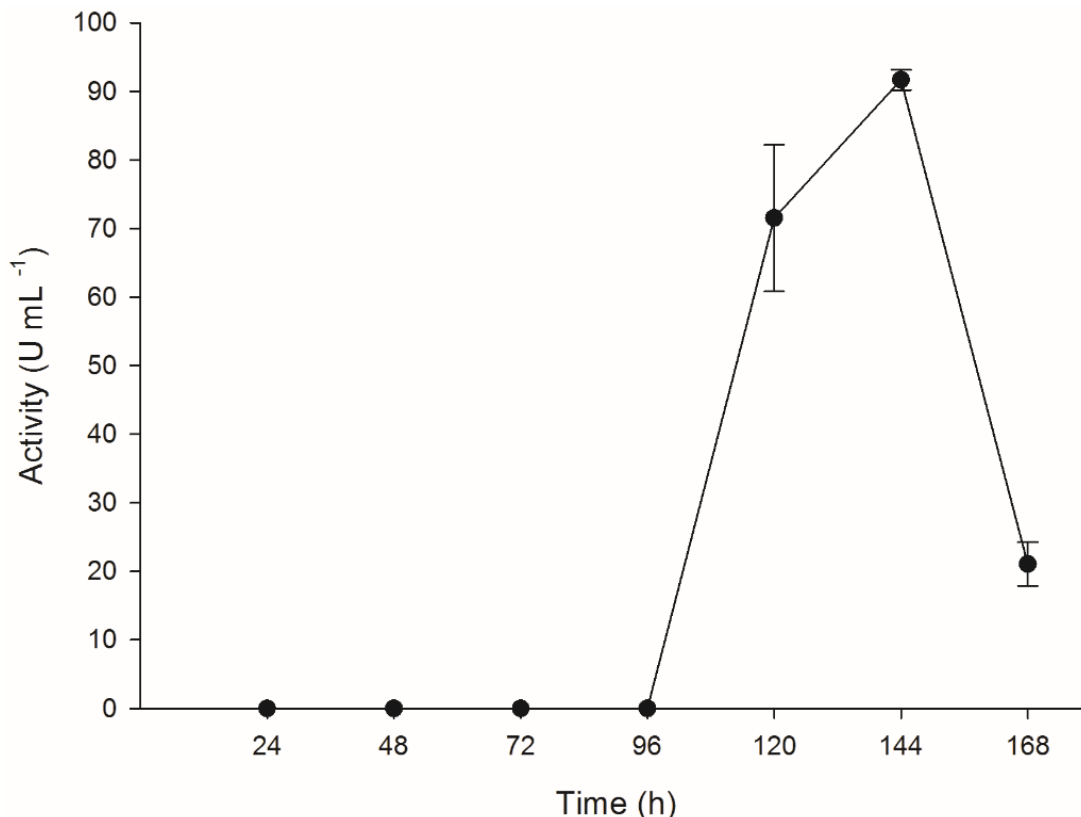


Figure 3. Assay of proteolytic activity during the incubation period of 168 h (7 days). Samples of culture medium were collected every day in order to measure the proteolytic activity along the days, to obtain the time of higher enzyme production.

nematodes have been performed with the species *P. ostreatus* (Okorie et al., 2011). However, based on previous studies involving other groups of nematophagous fungi, the present experimental assay has shown to be "elucidative" with regard to the reduction of nematodes in Petri dishes in medium 2% WA, since these plates were observed for three days and every 24 h. This premise is important to elucidate the predatory mechanism of this species on free-living nematodes (Thorn and Barron, 1984). The model organism *Panagrellus* sp. has been used for observation of predatory activity of fungi with potential nematophagous, since they are "agile" and provide the production of traps by fungi (Eren and Pramer, 1965). This type of model is important since it provides the basis for other experiments aimed at reducing nematodes that are harmful to animals, humans and plants (Jaffee and Muldoon, 1997; Tavela et al., 2012). From this point, comparatively, the carnivore fungus *Monacrosporium* spp., were shown to be effective in capturing and destroying *Panagrellus* sp. and *Meloidogyne incognita*, phytonematodes, under laboratory conditions (Gomes et al., 1999). In that work, the authors showed that *Panagrellus* sp., was the most susceptible ($p < 0.05$).

Those results are in agreement with the present work, demonstrating that the species *P. ostreatus* has reduced *Panagrellus* sp. larvae in Petri dishes by more than 90% at the end of 3 days, thus suggesting the enormous biocontrol potential of these species.

In another context, the literature has mentioned the realization of some studies with nematophagous fungi with potential for the production of extracellular enzymes with nematocidal activity (Braga et al., 2012b; 2013; Soares et al., 2012; 2013). In this study, it was observed that the fungus *P. ostreatus* (PLO 06) successfully produced proteases. The fifth day of incubation was that in which the major proteolytic activity was observed (Figure 3). Furthermore, highest enzyme activity occurred at pH 9 and 60°C (Figure 4). In the assay with the inhibitor, total proteolytic activity was inhibited by PMSF (Table 2), suggesting that proteases produced by addition to *P. ostreatus* are serine proteases. Shin and Choi (1998) have purified and characterized a cysteine protease from the fungus *P. ostreatus* after the formation of the fruiting body. In this study, the fungus in fruiting body stage was not used, suggesting that in different morphological stages there is the production of different proteases. The proteolytic profile of proteases produced

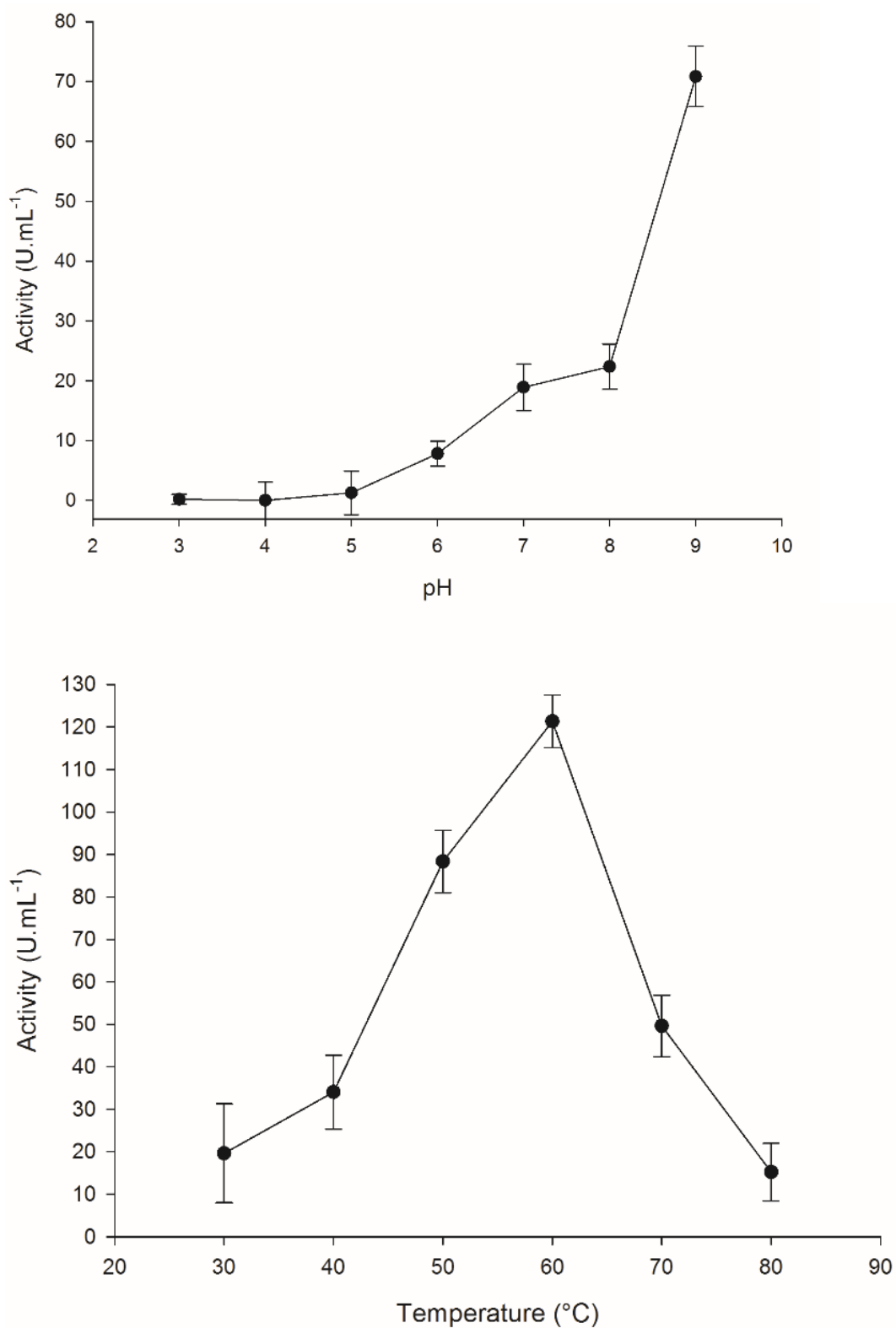


Figure 4. a. Effect of pH on activity of the *Pleurotus ostreatus* (PLO 06) proteases. Citrate-phosphate 50 mM buffer was used throughout the pH range. The assay was conducted at temperature of 40°C. b. Effect of temperature on activity of the *Pleurotus ostreatus* (PLO 06) proteases. The tested temperatures were: 30, 40, 50, 60, 70 and 80°C. The pH used in the test was the one with maximum activity obtained in the above test.

Table 2. Influence of inhibitor PMSF (1 mM) on the relative activity (%) of *Pleurotus ostreatus* (PLO 06) proteases in the optimum values of pH and temperature found in previous experiments.

Treatments	Relative activity (%)
Control ^a	100
PMSF	0

^a50 mM citrate - phosphate (pH 9,0; 60°C).

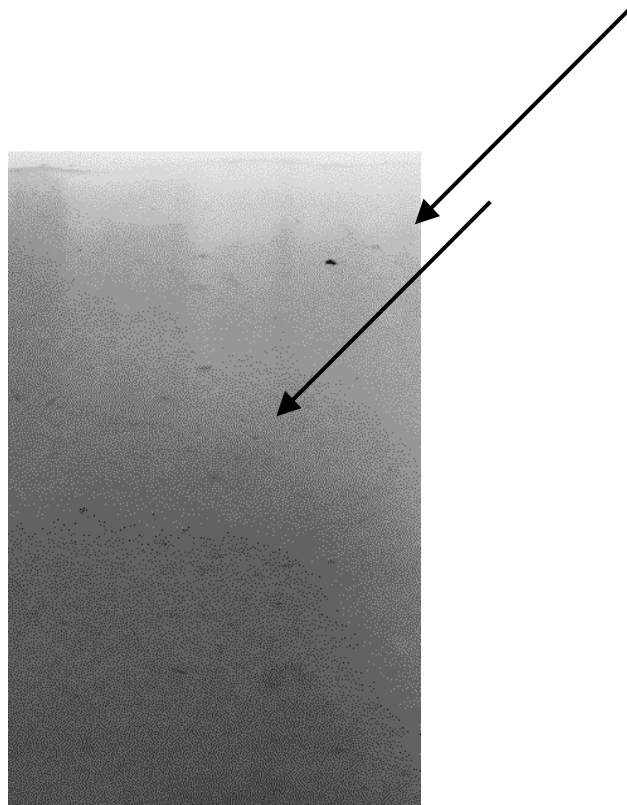


Figure 5. Zymogram of proteases produced by the fungus *Pleurotus ostreatus* (PLO 06) in polyacrylamide gel containing SDS. Arrows highlight the different halos of digestion, suggesting the existence of more than one protease.

by *P. ostreatus* was evaluated by a zymogram (Figure 5). The presence of different halos of digestion in the gel indicates the presence of more than one protease in the gel. This result confirms the experimental data obtained by Faraco et al. (2005), who demonstrated by zymography that the fungus *P. ostreatus* produces different proteases. In relation to the nematocidal assay of PLO 06 proteases, at the interval of 24 h, these enzymes have provided a significant percentage reduction ($p < 0.05$) of 42% on the number of *Panagrellus* sp. larvae, when compared to the control. Soares et al. (2013) have used the nematophagous proteases of *Monacrosporium*

thausasium (NF34) on larvae of *Panagrellus redivivus* and have observed that the purified enzymes destroyed more than 90% of the larvae subjected to the treatment. In the present work, the destruction was lower, however, it is worth mentioning that the fungus *M. thausasium* belongs to the predatory class of fungi, while the fungus *P. ostreatus* belongs to the toxin producers class (Yang et al., 2007). This fact is important because it is known that different classes of fungi produce different metabolites and in distinct concentrations (Braga et al., 2012).

In this study, comparing the percentage reduction of *P. ostreatus* (95%) with the action of its proteases (65.6%) on the larvae of *Panagrellus* sp, there was a higher value of the action of the fungus. However, this fact can be explained by means of the interaction-time difference with the larvae in the two assays (three days in the assay with the fungus and only one day in the assay with the enzymes). The results show that the fungus *P. ostreatus* (PLO 06) and its proteases have very effective predatory activity against nematode larvae of the *Panagrellus* genus, demonstrating great potential for use in integrated biological control. Moreover, this is the first report of the use of *P. ostreatus* (PLO 06) proteases against nematode larvae of the *Panagrellus* genus, thus much remains to be studied about the action and the mechanism of these enzymes nematocidal.

Conflict of interest

The authors do not have any financial or commercial conflicts of interest to declare.

ACKNOWLEDGMENTS

The authors would like to thank CAPES, FAPEMIG, FAPES and CNPq for the financial support.

