



African Journal of Biotechnology

November 2013 - Vol. 12 Num. 46

Submit manuscripts: www.ms.academicjournals.org

Editorial Office: gjb@academicjournals.org

URL: www.academicjournals.org

academicJournals

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-Ambria
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 Aydın

*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: © 2013, 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

Research Articles

Comparative effectiveness of inter-simple sequence repeat and randomly amplified polymorphic DNA markers to study genetic diversity of Indian *Garcinia*

Utpala Parthasarathy, O. P. Nandakishore, K. Nirmal babu, Senthil Kumar and V. A. Parthasarathy

Sensory evaluation of different preparations of cassava leaves from three species as a leafy vegetable

M. G. Umuhozariho, , N. B. Shayo, P. Y. K. Sallah and J. M. Msuya

Evaluation of pollen viability, stigma receptivity and fertilization success in *Lagerstroemia indica* L.

Fang Chen, Wei Yuan, XuePing Shi and YaoMei Ye

A review on trypanosomosis in dogs and cats

Nwoha, R. I. O.

Micropropagation of *Helianthemum lippii* L. var *Sessiliforuum* (Cistaceae) an important pastoral plant of North African arid areas

Amina Hamza, Maher Gtari and Neffati Mohamed

Partial dehydration of 'Niagara Rosada' GRAPES (*Vitis labrusca* L.) targeting increased concentration of phenolic compounds and soluble solids

Wesley Esdras Santiago, Rodolpho César Dos Reis Tinini, Rafael Augustos De Oliveira and Barbara Janet Teruel

Preliminary screening of plant essential oils against larvae of *Culex quinquefasciatus* Say (Diptera: Culicidae)

M. Ramar, M. Gabriel Paulraj and S. Ignacimuthu

The role of seed priming in improving seed germination and seedling growth of maize (*Zea mays* L.) under salt stress at laboratory conditions

Gebremedhn Yohannes and Berhanu Abraha

Table of Contents: Volume 12 Number 46, 13 November, 2013

Pathogenicity of *Beauveria bassiana* and production of cuticle-degrading enzymes in the presence of *Diatraea saccharalis* cuticle

Virgínia Michelle Svedese, Patricia Vieira Tiago, Jadson Diogo Pereira Bezerra, Laura Mesquita Paiva, Elza Áurea de Luna Alves Lima, Ana Lúcia Figueiredo Porto

A comparative study of pectinolytic enzyme production by *Bacillus* species

Torimiro N. and Okonji, R. E.

Bio-corrosion of water pipeline by sulphate-reducing bacteria in a mining environment

Z. Manafi, M. Hashemi, H. Abdollahi and Gregory. J. Olson

An investigation of the bioaccumulation of chromium and uranium metals by *Cynodon dactylon*: A case study of abandoned New Union Gold Mine Tailings, Limpopo, South Africa

K. Nelushi, J. R. Gumbo and F. A. Dacosta

In vitro cultivation of *Pleurotus ostreatus* and *Lentinula edodes* in lignocellulosic residues from Amazon

Ceci Sales-Campos, Diego A. Pires, Samira R. L. Barbosa, Raimunda Liége S. Abreu and Meire C. N. Andrade

Second derivative spectrophotometric determination of cyclophosphamide in pharmaceutical formulations

Nabil A. Fakhre, Hemn A. Qader and Alaadin M. Naqishbandi

Effects of hypoxia on serum hepatic chemistries of Tibet chicken and Shouguang chicken

Wenpeng Han, Meiling Song, Hui Yuan, Haigang Bao, Chong Liu, Changxin Wu and Chunjiang Zhao

Full Length Research Paper

Comparative effectiveness of inter-simple sequence repeat and randomly amplified polymorphic DNA markers to study genetic diversity of Indian *Garcinia*

Utpala Parthasarathy*, O. P. Nandakishore, K. Nirmal babu, Senthil Kumar and V. A. Parthasarathy

Indian Institute of Spices Research, Calicut-673 012, Kerala, India.

Accepted 10 October, 2013

A study to compare the effectiveness of inter-simple sequence repeats (ISSR) and randomly amplified polymorphic DNA (RAPD) profiling was carried out with a total of 65 DNA samples using 12 species of Indian *Garcinia*. ISSR and RAPD profiling were performed with 19 and 12 primers, respectively. ISSR markers generated a total number of 156 bands with 92 polymorphic bands, while RAPD markers produced a total of 134 bands with 80 polymorphic bands. Percentage of polymorphic loci in RAPD profiling was 60.4% while in ISSR profiling, it was 59.3%. Heterogeneity index was similar for the markers, 0.86 for ISSR and 0.89 for RAPD, indicating that both the marker systems are effective in determining polymorphism in *Garcinia*. ISSR markers showed clear distinction among the species whereas RAPD markers showed segregation based on geographical location as well as species based.

Key words: *Garcinia*, genetic diversity, inter-simple sequence repeats, randomly amplified polymorphic DNA, principal component analysis.

INTRODUCTION

Garcinia is a large genus with 240 species of evergreen trees and shrubs. About 35 species are reported in India, among which seven are endemic to Western Ghats, 5 in north-east Himalayas and six in Andaman Islands (Peter and Abraham, 2007). *Garcinia* is one among the few genera in angiosperms that shows a very high degree of diversity (Osman and Rahman, 2006). It is one of the potential under exploited multipurpose crops and recently gained a lot of attention as a popular means of weight loss because of the presence of (-) hydroxycitric acid in the fruit rind and leaves. The Plant List (2010) reports that 25.5% of the species names of *Garcinia* are

synonyms, 3.4% names are unplaced and 4.4% names are unassessed. The differences that distinguish one plant from another are encoded in the plant's genetic material, the DNA. So the difference among the species and within the species can be studied using various molecular markers. ISSR and RAPD are the two commonly used PCR based techniques to study genetic variation among the species. ISSR markers are highly polymorphic and are useful in studies on genetic diversity, phylogeny, gene tagging, genome mapping and evolutionary biology (Pradeep et al., 2002). This technique is useful for phylogeographic analyses or

*Corresponding author. E-mail: utpala@spices.res.in.

Abbreviations: BSI, Botanical Survey of India; ISSR, inter-simple sequence repeats; RAPD, randomly amplified polymorphic DNA; PMB, percentage of monomorphic bands; PPB, percentage of polymorphic bands; PIC, polymorphism information content; PCoA, principle coordinate analysis.

delimiting species. The RAPD technique has received a great deal of attention from population geneticists because of its simplicity and rapidity in revealing DNA-level genetic variation. Congiu et al. (2000) employed RAPD markers for individualization of strawberry. RAPD and ISSR markers were utilized for comparative analysis of genetic diversity in blackgram genotypes (Souframanien and Gopalakrishna, 2004). A work by Seyit et al. (2010) showed a comparison of RAPD and ISSR markers for the genetic characterization of seized *Cannabis sativa* L. The study shows the effectiveness of these molecular markers through various statistical analyses. Similarly, comparison of ISSR and RAPD markers was performed to analyze their efficacy in determining genetic diversity of pistachio (Ayda et al., 2010), apricots (Meetul et al., 2009) and barley (Ferdaous et al., 2012).

The objective of this study was to compare the effectiveness of ISSR, RAPD and ISSR-RAPD combined system to find out the molecular diversity of *Garcinia* species of various geographical regions in India.

MATERIALS AND METHODS

Plant materials and DNA extraction

Leaf samples from a total of 65 plant samples were collected from two distinct geographical locations, namely, North Eastern Himalayan foot hills (namely Assam, Nagaland and Meghalaya) and from Western Ghats (namely Kerala, Tamil Nadu, Karnataka and Maharashtra). The species are *G. indica* Choisy., *G. gummi-gutta* Robs., *G. cowa* Roxb., *G. kydia* Roxb., *G. lanceaeifolia* Roxb., *G. xanthochymus* Roxb., *G. pedunculata* Roxb., *G. morella* Desrouss., *G. nervosa* Miq., *G. dulcis* (Roxb.) Kurz., *G. cornea* Linn. and *G. spicata* (Wight & Arn.) Hook. Information relevant to samples are given in Table 1. Genomic DNA was isolated from the leaves of the plants by modified Doyle and Doyle (1990) method using 4% CTAB buffer containing 1% PVP. Purified DNA was quantified in agarose gel electrophoresis and Eppendorf BioPhotometer and suitably diluted.

Molecular marker profiling

Thirty (30) RAPD primers were used for initial screening, out of which 12 primers were found to be suitable for amplification. The sequences of the primers used are given in Table 2. All the primers were decamers. For ISSR profiling, 55 UBC primers available in the institute were first screened *in-silico*. The selected 19 primers were used for wet-lab studies. The selected primers were 18 and 20-mers of various GA and CA repeats. The sequences of the primers used are given in Table 3. The concentration of reaction mixture components and the PCR conditions were standardized by trials. Reactions were carried out in a total volume of 25 µl containing 50 ng DNA, 1 µl each of primer (20 pM) and dNTP (10 mM) solution, 2.5 µl 10xTaq buffer (without MgCl₂) and 0.2 µl of 5U Taq polymerase from Merck (Genei Pvt. Ltd., Bangalore) was used for the reaction. For RAPD, 1.5 µl of 25 mM MgCl₂ was used while 1 µl for ISSR. The rest of the volume was made up using nuclease free sterile water. For ISSR profiling, amplification was performed under the following conditions: 5 min at 94°C for 1 cycle, followed by 30 s at 94°C, 1 min at annealing temperature (depending on primer) and 1 min at 72°C for 35 cycles. Final extension was done at 72°C for

10 min. For RAPD profiling, denaturation step was performed for 1 min and final extension for 15 min. The rest steps remained unchanged.

PCR products were resolved by agarose gel (1.5% in 1xTAE buffer, containing 1% ethidium bromide) electrophoresis and then documented using Kodak gel documentation-scoring system.

Data analysis

Various molecular profile characteristics such as total number of bands, percentage of monomorphic and polymorphic bands, polymorphic information content (PIC) and Mantel correlation (r) were tabulated separately for RAPD and ISSR profiling. Rectangular binary data matrices were created for ISSR and RAPD profiling, with 1 for the presence of band and 0 for absence, which were used for further statistical studies. Binary matrices of ISSR and RAPD were combined (ISSR-RAPD) to facilitate an analysis of combined ISSR and RAPD markers together as well. Pair-wise similarity matrices were generated using SIMQUAL procedure of NTSYS statistical package. Principal coordinate analyses (PCA) and (PCI) of data were also performed using NTSYS. Cluster analyses for the matrices were performed by means of SAHN procedure via UPGMA to develop dendrograms. Mantel test was performed in order to compare ISSR versus RAPD, ISSR versus ISSR-RAPD and RAPD versus ISSR-RAPD.

RESULTS AND DISCUSSION

In this study, a total number of 19 UBC ISSR primers and 12 UBC RAPD primers were used for the detection of polymorphism. Figures 1 and 2 represent the good polymorphism with UBC -ISSR 810 and UBC-RAPD-PO5. Various molecular profiling characters, namely, percentages of monomorphic bands (PMB) and polymorphic bands (PPB) for each primer and their polymorphism information content (PIC) for ISSR profiling is given in Table 2 and that for RAPD analysis is given in Table 3. The ISSR and RAPD markers used allowed reproducible and informative polymorphisms (Figures 3, 5 and 7). Seyit et al. (2010) also recorded the same type of work with marijuana (*Cannabis sativa* L). Figures 1 and 2 represent the banding pattern by *Garcinia* species indicating considerable level of polymorphism. In ISSR profiling, largest number of monomorphic bands were produced by primers 810 and 815 (3 bands), whereas primers 816 and 848a produced only polymorphic bands. Primers 857a and 857b amplified highest numbers of polymorphic bands with an average of 5.3 and 4.8 bands per sample, respectively. The total number of bands was highest in 857a, producing an average of 7.2 bands per sample. ISSR primers generated a total of 157 with an average of 8 bands per primer, out of which 63 bands were monomorphic. Hakki et al. (2007) also reported effective segregation of species with ISSR. Kojoma et al. (2002) also used ISSR for studying DNA finger printing in *C. sativa*.

RAPD primers generated a total of 134 bands, average of 11.2 per primer, out of which 53 bands were monomorphic. Primers AO12 and AB16 produced largest number of monomorphic bands (two bands). AB01 primer

Table 1. *Garcinia* accessions and corresponding locations.

Sample number	Species	Location	Ecosystem
1, 2, 3, 4, 5, 6, 7, 8 and 9 38, 39 and 40	<i>G. xanthochymus</i>	Assam Meghalaya	
10, 11, 12 and 13 30, 31, 32, 33, 34 and 35 41, 42 and 43	<i>G. kydia</i>	Assam Meghalaya Nagaland	
29 36 and 37	<i>G. cowa</i>	Assam Meghalaya	N. E. Himalayan foothills
14, 15, 16 and 17 18, 19, 20, 21, 22 and 23 24 25 26 27 28	<i>G. pedunculata</i> <i>G. lanceaefolia</i> <i>G. nervosa</i> <i>G. spicata</i> <i>G. morella</i> <i>G. dulcis</i> <i>G. cornea</i>	Assam Assam Assam Assam Assam Assam Assam	
44, 53, 54, 55 and 56 47, 48, 49, 50, 51 and 52 45 and 46	<i>G. gummi-gutta</i>	Karnataka Kerala Tamil Nadu	Western Ghats
60, 64 and 65 61, 62, 63	<i>G. indica</i>	Karnataka Kerala	
57, 58 and 59	<i>G. xanthochymus</i>	Kerala	

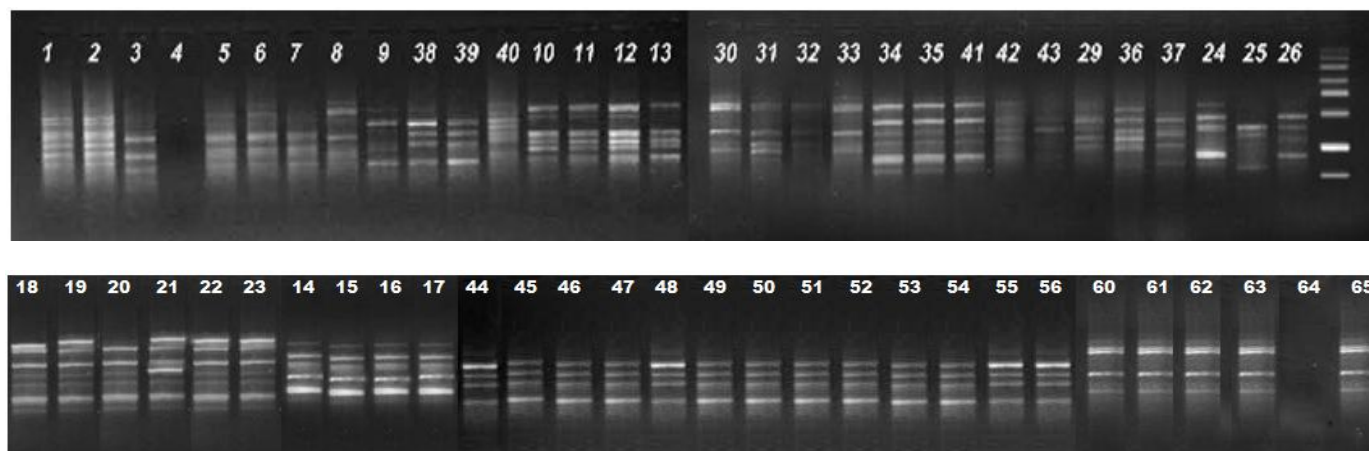
Table 2. ISSR primers and details of the profiling.

Code	Sequence	TNB	PMB	PPB	Band range (bp)	PIC _i
868	(GAA)6	8	56.56	43.44	394.7 - 1384	0.94
816	(CA)8T	7	0.00	100.00	238 - 875	0.90
810	(GA)8T	13	69.31	30.69	263.2 - 933.3	0.95
812	(GA)8A	7	31.11	68.89	217.4 - 730.8	0.74
815	(CT)8G	10	73.43	26.57	522.7 - 2031	0.95
835a	(AG)8CC	5	42.86	57.14	485 - 2000	0.93
835b	(AG)8TC	11	49.62	50.38	272 - 1366.7	0.90
841a	(GA)8CC	6	51.11	48.89	280.6 - 2000.5	0.87
841b	(GA)8TC	16	32.75	67.25	275.4 - 2100	0.92
848a	(CA)8AG	7	0.00	100.00	250 - 890.5	0.80
848b	(CA)8GG	5	24.18	75.82	250 - 933.2	0.90
860a	(TG)7AA	5	59.57	40.43	522.7 - 2200.2	0.95
860b	(TG)8GA	6	42.42	57.58	500.3 - 2100	0.98
861	(ACC)6	8	64.40	35.60	235 - 750.5	0.86
852a	(TC)8AA	8	46.46	53.54	250.8 - 730.6	0.93
857a	(AC)8CG	13	26.82	73.18	510.4 - 2100	0.75
809	(AG)8G	5	50.00	50.00	500 - 2000.2	0.79
840b	(GA)8TT	5	23.75	76.25	395 - 1530.6	0.90
857b	(AC)8TG	12	28.33	71.67	475.5 - 2200.3	0.75
Average		8.2	40.67	59.33		0.86

Table 3. RAPD primers and details of the profiling.

Code	Sequence	TNB	PMB	PPB	Band range (bp)	PIC _i
AB 01	CCGTCGGTAG	18	32.31	67.35	173.2 - 1500	0.90
AA 01	AGACGGCTCC	13	42.61	57.39	160.1 - 3214.2	0.91
AP 20	CCCGGATACA	8	46.54	53.46	200 - 1750.3	0.90
AV 03	TTTCGGGGAG	7	52.30	47.70	110 - 1277.7	0.91
AO 12	TCCCGGTCTC	12	58.78	41.22	525.4 - 3200	0.91
BB 18	CAACCGGTCT	8	31.41	68.59	200.5 - 1800	0.84
AB 16	CCCGGATGGT	12	51.24	48.76	295.6 - 2000	0.86
AB 11	GTGCGCAATG	14	25.33	74.67	300 - 1914.2	0.93
AA 11	ACCCGACCTG	10	33.82	66.18	388.4 - 2000	0.93
W 15	ACACCGGAAC	14	36.03	63.97	200 - 1777.3	0.90
PO 5	CCCGGTAAC	9	29.33	70.67	110 - 1714.3	0.92
AF 11	AAGACCGGGA	9	34.90	65.10	200.5 - 2285.5	0.79
Average		11.2	39.55	60.42		0.89

TNB - Total number of bands, PMB - percentage of monomorphic bands, PPB - percentage of polymorphic bands, PIC_i - polymorphic information content.

**Figure 1.** Gel picture for ISSR profiling using primer 810.

amplified highest number of polymorphic bands with average of 3.2 bands per sample. This primer also produced highest total number of bands (4.3 bands per sample). Percentage of polymorphic loci in RAPD detected was 60.42%, while in ISSR profiling it was 59.33%. Average percentages of monomorphic bands and polymorphic bands for ISSR profiling were 40.6 and 59.3 respectively and for RAPD they were 39.5 and 60.4, respectively. Though there were some variation in the total number of bands, PMB and PPB percentages were almost same. The dendrograms constructed using RAPD and ISSR marker profiling did not show considerable degree of variation with each other as revealed by Mantel correlation ($r = 0.558$). The similarity coefficient ranged 0.66 to 1.00 with ISSR, 0.60 to 0.96 with RAPD and 0.68 to 0.98 for the combined RAPD-ISSR dendrograms. Seyit

et al. (2010) reported a weak correlation of ($r = 0.03$) in ISSR and combined ISSR-RAPD matrix for marijuana of Turkey. A total of seven clusters were observed in dendrogram (Figure 3) for ISSR profiling. *G. xanthochymus* from both ecosystems formed cluster 1. The other clusters were of *G. pedunculata*, *G. lanceaefolia*, *G. kydia*, *G. cowa*, *G. gummi-gutta* and *G. indica*. The clusters of *G. gummi-gutta* and *G. indica* were distinct from the other clusters which contain the species of Himalayan origin. Species with single accessions such as *G. nervosa*, *G. spicata*, *G. morella*, *G. dulcis* and *G. cornea* (24 to 28) which were collected from BSI Sibpur, germplasm were separated out as cluster 3 in Figure 3. Similarly, in the PCoA scattered diagram (Figure 4), the Himalayan species were clustered nearby while Western Ghats species had separated out. Even though *G. xanthochymus*,

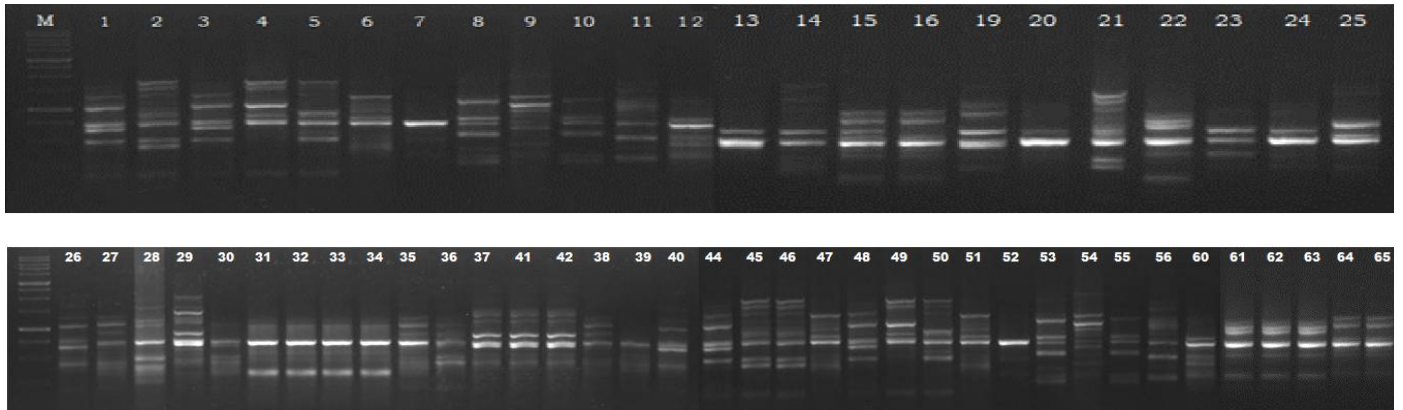


Figure 2. Gel picture for RAPD profiling using primer PO 5.

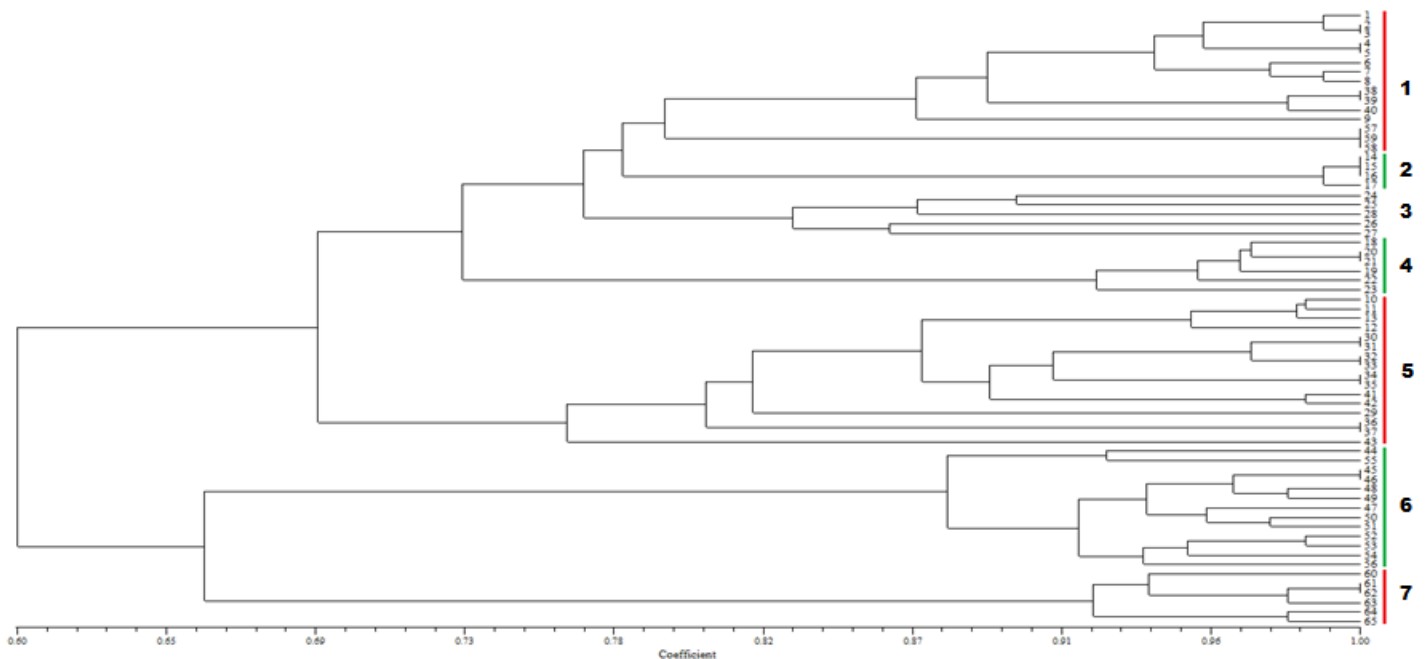


Figure 3. Dendrogram for ISSR profiling.

Cluster 1 - *G. xanthochymus* (1, 2, 3, 4, 5, 6, 7, 8, 9, 38, 39, 40, 57, 58 and 59), 2 - *G. pedunculata* (14, 15, 16 and 17), 3 - (24, 25, 26, 27 and 28 single accession collected from BSI, Kolkata), 4 - *G. lanceaefolia* (18, 19, 20, 21, 22 and 23), 5 - *G. kydia*, *G. cowa* (10, 11, 12, 13, 30, 31, 32, 33, 34, 35, 41, 42 and 43), 6 - *G. gummi-gutta* (44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, and 56) 7 - *G. indica* (60, 61, 62, 63, 64 and 65).

the species collected from Western Ghats was clustered together with the Himalayan species, it showed a slight segregation.

The dendrogram for RAPD profiling also produced 6 clusters (Figure 5), which showed species distinction. The PCoA analysis showed only 3 clusters. In the PCoA analysis, *G. xanthochymus*, of both the region came together while *G. gummi-gutta*/*G. indica* formed a combined cluster (Figure 6). The rest of the species separated out. Here the clustering pattern represented

their origin and natural habitat. In this case distinctions between species were not much clear. The species with single accession (*G. nervosa*, *G. spicata*, *G. morella*, *G. dulcis* and *G. cornea*) were randomly dispersed in the clusters with Himalayan species. Seyit et al. (2010) also got very clear segregation in the PCA analysis of the two (ISSR & RAPD) systems, in their studies on *Cannabis*. In case of the combined cluster of ISSR and RAPD profiling, the pattern of dendrogram (Figure 7) was found similar to that of ISSR profiling. The Western Ghat and Himalayan

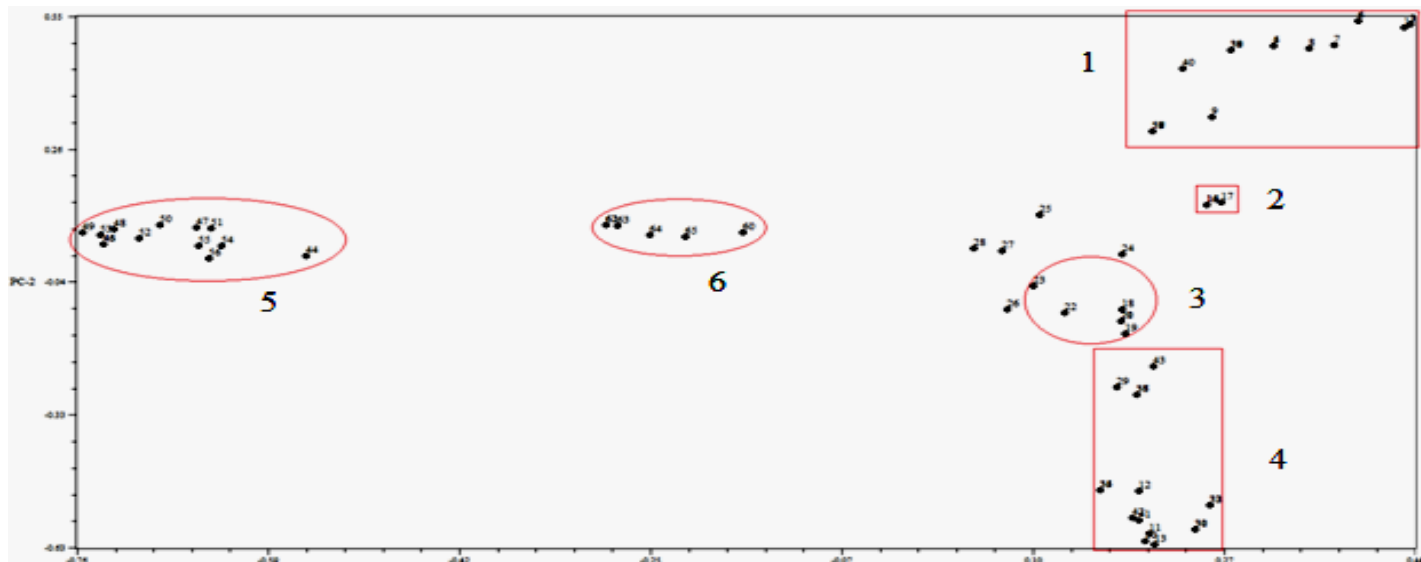


Figure 4. Principle coordinate analysis for ISSR profiling. 1 - *G. xanthochymus* (1, 2, 3, 4, 5, 6, 7, 8, 9, 38, 39, 40, 57, 58 and 59), 2 - *G. pedunculata* (14, 15, 16 and 17), 3 - *G. lanceaefolia* (18, 19, 20, 21, 22 and 23), 4 - *G. kydia*, - *G. cowa* (10, 11, 12, 13, 30, 31, 32, 33, 34, 35, 41, 42 and 43), 5 - *G. gummi-gutta* (44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, and 56), 6 - *G. indica* (60, 61, 62, 63, 64 and 65).