REFERENCES

- Ayres M, Ayres JRM, Ayres DL, Santos AS (2003). Aplicações estatísticas nas áreas de ciências biológicas. Belém: Sociedade civil mamirauá: Brasília CNPq, p. 290.
- Bonatti M, Karnopp P, Soares HM, Furlan SA (2004). Evaluation of *Pleurotus ostreatus* and *Pleurotus sajor-caju* nutritional characteristics when cultivated in different lignocellulosic wastes. Food Chem. 88:425-428.
- Braga FR, Araújo JV, Araújo JM, Silva AR, Carvalho RO, Ferreira SR, Benjamin LA (2010). Predatory activity of the nematophagous fungus *Duddingtonia flagrans* on horse *cyathostomin* infective larvae. Trop. Anim. Health Prod. 42:1161-1163.
- Braga FR, Araújo JV, Soares FEF, Araújo JM, Ferreira SR, Queiroz JH (2012a). Use of statistical tools in the study of the conditions of predation of *Duddingtonia flagrans* versus *Panagrellus* sp. Biocont. Sci. Technol. 22:559-565.
- Braga FR, Araújo JV, Soares FEF, Araújo JM, Tavela AO, Mello INK, Carvalho LM, Paula AT, Lelis RT, Queiroz JH (2013). Interaction of the nematophagous fungus *Duddingtonia flagrans* on *Amblyomma cajannense* engorged females and enzymatic characterisation of its

- chitinase. *Biocont. Sci. Technol.* 23:584-594.
- Braga FR., Araújo JV, Soares FEF, Genier HLA, Queiroz JH (2012b). An extracellular serine protease of an isolate of *Duddingtonia flagrans* nematophagous fungus. *Biocontrol Sci. Technol.* 22:1131-1142.
- Cohen R, Persky L, Hadar Y (2002). Biotechnological applications and potential of wood degrading mushrooms of the genus *Pleurotus*. *Appl. Microbiol. Biotechnol.* 58:582-594.
- Eira AF, Edible Fungi. In Espósito E, Azevedo JL (2004). *Fungi an introduction to biology, biochemistry and biotechnology*. Caxias do Sul: Educ. Cap. 12 Edible Fungi, p. 510.
- Eren J, Pramer D (1965). The most probable number of nematode-trapping fungi in soil. *Soil Sci.* 99: 285.
- Faraco V, Palmieri G, Festa G, Monti M, Sannia G, Giardina P (2005). A new subfamily of fungal subtilases: structural and functional analysis of a *Pleurotus ostreatus* member. *Microbiol.* 151: 457-466.
- Gomes APS, Araújo JV, Ribeiro RCF (1999). Differential in vitro pathogenicity of predatory fungi of the genus *Monacrosporium* for phytonematodes, free-living nematodes and parasitic nematodes of cattle. *Braz. J. Med. Biol. Res.* 32:79-83.
- Grønvald J, Henriksen SA, Larsen M, Nansen P, Wolstrup J (1996). Aspects of biological control with special reference to arthropods, protozoans and helminths of domesticated animals. *Vet. Parasitol.* 64:47-64.
- Hibbett DS, Thorn RG (1994). Nematode trapping in *Pleurotus tuberregium*. *Mycologia.* 86: 696-699.
- Hummel KM, Penheiter AR, Gathman AC, Lilly WW (1996). Anomalous estimation of protease molecular weights using gelatin containing SDS-PAGE. *Anal. Biochem.* 233:140-142.
- Jaffee BA, Muldoon AE (1997). Suppression of the root-knot nematode *Meloidogyne javanica* by alginate pellets containing the nematophagous fungi *Hirsutella rhossiliensis*, *Monacrosporium cionopagum* and *M. ellipsosporum*. *Biocontrol Sci. Technol.* 7:203-218.
- Kwok CH, Plattner R, Weisleder D, Wicklow DT (1992). A nematocidal toxin from *Pleurotus ostreatus* NRRL 3526. *J. Chem. Ecol.* 18:127-136.
- Larsen M (1999). Biological control of helminthes. *Int. J. Parasitol.* 29:139-146.
- Lopez-Llorca LV, Maciá-Vicente JG, Janssonm HB (2008). Mode of action and interactions of nematophagous fungi. In: Ciancio A, Mukerji KG, *Integrated Management and Biocontrol of Vegetable and Grain Crops Nematodes*. Springer, Dordrecht, pp. 51-76.
- Mendoza-de Gives P (1999). Interaction between nematodes and biocontrol agents with potential for use in biomanagement systems. PhD Thesis, University of Nottingham. p. 219.
- Okorie CC, Ononuju CC, Okwujiako IA (2011). Management of *Meloidogyne incognita* with *Pleurotus ostreatus* and *P. tuberregium* in Soybean. *Int. J. Ag. Biol.* 13:401-405.
- Parra JRP, Botelho PSM, Correa-Ferreira BS, and Bento JMS (2002). Biological control in Brazil: parasitoids and predators. Ed. Manole, São Paulo. P. 609.
- Satou T, Kaneko K, Li W, Koike K (2008). The toxin produced by *Pleurotus ostreatus* reduces the head size of nematodes. *Biol. Pharm. Bull.* 31: 574-576.
- Shin HH, Choi HS (1998). Purification and characterization of cysteine protease from *Pleurotus ostreatus*. *Biosci. Biotechnol. Biochem.* 62:416-418.
- Soares FEF, Braga FR, Araújo JV, Geniêr HLA, Gouveia AS, Queiroz JH (2013). Nematicidal activity of three novel extracellular proteases of the nematophagous fungus *Monacrosporium sinense*. *Parasitol. Res.* 112: 1557-65.
- Soares FEF, Braga FR, Araújo JV, Lima WS, Mozer LR, Queiroz JH (2012). In vitro activity of a serine protease from *Monacrosporium thaumasium* fungus against first-stage larvae of *Angiostrongylus vasorum*. *Parasitol. Res.* 110:2423-2427.
- Tavela AO, Araújo JV, Braga FR., Araujo JM, Magalhaes LQ, Silveira WF, Borges L.A (2012). In vitro association of nematophagous fungi *Duddingtonia flagrans* (AC001), *Monacrosporium thaumasium* (NF34) and *Pochonia chlamyosporia* (VC1) to control horse *cyathostomin* (Nematoda: Strongylidae). *Biocont. Sci. Technol.* 22: 607-610.
- Thorn RG, Barron GL (1984). Carnivorous mushrooms. *Sci.* 224:76-78.
- Yang JL, Tian B, Liang L, Zhang KQ (2007). Extracellular enzymes and the pathogenesis of nematophagous fungi. *Appl. Microbiol. Biotechnol.* 75(1):21-31.

Full Length Research Paper

Enhanced recombinant human blood coagulation factor VIII expression in the milk of goats using replication-defective adenovirus

Zengsheng Han^{1,*}, Lin Wang¹, Kun Li¹, Jian Li¹, Hongwei Zhao¹, Zhiwei Liu¹, Wenguang Cao² and Qingwang Li^{1,3}

¹Department of Biological Engineering, College of Environment and Chemical Engineering, Yanshan University, Qinhuangdao, Hebei Province 066004, China.

²Institute of Animal Science, Chinese Academy of Agricultural Sciences, Beijing 100094, China.

³College of Animal Science and Veterinary Medicine, Northwest A&F University, Yangling, Shaanxi Province 712100, China.

Received 11 February, 2015; Accepted 22 April, 2015

High-level expression of recombinant human blood coagulation factor in milk of farm animals at a large scale provides a powerful tool for protein production. However, a bottleneck in recent protein synthesis technologies is the high cost of current transgenic livestock system. Here, we report a simple, rapid, and low-cost protein production method based on a replication-defective adenoviral vector system. The recombinant hfVIII adenoviral vector was generated by using homologous recombination in bacteria and transfected the plasmids into the HEK293 packaging cell line. Goat's mammary glands at the different physiological stage were infected with the recombinant adenovirus containing a human blood coagulation factor VIII gene. The expression level of bioactive hfVIII from milk sample was confirmed by Western blot analysis and ELISA methods. The *hfVIII* gene was expressed as a protein of about 26 kDa and no recombinant hfVIII protein was detected in negative control treatments. The hfVIII was expressed with a high activity level up to 1.4 U/ml in the milk of goats. These data suggest that the approach established here could be a low cost and further increase expression efficacy for the recombinant human blood coagulation factor and other biopharmaceutical interest.

Key words: Adenovirus vector, human blood coagulation factor VIII, mammary gland, milk, goat, recombinant protein.

INTRODUCTION

Factor VIII (fVIII) is a large glycoprotein (300 kDa) that functions as an integral component of the intrinsic

pathway of blood coagulation. Mutations in the fVIII gene that result in decrease or defective fVIII protein give rise

*Corresponding author. E-mail: hanzs@126.com. Tel: +86-0335-8074662. Fax: +86-0335-8061569.

to the genetic disease, hemophilia A, which is phenotypically characterized by recurrent bleeding episodes. Treatment of hemophilia A entails intravenous infusion of either human plasma-derived or recombinant fVIII material. However, human blood, plasma, and plasma-derived products were discovered to be transmitting potentially deadly blood-borne viruses, including HIV and hepatitis C virus (HCV) (Chudy et al., 1999). While plasma-derived fVIII products may be considered effective, new recombinant and transgenic therapeutic fVIII products are an important new alternative. And until now, several attempts successfully expressed fVIII in mammalian cells and transgenic animals (Wood et al., 1984; Paleyanda et al., 1997; Niemann et al., 1999; Chen et al., 2002; Hiripi et al., 2003; Chrenek et al., 2007). The market for recombinant pharmaceutical proteins is expanding rapidly. More recently, a new commercial drug was available for hemophilia A (Goldenberg, 2014). However, the supply of conventional or rhfVIII preparations is by far insufficient to cover the worldwide demand.

Adenoviral vectors are among the most promising viral vectors for gene expression for several reasons (Nienhuis, 2013). They can be produced in large quantities, they transfer heterologous genes to a broad spectrum of cell types, and gene transfer is not dependent on active cell division. The direct *in vivo* infection of the mammary gland with recombinant adenoviruses was proposed as an effective and versatile alternative to target the expression of exogenous gene to the secretory mammary epithelial cells (Yang et al., 1995; Kerr et al., 1996; Sobolev et al., 1998; Hens et al., 2000; Russell et al., 2003). Previous study showed that the hfVIII transgene has been ectopically expressed in brain, heart, liver, spleen, kidney and salivary gland, although regulation sequences are specific for mammary gland expression (Niemann et al., 1999). This low expression level of hfVIII in the milk of transgenic animals is associated to the detrimental effects. Both the intrinsic toxicity of hfVIII and high costs associated to the generation of large transgenic animals seem to be the main drawbacks hampering such approach. To our knowledge, there are no reports in the literature related with the expression of hfVIII in the milk of goats by this method.

In this study, we investigated the ability of an adenoviral vector to express hfVIII via direct *in vivo* infection of secretory mammary epithelium. We successfully expressed hfVIII in goat milk and obtained the highest activity level of the recombinant protein up to 1.4 U/ml by using an ELISA-based method.

MATERIALS AND METHODS

Cell lines and culture

Unless otherwise stated, all chemicals used in this study were obtained from Sigma Chemical Co. (St. Louis, MO). Human embryonic

kidney (HEK) 293 cells were obtained from the cell center of Chinese Academy of Sciences (Beijing, China) and maintained in Dulbecco's Modified Eagle's medium (Invitrogen, Beijing, China), supplemented with 10% (v/v) fetal bovine serum (Invitrogen), 1% non-essential amino acid, 2 mM L-glutamate and 1% penicillin-streptomycin.

hfVIII cDNA amplification and cloning

A human fVIII cDNA (Accession No.: NM_019863) was directly amplified by PCR from Fuleng expression plasmid library (Guang Zhou Fuleng). The primers used in PCR amplification were designed as follows: forward primer: 5'-tttagatctatgcggatccaagacc-3', reverse primer: 5'-tttctcgagctagtagggtcctgt-3'. To facilitate downstream subcloning of PCR products, we added *Bgl* II and *Xho* I (underlined) sites into 5' ends of the primers, respectively. PCR was carried out under the following standard conditions: started at 94°C for 5 min, followed by 30 cycles of a denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 45 s, and ended at 72°C for 5 min. PCR products were cloned and sequenced.

Construction and preparation of recombinant adenovirus vector

To monitor the recombinant hfVIII gene expression *in vitro* and recombinant virus infection efficiency *in vivo*, we first designed an internal ribosome entrance site (IRES) vector (Clontech) to express hfVIII and green fluorescent protein (GFP) from the same promoter CMV. The pShuttle-hfVIII-gfp was generated as described previously. In brief, the plasmid p3.1-ires-gfp (6782 bp) was digested with *Eco*RI and blunt ended with Klenow polymerase (Promega), and then digested with *Eco*R V. The resulted 1366-bp fragment contain IRES-GFP sequence. The pShuttle-CMV vector (Stratagene 7453 bp) was first digested with *Xho*I and blunt ended with Klenow polymerase (Promega), and then digested with *Eco*R V. The resulted 7432-bp vector containing left and right arm sequence that allow homologous recombination with pAdEasy-1, was ligated with the above 1366-bp fragment containing IRES-GFP sequence. The 8798-bp recombinant plasmid containing pShuttle-cmv-ires-gfp was obtained. The pShuttle-cmv-ires-gfp was digested with *Bgl* II and *Sal* I. The resulted DNA fragment was about 8768 bp. The PCR product containing hfVIII sequence was digested with *Bgl* II and *Xho* I. The resulted DNA fragment was about 657 bp. Then this 657-bp and 8768-bp DNA fragments were ligated by *T*₄ DNA Ligase. The resulted plasmid was designated as pShuttle-cmv-fVIII-gfp (9443 bp) containing hfVIII cDNA and GFP gene sequence. Correct orientation and confirmation of intact cDNA was determined by diagnostic restriction digestion.

The replication defective adenovirus vector pAd-fVIII was generated as described previously (Luo et al., 2007). In brief, The AdEasy vector (Stratagene) containing the adenovirus type 5 genome deleted for E1 and E3 regions was first transformed into the ultra-competent *BJ5183* bacteria to obtain the AdEasy bacteria. The plasmid pshuttle-cmv-fVIII-gfp was then transformed to the AdEasy bacteria, yielding the pAd-hfVIII recombinant adenovirus vector. The pAd-fVIII vector was linearized with *Pac*I and purified by the commercial purification kits (Qiagen) according to the manufacturer's instructions. The resulted vector was stored at -20°C until use.

The pAd-fVIII viral stocks were produced by transient transfection of HEK 293 cells using lipofectamine 2000™ (Invitrogen) according to the manufacturer's instructions. To amplify further, the adenovirus stocks were diluted appropriately and added to 30 T75 flasks. The recombinant adenovirus was harvested 48 h post infection and the

cell pellets from all flasks were combined. The final cell pellet was resuspended in 5 ml sterile phosphate-buffered saline (PBS). The cells were lysed by four cycles of freezing/thawing. Cell lysate was centrifuged at 2500 *g* for 5 min at 4°C. The titer of the recombinant adenovirus preparation was determined by GFP expression on semi-confluent HEK 293 cells. The final virus stock was stored at -70°C until use.

Animals

Adult female goats (35 ± 0.5 kg) were bred from a local goat farm and kept in the animal facility at the school of biological engineering, the University of Yanshan. All animal procedures were approved by the Animal Care and Use Committee of Yanshan University.

In vivo expression assay

Twelve (12) goats were anesthetized for recombinant adenovirus infusion as described previously (Toledo et al., 2006). For all mammary infusions and sample collections, teats were routinely wiped with 70% alcohol and iodine to prevent mammary gland infection. The recombinant adenoviruses were infused directly into the left mammary glands of each goat. The left ventral mammary glands of each goat were infused with 200 µl of a solution of PBS containing the desired amount of the recombinant adenoviruses by using a 1-ml syringe. As an intra-animal control, the right mammary glands received the same volume of sterile PBS.

The treated goats were milked on the indicated days. Milk samples from the same experimental group were pooled for analysis. The milk was collected and immediately centrifuged at 10000 *g* for 20 min at 4°C and the upper lipid layer was removed. The samples were either subjected to further analysis or stored at -80°C until used.