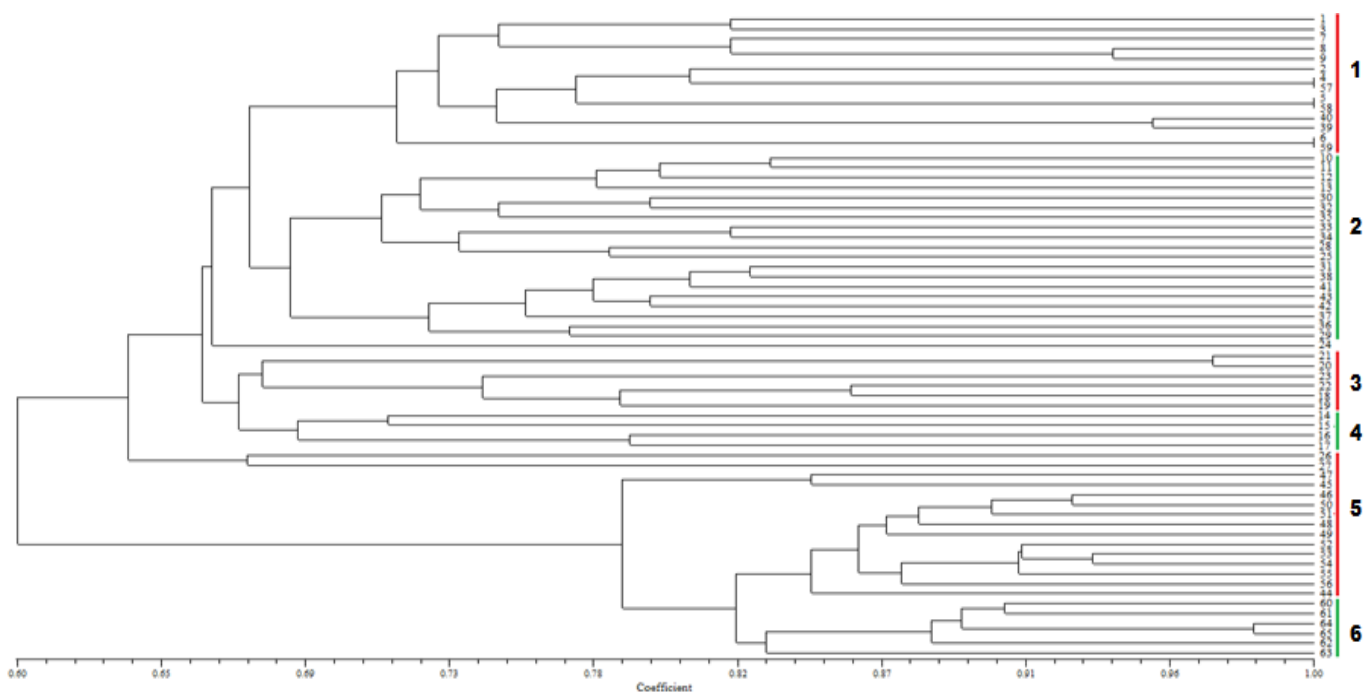


Figure 5. Dendrogram for RAPD profiling. Cluster 1 - *G. xanthochymus* (1, 2, 3, 4, 5, 6, 7, 8, 9, 38, 39, 40, 57, 58 and 59), 2 - *G. kydia*, *G. cowa* (10, 11, 12, 13, 30, 31, 32, 33, 34, 35, 41, 42 and 43), 3- *G. lanceaefolia* (18, 19, 20, 21, 22 and 23), 4- *G. pedunculata* (14, 15, 16 and 17), 5- *G. gummi-gutta* (44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, and 56), 6- *G. indica* (60, 61, 62, 63, 64 and 65).

species were clustered with clear distinction. In the case of PCoA analysis (Figure 8), *G. gummi-gutta* and *G. indica* formed one cluster which is distinctly separated from the clusters formed by other species.

Conclusion

The study indicates that in *Garcinia*, both the marker systems were equally effective. Both ISSR and RAPD

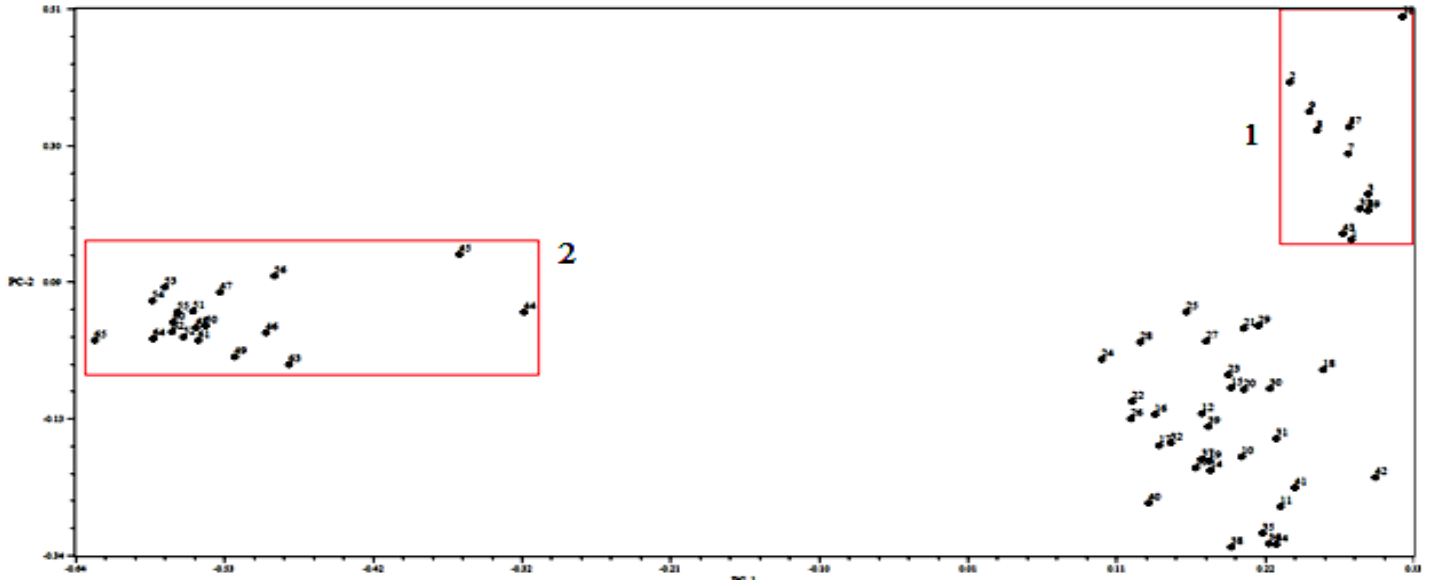


Figure 6. Principle coordinate analysis for RAPD profiling. Cluster 1- *G. xanthochymus* (1, 2, 3, 4, 5, 6, 7, 8, 9, 38, 39, 40, 57, 58 and 59), 2 - *G. gummi-gutta* and *G. indica* (44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 60, 61, 62, 63, 64 and 65).

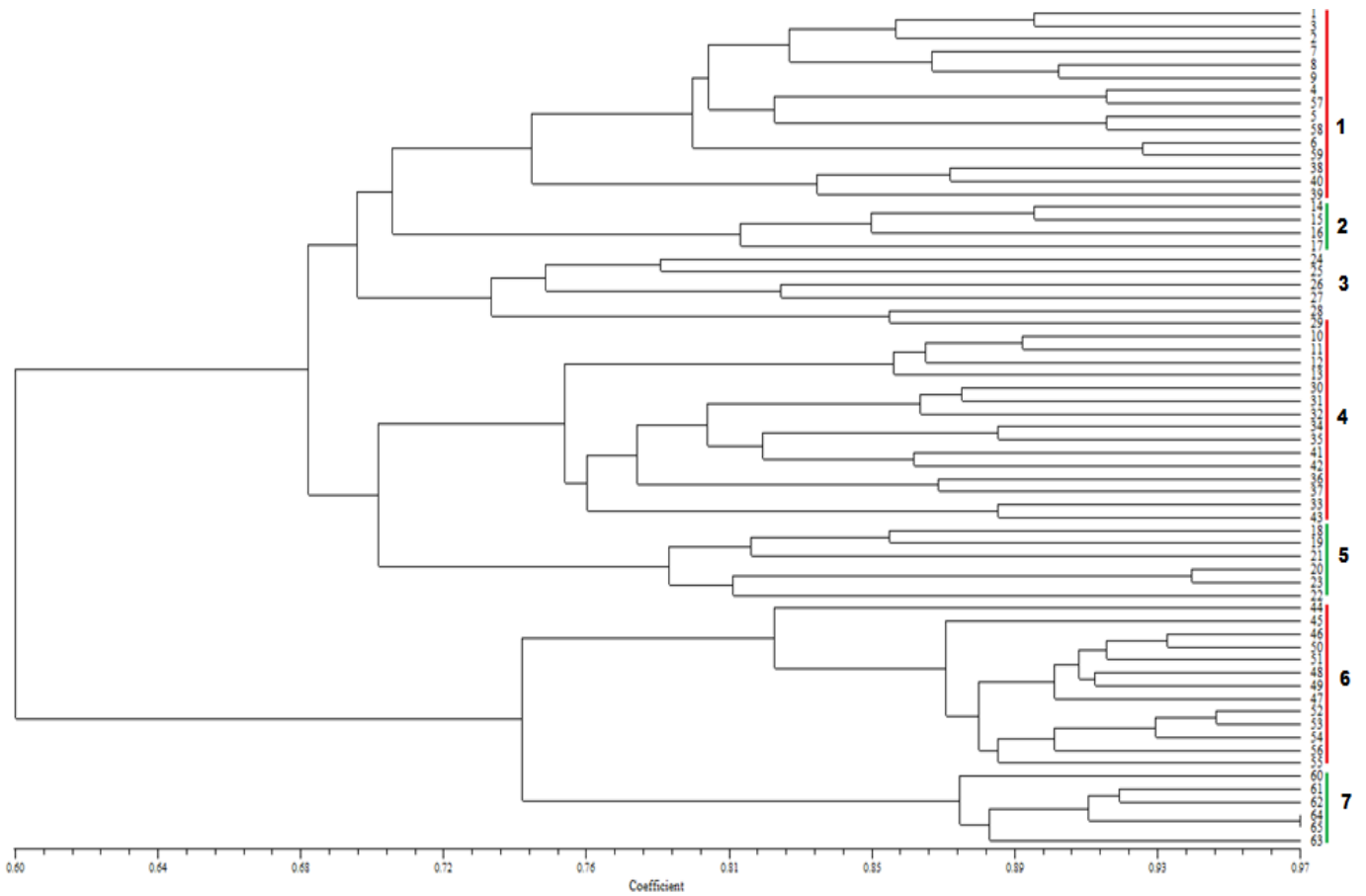


Figure 7. Dendrogram for RAPD-ISSR combined data matrix. Cluster 1 - *G. xanthochymus* (1, 2, 3, 4, 5, 6, 7, 8, 9, 38, 39, 40, 57, 58 and 59), 2 - *G. pedunculata* (14, 15, 16, and 17), 3 - (24, 25, 26, 27, and 28), 4 - *G. lanceaefolia* (18, 19, 20, 21, 22, and 23), 5 - *G. kydia*, *G. cowa* (10, 11, 12, 13, 30, 31, 32, 33, 34, 35, 41, 42, and 43), 6 - *G. gummi-gutta* (44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, and 56), 7 - *G. indica* (60, 61, 62, 63, 64 and 65).

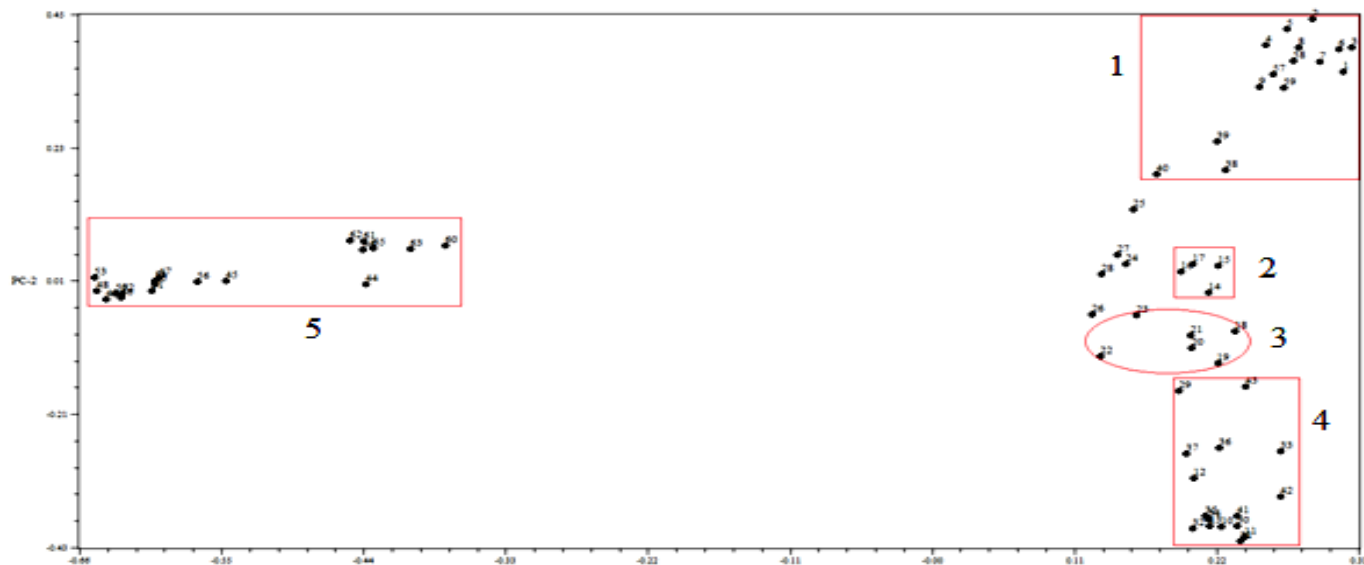


Figure 8. Principle coordinate analysis for RAPD-ISSR combined data. Cluster 1 - *G. xanthochymus* (1, 2, 3, 4, 5, 6, 7, 8, 9, 38, 39, 40, 57, 58 and 59), 2 - *G. pedunculata* (14, 15, 16 and 17), 3 - *G. lanceaefolia* (18, 19, 20, 21, 22 and 23), 4 - *G. kydia*, *G. cowa* (10, 11, 12, 13, 30, 31, 32, 33, 34, 35, 41, 42, and 43), 5 - *G. gummi-gutta*, *G. indica* (44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 60, 61, 62, 63, 64 and 65).

markers produced polymorphism and proved helpful for diversity studies. PCR based marker systems are best to analyze genetic relationship of crops (Milbourn et al., 1997; Patzak, 2001). It is interesting to note that average band for ISSR was 8.2, while that of RAPD was 11.2. The highest BP value in case of ISSR was 2200 kb, and 9 primers showed bands more than 2000 kb. In case of RAPD, the highest bp value was 3214 kb and in all the samples highest value is more than 1500 kb. In case of lowest bp value, RAPD showed 110 kb and ISSR was 235 kb. In comparison to ISSR, RAPD gave larger ranges of bands. ISSR primers could give a distinct segregation based on the species of the sample, whereas RAPD primers produced clustering based on geographical location of the samples (Jagadish et al., 1996). PIC values for both the marker systems are very close, indicating the suitability of both the marker types in molecular studies.

This study indicates that combined information of ISSR and RAPD markers could be a better tool in studying molecular diversity of *Garcinia*, especially as it does not have adequate genomic information to design specific markers (Seyit et al., 2010). It can be concluded from the present study that ISSR markers could be used to identify the unknown species in *Garcinia*, whereas RAPD markers can be used to determine the variation based on geographical or morphological features within the same species.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. R. K. Bhattacharya, Professor of Horticulture, Assam Agricultural University,

Jorhat for his help in arranging the visits to various places in Assam for the survey on *Garcinia*. Authors acknowledge CSIR-HRD group for granting fellowship to O. P. Nandakishore.