Western blot analysis

Proteins from milk samples were mixed with equal volumes of loading buffer (10% β-mercaptoethanol, 0.2% SDS), followed by boiling samples for 5 min. After separation in 10% SDS-polyacrylamide gel electrophoresis (PAGE), the proteins were transferred onto polyvinylidene fluoride (PVDF) membrane (Millipore) using a semi-dry transfer method. The membrane was blocked with 5% non-fat dried milk in PBS with 0.05% Tween-20 (PBST) for 1 h at room temperature, washed three times (each for 10 min) with PBST and incubated with PBST containing 5% of non-fat dried milk and mouse anti-human blood coagulation factor monoclonal antibody for 1 h at 37°C. The membrane was then washed three times (each for 10 min) with PBST followed by 1 h incubation of horseradish peroxidase (HRP) labeled goat anti-mouse IgG antibody at room temperature. After four times (each for 10 min) of washing with PBST, the hfVIII was detected by using a BeyoECL Plus Kit (Beyotime Inc. Hangzhou, China) according to the manufacturer's instructions.

Human fVIII activity assay

Human fVIII activity level was measured by using a Commercial ELISA kit IMUBIND® FVIII ELISA Kit (American Diagnostica Inc.) as described by the manufacturer. This Kit is a "sandwich" ELISA using a monoclonal antibody against human factor VIII as the capture antibody. Incubate samples in pre-coated micro-test wells and a second monoclonal antibody, HRP conjugated, is used to detect the bound hfVIII antigen. The addition of TMB substrate, and its subse-

quent reaction with the HRP creates a blue colored solution. Sensitivity is enhanced by addition of a 0.5 N sulfuric acid stop solution, yielding a yellow color. The hfVIII levels are determined by measuring solution absorbances at 450 nm and comparing the values to those of a standard curve generated using hfVIII standards. This assay permits a direct measurement of hfVIII activity. All samples were assayed in triplicate and the means calculated. The hfVIII activity values are reported as the mean value and the standard error of the mean.

Statistical analysis

All data for detection were shown as the mean standard error of the mean (SEM). Analysis of variance (ANOVA) was carried out to find the significance, and the statistical significance of rhfVIII was established at $p < 0.05$ or $p < 0.01$.

RESULTS AND DISCUSSION

Recombinant hfVIII adenoviral vectors production

The gene used in present study codes a putative small protein, isoform b, which consists primarily of the phospholipid binding domain of factor VIIIc. This binding domain is essential for coagulant activity (NCBI Reference Sequence: NM_019863.2). To monitor the recombinant hfVIII gene expression, an IRES sequence derived from the encephalomyocarditis virus was used in present study. The GFP and hfVIII genes were respectively cloned into the downstream and upstream of the IRES region under the control of a CMV promoter. The incorporation of GFP encoding sequence in this vector was designed to serve as a live marker for convenient assessment of adenovirus transduction efficiency. A schematic overview of the adenoviral hfVIII DNA construct is provided in Figure 1.

The initial recombinant adenovirus could be produced from HEK 293 cells, 2-4 µg of linearized adenoviral vector containing hfVIII gene, then transfected into 293 cells. GFP expression was visible 24 h after transfection in 10-30% of the cells. With the aid of GFP fluorescence, we could easily find cells in the center of foci to be lysed 8-10 days after transfection (Figure 2A and B). Since the GFP gene was cloned downstream of IRES sequence controlled by the same CMV promoter, the GFP fluorescence indicated the expression level of hfVIII (Huang and Gorman, 1990). About 12 days after the infection, cells were collected and lysed for further viral production. The results show that a significant amounts of virus were present as early as three days after inoculation (Figure 2C and D).

Goat mammary glands transduction *in vivo*

Next, we tested the ability of recombinant virus pAd-fVIII transfection into the goat mammary glands *in vivo*. As our previous studies suggested that the physiological stage of goats mammary glands plays an important role in

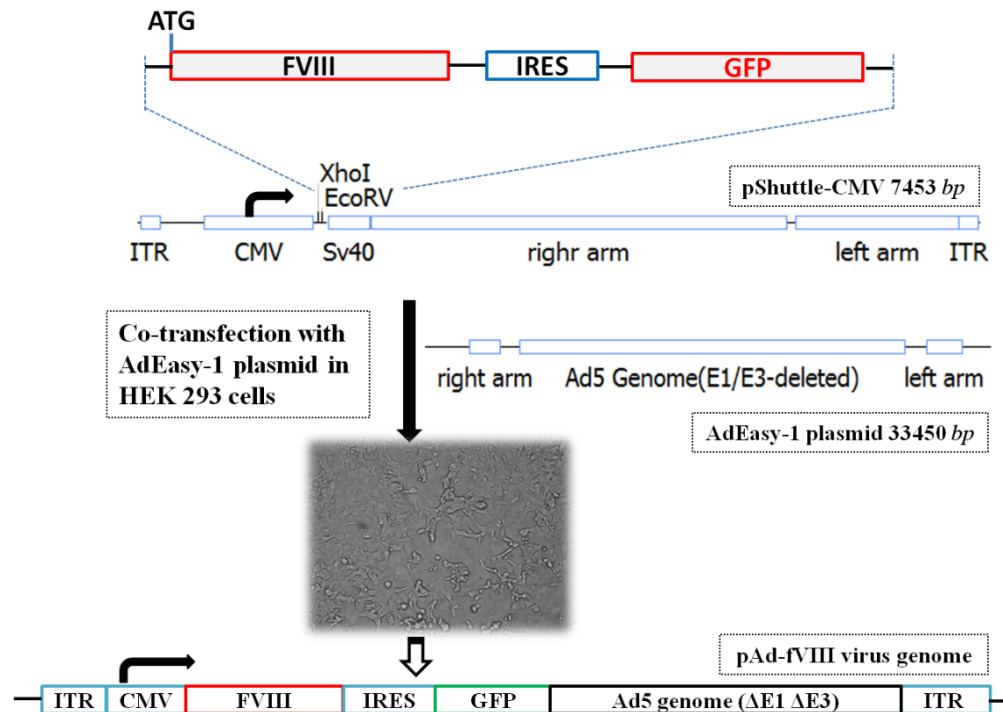


Figure 1. Construction of expression vector pAd-fVIII. The adenovirus vector used in this study is a second-generation, replication-defective vector, with deletions of E1 and E3. Incorporation of hfVIII cDNA in adenovirus vector was described elsewhere (materials and methods).

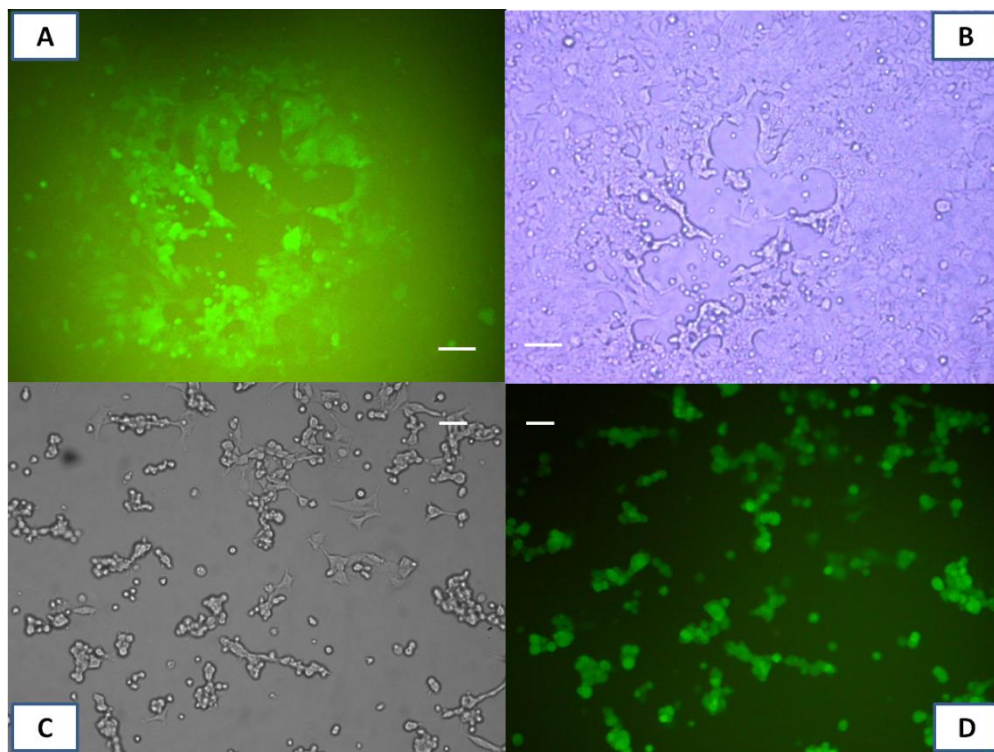


Figure 2. Recombinant adenovirus vector Ad-fVIII production in HEK 293 cells. The 293 cells were transfected with pAd-hfVIII vector encoding human coagulation factor VIII. The cells, at 50-70% confluence in a 25 cm² plate, were transfected with DNA (2 μg/well) using a transfection reagent lipofectamine 2000™ (Invitrogen) according to the manufacturer's instructions. In order to assess the efficiency of the transfection, cells were transfected with the pAd-hfVIII vector encoding GFP and human coagulation factor VIII under the same promoter. GFP expression was observed in cells after 24 h later with an inverted fluorescence microscope. Cells were examined under fluorescence field (A and D) or bright field (B and C). Scale bars=A and B 50 μm, C and D 30 μm.

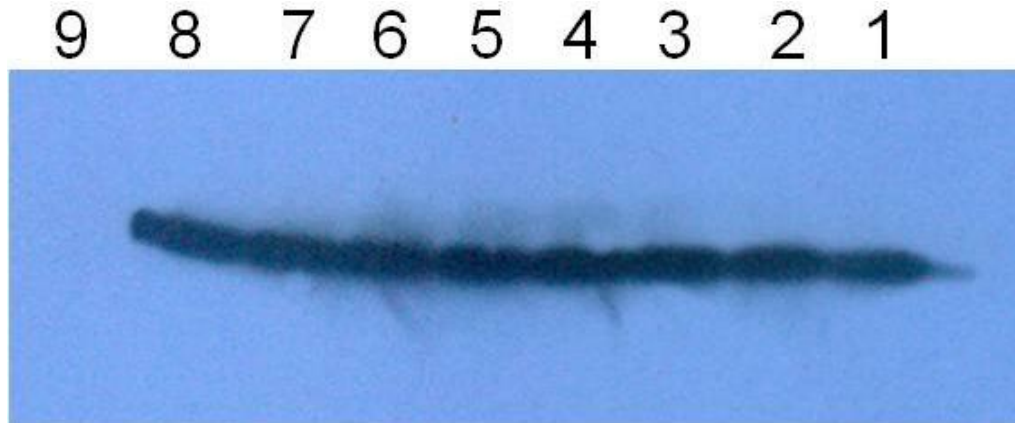


Figure 3. Western blotting analysis of Ad-hfVIII transduced mammary glands in milk of goats. The milk samples were subjected to sodium dodecylsulphate-polyacrylamide gel electrophoresis, and proteins were transferred to a polyvinylidene difluoride membrane. Twenty microliters of diluted milk samples were loaded in each well. The PVDF membrane was detected by ECL Chemiluminescence method. Lane 9, milk from PBS-treated mammary glands of goat; Lane 1, 2 and 3, milk from Ad-hfVIII vector infected mammary glands; Lane 4, 5 and 6, milk from Ad-hfVIII vector infusion supplemented with EDTA; Lane 7 and 8, milk from Ad-hfVIII vector infusion supplemented with BAPTA.

efficient adenovirus transduction, we designed three experimental groups to determine the optimal time for adenovirus administration. Mammary glands (MG) of goats were infused with 1×10^8 PFU per MG on day 3, 10 postpartum and day 27 of gestation, respectively. Each group consisted of at least three female goats. Milk was collected on day 2, 3, 4, 5, 6, 7, 8, 9 and 10 post-infused when lactation started. At the meanwhile, uninfected mammary glands were milked at the indicated days as a negative control.

To further investigate the hfVIII gene whether it could be expressed in milk of goat, we performed a Western blot analysis and ELISA assay. The results show that hfVIII gene was expressed as a protein of about 26 kDa. In contrast, no recombinant hfVIII protein was detected in negative control treatments (Figure 3).

The level of hfVIII protein expression in Ad-hfVIII treated MGs increased gradually after viral infusion and peaked at day 5, then dropped quickly. This decline in expression was likely due to a gradual loss of vector from the goat mammary epithelial during cell division. By using the ELISA method, the hfVIII concentrations were determined in the milk from all Ad-hfVIII treated MGs and ranged from 200 to 1400 mU/ml for the three goats at all evaluated groups. The results are also in agreement with previous reports showing that the use of the replication-defect adenoviral vector allowed only a transient expression due to the vector-induced toxicity (Yang et al., 1994; Toledo et al., 2006; Kron et al., 2011). Improved vectors could be used as a long-term transgene expression and protein production (Cots et al., 2013). Indeed, during the course of the experiments, none of the goats used in this study showed symptoms of mastitis.

Expression of rhfVIII in the milk of goats

Ours and previous studies showed that vector-mediated gene transfer to airway cells could be enhanced by opening epithelial junctions and allowing vector access to the basolateral membrane (Toledo et al., 2006). Agents that modulate tight junctions, such as ethyleneglycol-*bis*-(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 1,2-*bis* (2-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid (BAPTA), sodium caprate and ethylenediaminetetraacetic acid have been shown to increase the permeability of airway tight junction and also enhance gene transfer (Wang et al., 2000; Gregory et al., 2003; Oakland et al., 2012).

Based on this reason, eight goats were divided into four groups in this experiment, and each group had two animals at random. In three groups, the left mammary glands of goats were filled with a solution of virus supplemented with EGTA (20 mM), BAPTA (30 mM), and C10 (40 mM), respectively. In the fourth group, the left MGs were infected with recombinant vectors alone. At the meanwhile, the right MGs received the same volume of sterile PBS as a control in all four groups. As represented in Figure 4, the MGs of goats infected with the vectors containing EGTA/ BAPTA/ C10 showed a significantly higher expression of hfVIII compared to that of PBS-viral solution ($P < 0.05$). However, there is no significant difference among the three groups treated with EGTA/ BAPTA/ C10 ($P > 0.05$). Further experimental study with an optimal dose is needed in future.

In all the above experiments, no symptoms of mastitis were found in the mammary gland of goats. Milk appeared normal throughout the lactation (data no

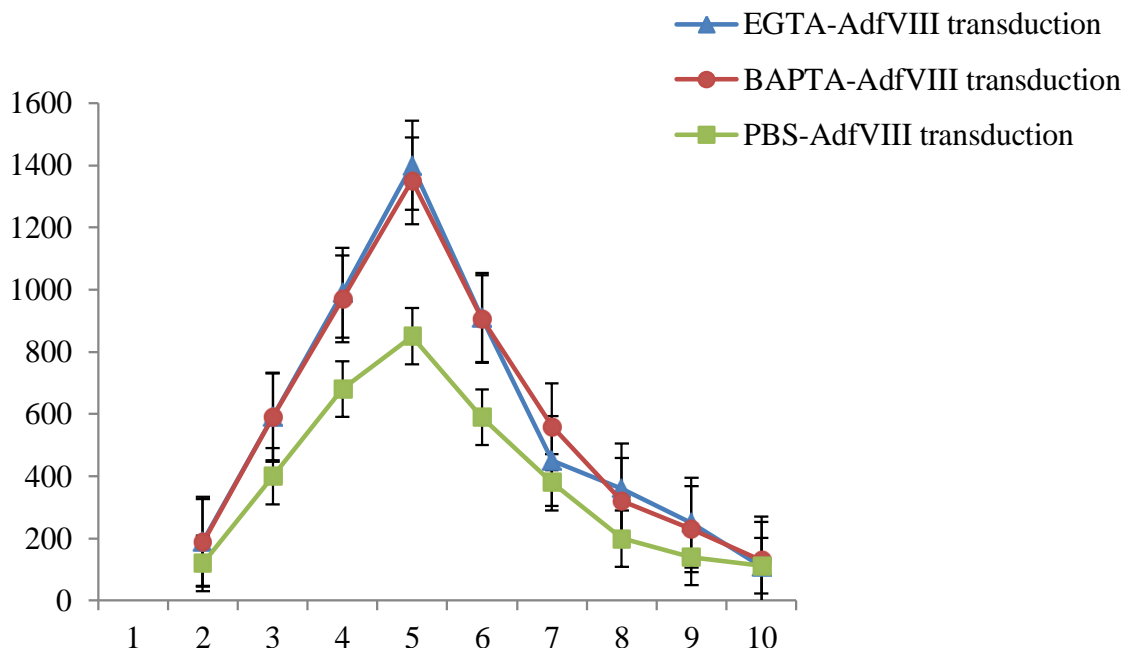


Figure 4. Expression of rhfVIII in the milk of goats. Milk samples were collected 2–10 days by hand-milking on indicated days. Thereafter, obtained milk was immediately centrifuged at 10,000 g for 10 min and the upper lipid layer was removed. The skim samples dilution were subjected to ELISA according to the manufacturer's instructions. Error bars Standard deviations calculated from the data ($n=3$)

shown). For all goats, the volume of milk obtained from each udder was about 50-150 ml on the first day of lactation and became normal gradually.

Biological systems for the expression of recombinant proteins have evolved from microorganisms to animal and plant cells. In these expression systems, the mammary gland has been identified as a suitable alternative to produce recombinant proteins (Samiec et al., 2011; Amiri et al., 2013). In previous attempts, transgenic pigs, sheep, goats and mice have been generated and variable levels of rhfVIII expression were obtained depending on the regulatory sequences employed (Paleyanda et al., 1997; Niemann et al., 1999; Chen et al., 2002; Hiripi et al., 2003). However, this transgenesis was technique sophisticated and time-consuming. In this article, we demonstrate successful targeting of AdV-5 vectors containing hfVIII cDNA to goat mammary gland and obtained high-level expression of recombinant human clotting factor VIII in the milk of goats. The methods established here is easy-handle and less time required (four months or less), and therefore could be more effective for production of hfVIII or other recombinant proteins.

In conclusion, a simple, fast, and efficient method for recombinant human coagulation expression in milk was established. Although the rhfVIII was transient expressed in milk of goat, it is probably one of the most economical and efficient ways for rhfVIII production in livestock. This method may also be suitable for the study of the

biological characteristics of recombinant proteins expressed in different species milk.

Conflict of interests

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

ACKNOWLEDGEMENTS

This study was partially supported by grants from the PhD Foundation of Yanshan University (B297), Hebei Province Grants (Z2010180; Z2012001; C2014203116) and Major Technology Project of Ministry of Agriculture (2011ZX8008-003).

REFERENCES

- Amiri YA, Dalman A, Eftekhari-Yazdi P, Sanati MH, Shahverdi AH, Fakheri R, Vazirinasab H, Daneshzadeh MT, Vojgani M, Zomorodipour A, Fatemi N, Vahabi Z, Mirshahvaladi S, Ataei F, Bahraminejad E, Masoudi N, Rezazadeh, Valojerdi M, Gourabi H (2013). Production of transgenic goats expressing human coagulation factor IX in the mammary glands after nuclear transfer using transfected fetal fibroblast cells. *Transgenic Res.* 22(1):131-142.
- Chen CM, Wang CH, Wu SC, Lin CC, Lin SH, Cheng WT (2002). Temporal and spatial expression of biologically active human factor VIII in the milk of transgenic mice driven by mammary-specific bovine alpha-lactalbumin regulation sequences. *Transgenic Res.* 11(3):257-268.

- Chrenek P, Ryban L, Vetr H, Makarevich AV, Uhrin P, Paleyanda RK, Binder BR (2007). Expression of recombinant human factor VIII in milk of several generations of transgenic rabbits. *Transgenic Res.* 16(3): 353-361.
- Chudy M, Budek I, Keller-Stanislawski B, McCaustland KA, Neidhold S, Robertson BH, Nübling CM, Seitz R, Löwer J (1999). A new cluster of hepatitis A infection in hemophiliacs traced to a contaminated plasma pool. *J. Med. Virol.* 57(2):91-99.
- Cots D, Bosch A, Chillón M (2013). Helper dependent adenovirus vectors: progress and future prospects. *Curr. Gene Ther.* 13(5):370-381.
- Goldenberg MM (2014). Pharmaceutical approval update. *P. T.* 39(4):553-566.
- Gregory LG, Harbottle RP, Lawrence L, Knapton HJ, Themis M, Coutelle C (2003) Enhancement of adenovirus-mediated gene transfer to the airways by DEAE dextran and sodium caprate *in vivo*. *Mol. Ther.* 7(1):19-26.
- Hens JR, Amstutz MD, Schanbacher FL, Mather IH (2000). Introduction of the human growth hormone gene into the guinea pig mammary gland by *in vivo* transfection promotes sustained expression of human growth hormone in the milk throughout lactation. *Biochim. Biophys. Acta* 1523(2-3):161-171.
- Hiripi L, Makovics F, Halter R, Baranyi M, Paul D, Carnwath JW, Bösze Z, Niemann H (2003). Expression of active human blood clotting factor VIII in mammary gland of transgenic rabbits. *DNA Cell Biol.* 22(1):41-45.
- Huang MT and Gorman CM (1990). Intervening sequences increase efficiency of RNA 3' processing and accumulation of cytoplasmic RNA. *Nucleic Acids Res.* 18(4):937-947.
- Kerr DE, Furth PA, Powell AM, Wall RJ (1996). Expression of gene-gun injected plasmid DNA in the ovine mammary gland and in lymph nodes draining the injected site. *Anim. Biotechnol.* 7(1):33-45.
- Kron MW, Engler T, Schmidt E, Schirmbeck R, Kochanek S, Kreppel F (2011). High-capacity adenoviral vectors circumvent the limitations of $\Delta E1$ and $\Delta E1/\Delta E3$ adenovirus vectors to induce multispecific transgene product-directed CD8 T-cell responses. *J. Gene Med.* 13(12):648-57.
- Luo J, Deng ZL, Luo X, Tang N, Song WX, Chen J, Sharff KA, Luu HH, Haydon RC, Kinzler KW, Vogelstein B, He TC (2007). A protocol for rapid generation of recombinant adenoviruses using the AdEasy system. *Nat. Protoc.* 2(5):1236-1247.
- Niemann H, Halter R, Carnwath JW, Herrmann D, Lemme E, Paul D (1999). Expression of human blood clotting factor VIII in the mammary gland of transgenic sheep. *Transgenic Res.* 8(3):237-247.
- Nienhuis AW (2013). Development of gene therapy for blood disorders: an update. *Blood* 122(9): 1556-1564.
- Oakland M, Sinn PL, McCray PB Jr (2012). Advances in cell and gene-based therapies for cystic fibrosis lung disease. *Mol. Ther.* 20(6):1108-1115.
- Paleyanda RK, Velander WH, Lee TK, Scandella DH, Gwazdauskas FC, Knight JW, Hoyer LW, Drohan WN, Lubon H (1997). Transgenic pigs produce functional human factor VIII in milk. *Nat. Biotechnol.* 15(1):971-975.
- Russell TD, Fischer A, Beeman NE, Freed EF, Neville MC, Schaack J (2003). Transduction of the mammary epithelium with adenovirus vectors *in vivo*. *J. Virol.* 77(10):5801-5809.
- Samiec M and Skrzyszowska M (2011). Transgenic mammalian species, generated by somatic cell cloning, in biomedicine, biopharmaceutical industry and human nutrition/dietetics recent achievements. *Pol. J. Vet. Sci.* 14(2):317-328.
- Sobolev AS, Rosenkranz AA, Smirnova OA, Nikitin VA, Neugodova GL, Naroditsky BS, Shilov IN, Shatski IN, Ernst LK (1998). Receptor-mediated transfection of murine and ovine mammary glands *in vivo*. *J. Biol. Chem.* 273(14):7928-7933.
- Toledo JR, Sánchez O, Seguí RM, García G, Montañez M, Zamora PA, Rodríguez MP, Cremata JA (2006). High expression level of recombinant human erythropoietin in the milk of non-transgenic goats. *J. Biotechnol.* 123(2):225-235.
- Wang G, Zabner J, Deering C, Launspach J, Shao J, Bodner M, Jolly DJ, Davidson BL, McCray PB Jr (2000). Increasing epithelial junction permeability enhances gene transfer to airway epithelia *in vivo*. *Am. J. Respir. Cell Mol. Biol.* 22(2):129-138.
- Wood WI, Capon DJ, Simonsen CC, Eaton DL, Gitschier J, Keyt B, Seeburg PH, Smith DH, Hollingshead P, Wion KL, Delwart E, Tuddenham EG, Vehar GA, Lawn RM (1984). Expression of active human factor VIII from recombinant DNA clones. *Nature* 312(5992):330-337.
- Yang J, Tsukamoto T, Popnikolov N, Guzman RC, Chen X, Yang JH, Nandi S (1995). Adenoviral-mediated gene transfer into primary human and mouse mammary epithelial cells *in vitro* and *in vivo*. *Cancer Lett.* 98(1):9-17.
- Yang Y, Nunes FA, Berencsi K, Furth EE, Gönczöl E, Wilson JM (1994). Cellular immunity to viral antigens limits E1-deleted adenoviruses for gene therapy. *Proc. Natl. Acad. Sci. USA* 91(10):4407-4411.

Full Length Research Paper

Insights on predominant edible bamboo shoot proteins

Sayanika Devi Waikhom¹, Louis Bengyella^{1,2,3*}, Pranab Roy⁴ and Narayan Chandra Talukdar^{1*}¹Institute of Bioresources and Sustainable Development, Takyelpat, Imphal, Manipur 795001, India.²Department of Biochemistry, University of Yaoundé I, BP812, Yaoundé, Cameroon.³Department of Biotechnology, The University of Burdwan, 713104 Golapbag More, West Bengal, India.⁴Department of Biotechnology, Haldia Institute of Technology, 721657 Haldia, West Bengal, India.

Received 14 January, 2015; Accepted 13 April, 2015

Juvenile bamboo shoots have become a US\$ 18 billion industry and have lured interest worldwide for its nutritive value and health enhancing properties; making it a suitable candidate for food security. Quantitative analysis revealed that juvenile bamboo shoots are a good source of proteins. However, there is no qualitative analysis describing unique proteins present in the edible bamboo shoots. In order to provide the identity of predominant proteins present in edible bamboo shoot, 13 edible bamboo species were analysed. SDS-polyacrylamide gel electrophoresis revealed that high level of peptides polymorphism among 13 bamboo species was within the range of 20.10-15.50 and 66.50-29 KDa. Gel analysis shows that *Bambusa oliveriana* expressed the maximum number of diverse peptides while *Bambusa nutans* expressed the minimum number of peptides. Importantly, MS/MS data revealed that abundant peptides in bamboo shoots are histone-like related (H2A, H3 and H4) which generally form the nucleosome core and can participate in defense, stress and development. This study is the first qualitative data on protein components of bamboo shoots which harmonize the existing quantitative data that edible bamboo species as healthy food and a rich source of protein.

Key words: Bamboo shoot proteins, histone-likely proteins, peptide polymorphism, SDS-PAGE-MS/MS, *Dendrocalamus hamiltonii*.

INTRODUCTION

Over two million tonnes of juvenile bamboo shoots are consumed in the world annually (Yang et al., 2008). The USA alone imports over 14.5% of the world bamboo shoots mostly from Asia, making an estimated US\$ 18 billion trade industry (Daphne, 1996; Lobovikov, 2003). In the North Eastern States of India, 1979 tonnes of fermented bamboo shoots are consumed annually (Bhatt et al., 2004) with a price tag of US\$ (0.66 - 0.88) per kg (Singh et al., 2010). Due to the high demand for bamboo

shoots, efficient protocols for cultivation of edible bamboo species have been developed to balance future demand (Brar et al., 2013; Devi et al., 2012; Singh et al., 2012; Waikhom and Louis, 2014).

Essentially, juvenile bamboo shoots are consumed as vegetable or pickled, but can be processed by fermentation or deep frying, as shredded chips and canned into more palatable forms (Choudhury et al., 2011; Waikhom et al., 2013). Also, bamboo shoots contain high

*Corresponding author. E-mail: bengyellalouis@gmail.com; nctalukdar@yahoo.com.

level of phytosterols, playing a key role in lowering blood cholesterol and high levels of cellulosic content, an important appetizer (Nirmala et al., 2011), anti-fatigue activity (Akao et al., 2004), high levels of antioxidant activity, microminerals, macrominerals and high protein levels per gram of dry weight (Waikhom et al., 2013).

Notwithstanding of the rich dietary and therapeutic traits reported for bamboo shoots of several bamboo species (Akao et al., 2004; Waikhom et al., 2013), some species are rich in toxic cyanogenic-like taxiphyllin, significantly associated with neurological disorder called Konzo (Nzwalo and Cliff, 2011; Schwarzmaier, 1997; Waikhom et al., 2013).

Furthermore, only quantitative data on bamboo shoots proteins have been generated thus so far (Nirmala et al., 2008; Waikhom et al., 2013). It is unclear which of the reported species of bamboo shoots is endowed with diverse proteins which can only be determined qualitatively. Furthermore, the identity of abundant bamboo shoot proteins has not been determined. Therefore, we set as objective to profile the crude proteins of edible bamboo shoots of 13 bamboo species and identify prominent proteins.

MATERIALS AND METHODS

Plant material

We followed the collection procedures as defined in Waikhom et al. (2013) from the field to the laboratory for edible bamboo species located at different altitudes of Manipur, India (23°47' -25°41' NL; 92°58' to 94°47' EL), during July to August of 2012 to 2013. The 13 bamboo species studied were authenticated morphologically by the Botanical Survey of India (BSI), Kolkata and voucher specimens are deposited at the Central National Herbarium in BSI (Table 1). These edible bamboo species have been identified by Waikhom et al. (2013) on the basis of the trnL-F intergenic spacer and the sequences are available at NCBI DNA nucleotide sequences database. These juvenile bamboo shoots of the 13 bamboo species were selected for the study because they are available in the local markets throughout the year in the North Eastern States of India.