REFERENCES

- Ayda T, Jafar A, Rahim H, Mahdi Z (2010). A comparative analysis of ISSR and RAPD markers for studying genetic diversity in Iranian pistachio cultivars. *Iranian J. Genet. Plant Breed.* 1(1):9-13.
- Congiu L, Chicca M, Cella R, Rossi R, Bernacchia G (2000). The use of random amplified polymorphic DNA (RAPD) markers to identify strawberry varieties: A forensic application. *Mol. Ecol.* 9(2):229-232.
- Ferdaous G, Walid E, Hédia H (2012). The Use of ISSR and RAPD Markers for Genetic Diversity among South Tunisian Barley. *ISRN Agronomy.*, Article ID 952196: 10.
- Hakki EE, Kayis SA, Pinarkara E and Sag A (2007). Inter simple sequence repeat separate efficiently Hemp from Marijuana (*Cannabis sativa*). *Elect. J. Biotechnol.* 10(4):570-581.
- Hedrick P (1992). Shooting the RAPDs. *Nature* 355:679-680.
- Jagadish V, Robertson J, Gibbs A (1996). RAPD analysis distinguishes *Cannabis sativa* samples from different sources. *Forensic Sci. Int.* 79(2):113-121
- Kojoma M, Iida O, Makino Y, Satake M (2002). DNA fingerprinting of *Cannabis sativa* using inter-simple sequence repeat amplification. *Planta Medica* 68(1):60-63.
- Meetul K, Gyan PM, Raghwendra S, Jitendra K, Pradeep KN, Shashi BS (2009). Correspondence of ISSR and RAPD markers for comparative analysis of genetic diversity among different apricot genotypes from cold arid deserts of trans-Himalayas. *Physiol. Mol. Biol. Plants.* 15(3):225-236.
- Milbourn D, Meyer R, Bradshaw JE, Braid E, Bonar N, Waugh R (1997). Comparison of PCR based marker systems for analyzing of genetic relationships in cultivated potato. *Mol. Breed.* 3(2):127-136.
- Osman MB, Rahman MA (2006). *Mangosteen-Garcinia mangostana*. Southampton Centre for Underutilised Crops, University of Southampton. pp. 17-28.
- Patzak J (2001). Comparison of RAPD, STS, ISSR, and AFLP molecular methods used for assessment of genetic diversity in hop

- (*Humulus lupulus* L.) Euphytica, 121(1):09-18.
- Peter KV, Abraham Z (2007). Biodiversity in Horticultural Crops. Volume 1. Daya Books, New Delhi. pp. 264-267.
- Pradeep RM, Sarla N, Siddiq EA (2002). Inter simple sequence repeat (ISSR) polymorphism and its application in plant breeding. Euphytica 128:9-17.
- Seyit AK, Erdogan EH, Emine P (2010). Comparison of effectiveness of ISSR and RAPD markers in genetic characterization of seized marijuana (*Cannabis sativa* L.) in Turkey. Afr. J. Agri. Res. 5(21):2925-2933.
- Souframanien J, Gopalakrishna T (2004). A comparative analysis of genetic diversity in blackgram genotypes using RAPD and ISSR markers. Theor. Appl. Genet. 109(8):1687-1693.
- The Plant List (2010). Version 1. Published on the Internet; <http://www.theplantlist.org>

Full Length Research Paper

Sensory evaluation of different preparations of cassava leaves from three species as a leafy vegetable

M. G. Umuhozariho^{1,2}, N. B. Shayo¹, P. Y. K. Sallah² and J. M. Msuya¹

¹Sokoine University of Agriculture, Faculty of Agriculture, Department of Food Science and Technology, P.O. Box 3006, Morogoro, Tanzania.

²National University of Rwanda, Faculty of Agriculture, P.O. Box 117, Huye, Rwanda.

Accepted 17 October, 2013

Cassava leaves are largely consumed in Africa and are among the top three African indigenous vegetables rich in nutrients. Leaves from bitter (*Manihot utilissima*), sweet (*Manihot dulcis*) and wild (*Manihot glaziovii*) species of cassava were cooked by boiling in salted (sodium bicarbonate and table salt) water with the addition of palm oil and ground-nut paste, following processing by “pounding”, “pounding and then drying” and, “drying and then pounding”. The drying was done in tunnel solar drier at temperature of 65°C on average. Nine samples (three species x three processing methods) were evaluated by 31 panelists, using a five point hedonic scale, where 5 = like very much and 1= dislike very much. Cassava species affected significantly ($p = 0.0047$; 0.0206) scoring for texture and overall acceptability, respectively, but not for colour, aroma and taste. Processing method highly significantly ($p < 0.0001$) affected all the sensory attributes scoring. Leaves from all three species were liked as leafy vegetable, except when pounded after drying.

Key words: Cassava leaves, cassava species, sensory characteristics, tunnel solar drying, processing methods, Rwanda.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is one of the staple food crops in Africa, it is a rainy fed crop grown mainly in humid and sub humid regions, but being particularly suited to conditions of low nutrients availability of soils and able to survive drought, Cassava became one of the most important food crops in almost entire countries within the tropics (Huzsvai and Rajkai, 2009; El-Sharkawy, 2007). For instance in Rwanda, cassava is the third among main food crops after banana and sweet potatoes, and is one of the priority crops that are being promoted for economic development and poverty reduction in the agricultural sector (MINECOFIN, 2007). In some countries of West and Central Africa, cassava roots and leaves are the basic foods. The Congolese consider the cassava as “all sufficient” because the cas-

sava starchy roots are complemented nutritionally by cassava leaves, which are good source of protein, vitamins and mineral (Bradbury and Denton, 2011). Austin et al. (2009) after realizing the value and utilization of cassava leaves as green vegetable, he placed them among Rwanda's high value vegetables that have potential on domestic, regional and international markets.

Cassava leaves are among the top three African indigenous vegetables rich in nutrients. They are the second in β -carotene after *Moringa oleifera*, the second in vitamin C after *Moringa stenopetala*, the third in vitamin E after *M. stenopetala* and *M. oleifera*, the third in zinc after *Pterocarpus mildbraedii* and *M. oleifera*, the third in antioxidant activity after *Adansonia digitata* and *Rorippa madagascariensis* and the third in total phenolic after

R. madagascariensis and *A. digitata* (Shackleton et al., 2009). Micronutrients, as well as the many non-nutrient phytochemicals in vegetables are associated with health maintenance and prevention of chronic diseases (Yang and Keding, 2009; Steinmetz and Potter, 1996). For that healthy and nutrition importance, cassava leaves consumption may be improved by preservation methods that can increase its availability and quality.

Cassava leaves, like many other leafy vegetables, are generally seasonal with surpluses in the rainy season and scarcity with high costs in the dry season. Their perishability causes a considerable amount of post-harvest losses in rainy season. To minimize the losses and stabilize availability and price in different markets and seasons, appropriate preservation methods, affordable to rural communities, are needed. According to the study of Thomas (2008), spoilage of food is due to three main causes: Microorganisms, enzymes and chemical reactions. Drying, especially sun-drying is among the oldest and cheapest preservation methods that slow down or completely stop food deterioration by removing available water, the principal factor of food deterioration. As reported by Umuhozariho et al. (2011), in some rural areas of Rwanda, especially in dry regions, where cassava is a main food crop, leaves are dried on mats in open air, either pounded or un-pounded, for increasing shelf life. However, only fresh cassava leaves are sold in different local markets in villages, cities and super markets, the dry products being consumed at family levels. To stimulate people to process market oriented product, the dry cassava leaves need to be tested for physical quality and acceptability.

The direct exposure to sunlight is known to reduce the quality (colour and vitamin contents) of the final product (MMA, 2008). The product is open to various contaminations such as dust, insects, wetness and rain. Moreover, the process is very dependent on good weather, and the very slow drying rates of the process create the danger of mould growth. In contrast, solar dryers are simple installations that can eliminate the negative effects of open air sun drying and thus seems to be the most promising. Solar drying offers the following advantages over sun drying when correctly designed: Faster drying rate, greater retention of vitamins, especially vitamins A and C, minimizing damage from rain, protection against infection among others, and also some advantages over the conventional drying with respect to cost and adaptability to small scale farmers (Eze, 2010; Ferreira et al., 2008). According to literatures, solar drying gives faster drying rates by heating the air to 10-30°C above ambient temperature, using solar energy collectors with natural or forced airflow inside the dryers, and thus increasing their efficiency (Eze, 2010; Lotfalian et al., 2010; Ferreira et al., 2008). In addition, solar dryers are a promising means for tropical countries to meet their requirements as the available amount of solar energy in most cases is sufficient to cover the energy requirements for small

dryers (Eze, 2010).

The present study was conducted to assess the usefulness of tunnel solar drying for preserving sensory qualities of cassava leaves and determine which cassava species and processing procedures are preferable for better physical properties and acceptability, as food relishes after leaves are cooked.

MATERIALS AND METHODS

Materials

Collection of cassava leaves

In April 2012, tender cassava leaves were harvested from three species of cassava, bitter (*Manihot utilissima*), sweet (*Manihot dulcis*) and wild (*Manihot glaziovii*). Varieties named Igicucu, Seruruseke (5280) and ISAR 1961 were chosen for wild, sweet and bitter cassava species respectively (Figure 1). In order to minimize the effects of age, environment and soil types on sensory characteristics, leaves samples of the same age were selected from the same field, Rwanda Agricultural Board (RAB)'s field at the Karama Research Station, in Bugesera District of Eastern Province of Rwanda.

Experimental design of sample preparation

The three cassava species as source of vegetable and three processing methods in a completely randomized design (CRD) were evaluated for sensory attributes in one session. The preparation procedures used to prepare leaves from each cassava species before cooking were: Pounding fresh leaves, drying pounded leaves, and drying un-pounded leaves (Figure 2). In this study, both fresh and dried leaves were pounded using traditional woody mortar and pestle. In addition, leaves were blanched before drying and blanching was carried out according to the method described by Kendall et al. (2010). Thus, leaves were submerged in boiling water for 4 min, and then immediately cooled in tap water at ambient temperature. Before drying, pounded and un-pounded leaves were kept in closed polyethylene bags and were stored on ice in an ice-chest for direct transportation to the solar drying station at Sokoine University of Agriculture, Morogoro, Tanzania.

Drying procedures

Samples of pounded and un-pounded leaves from bitter, sweet and wild cassava were dried to brittle using a tunnel solar dryer (Figures 3 and 4). The complete drying was when sample leaves became entirely brittle. Green vegetables contains less sugar, and can be dried to brittle and water content 4-8%, depending on the type of vegetable (James and Kuipers, 2003). The time taken to complete drying for each sample was noted. The temperature inside the tunnel solar dryer was recorded at 8 a.m., noon and 8 p.m. each day. After drying, samples were packed in plastic materials, sealed and stored in opaque cartons at ambient temperature before cooking for sensory evaluation.

Cooking procedures

Similar cooking procedures were followed for all the nine samples. Thus, processed samples were boiled in salted (sodium bicarbonate and table salt) water, with the addition of palm oil and ground-



Figure 1. Sample leaves from sweet (left), wild (middle) and bitter (right) cassava species.

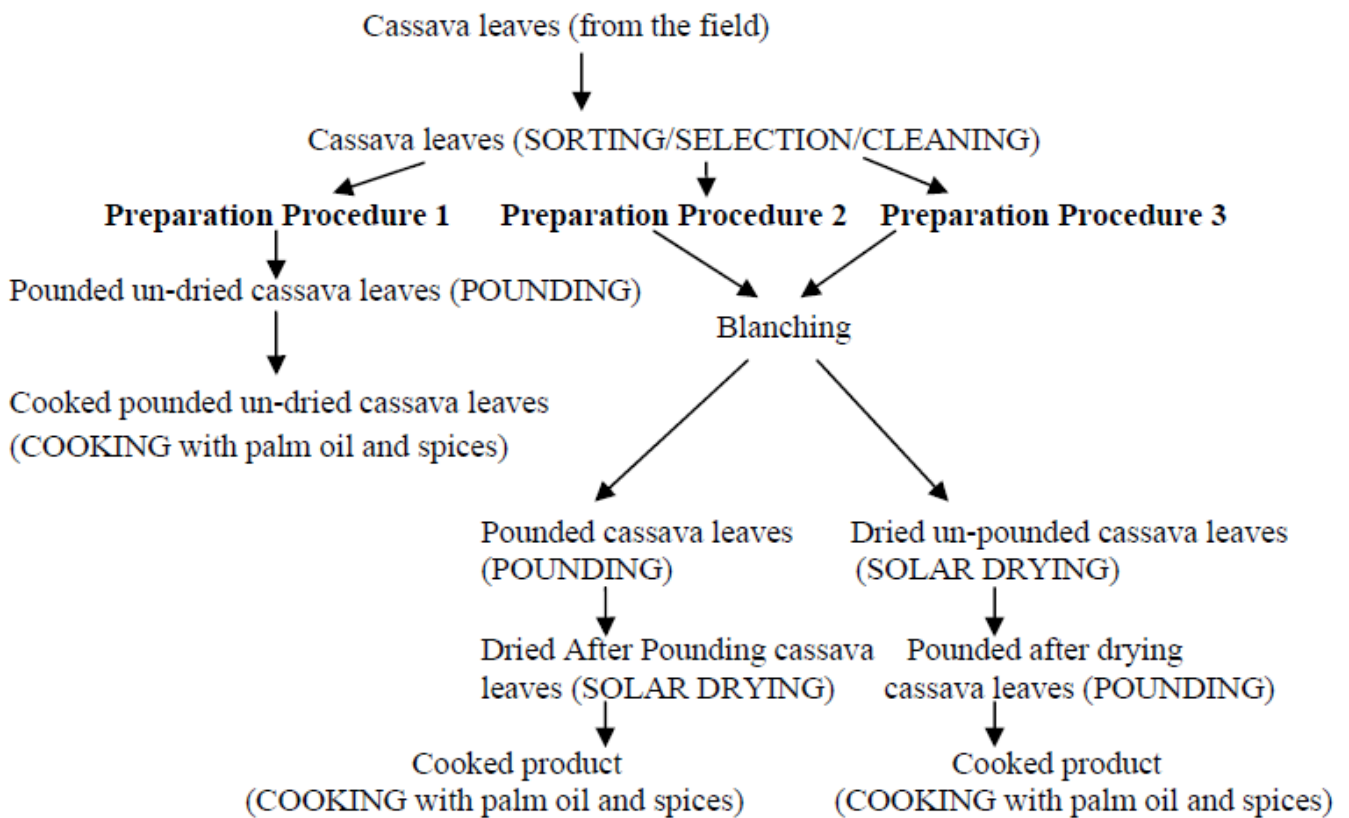


Figure 2. Flow diagram illustrating preparation procedures of cassava leaves.



Figure 3. Tunnel solar dryer covered after placing samples for drying.

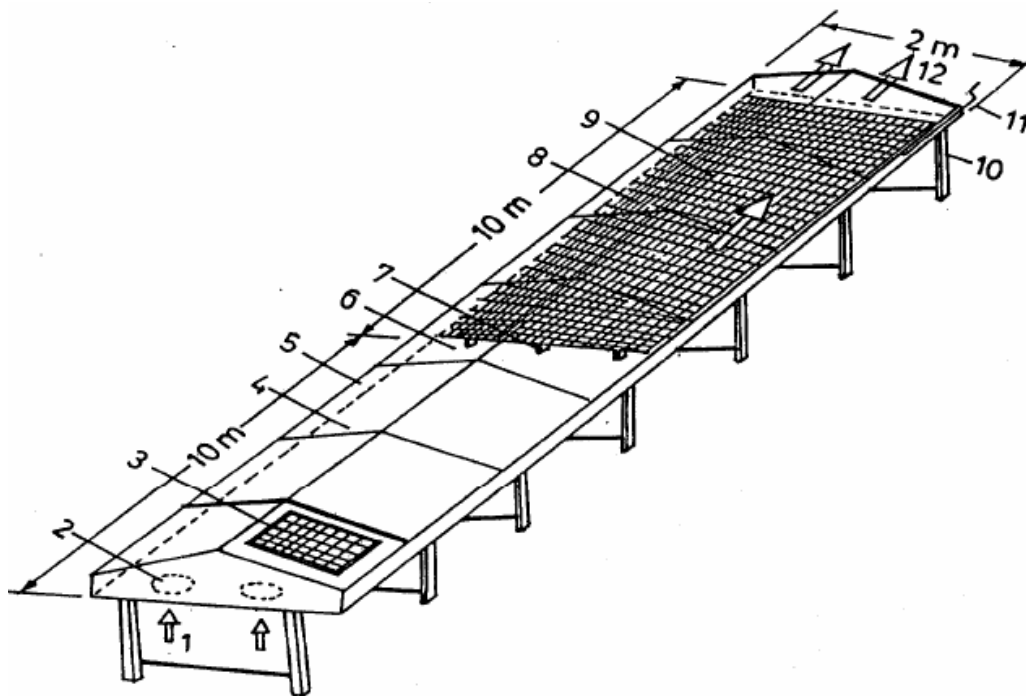


Figure 4. Diagram of the tunnel solar drier. 1, Air inlet; 2, fan; 3, solar module; 4, solar collector; 5, side metal frame; 6, outlet of the collector; 7, wooden support; 8, plastic net; 9, roof structure for supporting the plastic cover; 10, base structure for supporting the tunnel drier; 11, rolling bar; 12, outlet of the tunnel drier.