Protein extraction

The sheaths of the bamboo shoots were removed (Figure 1A). The inner edible shoot stem was measured with a caliper and cut into three equal portions, that is, the tip, middle and bottom (Figure 1B). Based on data from Waikhom et al. (2013) which showed that the tips of bamboo shoots are rich in toxic total cyanogen content (TTC) and that consumer prefer the crispy taste of the middle portion of the bamboo shoots, only the middle portion was used in this study. The fresh bamboo shoots were crushed in 10 mM CaCl₂ solution containing 0.25% Triton-X-114 (Sigma-Aldrich®, Missouri, USA) and 1% of dithiothreitol (DTT, Sigma-Aldrich®, Missouri, USA) as described in Louis et al. (2014). Protein was washed with ReadyPrep™ 2-D cleanup Kit® (Bio-Rad, Hercules, CA, USA) following the manufacturer instructions. Proteins were dissolved in ReadyPrep™ rehydration buffer consisting of 8 M urea, 2% CHAPS, 50 mM DTT, 0.2% (w/v), Bio-Lyte® 3/10 ampholytes, and traces of Bromophenol Blue (Bio-Rad®, Hercules, CA, USA). Additionally, the protein content of 10 µl aliquots was quantified spectrophotometrically at 595 nm by the dye-binding method (Bradford, 1976) using bovine serum albumin as standard.

Table 1. Morphological authentication of 13 species of bamboo shoots by the Botanical Survey of India (BSI), Kolkata with voucher specimens at BSI Central National Herbarium

GenBank accession/bamboo species	BSI Voucher accession
KC013282/ <i>Chimonobambusa callosa</i>	IBSD/WS/019
KC013285/ <i>Bambusa cacharensis</i>	IBSD/WS/020
JX564900/ <i>Bambusa manipureana</i>	IBSD/WS/008
JX564901/ <i>Bambusa nutans</i>	IBSD/WS/023
JX507132/ <i>Bambusa tulda</i>	IBSD/WS/022
JX507131/ <i>Bambusa oliveriana</i>	IBSD/WS/010
JX564902/ <i>Dendrocalamus giganteus</i>	IBSD/WS/001
JX564903/ <i>Dendrocalamus hamiltonii</i>	IBSD/WS/004
JX564904/ <i>Dendrocalamus hookeri</i>	IBSD/WS/005
JX564905/ <i>Dendrocalamus manipureanus</i>	IBSD/WS/002
JX507133/ <i>Melocanna baccifera</i>	IBSD/WS/018
JX507134/ <i>Schizostachyum dullooa</i>	IBSD/WS/003
JX564906/ <i>Bambusa</i> sp.	IBSD/WS/024
JX564907/ <i>Bambusa</i> sp.	IBSD/WS/007
KC013288/ <i>Bambusa tuldoidea</i>	IBSD/WS/006

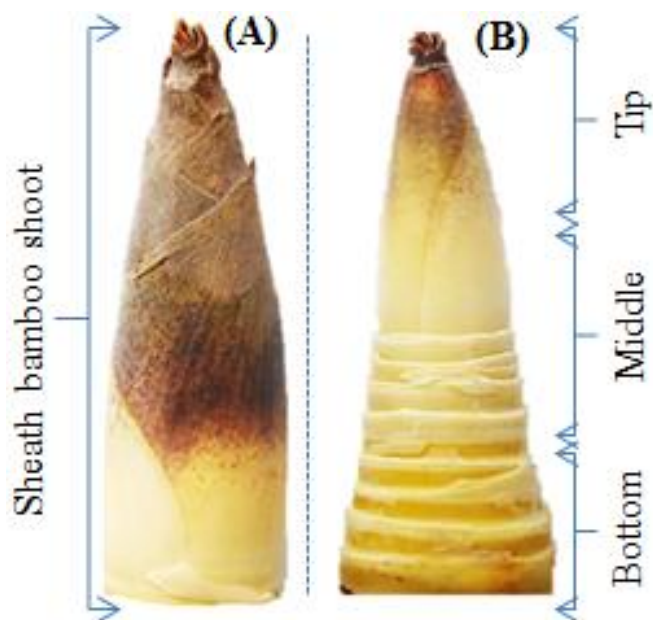


Figure 1. Juvenile edible bamboo shoot of *Bambusa oliveriana* (GenBank accession JX564901). (A) A sheathed bamboo shoot. (B) A bamboo shoots without sheath.

SDS-polyacrylamide gel electrophoresis and analysis

Using standard one dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE), 15 µg protein for each species was profiled on a 15% SDS-polyacrylamide gel. SDS-PAGE was performed at 170 V in a 1X Tris-glycine-SDS, pH 8.3 (25 mM Tris-HCl, 200 mM glycine, 0.1% SDS) running buffer in PowerPac™ Basic 300 V system (Bio-Rad®, Hercules, CA, USA). A

solution of 0.35% Coomassie brilliant blue R250 (SRL, Mumbai, India) was used for staining overnight. Destaining was performed in a solution containing 50% methanol and 10% acetic acid until visible bands were observed. Triplicate gels and two biological repeats (for July-August 2012 - 2013) were scanned and subjected for analysis in Phoretix 1D v.10.4 algorithm (TotalLab Ltd, Newcastle, UK). Following background subtraction, bands were automatically detected based on normalized pixel-to-pixel intensity threshold. Peptides banding polymorphism among the bamboo shoots of 13 species was established using the neighbor-joining method (Saitou and Nei, 1987), based on the relative mobility of bands.

Peptide fingerprinting and database searching

Prominent bands were manually excised and subjected to trypsin digestion and elution as earlier described (Shevchenko et al., 2006). The digested protein solution (0.45 µl) was sandwiched in 5 mg/ml α-cyano-4-hydroxy-cinnamic acid (diluted in 0.1% trifluoroacetic acid, 50% acetonitrile) on a matrix assisted laser desorption/ionization (MALDI) target plate (Applied Biosystems, Vernon Hills, IL, USA). MALDI-TOF/TOF MS/MS was performed in SCIEX4800 MALDI TOF-TOF proteomics at an accelerating voltage of 20 kV, and mass resolution was maximized at 1600 Da. All the acquired spectra were processed with the 4700 Explore™ software (Applied Biosystems, Vernon Hills, IL, USA) at default settings. NCBI nr and green plant MSDB sequence databases were searched against all updated entries via the in-house MASCOT server (v.2.3 MatrixScience, London, UK). Search parameters were set as follows: enzyme, trypsin; fixed modifications, carbamidomethyl (C); variable modification, oxidation (M); peptide mass tolerance, 40-100 ppm; maximum missed cleavages, 2. The accepted MOWSE score threshold was inferred at $P < 0.05$. A false-discovery rate (FDR) (Elias et al., 2005) for the peptide search match was calculated using a decoy database at a cut-off $FDR \leq 1\%$. To determine the biological signatures and putative domains for the peptides, KEGG Orthology Based Annotation System (KOBAS) 2.0 (Xie et al., 2011) and NCBI Conserved Domains Database (<http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml>) servers were used.

RESULTS AND DISCUSSION

Bamboo shoots are usually pigmented with secondary metabolites (Figure 1B); as a result the proteins are pigmented. Bamboo shoots are also hard to fine-crush and pose difficulties in protein extraction. We observed that bamboo shoot proteins extracted in buffers containing SDS and urea often produced poor banding on polyacrylamide gel, marked by vertical streaking, smearing and colouring of the gels due to pigmentation (data not shown). Pigmentation was reduced by washing the extracted protein repeated with a clean-up kit (Bio-Rad, Hercules, CA, USA). By using 10 mM CaCl₂ solution containing 0.25% Triton-X-114 extraction buffer as previously described (Louis et al., 2014), smearing and streaking problems were eliminated.

Compelling quantitative data on freshly harvested, fermented and canned bamboo shoots of *Dendrocalamus giganteus* (Nirmala et al., 2008) revealed that bamboo shoots are good source of proteins. Furthermore, Nirmala et al. (2008) found that 100 g of fermented and fresh bamboo

shoot of *D. giganteus* contained 2.17 and 3.11 g of proteins, respectively. Based on the analysis of 15 edible bamboo species, Waikhom et al. (2013) reported that bamboo shoots are a rich source of nitrogen. For instance, the juvenile shoot of *C. callosa* was found to contain 1153 mg of nitrogen per 100 g dry weight (Waikhom et al., 2013). However, on the basis of these pioneering quantitative studies (Nirmala et al., 2008; Waikhom et al., 2013), it is difficult to tell which species of bamboo produce shoots rich in diverse proteins given that all species contain TTC and other valuable nutritional attributes. Qualitative proteomics analysis can help stakeholders in the bamboo industry to select bamboo shoots in a quest for diverse protein source.

SDS-PAGE profile of bamboo shoots crude proteins for 13 species revealed high level peptide banding polymorphism within the range of 66.5 to 29.10 KDa and 20.10 to 15.50 KDa (Figure 2). It is worth noting that above 29.10 KDa, polymorphism was less because only banding intensities varied among species (Figure 2). On the contrary, below 20.10 KDa, high level polymorphism was observed, hallmarked by new prominent bands reflected by variations in relative mobility in the dendrogram (Figure 3). On this basis, it is concluded that the major proteins of bamboo shoots have a low molecular weight ranging between 20.10 to 15.50 KDa. Irrespective of the lane used for rooting the dendrogram, two main clades (I and II) were generated, viz. Clade I (*D. manipureanus*, *D. giganteus*, *B. oliveriana*, and *Bambusa* sp., *S. dullooa*) and Clade II (*B. caharensis*, *Bambusa* sp., *B. tuldiodes*, *B. manipureana*, *B. tulda*, *C. callosa*, *D. hamiltonii* and *M. baccifera*) (Figure 3). Only internal branch length for *B. tuldooides* (lane 11, length = 0.128) and *D. hamiltonii* (lane 7, length = 0.128) matched at 100% revealing a high level of polymorphism among the peptides of other species.

Taxonomic placement of bamboo species has suffered a great deal in the last decade (Baldwin et al., 1995). Previous report based on morphological descriptors discerned 15 edible bamboo species into two clades (Waikhom et al., 2013). Using the same set of bamboo specimens, Random Amplified Polymorphic DNA (RAPD) and TrnL-F intergenic spacer analyses generated three and two clades, respectively (Waikhom et al., 2013). Based on previous taxonomical placement (Waikhom et al., 2013) and the present finding based on peptide polymorphism, it is tempting to suggest that the placement of bamboo species is a function of the experimental approach. Dominant morphological characters in bamboo shoots such as colour, shape, presence of hair in the culm sheath significantly compromises taxonomic placement (Waikhom et al., 2013). Furthermore, rapid concerted evolution because of high level transition-transversion at the rDNA and trnL-F loci impedes accurate phylogenetic inference of bamboo species (Baldwin et al., 1995; Nieto-Feliner and Rossello, 2007). Although abundant peptides could

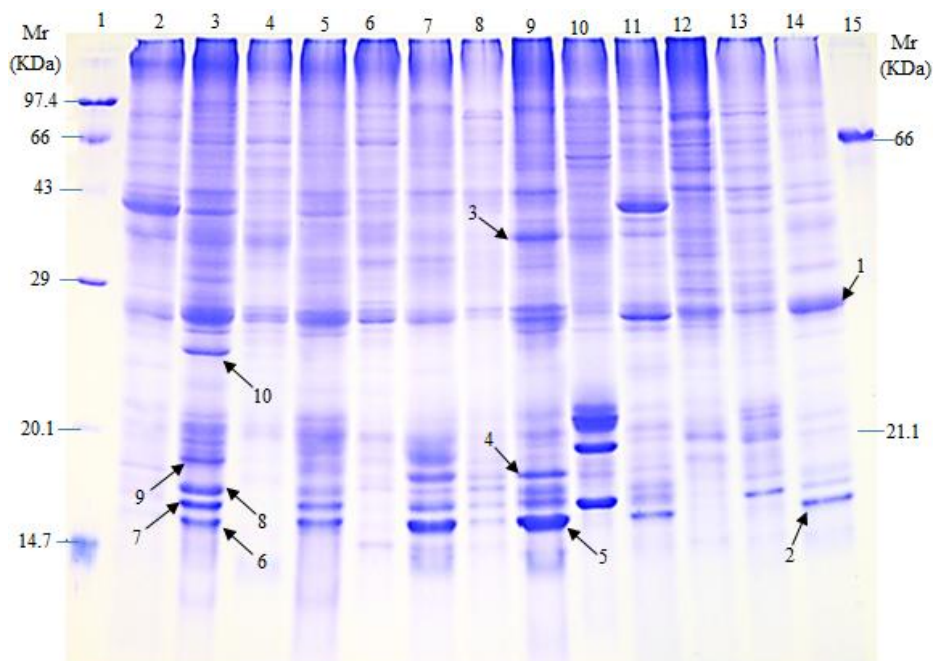


Figure 2. Juvenile edible bamboo shoots crude proteins (15 µg per lane) polymorphism- profiled on a 15% SDS-PAGE gel. Lane 1, Standard medium range molecular mass markers; lane 2, *Bambusa* sp. JX564906; lane 3, *B. oliveriana* JX564901; lane 4, *B. tulda* JX507132; lane 5, *Bambusa* sp. accession JX564907; lane 6, *B. manipureana* accession JX564900; lane 7, *D. giganteus* accession JX564902; lane 8, *D. hamiltonii* accession JX564903; lane 9, *D. manipureanus* accession JX564905; lane 10, *C. callosa* accession KC013282; lane 11, *S. dullooa* accession JX507134; lane 12, *B. tuldooides* accession KC013288; lane 13, *B. cacharensis* accession KC013285; lane 14, *M. baccifera* accession JX507133; lane 15, bovine serum albumin, respectively.

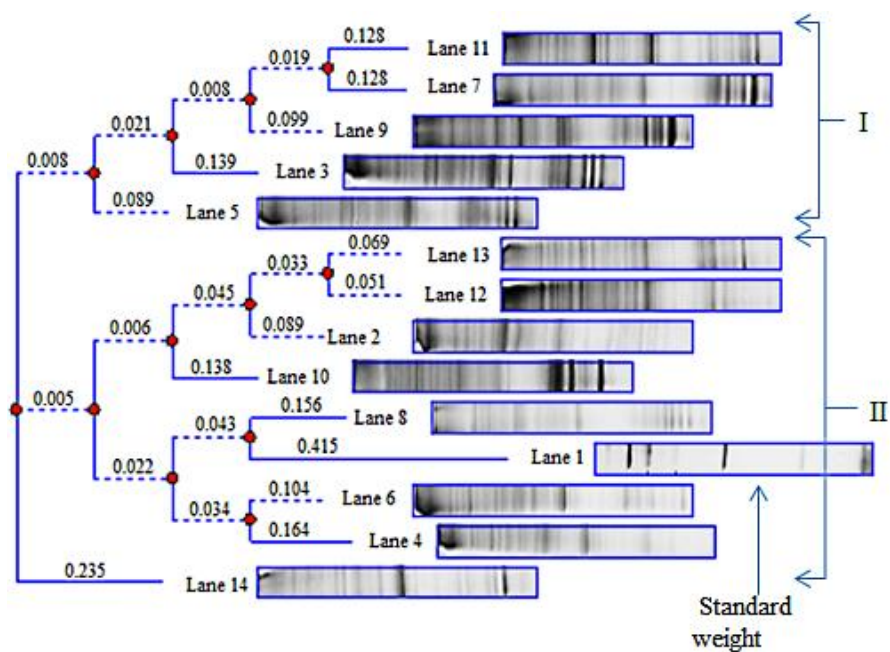


Figure 3. Dendrogram depicting diversity in proteins of species of edible bamboo shoots based on Neighbour joining generated in Phoretix 1D v.10.4 algorithm (Totalab Ltd, Newcastle, UK). Lane 1- Standard medium range molecular mass markers, lane 2 - *Bambusa* sp. JX564906, lane 3 - *B. oliveriana* JX564901, lane 4 - *B. tulda* JX507132, lane 5 - *Bambusa* sp. accession JX564907, lane 6 - *B. manipureana* accession JX564900, lane 7 - *D. giganteus* accession JX564902, lane 8 - *D. hamiltonii* accession JX564903, lane 9 - *D. manipureanus* accession JX564905, lane 10 - *C. callosa* accession KC013282, lane 11 - *S. dullooa* accession JX507134, lane 12 - *B. tuldooides* accession KC013288, lane 13 - *B. cacharensis* accession KC013285, and lane 14 - *M. baccifera* accession JX507133, respectively.