Table 1. Significance levels from the analyses of variance and for main factors and their interactions on sensory characteristics of cooked cassava leaves as vegetable.

Parameter	Sensory attributes and their p-values				
	Color	Taste***	Aroma	Texture	Overall acceptability
Cassava species	1.1308	0.4890	0.0860	0.0047	0.0206
Processing methods	<.0001	<.0001	<.0001	<.0001	<.0001
Species x methods	0.7526	-	0.0312	0.0733	0.0502
Age group	0.0010	0.1581	<.0001	0.0013	0.0007
Gender	0.9012	0.5898	0.8476	0.0003	0.9040
Age group x Gender	0.1246	-	0.0081	0.0597	0.0908
R ²	0.78	-	0.85	0.90	0.88
CV	9.36	-	10.70	6.65	9.50

R²: Coefficient of determination; CV: coefficient of variation; *Kruskal Wallis test rather than ANOVA and thus no R² nor CV.

nut paste, for about 45 min.

Sensory evaluation

A panel of 31 adults (aged above 21 years old), were purposively selected for sensory evaluation. All the participants were familiar with cassava leaves meals even if they were oriented on making inferences and recording the scores for each sample. The panel comprised of females and males of age ≥ 21 years old. A five point hedonic scales as described by Larmond (1977) were used, where 5 = like very much, 4 = like moderately, 3 = neither like nor dislike, 2 = dislike moderately and 1 = dislike very much. The nine samples were served in identical containers, coded with 3 digit random numbers and presented to panellists in one session. The sensory attributes of interest were colour, taste, texture or mouth feel, aroma and overall acceptability. Necessary precautions were taken to reduce crossover effects by selecting greater number of interested panellists rather than motivating panellists and using small number and repeating preparations. In addition, panellists rinsed their mouths with water before tasting the next sample. Out of the 31 panellists, 61% were females and 39% were males. Their ages ranged between 21 and 57 years. Out of 19 females, 47% were young (< 35years) and 53% aged (≥ 35 years) while among males, 75% were young and 25% aged. The session was held in one of the laboratories of the Faculty of Agriculture, National University of Rwanda (NUR), from 11 a.m. to 14 p.m., in a uniform and natural lighting environment.

Statistical analysis

Data analysed included time of drying, temperature inside the tunnel solar dryer and scores for different sensory attributes per sample. After averaging the scores across the five sensory attributes, the "Kolmogorov-Smirnov" test to assess the normality of the averages was carried out as suggested by Kutner et al. (2005). Multiple ways analysis of variance (ANOVA) was applied to sensory attributes with normally distributed averages and, Kruskal Wallis nonparametric test was done for sensory characteristics with strong departure from normal distribution (Kutner et al., 2005). Statistical Analysis Systems (SAS) software, version 9.2 (SAS Institute, 2008) was used to compute ANOVA (F-test statistics), Fisher's Least Significant Difference (LSD) to separate means, and Kruskal Wallis test. The treatments were judged significantly different and highly significantly different at $p < 0.05$ and $p < 0.01$, respectively.

RESULTS AND DISCUSSION

On average, the temperature inside the tunnel solar dryer was 65°C even if the mean monthly temperature of the area was 25°C. The duration of drying was 14 and 16 h under sunny conditions for un-pounded and pounded cassava leaves, respectively. These times of drying of less than one day under the sunny conditions concurred with reports from several studies that solar dryers improve efficiency and quality of the dried food products by increasing temperature of drying and decreasing period of drying. For a tunnel solar dryer, products receive energy both from hot air supplied from the collectors and from incident solar radiation. This increases the temperature inside the dryer and accelerates the drying process (Almhanna, 2012; Banout and Ehl, 2010; Lotfalian et al., 2010; Medugu, 2010; Ferreira et al., 2008).

The effect of processing methods, cassava species, panellists' gender and ages, and their pair wise interactions on various sensory attributes are shown in Table 1. Kolmogorov-Smirnov test indicated that only taste score averages showed a strong departure from normality and Kruskal Wallis test was done. Processing method had high ($p < 0.0001$) significant effects on all the sensory characteristics (colour, taste, aroma, texture and overall acceptability). Cassava species did not significantly influence colour, aroma and taste. In contrast, the species significantly ($p = 0.0206$) influenced overall acceptability and highly significantly ($p = 0.0047$) influenced texture of cooked relishes. Cassava species and processing methods interaction effects were significant for aroma with $p = 0.0312$. Age group of panellists had considerable effects on scoring of different sensory attributes with p-values of 0.0010, < 0.0001, 0.0013 and 0.0007 for color, aroma, texture and overall acceptability, respectively. Gender did not have any significant effect on the averages of the colour, taste, aroma and overall acceptability scoring but highly

Table 2. Means for sensory characteristics for leaves from different cassava species and processing methods.

Parameter		Sensory attributes and their scores				
		Colour	Taste	Aroma	Texture	Overall acceptability
Species	Bitter	4.04 ^a	3.72 ^a	3.71 ^a	3.79 ^a	3.77 ^a
	Sweet	4.03 ^a	3.95 ^a	3.95 ^a	3.86 ^a	3.85 ^a
	Wild	3.76 ^a	3.57 ^a	3.57 ^a	3.64 ^b	3.46 ^b
Processing methods	Dried and then pounded	3.40 ^c	2.90 ^c	2.90 ^c	3.06 ^c	2.87 ^c
	Fresh	4.58 ^a	4.36 ^a	4.38 ^a	4.28 ^a	4.45 ^a
	Pounded and then dried	3.86 ^b	3.94 ^b	3.95 ^b	3.65 ^b	3.76 ^b
Age groups	Aged (≥ 35)	4.17 ^a	3.91 ^a	4.07 ^a	3.81 ^a	3.91 ^a
	Young (<35)	3.71 ^b	3.55 ^b	3.41 ^b	3.52 ^b	3.48 ^b
Gender	Female	3.93 ^a	3.75 ^a	3.73 ^a	3.83 ^a	3.70 ^a
	Male	3.95 ^a	3.71 ^a	3.76 ^a	3.49 ^b	3.69 ^a

For each sensory characteristic, within column, values with the same letter were not significantly different ($p < 0.05$).

influenced texture scoring with a p-value of 0.0003. Age group and gender interaction effects were highly significant ($p = 0.0081$) for only aroma.

Cassava species were equally liked for colour, taste and aroma by panellists (Table 2). Texture and overall acceptability were rated differently with sweet and bitter species being equally liked and the wild species the least preferred (Table 2). During pounding, it was observed that fresh wild cassava leaves were juicier when compared to sweet and bitter. The crude water made pounding hard and particles larger. The less liked texture has been attributed to the larger particles after pounding and cooking. The study showed that leaves from sweet and bitter cassava species were liked more than those from the wild species, although Umuhozariho et al. (2011) reported earlier that leafy vegetable of wild cassava species were more utilized as human food in Rwanda. The reason may be that authors were concerned with consumer habits with respect to cassava leaves which were more subjective, and one of the reported reasons was the availability of wild cassava leaves as the farmers are more interested in root production than leaves for bitter and sweet species. This is the first study of sensory comparison of leaves from different species of cassava in Rwanda, clearly showing leaves from wild cassava species are less preferred by panellists compared to those from cultivated species.

Panellists above 35 years of age liked all preparations of the vegetables for all the sensory attributes more than those less than 35 years (Table 2). This was not surprising as Larmond (1977) mentioned age and sex of panellist among important factors that can influence result in sensory test.

Colour of the different vegetable preparations differed significantly according to processing methods, but not to species (Table 2). In this study, all samples were cooked in salted (sodium bicarbonate and table salt) water, but blanching had been done in un-salted water before

drying. Cooked fresh leaves became bright-green while dried ones were dark-green. Heating green vegetables in an alkaline solution such as sodium bicarbonate (NaHCO_3) make the cooking water slightly basic, the magnesium ion is retained in the chlorophyll and the colour is a bright-green. In contrast, when vegetables are heated without the alkaline, such as blanching before drying as in the present study, part of their cells are disrupted and some organic acids are released and react with chlorophyll. The reaction with the acids replaces the magnesium atom (Mg) of chlorophyll with a hydrogen atom (H) to form an unattractive dark-green pigment pheophytin (FAO, 1995). The unlikeable colour was probably due to the formation of pheophytin in dry cooked cassava leaves.

Fresh cassava leaves were the most liked, followed by the "pounded and then dried". In another investigation, Mepba et al. (2007) found that panellists preferred fresh to dry vegetable soup. "Dried before pounding" was the least liked for all sensory attributes (Table 2). The poor rating of "dried before pounding" products for all sensory characteristics were attributed to their bitterness as commented by panellists.

Taste and aroma were highly correlated with overall acceptability, with $r = 0.93$, $p < 0.0001$ and $r = 0.91$, $p < 0.0001$, respectively. Taste and aroma were also much correlated ($r = 0.90$, $p < 0.0001$) (Table 3). High correlation between taste, aroma and overall acceptability is not surprising. Taste perception has been suggested to play a key role in determining individual food preferences and dietary habits (IUFoST, 2012; Garcia-Bailo et al., 2009). Equally, Clark (1998) reported a similar relation of a strong influence of taste and aroma (odour and flavour) on food acceptability, and that the two sensory characteristics are considered the key of food choice. The high positive correlation among the sensory characteristics, especially taste, aroma and overall acceptability (Table 3), suggests that the unlikeable bitter

Table 3. Linear correlation among sensory characteristics of processed leafy vegetables from three cassava species.

Sensory attribute		Colour	Taste	Aroma	Texture	Overall acceptability
Colour	r	1	0.8198	0.7764	0.7619	0.8881
	Prob > r		<.0001	<.0001	<.0001	<.0001
Taste	r		1	0.9059	0.8673	0.9393
	Prob > r			<.0001	<.0001	<.0001
Aroma	r			1	0.7949	0.9109
	Prob > r				<.0001	<.0001
Texture	r				1	0.8569
	Prob > r					<.0001
Overall acceptability						1

r, Linear correlation coefficient; Prob > |r|, probability of having a correlation factor equal to or larger than the obtained r.

taste could be the only source of “dried before pounding” vegetables rejection. Also when leaves were pounded after they were dried, particles were very fine and gave a soup-like texture after cooking, which was less liked by panellists.

Bitterness in the “dried before pounding” cassava leaves may possibly be attributed to high levels of residual cyanogens. Karlun et al. (2004) demonstrated a strong correlation between bitter taste and cyanogen (HCN) potential in cassava. Awoyinka et al. (1995) reported that blanching does not change HCN-potential, but pounding or grinding reduces both HCN-potential and tannins. As in the present study, the leaves were blanched to limit degradation of nutrients and colour, endogenous enzymes, linamarase and hydroxynitrile lyase, important in linamarin and acetone cyanohydrin hydrolysis, were also deactivated. But significantly, linamarin and the break down product of linamarin, cyanohydrin can decompose spontaneously at high temperatures or pH 4 and above, to release HCN, harmful to human health, but volatile during preparations (Cereda and Mottos, 1996; Mkpong et al., 1990). Cyanogen levels were not analysed in this study, an additional study for safety of the products is necessary.

Conclusion

Leaves from all the three species of cassava found in Rwanda (bitter, sweet and wild) were liked for food as green vegetables. However, leaves from wild species were less preferred for texture and overall acceptability than those from sweet and bitter species. Processing methods were a strong source of differences in sensory attributes and fresh leaves were the best, followed by “pounded before drying” leaves for all the sensory attributes. Therefore, for marketability and preservation issues, cassava leaves can be processed by solar drying, and preferably pounded before they are dried for a better

taste and texture. Blanching in alkaline water is appropriate to preserve the light-green colour, preferred by consumers. Though un-pounded leaves dried faster and pounding after drying was easier, they were poorly rated, especially for taste, aroma and overall acceptability. An additional study on cyanide and nutrients of the processed cassava leaves is highly recommended to ensure nutritional quality and safety of the products for human consumption.

ACKNOWLEDGEMENTS

This study was supported by grant from the Swedish International Development Cooperation Agency (Sida) through the Research Commission of the National University of Rwanda. They are acknowledged. The authorizations for using Sokoine University of Agriculture’s solar drying station and Rwanda Agricultural Board’s cassava field are gratefully acknowledged.

REFERENCES

- Almhanna EA (2012). Utilization of a solar greenhouse as a solar dryer for drying dates under the climatic conditions of Eastern province of Saudi Arabia. *J. Agric. Sci.* 3 (4):237-246.
- Austin J, Ravry C, Ronchi L, Turner A, Norton R, Bicali V, Kyambadde S, Webber M, Jones C, Cavanaugh G (2009). Study on marketing, post-harvest and trade opportunities for fruits and vegetables in Rwanda. World Bank. Kigali. Rwanda.
- Awoyinka AF, Abegunde VO, Adewusi SRA (1995). Nutrient content of young cassava leaves and assessment of their acceptance as green vegetable in Nigeria. *Plant Foods Hum. Nutr.* 47:21-28.
- Banout J, Ehl P (2010). Using a double-pass solar dryer for drying of bamboo shoots. *J. Agric. Rural Dev. Trop. Subtrop.* 2 (111):119-127.
- Bradbury JH, Denton IC (2011). Mild methods of processing cassava leaves to remove cyanogens and conserve key nutrients. *Food Chem.* 127:1755-1659.
- Cereda MP, Mattos MCY (1996). Linamarin: the toxic compound of cassava. *J. Venom. Anim. Toxins* 2 (1):6-12.
- Clark JE (1998). Taste and flavour: their importance in food choice and acceptance. *Proc. Nutr. Soc.* 57:639-643.

- El-Sharkawy MA (2007). Physiological characteristics of cassava tolerance to prolonged drought in the tropics: Implications for breeding cultivars adapted to seasonally dry and semiarid environments. Centro Internacional de Agricultura Tropical (CIAT). Cali, Colombia.
- Eze JI (2010). Evaluation of the efficacy of a family sized solar cabinet dryer in food preservation. *Am. J. Sc. Ind. Res.* 1(3):610-617.
- FAO (1995). Fruit and vegetable processing. FAO Agricultural Services Bulletin no 119. Rome, Italy.
- Ferreira AG, Maia CB, Cortez MFB, Valle RM (2008). Technical feasibility assessment of a solar chimney for food drying. *Sol. Energ.* 82:198-205.
- Garcia-bailo B, Toguri C, Eny KM, El-Sohehy A (2009). Genetic variation in taste and its influence on food selection. *OMICS* 13 (1):69-80.
- Huzsvai L, Rajkai K (2009). Modeling of plant adaptation to climatic drought induced water. Institute of Botany. *VERSITA Biologia* 64:536-540.
- IUFoST (International Union of Food science and technology) 2012. Nutrigenomics. IUFoST Scientific Information Bulletin (SIB).
- James IF, Kuipers B (2003). Preservation of fruit and vegetables. 4th edition. Agromisa Foundation. Wageningen.
- Karlun LC, Brimer L, Saka JDK, Mhone AR, Mkumbira J, Johansson L, Bokanga M, Mahugu NM, Rosling H (2004). Bitter taste in cassava roots correlates with cyanogenic glucoside levels. *J. Sci. Food Agric.* 84 (6):581-590.
- Kendall P, DiPersio P, Sofos J (2010). Drying vegetables. Colorado State University Extension Ltd.
- Kutner MH, Nachtsheim CN, Neter J, Li W (2005). Applied linear statistical models. 4th edition. Mc Graw-Hill.
- Larmond E (1977). Laboratory Methods for Sensory Evaluation of Food. Research Branch, Canada, Department of Agriculture, Ottawa Publication.
- Loffalian A, Ghazavi MA, Hosseinzadeh B (2010). Comparing the performance of two type collectors on drying process of lemon and orange fruits through a passive and indirect solar dryer. *J. Am. Sci.* 6(10):248-251.
- Medugu DW (2010). Performance study of two designs of solar dryers. *Arch. Appl. Sci. Res.* 2(2): 136-148.
- Mepba HD, Eboth, Banigo DEB (2007). Effects of processing treatments on the nutritive composition and consumer acceptance of some Nigerian edible leafy vegetables. *Afr. J. Food Agric. Nutr. Dev.* 7 (1): 1-18.
- Mkpong OE, Yan H, Chism G, Sayre RT (1990). Purification, characterization and localization of linamarase in cassava. *Plant Physiol.* 93:176-181.
- MMA (Match Makers Associates limited) (2008). Dried fruits and vegetables for urban and export markets. Sub sector and value chain analysis. Tanzania. Final report.
- Rwandan Ministry of Finance and Economic Planning (MINECOFIN) (2007). Economic Development and Poverty Reduction Strategy, 2008-2012 (EDPRS). Government Printer, Kigali, Rwanda.
- SAS Institute (2008). Software 9.2. University of Notre Dame-T&R, Cary, NC, USA.
- Shackleton CM, Pasquini MW, Drescher AW (2009). African indigenous vegetables in urban agriculture. Earthscan. London.UK.
- Steinmetz KA, Potter JD (1996). Vegetables, fruit and cancer prevention:A review. *J. Am. Diet. Assoc.* 96:1027-1039.
- Thomas SCL (2008). Vegetable and fruits. Nutritional and therapeutic values. CRC Press. Taylor & Francis Group. London. UK.
- Umuhozariho MG, Shayo NB, Msuya JM, Sallah PYK (2011). Utilization of cassava leaves as a vegetable in Rwanda. *Rw. J. Agric. Sci.* 24 (Series E): 15-27.
- Yang RY, Keding GB (2009). Nutritional contribution of important African indigenous vegetables. In Shackleton CM, Pasquini MW, Drescher AW (eds). African indigenous vegetables in urban agriculture. Earthscan. London.UK.

Full Length Research Paper

Evaluation of pollen viability, stigma receptivity and fertilization success in *Lagerstroemia indica* L.

Fang Chen, Wei Yuan^{1,2}, XuePing Shi² and YaoMei Ye^{2*}

¹The China (Zhengzhou Henan) Greening Expo Garden, Zhengzhou 451460, Henan Province, China.

²Key Laboratory of Horticultural Plant Biology, Laboratory of Silviculture and Tree Breeding, College of Horticulture and Forestry Sciences, Huazhong Agricultural University, Wuhan 430070, Hubei Province, China.

Accepted 10 April, 2012

To provide theoretical basis for artificial pollination in *Lagerstroemia indica* L., pollen viability and stigma receptivity were tested and the morphological change of stigma was observed. Pollen viability tested by *in vitro* culture, stigma receptivity examined by benzidine-H₂O₂ testing and fruit set estimated by field artificial pollination were analyzed in this study. The maximum pollen viability was observed at 10:00 am one day of anthesis (DA), of which 'Hong Wei' (46.2%) was significantly lower than that of 'Yin Wei' (56.8%) and 'Zi Wei' (62.5%). The stigma receptivity of the three crape myrtle cultivars was sustained for eight days, which was 95.7 to 96.9% at 1 DA to two days after anthesis (DAA), then declined to 75.5 to 79.9% at 3 to 4 DAA and 50.6 to 59.7% at 5 to 6 DAA, and only 29.5% at 7 DAA. Higher stigma receptivity was associated with columnar style, upward stigma, green and wet papillae and copious exudates at stage 1 (1 DA to 2 DAA). Frequencies of fruit set at stage 1 (74.4%) and stage 2 (3 to 4 DAA) (78.9%) were significantly higher than that at stage 3 (5 to 6 DAA) (21.9%). So, selecting pollen at 10:00 am 1 DA and stigma of 1 DA to 4 DAA was a strategy to enhance fruit set in the future artificial hybridizations for crape myrtle.

Key words: *Lagerstroemia indica* L., dimorphic pollen, pollen viability, stigma receptivity, fruit set.

INTRODUCTION

Lagerstroemia indica (commonly known as crape myrtle) is a deciduous shrub or small woody species native to China, having been cultivated for 1500 years (Zhang, 1991; Chen, 2001). Due to its excellent traits of long-lasting bloom in mid- to late- summer, striking flower color, sinuate trunks, good drought tolerance, and ease of production and cultivation, crape myrtle has been widely introduced to many regions in Australia (Egolf and Andrick, 1978), America (Egolf, 1985), Europe (Attorre et al., 2000) and Turkey (Göre, 2009) for serving as an ornamental plant. In the sexual reproduction of higher plants, double fertilization success is determined by a

Number of factors including pollen viability, female receptivity and environmental influences, in which pollen and stigma fulfill important functions (Russell, 1992). Any success in breeding experiments or artificial pollination procedures should be accompanied by tests on pollen viability and timing and duration of the stigma receptivity (Lavithis and Bhalla, 1995; Stone et al., 1995; Nautiyal et al., 2009). Currently, systematic studies on pollen viability and stigma receptivity have been reported in a variety of genera, for example, pollen longevity of *Tectona grandis* (Tangmitcharoen and Owens, 1997a, b) and *Grevillea robusta* (Kalinganire et al., 2000) remained for 3 and 4

*Corresponding author. E-mail: yymld@mail.hzau.edu.cn. Tel: +86 27 8728 2435. Fax: +86 27 8728 2010.

days, and their viability reached a maximum of 92.2% at 1 day of anthesis (DA) and 95% at 1 day after anthesis (DAA), respectively. Stigma receptivity of *G. robusta* (Kalinganire et al., 2000) and *Zinnia elegans* (Ye et al., 2007) which exhibited peak value at 2 DAA with the morphological characters of taller papillae, abundant exudates and increased stigmatic groove was maintained for 4 and 10 days, and at 2 to 3 DAA with yellow stigma and two lobes on the tip of stigma like γ , respectively. However, integrated study on pollen viability and stigma receptivity in *L. indica* has not been reported previously. There were only a few reports on pollen viability in *L. indica* (Nepi, et al., 2003; Zhang et al., 2008; Ye et al., 2010). The purpose of the present study was to provide theoretical basis for enhancing fruit set and developing new varieties in *L. indica* crossbreeding, concentrating on the pollen viability, pollen longevity, timing and duration of stigma receptivity and fruit set under defined dimorphic pollen and stigmatic developmental stages.

MATERIALS AND METHODS

Plant materials

The 3-4-year-old plants of three crape myrtle cultivars ('Hong Wei', 'Zi Wei' and 'Yin Wei') were obtained from the experimental field site of Huazhong Agricultural University, Wuhan, China. Field trials were carried out during 2007 to 2008 in the experimental field site of Huazhong Agricultural University. Laboratory tests were carried out with the facilities in the Laboratory of Silviculture and Tree Breeding, Ministry of Education, Huazhong Agricultural University, China.

In vitro pollen viability

As *L. indica* came into bloom, inflorescences at early flowering stage were excised from the tested plants and kept temporarily in small vials full of tap water. At nightfall before testing, opened florets were manually removed to avoid mixing with the tested florets. During 1 DA to 2 DAA, pollen grains were collected 11 times from 7:00 to 16:00 at 3 h intervals except 16:00 of 2 DAA. Pollen viability was tested on *in vitro* germination medium ME₃ (Leduc et al., 1990) supplemented with 16% (w/v) PEG4000 and 12% (w/v) sucrose (Sinopharm Chemical Reagent Co., Shanghai) for 0.5 h (preliminary experiments showed that this was the optimal medium for pollen germination in this species, data not shown). For each sample, germinations of five no-overlapped views were recorded with at least 50 pollen grains per view. Pollen was viewed as germinated when the length of the pollen tube exceeded the diameter of its pollen grain. The experiments were replicated three times. Pollen viability was estimated by pollen germination rate which was calculated as the ratio of the number of germinated pollen grains to the total number of pollen grains.

Fruit set of different pollen types by artificial pollination

Using two-factor randomized design, 3 cultivars ('Hong Wei', 'Zi Wei' and 'Yin Wei') × 3 pollen types (real pollen, feed pollen and mixture pollen), 9 cross combinations were studied. When inflorescences came into bloom, all florets to be pollinated were emasculated and isolated by transparent sulfuric acid paper bag on

the day before anthesis to avoid self-pollination. Subsequently, between 8:00 and 10:00 am, florets were hand pollinated with fresh pollen of the corresponding cultivar using a small paintbrush, enclosed within transparent sulfuric acid paper bags, and the number of the pollinated florets in every combination with 10 to 15 replicates were labeled and recorded. To facilitate the development of capsules, the bags were removed a week later and the capsules were harvested after a month. Fruit set was expressed as the ratio of harvested capsules to pollinated florets.

Observation of stigmatic morphological characters

Samples of the three plants were randomly collected for each of the three crape myrtle cultivars for conducting the observation. On the day before anthesis, a total of 120 buds for three cultivars (at least 40 buds per cultivar) were selected to emasculate and isolated by transparent sulfuric acid paper bag individually. At 8:00 am of each day in the following eight days, five pistils per cultivar were excised to observe the morphological characters with the style performance and stigma performance, including the bent degree and color of style, the orientation, color and papillae of stigma and the amount of exudates.

Stigma receptivity by benzidine-H₂O₂ test

Florets were isolated by transparent sulfuric acid paper bag at 1 DA to 7 DAA. The pistils of the three cultivars were collected at 8:00 am and tested by benzidine-H₂O₂ (1% benzidine: 3% H₂O₂ hydrogen peroxide: water = 4:11:22, v/v) (Sinopharm Chemical Reagent Co., Shanghai) respectively (Dafni and Motte, 1998). The collected pistils were stained for 10 to 15 min at 28°C and a pistil was regarded as receptive when more than 2/3 of the stigmatic area were stained dark blue and associated with some amount of bubbles. Each treatment was carried out with ten pistils in three replicates. Stigma receptivity was evaluated by dyeing rate which was calculated as the ratio of the number of stained stigmas to the total number of tested stigmas.

Fruit set at different stigma stages by artificial pollination

Using two-factor randomized design, 3 cultivars ('Hong Wei', 'Zi Wei' and 'Yin Wei') × 3 stigma stages (1DA to 2 DAA, 3 to 4 DAA and five to six DAA classified by the results of benzidine-H₂O₂ examination), a total of 9 cross combinations were studied. The process of pollination and the calculation of fruit set were as previously stated.

Statistical analysis

Percentage data as pollen viability, stigma receptivity and fruit set were transformed via arcsine before analysis. Statistical analysis was performed by SAS software followed by Duncan's multiple comparisons at the level of 0.05 and 0.01 (SAS, 2000).

RESULTS

Pollen viability

Results of single factor variance analysis showed that pollen-collecting time had a very significant ($p=0.01$) effect on viability of real pollen or feed pollen in all three crape myrtle cultivars (Figure 1).

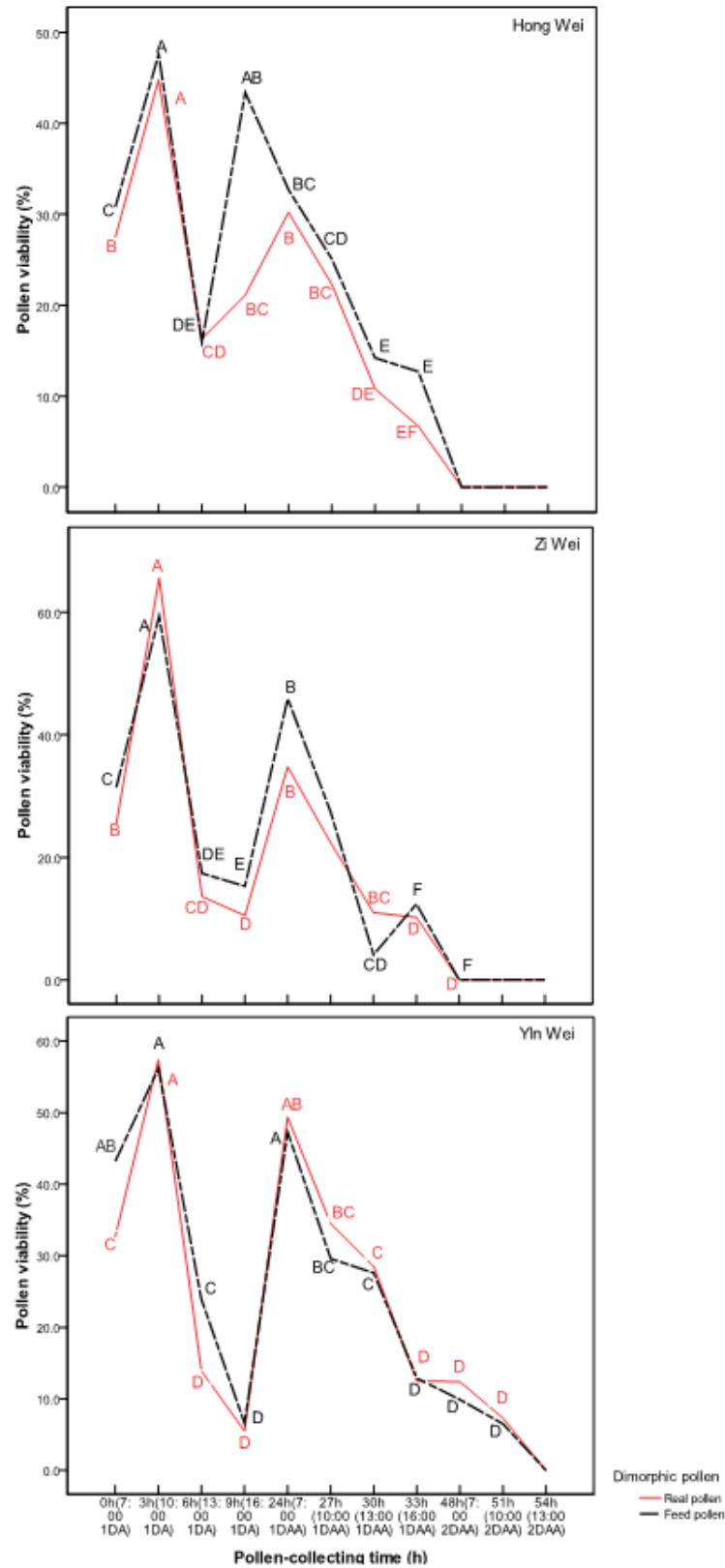


Figure 1. The changing trend of pollen viability with collecting time in three *L. indica* cultivars. *0 h stands for the pollen-collecting time of 7:00 am 1 DA, and other times equal to the time of subtracting 7:00. The black capital letters stand for the results of multi-comparison of feed pollen (p=0.01), while the red capital letters for the real pollen (p=0.01).

Table 1. The relationship between cultivar, dimorphic pollen type and pollen viability (10:00 am 1 DA) of *L. indica*.

Dimorphic pollen type	Germination rate (%)	Cultivar	Germination rate (%)
Real pollen	55.9±3.7 ^A	Hong Wei	46.2±2.1 ^{bB}
Feed pollen	54.4±2.5 ^A	Zi Wei	62.5±3.2 ^{aA}
		Yin Wei	56.8±2.5 ^{aAB}

Table 2. Multi-comparison of fruit set among different combinations of *L. indica* cultivars and three pollen types.

Combination	Feed pollen(% of fruit set)	Real pollen(% of fruit set)	Mixture pollen(% of fruit set)
Hong Wei	72.9 ± 7.0 ^{abAB}	56.9 ± 11.8 ^{bcAB}	49.4 ± 5.3 ^{bcAB}
Zi Wei	35.5 ± 2.9 ^{cB}	84.1 ± 4.7 ^{aA}	34.7 ± 2.8 ^{cB}
Yin Wei	56.9 ± 14.5 ^{bcAB}	57.8 ± 13.4 ^{bcAB}	59.7 ± 9.3 ^{bcAB}

**Figure 2.** Morphological characters of stigma.

The pollen viability of three cultivars were maintained for 33 h in 'Hong Wei', 33 h in 'Zi Wei' and 51 h in 'Yin Wei'. In the natural environment, the pollen viability was less than 10% (data not shown) at 5:00 am when the corolla just surpassed the calyx. In 'Hong Wei', the real pollen viability was 27.5% at 7:00 am 1 DA, which increased very significantly to the peak (44.8%) at 10:00 am and decreased to 16.3% till 13:00 pm, 21.1% at 16:00 pm of 1 DA. It later increased significantly to 30.2% at 7:00 am of 1 DAA, then decreased to 22.3% at 10:00 am of 1 DAA and decreased very significantly to 10.8% at 13:00 pm of 1 DAA, before it lost vigor at 16:00 pm of 1 DAA.