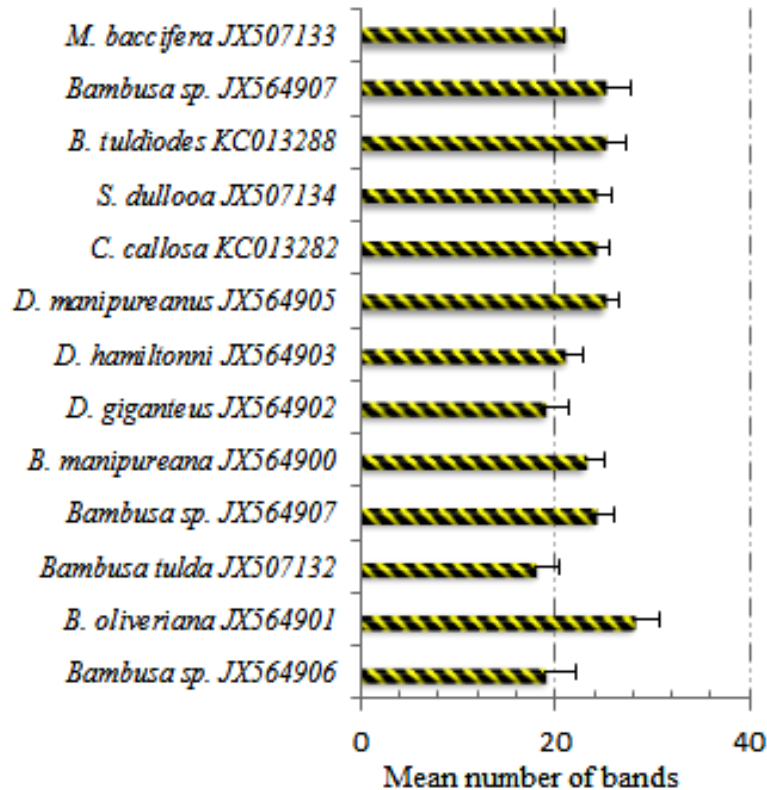


Figure 4. Mean number of bands observed among edible bamboo species over two seasons of July to August of 2011 - 2013 reveal *B. oliveriana* encodes diverse proteins and bars represent standard deviations.

mask others and compromise resolution in SDS-PAGE, the approach allows for many samples on the same gel and subsequent accurate analysis of band patterns, an advantage over two-dimensional SDS-PAGE.

Using normalized threshold values for triplicates and two biological repeats, the mean of bands detected varied among species but consistent for each given species (Figure 4). The data revealed that *B. oliveriana* expressed diverse peptides with the highest mean band number of 28, while the lowest banding of 18 was observed in *B. tulda* (Figure 4). From previous data on toxicity content in bamboo shoots of different edible bamboo species (Waikhom et al., 2013) and other nutritional components of *D. giganteus* (Nirmala et al., 2008) and the present findings, it is clear that bamboo shoots are a rich source of protein and could help in areas with poor protein diet. Based on MALDI-TOF/TOF MS/MS results (Table 2, Figure 2), the predominant bands were identified as histone-like related proteins. Although other peptides were identified at MOWSE score ($P < 0.05$) they failed the FDR test at cut-off value $\leq 1\%$ and are not reported. Histones are highly alkaline proteins, located in the nucleus and associated with DNA to form chromatin, highly organized into nucleosome cores. Five core histones are H1, H2A, H2B, H3, and H4

and are extremely conserved throughout evolution and are modified under stress conditions (Pawlak and Deckert, 2007). So far, it has been shown that histones undergoes numerous covalent modification such as acetylation, methylation, phosphorylation and ubiquitination, and these modifications controls chromatin functions mediated by histones (Kouzarides, 2007). An important line of evidence showed that trimethylation of histone H3 at lysine 27 (H3K27me3) is involved in cold adaptation in planta (Kwon et al., 2009; Zhu et al., 2012). Furthermore, vigorous temperature fluctuations during the day and night are suggested to influence nucleosome assembly/disassembly and provide a gateway for rapid chromatin configuration to adapt to ambient temperatures (Zhu et al., 2012).

In *Arabidopsis*, cold stress also triggered rapid and transient upregulation of histone H3 Ser-10 phosphorylation, H3 phosphoacetylation, and H4 acetylation followed by stress-type-specific gene expression (Sokol et al., 2007). In the present study H4, H3 and H2A were predominant histones-like proteins identified (Figure 5, Table 2). Since the bamboo shoots studied herein were collected in month of July and August which falls within the cold monsoon season in the North Eastern States of India, fluctuations in temperature

Table 2. Identified predominant peptide bands (of figure 2) from 13 species of edible bamboo shoots.

Name of species/lane	Band	Accession	Organism	Name of protein	Putative function	N M	Exp. Mr (KDa)	PS	Co.	N-terminal amino acids
<i>M. baccifera</i> / 14	1	gi 41387680	<i>Chlamydomonas reinhardtii</i>	Phosphoenol pyruvate carboxylase	Carbohydrate metabolism	0	28.81	63	248	GDAGASDMLSHR
	2	gi 70772	<i>Triticum aestivum</i>	Histone H4	Forms nucleosome core	4	16.31	180	206	TVTAMDVVYALKR
<i>D. hamiltonii</i> / 9	3	gi 122084	<i>Triticum aestivum</i>	Histone H3	Forms nucleosome core	13	36.21	179	164	RVTIMPK
	4	gi 70772	<i>Triticum aestivum</i>	Histone H4	Forms nucleosome core	8	17.61	223	134	TVTAMDVVYALKR
	5	gi 81906	Garden pea	Histone H2A	Forms nucleosome core	6	15.23	75	291	HLCLAIR
<i>B. oliveriana</i> / 2	6	gi 34902360	<i>Oryza sativa</i> L.	Hypothetical protein	Unknown	1	14.92	51	995	EMEGVVRAIR
	7	gi 19611	<i>Medicago sativa</i>	Histone H3	Forms nucleosome core	10	16.25	129	248	VTIMPKDIQLAR
	8	gi 22217761	<i>Daucus carota</i>	Histone H4	Forms nucleosome core	9	16.84	212	133	DNIQGITKPAIR
	9	gi 70772	<i>Triticum aestivum</i>	Histone H4	Forms nucleosome core	9	18.63	225	206	TVTAMDVVYALKR
	10	gi 3775995	<i>Arabidopsis thaliana</i>	RNA helicase	Unwind RNA	0	23.89	52	716	QSMMFSATMPSWIRSLTK

NM - Number of matches, Exp. Mr - experimental molecular weight kilo Dalton, PS - protein score reported by MASCOT at $P \leq 0.05$, Cov. - Amino acid coverage.

might justify the predominance of histone related proteins. Histones are understood not to only expand the storage capacity of DNA, but also offer fast reversible changes in chromatin accessibility to adjust with changing internal and external stimuli. The exact role of histones in bamboo shoots is yet to be determined and requires further studies. Nonetheless, acetylated histones play key role in plant development, defense, and adaptation (Chua et al., 2003; Zhou et al., 2005).

Conclusion

Extraction of quality proteins is crucial for downstream proteomics analysis. The protocol for protein extraction in the present study hallmarks the introduction of bamboo into the proteomics era. Based on the nutritive value and health enhancing properties, bamboo shoots are becoming popular food worldwide. While considering the nutraceutical values of edible bamboo shoots and the toxicity implications,

dietary intake of proteins can be a deciding factor in region of low protein diet. Hence, an appropriate selection of species of juvenile edible bamboo shoots is the solution for food security as well as a potential source for proteins. In the present study, juvenile edible bamboo shoots expressed diverse peptides, predominantly of low molecular weight (20.10 to 15.50 KDa) histone-like proteins. This first qualitative proteomics analysis on species of bamboo shoots tells a consumer could benefit differently from bamboo

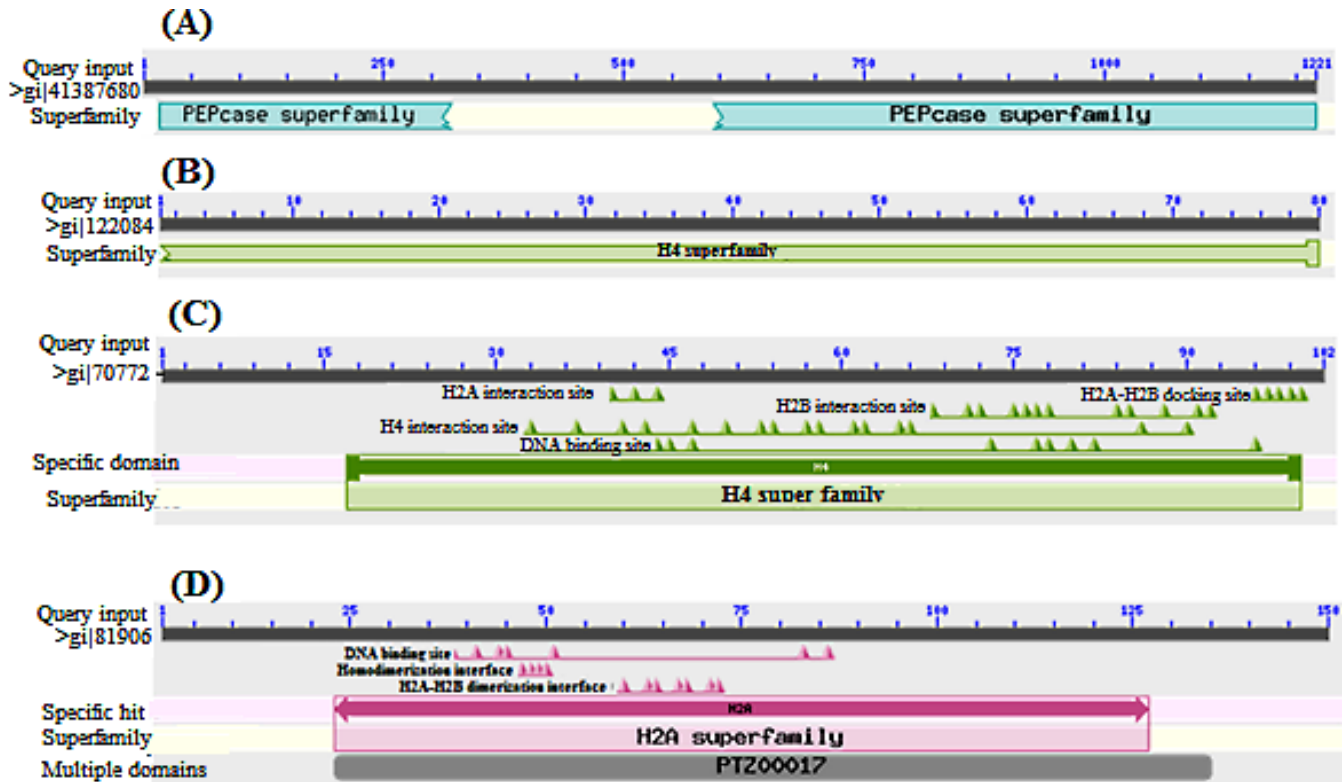


Figure 5. Authentication of identified peptides in NCBI Conserved Domain Database. (A) Phosphoenolpyruvate carboxylase, (B) histone 3, (C) histone 4 and (D) histone H2A, respectively.

protein intake as a function of a chosen edible bamboo species.

Conflict of interests

The authors did not declare any conflict of interest.

ACKNOWLEDGEMENTS

The author, SDW, is thankful to Department of Biotechnology (DBT) for the award of Post doctoral Fellowship. BL, is grateful to The Academy of Sciences for Developing World (TWAS) and DBT, Government of India (program no. 3240223450), for his fellowship.

REFERENCES

Akao Y, Seki N, Nakagawa Y, Yi H, Matusumoto K, Ito Y, Ito, K, Funaoka M, Maruyama W, Naoi M, Nozawa Y (2004). A highly bioactive lignophenol derivative from bamboo lignin exhibit a potent activity to suppress apoptosis induced by oxidative stress in human neuroblastoma SH-SY5Y cells. *Bioorg. Med. Chem.* 12:4791-4801.
 Baldwin BG, Sanderson MJ, Wojciechowski MF, Campbell CS, Donoghue MJ (1995). The ITS region of nuclear ribosomal DNA: a valuable source of evidence on angiosperm phylogeny. *Ann. Mo. Bot. Gard.* 82:247-277.
 Bhatt BP, Singha LB, Sachan MS, Singh K (2004). Commercial edible

bamboo species of the North-Eastern Himalayan Region, India. Part I: young shoot sales. *J. Bamboo Rattan* 3:337-364.
 Bradford MM (1976). A rapid and sensitive method for the quantification of microgram quantities using the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.
 Brar J, Anand M, Sood A (2013). *In vitro* seed germination of economically important edible bamboo *Dendrocalamus membranaceus* Munro. *Ind. J. Exp. Biol.* 51: 88-96.
 Choudhury D, Sahu JK, Sharma GD (2011). Bamboo shoot based fermented food products review. *J. Sci. Ind. Res.* 70:199-203.
 Chua YL, Watson LA, Gray JC (2003). The transcriptional enhancer of the pea plastocyanin gene associates with the nuclear matrix and regulates gene expression through histone acetylation. *Plant Cell* 15:1468-1479.
 Daphne L (1996). Bamboo shoots: delicious to eat, easy to sell. *Washington Tith, Autumn* 7-9.
 Devi WS, Louis B, Sharma GJ (2012). *In vitro* seed germination and micropropagation of edible *Dendrocalamus giganteus* Munro using seeds. *Biotechnology* 11:74-80.
 Elias JE, Haas W, Faherty BK, Gygi SP (2005). Comparative evaluation of mass spectrometry platforms used in large-scale proteomics investigations. *Nat. Methods* 2:667-675.
 Kouzarides T (2007). Chromatin modifications and their function. *Cell* 128:693-705.
 Kwon CS, Lee D, Choi G, Chung WI (2009). Histone occupancy-dependent and -independent removal of H3K27 trimethylation at cold-responsive genes in *Arabidopsis*. *Plant J.* 60:112-121.
 Lobovikov M (2003). Bamboo and rattan products and trade. *J. Bamboo Rattan* 2(4):397-406.
 Louis B, Waikhom SD, Roy P, Bhardwaj PK, Singh MW, Sharma KC, Talukdar NC (2014). Invasion of *Solanum tuberosum* L. by *Aspergillus terreus*: a microscopic and proteomics insight on pathogenicity. *BMC Res. Notes* 7:350.
 Nieto-Feliner G, Rosssello JA (2007). Better the devil you know?