Two-factor analysis of variance showed that cultivar and the interaction of cultivar and dimorphic pollen type had highly significant effect on the peak pollen viability (10:00 am 1 DA). Further multi-comparison on cultivars (Table 1) indicated that the pollen viability of 'Zi Wei' (62.5%) was significantly higher than that of 'Hong Wei' (46.2%), while that of 'Yin Wei' (56.8%) had no significant difference with them.

Fruit set of different pollen types

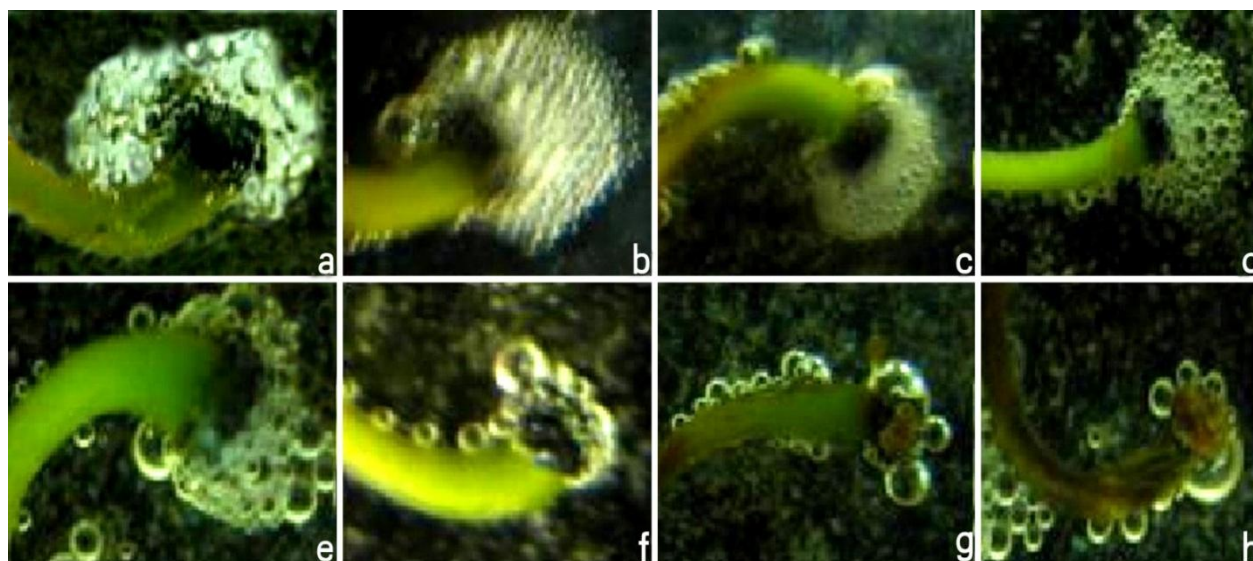
Two-factor analysis of variance showed that cultivar and pollen type had no significant effect, but their interaction had highly significant effect on the fruit set. The dimorphic pollen in three cultivars all had fertility. Further multi-comparison (Table 2) revealed the fruit set of three pollen types showed no difference either in 'Hong Wei' or 'Yin Wei'. However, the fruit set of real pollen (84.1%), to a great extent, was significantly higher than that of feed pollen (35.5%) and mixture pollen (34.7%) in 'Zi Wei'.

Morphological characters of stigma

The stigmatic morphological characters during the period of 1 DA to 7 DAA greatly changed in the developmental process (Figure 2). At 1 DA to 2 DAA, the style was columnar or slightly-bent about 30° at the front end, light green, and the stigma was characterized by upward,

Table 3. The relationship between cultivar, growth day of stigma and stigma receptivity of *L. indica*.

Cultivar	Dyeing rate (%)	Growth day of stigma	Dyeing rate (%)
Hong Wei	73.8 ± 4.9 ^A	1 DA	97.9 ± 1.1 ^A
Zi Wei	68.9 ± 6.2 ^A	1 DAA	97.4 ± 1.5 ^A
Yin Wei	77.1 ± 4.1 ^A	2 DAA	95.7 ± 1.7 ^A
		3 DAA	79.9 ± 2.9 ^B
		4 DAA	75.5 ± 3.6 ^B
		5 DAA	59.7 ± 3.6 ^C
		6 DAA	50.6 ± 2.3 ^C
		7 DAA	29.5 ± 4.8 ^D

**Figure 3.** Stigmatic reaction to benzidine-H₂O₂ (a to h stands for the 1 DA to 7 DAA).

green, wet papillae and accumulative copious exudates. At 3 to 4 DAA, the front end of style became slightly curled at about 45°, dark green, and the stigma was characterized by obliquely downward, light green, wet papillae and a few exudates. At 5 to 6 DAA, the style bended at about 90° from the upper middle part in a semicircle-like, wilted from the base and brown, and the stigma was downward, yellow green, slightly wet papillae and few remnants of exudates, suggesting decrease in the support to pollen adhesion. Up to 7 DAA, the style further curled at the front end withered at the base, the stigma looked introflexed, wilted and collapsed as the brown papillae.

Stigma receptivity

Stigma receptivity had no significant difference among the three *L. indica* cultivars which were 73.8% in 'Hong Wei', 68.9% in 'Zi Wei' and 77.1% in 'Yin Wei', while significant differences were observed among growth days

(Table 3, Figure3). At 1 DA, the stigmatic surface was not only inundated by more bubbles around its peripheral areas, but completely stained blue, with the stigma receptivity of 97.9%, indicating that the stigma was extremely receptive to pollen (Table 3, Figure 3a). At 1 to 2 DAA, the stigmatic surface accumulated many small and compact bubbles, and was still entirely stained with the stigma receptivity of 97.4 and 95.7% (Table 3; Figure 3b, c). As the stigma developed into 3 to 4 DAA, the bubbles became significantly decreased in amount and density; the staining extent weakened and the stigma receptivity obviously decreased to 75.5 to 79.9% (Table 3; Figure 3d, e). At 5 to 6 DAA, the bubbles further decreased, the staining extent further weakened, and the stigma receptivity significantly declined to 50.6 to 59.7% (Table 3; Figure 3f, g). Until 7 DAA, only few visual bubbles and scarcely staining area were observed and the stigma receptivity was only 29.5% (Table 3; Figure 3h).

Based on the detailed observation of stigmatic morphological character and examination of stigma receptivity on

Table 4. The relationship between cultivar, stigma stage and fruit set of *L. indica*.

Cultivar	Fruit set (%)	Stigma stage	Fruit set (%)
Hong Wei	46.9 ± 17.7 ^b	Stage 1 (1 DA-2 DAA)	74.4 ± 12.5 ^A
Zi Wei	60.9 ± 20.2 ^a	Stage 2 (3-4 DAA)	78.9 ± 8.9 ^A
Yin Wei	53.4 ± 18.6 ^a	Stage 3 (5-6 DAA)	21.9 ± 5.9 ^B

a subset of stigmas, four developmental stages could be classified in the stigmatic developmental process, that is, stage 1 (1 DA to 2 DAA), stage 2 (3 to 4 DAA), stage 3 (5 to 6 DAA) and stage 4 (7 DAA).

Fruit set at different stigma stages

Results of field pollination showed that stigma stage as well as cultivars had highly significant effect on fruit set, but their interaction had no significant effect. Further multiple comparisons (Table 4) revealed that fruit set of 'Zi Wei' (60.9%) and 'Yin Wei' (53.4%) had no significant difference, but that of these two cultivars were significantly higher than that of 'Hong Wei' (46.9%). Fruit set of stage 1 (74.4%) and stage 2 (78.9%) had no significant difference, but that of these two stages were highly significantly higher than that of stage 3 (21.9%).

DISCUSSION

Pollen viability

The pollen viability for three cultivars exhibited similar trend of fluctuant decline with prolonged flowering time (Figure 1). This fluctuant trend of pollen viability with prolonged flowering time was caused by its physiological change as well as the real-time temperature and relative humidity (RH). On a sunny day in August, the environment conditions at 7:00 am were of relatively low temperature and high RH. The temperature gradually rose from 10:00 am, reached the peak till 13:00 pm, gradually decreased after 16:00 pm and maintained this low temperature at night, while RH went up at night. So, the pollen viability was greatly decreased at 13:00 pm 1 DA under the conditions of relative high temperature and low RH, while it increased to sub-peak at 7:00 am 1 DAA under the conditions of relative temperature and higher RH. The performance of pollen viability being affected by different conditions has been clearly reported in many plants. Pollen viabilities of *Oryza sativa* and *Zea mays* when exposed to higher temperature and lower RH, >35°C/65 to 70% and 27.2°C/42%, were maintained for 30 and 43 min, while when subjected to lower temperature and higher RH, 25 to 28°C/70 to 80% and 22.3°C/54.3%, they were prolonged to 60 and 72 min, respectively (Li et al., 2002; Fonseca and Westgate, 2005). Pollen viability of *Rosmarinus officinalis* when treated with 38°C/48% were maintained for only 10%, but

significantly increased to 20% after being stored at 4°C/100% for 1 h (Aronne et al., 2006). Thus, loss of pollen viability seems to be a general response due to higher temperature and lower RH. Hence, the fluctuant trend of pollen viability in *L. indica*, which decreased at 13:00 pm 1 DA and increased at 7:00 am 1 DAA, is ascribed partly to the real-time temperature and RH in the study.

The pollen viability of three cultivars 'Zi Wei' (62.5%), 'Hong Wei' (46.2%) and 'Yin Wei' (56.8%) (Table 1) was higher than our previous report of 38.9, 30.1 and 42.8%, respectively (Ye et al., 2010). These inconsistent results might be because the experimental seedlings sown in two different years were different in genotype and plant physiology and growth. Additionally, the pollen grain tested in this study underwent no treatment, while that of Ye et al. (2010) were collected from the dried bud at 25°C for 2 h in homoeothermic incubator. These previous results may demonstrate that the pollen viability of crape myrtle has close relationship to genotype, pollen-collecting time, pollen grain treatment and medium components, and even the real-time and site-specific environmental conditions may play a key role in the pollen viability.

The present study shows that all the dimorphic pollen had viability, and there was no significant difference in the pollen viability (10:00 am 1 DA) and longevity for each crape myrtle cultivar (Figure 1; Table 1). Similar results were reported in two crape myrtle varieties where the dimorphic pollen viability for each one had no significant difference (Zhang et al., 2008). However, Nepi et al., (2003) presented that at anthesis, the viability of real pollen (90%) was higher than that of feed pollen (65%) tested by fluorochromic reaction (FCR) assay for crape myrtle. According to the present results, the dimorphic pollen viabilities varied with crape myrtle materials (genotypes). This trait also existed in genus *Senna* of *Leguminosae* family; the dimorphic pollen viabilities for either *Senna alata* or *Senna bicapsularis* had high significant difference, but no significant difference for *Senna surattensis* (Luo et al., 2009).

Stigmatic morphological traits, stigma receptivity and fruit set

Morphological traits of stage 1 (columnar style, upward and green stigma, wet papillae and copious exudates) and stage 2 (slightly curled style, obliquely downward and

yellow green stigma, wet papillae and a few exudates) were associated with higher stigma receptivity of above 95 and 75.0%, respectively (Figure 2; Table 3). This positive correlation between stigmatic morphological traits and higher stigma receptivity has been reported in many plants, such as straight style, enlarged stigma and turgid stigmatic papillae in teak (Tangmitcharoen and Owens, 1997a), widely-opened stigmatic grooves, taller stigmatic papillae and abundant secretion in silky oak (Kalinganire et al., 2000), and elongation of stigmatic papillae and increased amounts of stigmatic exudate in almond (Yi et al., 2006). These reports indicated that we could primarily judge the stigma receptivity by the stigmatic morphological traits, which would provide some reference for the judgment of the effective pollination period (EPP) (Williams, 1965; Galen and Plowright, 1987).

The results of Table 4 suggest choosing the stigma of two previous stages (stage 1 and stage 2) to be pollinated could obtain higher fruit set. The stigma with the morphological characters of columnar or slightly curled style, upward and green stigma, wet papillae and copious exudate was the preferred choice for pollination. Furthermore, the descending order of stigma receptivity was different with varying examination methods, that is, stage 1 > stage 2 > stage 3 (dyeing rate) by benzidine-H₂O₂ testing and stage 1 and stage 2 > stage 3 (fruit set) by field artificial pollination. To sum up, fertilization success does not merely depend on the high stigma receptivity, but also the pollen viability, pollen-stigma interaction, the fertilization process, the seed development process, the seasonal factors and the environment conditions. Further investigations are required to expound this observation.

Meanwhile, three different methods of stigmatic morphological observation, benzidine-H₂O₂ testing and field artificial pollination were adopted and successfully examined the stigma receptivity of three crape myrtle cultivars. Comprehensive methods which detect stigma receptivity have been reported in many plants, such as stigma morphology and pollination in *Prunus dulcis* (Yi et al., 2006), MTT staining and pollination in *Nolana humifusa* and *Nolana laxa* (Amy and Rosanna, 2010) and three integrated methods in *Zinnia elegans* (Ye et al., 2007).

In these examination methods, benzidine-H₂O₂ staining was destructive to stigma, was artificial pollination time-consuming, unrepeatable during same period and hard to handle the reference standards, while the stigmatic morphological observation was intuitive, simple and rapid. So, determining the stigma receptivity with stigmatic morphological observation is a feasible technique to promote the artificial pollination efficiency, which has important practical significance in *L. indica* crossbreeding.

Conclusion

Under field conditions, the maximum pollen viability were

observed at 10:00 am 1 DA. The dimorphic pollen of three cultivars all had viability and fertility. The stigma receptivity of three cultivars could sustain 8 d and be subdivided into four developmental stages: stage 1 (1 DA to 2 DAA), stage 2 (3 to 4 DAA), stage 3 (5 to 6 DAA) and stage 4 (7 DAA). Higher stigma receptivity was associated with columnar or slightly curled style, upward or obliquely downward stigma, green and wet papillae and copious exudates at stage 1. Frequencies of fruit set at stage 1 (74.4%) and stage 2 (78.9%) were significantly higher than that at stage 3 (21.9%). So, in view of our study, strategies to enhance fruit set in the artificial pollination should select the pollen of 10:00 am 1 DA and the stigma of 1 DA to 4 DAA. This information is especially valuable for breeders to developing new varieties in *L. indica* crossbreeding.