- Guidelines for insightful utilization of nrDNAITS in species level evolutionary studies in plants. *Mol. Phylogenet. Evol.* 44:911-919.
- Nirmala C, Bisht MS, Sheena H (2011). Nutritional properties of bamboo shoots: potential and prospects for utilization as a health food. *Compr. Rev. Food Sci. Food Saf.* 10:153-165.
- Nirmala C, Sharma ML, David E (2008). A comparative study of nutrients components of freshly harvested, fermented and canned bamboo shoots of *Dendrocalamus giganteus* Munro. *J. Am. Bamboo Soc.* 21:33-39.
- Nzwalo H, Cliff J (2011). Konzo: from poverty, cassava, and cyanogen intake to toxico-nutritional neurological disease. *PLoS Negl. Trop. Dis.* 5:6: e1051.
- Pawlak S, Deckert J (2007). Histone modifications under environmental stress. *Biol. Lett.* 44:65-73.
- Saitou N, Nei M (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4:406-425.
- Schwarzmaier U (1997). Cyanogenesis of *Dendrocalamus*: taxiphyllin. *Phytochemistry* 16:1599-1600.
- Shevchenko A, Tomas H, Havlis J, Olsen JV, Mann M (2006). In-gel digestion for mass spectrometric characterisation of proteins and proteomes. *Nat. Protoc.* 1:2856-2860.
- Singh PK, Devi SP, Devi KK, Ningombam DS, Athokpam P (2010). *Bambusa tulda* Roxb. in Manipur State, India: Exploring the local values and commercial implications. *Not. Sci. Biol.* 2:35-40.
- Singh SR, Dalal S, Singh R, Dhawan AK, Kalia RK (2012). Micropropagation of *Dendrocalamus asper* (Schult.&Schult.F) Backer ex k. Heyne): an exotic edible bamboo. *J. Plant Biochem. Biotechnol.* 21(2):220-228.
- Sokol A, Kwiatkowska A, Jerzmanowski A, Prymakowska Bosak M (2007). Up-regulation of stress-inducible genes in tobacco and *Arabidopsis* cells in response to abiotic stresses and ABA treatment correlates with dynamic changes in histone H3 and H4 modifications. *Planta* 227:245-254.
- Waikhom SD, Louis B (2014). An effective protocol for micropropagation of edible bamboo species (*Bambusa tulda* and *Melocanna baccifera*) through nodal culture. *Sci. World J.* 2014, Volume 2014, Article ID 345794, <http://dx.doi.org/10.1155/2014/345794>.
- Waikhom SD, Louis B, Sharma CK, Kumari P, Bharat GS, Singh WM, Talukdar NC (2013). Grappling the high altitude for safe edible bamboo shoots with rich nutritional attributes and escaping cyanogenic toxicity. *BioMed Res. Int.* 2013, Volume 2013, Article ID 289285, <http://dx.doi.org/10.1155/2013/289285>.
- Xie C, Mao X, Huang J, Ding Y, Wu J, Dong S, Kong L, Gao G, Li C, Wei L (2011). KOBAS 2.0: a web server for annotation and identification of enriched pathways and diseases. *Nucleic Acids Res.* 39:316-322.
- Yang Q, Duan Z, Wang Z, He K., Sun Q, Peng, Z (2008). Bamboo resources, utilization and ex-situ conservation in Xishuangbanna, South-Eastern China. *J. For. Res.* 19:79-83.
- Zhou C, Zhang L, Duan J, Miki B, Wu K (2005). Histone deacetylase19 is involved in jasmonic acid and ethylene signaling of pathogen response in *Arabidopsis*. *Plant Cell* 17:1196-1204.
- Zhu Y, Aiwu Dong, Wen-Hui Shen (2012). Histone variants and chromatin assembly in plant abiotic stress responses. *Biochim. Biophys. Acta* 1819:343-348.

Full Length Research Paper

Effect of the somatic cell count on physicochemical components of milk from crossbred cows

Bruna Ariel Dias Guariglia, Priscila Alonso dos Santos, Liomar de Souza Araújo, Cristiane Isabo Giovannini, Rodrigo Balduino Soares Neves, Edmar Soares Nicolau and Marco Antônio Pereira da Silva

Instituto Federal Goiano (IF Goiano), PostOffice Box 66, 75901-970, Rio Verde, GO, Brazil.

Received 3 March, 2015; Accepted 28 April, 2015

The aim of the present work was to evaluate the effect of somatic cell count on the composition of milk in individual samples from crossbred cows at the Rio Verde municipality. Data from 2,730 individual samples of milk from crossbred cows were used. The samples were collected between February and April 2012 and analyzed at the Milk Quality Laboratory (Laboratório de Qualidade do Leite) in the Food Research Center (Centro de Pesquisa em Alimentos) of the School of Veterinary Medicine and Animal Science of the Federal University of Goiás (Escola de Veterinária e Zootecnia da Universidade Federal de Goiás). Protein, fat, lactose, casein, urea, defatted dry extract and somatic cell counts (SCC) were analyzed. A completely randomized experimental design was used. An analysis of variance was performed, and individual means were compared using Tukey's test ($p < 0.05$). The urea content did not vary with increasing SCC. The lactose levels decreased with increasing SCC. The defatted dry extract was minimally influenced by increasing SCC.

Key words: SCC, percent composition, milk quality, casein, urea.

INTRODUCTION

Mastitis has an extremely detrimental effect on the composition and physicochemical characteristics of milk, which is accompanied by an increase in the somatic cell counts (SCC) in milk. Andrade et al. (2007) reported that mastitis is one of the main causes of decreased milk quality and quantitative losses in milk production, and it is the disease causing the greatest loss for dairy farming, both in Brazil and worldwide. Mastitis is responsible for increasing the levels of somatic cells, in addition to causing lesions in the milk-producing epithelial cells and increasing the vascular permeability, which results in a

greater passage of substances from the blood to the milk, such as sodium, chlorine, immunoglobulins, and other serum proteins (Kitchen, 1981). From the point of view of the industry, a high SCC results in problems during milk processing and in decreased yields as a consequence of the low casein, fat, and lactose contents, which result in products of low quality and stability (Brito, 1999b).

Protein has a high nutritional relevance and economic importance in payment-for-quality programs, mostly because casein is the main component in the manufacture of dairy products (Emmons et al., 2003).

*Corresponding author. E-mail: bruna.guariglia@hotmail.com. Tel: +55 64 9264-9741.

Urea nitrogen has been used in herd nutrition assessments because it is directly correlated to the urea levels in plasma and blood (Depeters and Ferguson, 1992). According to Chase (1994), dairy cows secrete an average of 25 to 35% of the nitrogen they consume into their milk. Almost all remaining nitrogen is excreted in urine and feces. Using the urea nitrogen content of milk to adjust the contents of feed protein and energy reduces feeding costs, and results in increased animal performance (Nelson, 1995). Increase in protein nitrogen concentrations in milk result in decreased industrial yield in cheese production because the serum proteins and casein are replaced by non-protein nitrogen, which increases the time for cheese curdling (Ferreira et al., 2006). Hojman et al. (2004) reported that the urea nitrogen concentration of milk is lower in samples with a high SCC.

Most of the dairy industries have based milk payments on quality criteria, with the aim of encouraging producers to seek to improve the milk quality. This practice increases the industry's production yield, with the producer receiving a bonus for the supplied product, and a better-quality product reaches the consumer.

In Brazil, as in other countries, the highest valued milk constituents in terms of the payment-for-quality programs are fat and protein because they are related to a higher industrial yield. The SSC in milk is a diagnostic method of subclinical mastitis, and it is internationally accepted as the standard method to determine the quality of raw milk (Ribas, 1999). Magalhães et al. (2006) reported that a high SCC results in decreased economic profit for the producer due to reduced production, higher expenses for medical drugs, and penalties applied by dairies.

The goal of the present study was to evaluate the influence of the SSC on the chemical components of milk originating from individual samples, which were collected from crossbred cows in the Rio Verde municipality in the state of Goiás (GO).

MATERIALS AND METHODS

Data from 2,730 individual samples of milk from Holstein-Zebu crossbred cows were used. The samples were collected in the Rio Verde municipality, in the state of Goiás, between February and April, 2012 and were analyzed at the Milk Quality Laboratory (Laboratório de Qualidade do Leite- LQL) in the Food Research Center (Centro de Pesquisa em Alimentos - CPA) of the School of Veterinary Medicine and Animal Science of the Federal University of Goiás (Escola de Veterinária e Zootecnia da Universidade Federal de Goiás- EVZ/UFG). Protein, fat, lactose, casein, urea, defatted dry extract (DDE), and SCC were analyzed.

The samples were collected following the completion of milking with individual measurers via a valve at the bottom that, prior to sample collection, was positioned in the agitation mode for approximately five seconds for milk homogenization. The milk was then transferred to a 40-mL collection vial containing the preservative Bronopol®.

The vials were labeled with a barcode identifying the respective farm and animal, and immediately placed in an isothermal box containing ice. The technical reports from the forwarded samples

were received, and the data referring to the milk composition were organized according to the SCC levels.

Analyses of the SCC, fat, protein, lactose, urea, casein, and DDE were performed.

Analysis of somatic cell count

The somatic cell count was analyzed by flow cytometry, with a Fossomatic 5000 Basic (Foss Electric A/S. Hillerod, Denmark). The results were expressed in somatic cells per milliliter (SC/mL).

Analysis of chemical composition

Fat, protein, lactose and DDE contents were determined by the analytical principle, with a Milkoscan 4000 (Foss Electric A/S. Hillerod, Denmark). This method is based on the differential absorption of infrared waves by the different milk components. The results were expressed as percentages (%).

Analysis of urea and casein

Urea (mg/dL) and casein (%) contents were determined using the analytical principle, with a Lactoscope (Delta Instruments). This method is based on the differential absorption of Fourier transform infrared (FTIR) spectroscopy.

Statistical analysis

A completely randomized experimental design (CRD) was used. The variables fat, protein, lactose, DDE, urea, and casein were compared according to the levels of SCC and subjected to analysis of variance. Individual means were compared using Tukey's test at a $p < 0.05$ probability level. The minimum and maximum values and coefficient of variance of milk components were determined using the WebCalc (2012) statistical software.

RESULTS AND DISCUSSION

The values of fat, protein, casein, urea, lactose and DDE were organized into different categories according to the SCC levels, as follows: under 200 thousand SC/mL, from 201 to 400 thousand SC/mL, from 401 to 600 thousand SC/mL, and above 601 thousand SC/mL (Table 1).

The minimum fat, protein and DDE levels, as percentages, that were set by Normative Instruction (Instrução Normativa - IN) no. 62 are 3.0, 2.9, and 8.4, respectively. The data reported in the literature are contradictory regarding the variation of milk fat content with an increasing SCC. The fat values were found to be above the minimum levels set by IN 62.

The percentage of fat increased with increasing SCC. The same result was observed by Rangel et al. (2009), when the authors evaluated the correlation between the SCC and milk components. These findings confirm that the animals were receiving adequate dietary fiber levels because the milk fat levels tend to decrease with decreasing dietary fiber content and increasing levels of non-fibrous carbohydrates (concentrate).

Bueno et al. (2005) evaluated the relationship between

Table 1. Variation in the chemical composition of milk of crossbred cows, according to the somatic cell counts.

Parameter	Up to 200 thousand SC ¹ /mL	201 thousand to 400 thousand SC/mL	401 thousand to 600 thousand SC/mL	Above 601 thousand SC/mL	CV ³ (%)
	N ² = 1317	N = 595	N = 227	N = 591	
Fat (%)	3.53 ^b	3.57 ^b	3.65 ^{a, b}	3.72 ^a	21.19
Protein (%)	3.27 ^b	3.36 ^a	3.38 ^a	3.41 ^a	11.18
Casein (%)	2.52 ^b	2.60 ^a	2.61 ^a	2.62 ^a	13.14
Urea (mg/dL)	15.50 ^a	15.41 ^a	15.14 ^a	16.01 ^a	32.55
Lactose (%)	4.67 ^a	4.58 ^b	4.50 ^c	4.33 ^d	5.55
DDE ⁴ (%)	8.91 ^a	8.93 ^a	8.87 ^a	8.74 ^b	5.39

Averages within the same row followed by the same letter are not significantly different according to Tukey's test ($P < 0.05$). ¹SC = somatic cells. ²N = number of collected samples. ³CV = coefficient of variation. ⁴DDE = defatted dry extract.

the SCC and components of raw milk stored in direct-expansion cooling tanks for individual use in the state of Goiás, and they found that the fat levels were similar for the group with an SCC above 601 thousand SC/mL and higher for the other groups, with values ranging from 3.71 to 3.75%. This finding is in agreement with Machado et al. (2000), who observed that an increased SCC in milk stored in tanks resulted in increased fat percentages.

This increase in fat content with an increased SCC can be explained by a decrease in the milk production by the animals and possibly in the concentration of this component. A potential explanation for this result can be found in the study by Pereira et al. (1999), who reported that, although the fat percentage generally decreases with increasing SCC, a reduction in milk production, which is more pronounced than the decrease in fat production, will result in higher fat concentrations.

Fat is the milk component that presents the highest variation. It varies according to cow breed, lactation stage, and animal diet. The genetic component and the lactation period also affect the milk lipid concentrations (González et al., 2001).

The lowest milk protein value observed was 3.27% for the group with an SCC level below 200 thousand SC/mL. The milk protein value significantly differed from the remaining groups, for which the recorded values were 3.36, 3.38, and 3.41% for the groups with an SCC from 201 to 400 thousand SC/mL, 401 to 600 thousand SC/mL and above 601 thousand SC/mL, respectively.

The protein values found in the present study were higher than the ones reported by Bueno et al. (2005), who observed protein levels between 3.18 and 3.35% for the different SCC levels studied. The milk protein concentrations increased with increasing SCC. This result is in accordance with Bueno et al. (2005), who evaluated the relationship between the SCC and components of raw milk stored in direct-expansion cooling tanks for individual use in the state of Goiás. However, Taffarel et al. (2010) found lower protein values than the ones found in the present study (between 3.15%

and 3.17%) while evaluating the effect of five SCC intervals on the milk composition.

The plasma proteins have a strong influence on the protein concentration of milk. These proteins migrate to the site of inflammation to fight the infection, increasing the milk protein concentration. However, presence of plasma proteins should not be considered favorable when evaluating the milk quality (Pereira et al., 1999). Noro et al. (2006) observed that an increase in the somatic cell score (SCS) resulted in an increase in the protein concentration of the milk.

The casein values were similar for the groups with SCC levels from 201 to 400 thousand SC/mL, 401 to 600 thousand SC/mL and above 601 thousand SC/mL (2.60, 2.61 and 2.62%, respectively) and were different from the group with an SCC below 200 thousand SC/mL (2.52%).

Santos and Fonseca (2006) reported a decrease in the casein content due to degradation by bacterial and leukocyte proteases and to decreased casein synthesis, which constitutes an undesirable effect. Decreased casein concentrations have a direct influence on dairy product production, decreasing its yield. For the dairy industry, this is a determining factor for the cheese manufacturing process.

The urea content was similar for all studied intervals, with values of 15.50 mg/dL for an SCC under 200 thousand SC/mL; 15.41 mg/dL for an SCC from 201 to 400 thousand SC/mL; 15.14 mg/dL for an SCC from 401 to 600 thousand SC/mL; and 16.01 mg/dL for an SCC above 601 thousand SC/mL. These values are within levels considered optimal for cows with a good dry matter intake, which are between 12 and 18 mg/100 mL milk (Torrent, 2000). Although the urea values were not significantly different between the different SCC levels, they did not vary greatly between groups.

This variation could be explained by unbalanced diets, especially regarding the concentrate and forage, although the values were within levels considered optimal by Gaona (2002). Urea levels between 10 and 16 mg/dL milk are considered normal. The fact that the recorded

Table 2. Mean, minimum and maximum values of fat, protein, lactose, DDE, urea and casein.

Parameter	Mean	Minimum value	Maximum value	CV ¹ (%)
Fat (%)	3.59	2.0	8.4	0.21
Protein (%)	3.33	2.38	4.93	0.11
Lactose (%)	4.56	2.12	5.21	0.06
DDE ² (%)	8.87	7.03	10.98	0.05
Urea (mg/dL)	15.5	1.4	71	0.32
Casein (%)	2.57	1.73	3.86	0.13

¹CV = coefficient of variation. ²DDE = defatted dry extract.

Table 3. Percentage of samples outside the standard levels established by IN 62.

Requisite	Samples outside standard levels (%)
Fat	20.4
Protein	12.0
DDE ¹	15.7
SCC ²	21.6

¹DDE = defatted dry extract. ²SCC = somatic cell counts.

urea values did not vary with the studied variables indicates that the studied animals were receiving a diet with adequate, and similar, protein values. The urea content in the blood plasma, urine and milk increases linearly with increasing levels of metabolizable protein in the diet (Wang et al., 2007). The lactose content was different among all levels of SCC, with the highest value observed for the group with an SCC under 200 thousand SC/mL and decreasing values with increasing SCC levels thereafter.

The decrease in lactose content could be caused by a loss of lactose from the mammary gland into the blood due to changes in membrane permeability (Rangel et al., 2009). Decrease in milk lactose compromise milk production because lactose is responsible for attracting water into the mammary gland, and a decrease in its concentration leads to a decrease in milk production.

The milk lactose concentration decreased with an increasing SCC. This result was in accordance with Machado et al. (2000), who observed a decrease in lactose concentrations with an increasing SCC in milk stored in expansion tanks in the states of São Paulo and Minas Gerais.

The lactose levels varied, with the lowest values being found in the group of animals with an SCC above 601 thousand SC/mL. Base on the fact that lactose is an osmotic component, which is responsible for the increase or decrease of milk production, these animals were likely to be infected with subclinical mastitis and had a lower milk production. The DDE values were similar for all the SCC levels except for the group with SCC above 601 thousand SC/ml which had 8.74%.

Beloti et al. (2011) found similar values of non-fat solids

(8.9%) while evaluating the physicochemical quality of raw milk samples collected in the north of Paraná state, in the Sapopema municipality. As the levels of SCC increased, the milk DDE levels decreased slightly. The decrease in DDE may have been influenced by the increase in mammary gland permeability and possible leakage of milk components into the blood.

Ventura et al. (2006) evaluated the correlation between the SCC and milk components, and observed a decrease in DDE with an increased SCC. Rangel et al. (2009) observed a positive correlation between the SCC and DDE, with the levels of DDE increasing with increasing SCC. This finding is in contrast to the present study because the DDE level increased with the SCC levels up to 201 to 400 thousand SC/mL and then decreased at higher SCC levels.

Although there was a slight decrease in the DDE at SCC levels above 201 thousand SC/mL, this component was not greatly influenced by the SCC. This was expected because the DDE is determined by all solid components except for fat, which is the nutrient with the highest variation. From the point of view of the industry, a lower DDE content is associated with a higher dairy production yield, and this component is sometimes used as the criterion for establishing payments and bonuses to the milk producers. The data obtained for the milk components of the crossbred cows, including the mean, maximum and minimum levels and coefficient of variation, are shown in Table 2.

The mean values of fat were within the minimum levels set by IN62 (Table 3). The variation between the minimum and maximum fat values was 21%, confirming that fat is the milk component with a greater variation.

The protein levels had an 11% variation, 4.93% maximum value and 3.33% minimum value which were within the limits set by IN 62. The minimum value observed was below the adequate levels and may have been influenced by known factors such as the cow breed, stage of lactation or mammary gland diseases. There are no legal recommendations regarding lactose levels. However, it is believed that this value should be approximately 4.6%. There was little variation of the maximum and minimum values, which was expected because lactose is the milk component that varies the least. Low levels of milk lactose compromise the milk production because lactose is responsible for water absorption due to its osmotic behavior.

The mean and maximum levels of DDE agree with the levels established by the legislation. The DDE displayed the lowest coefficient of variation, confirming that the milk components that are a part of the DDE were within the levels considered optimal. The minimum value found was below the value established by the legislation. Although there are no established maximum and minimum values for the urea concentrations in milk, it is considered that in well-fed cows that receive optimal dietary fiber levels, the milk urea concentrations remain close to 12 to 16 mg/dL milk. The mean values observed are within those levels; however, the minimum and maximum values were outside the levels considered to be optimal. A high milk urea content indicates protein supplementation that exceeds the required amount (Torrent, 2000).

The mean value of casein was 2.57%. There are no minimum and maximum casein levels established by the legislation. Philpot (1998) reported that inflammatory processes in the mammary gland increase with increasing concentrations of the plasmin enzyme in the milk, thereby increasing casein degradation and decreasing stability.

The values established by IN 62 for fat, protein, DEE and SCC are shown in Table 3. Although the number of samples studied was large, a great part of those samples exhibited fat values outside the standard levels. Approximately 20.4% of the samples had less than 3.0% fat, although the fat content displayed a great variation. This fact confirms that the studied animals were receiving a diet which was low in fiber.

The lower values found for protein content were outside the standard levels, amounting to 12.0% of the samples. The remaining 88% of the studied samples had protein levels higher than 2.9%, which is the lower limit established by the legislation. Because the DDE comprises all solid components of milk except for fat, the number of samples with values outside the standard levels (15.7%), even though fat had no influence, indicates that the milk composition was affected by the SCC. The SCC might have affected the permeability of the mammary gland membranes, causing the milk components to disperse into the blood.

The SCC was a determinant factor for the milk com-

position and for the percentage of samples that fell outside the standard levels because approximately 21.6% of the samples had SCC levels above 600 thousand SC/mL. This is considered to be very high and indicative of subclinical infection of mastitis in the animals. Attention should be given to these animals because they may increase the SCC levels of the herd's tank, which would result in lower returns from the industry to the producer.

Conclusions

Urea concentrations did not vary with increasing somatic cell counts. Lactose decreased with increasing somatic cell counts. Defatted dry extract was minimally influenced by increasing somatic cell counts. The results were found while considering the somatic cell levels within the standards established by IN 62/2011.

Conflict of interests

The authors did not declare any conflict of interest.

REFERENCES

- Andrade LM, El Faro L, Cardoso VL, Albuguerque de LG, Cassoli LD, Machado PF (2007). Efeitos genéticos e de ambiente sobre a produção de leite e a contagem de células somáticas em vacas holandesas [Genetic and environmental effects on milk yield and somatic cell count of Holstein cows]. *R. Bras. Zootec.* 36:343-349.
- Beloti V, Riberio Júnior JC, Tamanine R, Yamada AK, Cavaletti L, Shecaira CL, Novaes DG, Silva FF (2011). Qualidade microbiológica e físico-química do leite cru refrigerado, produzido no município de SAPOPEMA/PR [Microbiological quality and physical chemistry of raw refrigerated milk produced in the city SAPOPEMA/PR]. *R. Cient. Eletrôn. Med. Vet.* 9:16.
- Brito MAVP (1999b). Influência das células somáticas na qualidade do leite [Influence of somatic cells in milk quality]. Pages 41-46. In: *Qualidade do Leite e Produtividade dos Rebanhos Leiteiros [Milk Quality and Yield of Dairy Herds]*. C. E. Martins, C. N. Costa, J. R. F. Brito, L. C. T. Yamaguchi, M. Pires, and F. A. Minas Leite, ed. Embrapa Gado de Leite, Juiz de Fora, Brazil.
- Bueno VFF, Mesquita AJ, Nicolau ES, Oliveira AN, Oliveira JP, Neves RBS, Mansur JRG, Thomaz LW (2005). Contagem celular somática: relação entre a composição centesimal do leite e o período do ano no Estado de Goiás [Somatic cell count: relationship to milk composition and period of the year in Goiás State, Brazil]. *Cienc. Rural* 35:848-854.
- Chase LF (1994). Environmental considerations in developing dairy rations. *Cornell Nutr. Conf.*, Rochester, NY, Cornell University Press, Ithaca, NY. pp. 56-62.
- Depeters EJ, Ferguson JD (1992). Nonprotein nitrogen and protein distribution in the milk of cows. *J. Dairy Sci.* 75:3192-3209.
- Emmons DB, Dubé C, Modler HW (2003). Transfer of protein from milk to cheese. *J. Dairy Sci.* 86:469-485.
- Ferreira MG, Souza LT, Peleja L, Corassin CH, Gratão PR (2006). Ureia e qualidade do leite [Urea and milk quality]. *R. Cient. Eletrôn. Med. Vet.* 3:6.
- Gaona RC (2002). Alguns indicadores metabólicos no leite para avaliar a relação nutrição: fertilidade [Some metabolic indicators in milk for the evaluation of the nutrition:fertility ratio]. 29^o Congresso Nacional de Medicina Veterinária [29th Congress of Veterinary Medicine],

- Conbravet, Gramado, Brazil. pp. 40-48.
- González FHD, Dürr JW, Fontaneli RS (2001). USO do Leite para Monitorar a Nutrição e o Metabolismo de Vacas Leiteiras [Using Milk to Monitor Dairy Cow Nutrition and Metabolism]. Gráfica UFRGS, Porto Alegre, Brazil.
- Hojman D, Kroll O, Adin G, Gips M, Hanochi B, Ezra E (2004). Relationships between milk urea and production, nutrition and fertility traits in Israeli dairy herds. *J. Dairy Sci.* 87:1001-1011.
- Kitchen BJ (1981). Reviews of the progress of dairy: milk compositional changes and related diagnostic tests. *J. Dairy Res.* 48:167-188.
- Machado PF, Pereira AR, Sarríes GA (2000). Composição do leite de tanque de rebanhos brasileiros distribuídos segundo sua contagem de células somáticas [Bulk tank milk composition from Brazilian herds distributed by its somatic cell count]. *R. Bras. Zootec.* 29:1883-1886.
- Magalhães HR, Faro LE, Cardoso VL, Paz CCP, Cassoli LD, Machado PL (2006). Influência de fatores de ambiente sobre a contagem de células somáticas e sua relação com perdas na produção de leite de vacas da raça Holandesa [Effects of environmental factors on somatic cell count and reduction of milk yield on Holstein cows]. *R. Bras. Zootec.* 35:415-421.
- Nelson AJ (1995). Practical application of MUN analysis. 4th Annual Northeast Dairy Production and Medicine Symposium, Syracuse, NY. pp. 35-45.
- Noro G, González FHD, Campos R, Dürr GW (2006). Fatores ambientais que afetam a produção e a composição do leite em rebanhos assistidos por cooperativas no Rio Grande do Sul [Effects of environmental factors on milk yield and composition of dairy herds assisted by cooperatives in Rio Grande do Sul, Brazil]. *R. Bras. Zootec.* 35(Suppl. 3):1129-1135.
- Pereira AR, Silva LFP, Molon LK, Machado PF, Barancelli G (1999). Efeito do nível de células somáticas sobre os constituintes do leite I-gordura e proteína [Effects of somatic cell levels on milk components I-fat and protein]. *Braz. J. Vet. Res. Anim. Sci.* 36:121-124.
- Philpot WN (1998). Programas de qualidade do leite no mundo [Worldwide milk quality programs]. Simpósio Internacional Sobre Qualidade do Leite [International Symposium on Milk Quality], Curitiba, Brazil. p.16.
- Rangel AHN, Medeiros HR, Silva JBA, Barreto MLJ, Lima Jr. DM (2009). Correlação entre a contagem de células somáticas (CCS) e o teor de gordura, proteína, lactose e o extrato seco desengordurado do leite [Analysis of correlation between the somatic cell count (scc) and the fat, protein, lactose and dry extract of milk content]. *R. Verde* 4:57-60.
- Ribas NP (1999). Importância da contagem de células somáticas para a saúde da glândula mamária e qualidade do leite [Importance of somatic cell counts for mammary glands health and milk quality]. 4th Simpósio Internacional Sobre Produção Intensiva de Produção de Leite [4th International Symposium on Intensive Milk Production Systems], Instituto Fernando Costa, Caxambu, São Paulo. pp. 77-87.
- Santos MV, Fonseca LFL (2006). Estratégias para controle de mastite e melhoria da qualidade do leite [Strategies for mastitis control and milk quality improvement]. Editora Manole, Barueri, Brazil.
- Taffarel LE, Costa PB, Tsutsumi C, Braga GC, Zonin WJ, Portugal EF, Lins AC (2010). Variação de componentes do leite em função de níveis de células somáticas [Variation of milk composition according to the somatic cell levels] in IV Congresso Brasileiro de Qualidade do Leite [IV Brazilian Congress on Milk Quality], Florianópolis, SC, Brazil.
- Torrent J (2000). Nitrogênio ureico no leite e qualidade do leite [Urea nitrogen in milk and milk quality]. Simpósio Internacional Sobre Qualidade do Leite [International Symposium on Milk Quality]. Universidade Federal do Paraná [Federal University of Paraná], Curitiba, Brazil. pp. 27-29.
- Ventura RV, Leme TARP, Mendonça LC, Dias MS, Amorim MA (2006). Contagem de células somáticas e seus efeitos nos constituintes do leite. In: Congresso Brasileiro de Qualidade do Leite. Goiânia, II, Anais... Goiânia: 2006. P187-189.
- Wang C, Liu JX, Yuan ZP, Wu YM, Zhai SW, Ye HW (2007). Effect of level of metabolizable protein on milk production and nitrogen utilization in lactating dairy cows. *J. Dairy Sci.* 90:2960-2965.
- WebCalc (2012). Accessed Dec. 5, 2012. <http://www.webcalc.com.br/matematica/estatistica.html>

African Journal of **Biotechnology**

Related Journals Published by Academic Journals

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

academicJournals