REFERENCES

- Amy CD, Rosanna F (2010). Floral development, stigma receptivity and pollen viability in eight *Nolana* (Solanaceae) species. *Euphytica*, 174:105-117.
- Aronne G, De Micco V, Scala M (2006). Effects of relative humidity and temperature conditions on pollen fluorochromatic reaction of *Rosmarinus officinalis* L. (Lamiaceae). *Protoplasma*, 228:127-130.
- Attorre F, Bruno M, Francesconi F, Valenti R, Bruno F (2000). Landscape changes of Rome through tree lined roads. *Landsc. Urban Plan.* 49:115-128.
- Chen JY (2001). The Chinese flower species taxonomy. Chinese Forestry Publishing House, Beijing, China.
- Dafni A, Motte MM (1998). A rapid and simple procedure to determine stigma receptivity. *Sex. Plant Reprod.* 11:177-180.
- Egolf DR (1985). 'Biloxi', 'Miami' and 'Wichita' join the National Arboretum's list of crape myrtle introductions. *American-Nurseryman*. 20(6):1144-1145.
- Egolf DR, Andrick AO (1978). The Lagerstroemia handbook/checklist. Ameri. Association, Botanical Gardens, Arboreta, Inc.
- Fonseca AE, Westgate ME (2005). Relationship between desiccation and viability of maize pollen. *Field Crop. Res.* 94:114-125.
- Galen C, Plowright RC (1987). Testing accuracy of using peroxidase activity to indicate stigma receptivity. *Can. J. Bot.* 65:107-111.
- Göre ME (2009). Powdery mildew caused by *Erysiphe* sp. on *Lagerstroemia indica* newly reported in Turkey. *Plant Pathol.* 58:397.
- Kalinganire A, Harwood CE, Slee MU, Simons AJ (2000). Floral structure, stigma receptivity and pollen viability in relation to protandry and self-incompatibility in silky oak (*Grevillea robusta* A. Cunn.). *Ann. Bot.* 86(1):133-148.
- Lavithis M, Bhalla PL (1995). Esterases in pollen and stigma of *Brassica*. *Sex. Plant Reprod.* 8:289-298.
- Leduc N, Monnier M, Douglas GC (1990). Germination of trinucleated pollen: formulation of a new medium for *Capsella bursapastoris*. *Sex. Plant Reprod.* 3:228-235.
- Li XZ, Liang MZ, Zhou GQ, Chen LB (2002). Effect of environment condition on pollen vigor and seed set during flowering time of rice. *Acta Agron. Sin. Chinese*, 28(3):417-420.
- Luo ZL, Gu L, Zhang DX (2009). Intrafloral differentiation of stamens in heterantherous flowers. *J. Syst. Evol.* 47(1): 43-56.
- Nautiyal BP, Nautiyal MC, Khanduri VP, Rawat N (2009). Floral Biology of *Aconitum heterophyllum* Wall: A Critically Endangered Alpine Medicinal Plant of Himalaya, India. *Turk. J. Bot.* 33:13-20.
- Nepi M, Guarneri M, Pacini E (2003). "Real" and Feed Pollen of *Lagerstroemia indica*: Ecophysiology. Differences, *Plant Biol.* 5(3):311-314.
- Russell SD (1992). Double fertilization. *Internat. Rev. Cytol.* 140:357-388.
- SAS, 2000. SAS/STAT User Guide Version 8.1, SAS Institute Inc., Cary, NC, USA.

- Stone JL, Thomson JD, Dent-Acosta SJ (1995). Assessment of pollen-viability in hand-pollination experiments: a review. *Am. J. Bot.* 82:1186-1197.
- Tangmitcharoen S, Owens JN (1997a). Floral biology, pollination, pistil receptivity and pollen tube growth of teak (*Tectona grandis* Linn f.). *Ann. Bot.* 79: 227-241.
- Tangmitcharoen, S, Owens, JN (1997b). Pollen viability and pollen-tube growth following controlled pollination and their relation to low fruit production in teak (*Tectona grandis* Linn f.). *Ann. Bot.* 80:401-410.
- Williams RR (1965). The effect of summer nitrogen applications on the quality of apple blossom. *J. Hort. Sci.* 40:31-41.
- Ye YM, Tong J, Shi XP, Yuan W, Li GR (2010). Morphological and cytological studies of diploid and colchicine-induced tetraploid lines of crape myrtle (*Lagerstroemia indica* L.). *Sci. Hortic.* 124:95-101.
- Ye YM, Zhang JW, Qin YC, Chen TH, Bao MZ (2007). Studies on stigma receptivity and pollen viability of *Zinnia elegans*. *Scientia, Agric. Sin. Chin.* 40(10):2376-2381.
- Yi W, Law E, McCoy D, Wetzstein HY (2006). Stigma development and receptivity in almond (*Prunus dulcis*). *Ann. Bot.* 97(1): 57-63.
- Zhang QX (1991). Study on Cultivars of crape myrtle (*Lagerstroemia indica*) and their uses in urban greening. *J. Bering Forestry, Univ.* 13(4): 57-66.
- Zhang QY, Luo FX, Liu L, Guo FC (2008). Pollen viability of dimorphic anthers in *Lagerstroemia indica*. *Acta Hortic. Sin. Chin.* 12:1741.

Review

A review on trypanosomosis in dogs and cats

Nwoha, R. I. O.

Department of Veterinary Medicine, Micheal Okpara University of Agriculture Umudike. P. O. Box 824, Nigeria.

Accepted 16 September, 2013

Trypanosoma brucei brucei, *Trypanosoma cruzi* and *Trypanosoma congolense* were initially thought to be the only species of trypanosomes capable of causing diseases in dogs and cats. However, dogs and cats are challenged by diverse species of trypanosomes with varying virulence and pathogenicity. Dogs may develop clinical trypanosomosis by infection with *Trypanosoma evansi* but are refractory to *Trypanosoma rangeli* of man. Recently, a new species, *Trypanosoma caninum*, of unknown pathogenicity and mode of transmission has been reported in dogs. This review describes canine trypanosomosis as an entity of two types, African and American trypanosomosis. It describes the different species involved in each type of the disease condition, the emerging strains, the biological cycle, distribution, clinical symptoms, the pathology and treatment of various species of canine trypanosomes. It also describes different basic diagnostic techniques currently in use and progress towards development of vaccine.

Key words: Dogs, cats, *Trypanosoma brucei*, *Trypanosoma congolense*, *Trypanosoma cruzi*, *Trypanosoma caninum*, *Trypanosoma evansi*, *Trypanosoma rangeli*.

INTRODUCTION

Animal trypanosomosis has profound social, economic and biological implications for the affected regions (WHO, 2006; OIE, 2008; Finelle, 1973). Jones (2000) described canine trypanosomosis as a disease caused by *Trypanosoma cruzi* and *Trypanosoma evansi*. Today different species of trypanosomes have been implicated in causing trypanosomosis in dogs. Hence, canine Trypanosomosis is a disease caused by hemoprotozoan parasites: *Trypanosoma brucei brucei* and *Trypanosoma congolense*. The disease can also be caused by *T. cruzi* the cause of American Trypanosomosis known as Chagas disease in humans (Jimenez-Coelle et al., 2010; Tola and Muniz, 2010; Cohen and Gurtler, 2001; Doyle, 2006). Dogs are also infected by *Trypanosoma b. rhodesiense* and *Trypanosoma b. gambiense* of man (Samdi et al., 2006). It serves as reservoirs and maintenance of infection in humans vice versa. *Trypanosoma rangeli* is a non-pathogenic trypanosome

of humans which also infect dogs (CVBD, 2010). Its importance is mainly for creating confusion during diagnosis in cases of mixed infection with *T. cruzi* (CVBD, 2010). *T. evansi* is found sporadically in dogs causing severe and possibly fatal disease (Uilenberg, 1998; Finelle, 1973). Recently, *Trypanosoma caninum* of unknown pathogenicity has been isolated from an intact skin of a dog along with leishmania in south eastern Brazil (Madeira et al., 2009; Barros et al., 2012). Its mode of transmission is not yet known as efforts to infect triatomids failed (CVBD, 2010).

Canine trypanosomes cause infections of varying severities in dogs. The infection ranges from acute, sub-acute to chronic. Infections from *T. b. brucei* and *T. congolense* are found mainly in sub-Saharan Africa and the disease is relatively common in Nigeria because of the high prevalence of *Glossina* spp. in most parts of the country (Ahmed, 2007) due to lots of vegetation along

Table 1. Species of African and American canine trypanosomosis, predilection sites, therapeutic and management protocols.

African canine trypanosomosis	Name	Species involved	Predilection site	Treatment (Diminazene aceturate)
		<i>T. brucei brucei</i>	Blood vessels and tissue	7 mg/kg stat
		<i>T. congolense</i>	Blood vessels and tissue	3.5 mg/kg stat
	Surra/ Nagana	<i>T. b. rhodesiense</i>	Blood vessels	7 mg/kg stat
		<i>T. b. gambiense</i>	Blood vessels and tissue	7 mg/kg stat
		<i>T. evansi</i>	Blood vessels and tissue	7 mg/kg stat
		<i>T. rangeli</i>	Blood vessels	3.5 mg/kg stat
		<i>T. caninum</i>	Intact skin	3.5 mg/kg stat
American canine trypanosomosis	Chagas	<i>T. cruzi</i>	Blood vessels and tissue	7 mg/kg stat beta adrenergic blockers eg carvedolol, propanolol and atenolol, (ACEIs) eg enalapril, veno- or iono-dilators like prazosin or pimobendan,

rivers and lakes and large wooded savannah landmass. The disease in dogs is often as a result of a bite from an infected tse-tse fly. However, dogs can also get the infection by ingestion of fresh animal carcasses that died recently from trypanosomosis and through oral experimental infection (Raina et al., 1985; Uilenberg, 1998). The disease is more common in free roaming dogs than in those housed in tsetse fly net protected kennels.

IDENTIFICATION OF *TRYPANOSOMA* SPECIES THAT INFECT DOGS AND CATS

Trypanosoma species that affect dogs and cats can be (Table 1) identified by the following morphological characteristics. *T. b. brucei* may be found in two different forms (WHO, 2006): the long slender form measuring about 17 to 33 µm long and about 3.5 µm wide, and the undulating membrane which is conspicuous with a free flagellum at the anterior end. Its posterior end is pointed with a small and sub-terminal kinetoplast (Francois et al., 2005; OIE, 2008; Turnbull, 2009). The short stumpy form measures about 17 to 22 µm long and about 3.5 µm wide with a conspicuous undulating membrane. This form possesses a free flagellum and a pointed posterior end with a small and sub-terminal kinetoplast (OIE, 2008). *T. congolense* have its small forms measuring 8 to 25 µm with an obvious undulating membrane (Uilenberg, 1998). The posterior end is rounded with no free flagellum. The kinetoplast is medium sized and terminal, often laterally positioned.

Although considered monomorphous, a degree of morphological variation is sometimes observed which includes (savannah, forest, kilifi, tsavo) sub groups of *T. congolense* with different pathogenicity (Bengaly et al., 2002; Masumu et al., 2006; OIE, 2009). *T. cruzi* measures about 10 µm long, slender, thin with an irregular shaped undulating membrane. Its nucleus is

centrally positioned and the kinetoplast is posterior (Uilenberg, 1998; Hunt, 2010). The free flagellum runs through the remainder of the parasite and also extends beyond it. Visualized in stained sample, the parasite assumes a C or U shape (De Souza, 1999). *T. rangeli* has a similar morphology to *T. cruzi* (OIE, 2008). *T. caninum* are morphologically distinct from Salivarian trypanosomes. It differs from *T. cruzi* mainly by the size of its trypomastigote forms and kinetoplasts and absence of infectivity for macrophages and triatomine bugs (Madeira et al., 2009). This means that parasite may not be transmitted through tsetse bite.

Host affected

Indigenous pure breed, foreign breeds and cross breed of dogs are susceptible to trypanosomosis (Annette et al., 2006; Akpa et al., 2008).

Intermediate host

Glossina spp. (tse-tse fly) and haematophagus flies in the genus *Tabanus*, *Stomoxys* and Triatomids bugs are vectors for the transmission of trypanosomosis in dogs (Uilenberg, 1998).

Distribution

This is dependent on the distribution of tse-tse flies which are the primary vectors responsible for the transmission of African trypanosomosis and triatomine transmitters of American trypanosomosis in dogs (WHO, 2013; Serap et al., 2003; Hunt, 2010). Tse-tse flies are currently restricted between 14° latitude north and 29° latitude south of sub-Saharan Africa, affecting 10 million square kilo-

kilometers of landmass (Molyneux, 1997; Serap, 2003; WHO, 2010). African canine trypanosomosis have found its way beyond boundaries into Europe where apparently is devoid of tse tse fly. This is due to the diverse clinical forms of the different infecting parasites. *T. congolense* having three main strains of unequal pathogenicity causes diverse clinical conditions in the dog. The savannah strain of *T. congolense* produces asymptomatic clinical condition in infected dogs and has been implicated as the cause of the first ever recorded African canine trypanosomosis in the UK (Gow et al., 2007). Unknown to the importer, a six year old neutered male jack Russell Terrier was harboring the Savanna strain of *T. congolense* and therefore was passed undetected after long days of quarantine. However few days after arrival in the UK, Jack developed signs of anaemia and before long it died. Asymptomatic strains therefore enhance the distribution of canine trypanosomosis beyond geographic boundaries. *T. evansi* is found mainly in Northern Africa, Near East, Far East, and Central and Southern America. It was spread mechanically by several haematophagus biting flies and vampire bats in Latin America (William and Deborah, 1997; FAO, 2007). *T. rangeli* in dogs is endemic in Latin America as a parasite of man while *T. caninum* has only been identified in south-eastern Brazil (CBVD, 2010; Barros et al., 2012).

American canine trypanosomosis (Chagas) is found mainly in the southwestern United States and sporadically in southern United States (Masumu, 2006). The disease spread to Latin American populations following human migration in the last three centuries into natural habitats of triatomines species commonly known as "kissing bugs" (Amoro, 2004). The distribution of Chagas disease in dogs beyond triatomine zones is greatly influenced by the rise in canine blood transfusion (Rosypal et al., 2007). And in 1996, Chagas disease was recorded in the municipal areas in Brazil (Maywald et al., 1996).

Transmission

T. b. brucei and *T. congolense* are transmitted to susceptible dogs through tse-tse flies' bite during their feeding (Luckins, 1973; CBVD, 2010). *T. evansi* is transmitted mechanically in South America through vampire bats and by ingestion of infected herbivore meat (Steverding, 2008; Uilenberg, 1998). *T. cruzi* and *T. rangeli* are both transmitted by triatomines, such as kissing bugs. Dogs and cats can become infected with *T. cruzi* and *T. evansi* through ingestion of the vector's excreta or ingestion of the entire infected vector (Cohen and Gurtler, 2001; Eloy and Lucheis, 2009). Infections may also be through penetration of dogs' intact or abraded skin by metacyclic forms of *T. cruzi* (Uilenberg, 1998). In-utero and colostral, infections rarely occur

(Uilenberg, 1998). Infection with *T. rangeli* may be through contamination of tritomine feeding sites on the body of the dog with infected saliva or through the vectors' excreta (CVBD, 2010). The mode of transmission of *T. caninum* is yet unknown.

Pathogenesis

The pathology associated with canine trypanosomosis depends on the infecting species of *Trypanosome* (Hunt, 2010). However, irrespective of the species, there is formation of chancre within few days of a tsetse fly bite. Chancre formation is a local skin inflammatory reaction elicited as trypanosomes gain entry through the skin barrier (Manson-Bahr, 1931; FAO, 1998). The size of chancre is determined by the dog's immune status, the virulence of the infecting *Trypanosoma* species and the inoculation dose. Rapidly dividing parasites inside the chancre enter the regional lymph nodes to the afferent lymphatics to the thoracic lymph duct and finally the blood (Mario et al., 1997).

The incubation period for canine trypanosomosis caused by *T. b. brucei* is from four to eight days post infection (Anene et al., 1989; CBVD, 2010). From the blood, trypanosomes, especially *T. b. brucei* and *T. evansi*, are disseminated to various tissues and organs of the body while other species such as *T. congolense* remain within the blood vessels (Losos, 1986; Abubakar et al., 2005; Mario et al., 1997). However, it is been observed that *T. congolense* can invade tissues under certain conditions (Adah et al., 1992). An infection with *T. evansi* produces similar clinical manifestation as in *T. brucei brucei* (Mario et al., 1997). The presence of parasites in the lymph nodes causes profound enlargement of the tissues due to cellular proliferation in B-cell areas and migration of leucocytes from the chancre. Soon after, there is sequestration of the parasite in several organs such as the heart, liver and spleen (Akpa et al., 2008). Splenomegaly is a feature of the acute or parasitemic phase of infection and is mainly the result of the red cell and lymphocyte sequestration and an expanded macrophage population (Murray and Dexter, 1988).

In the liver, the Kupffer cells phagocytose the parasites that are bound by the infected dog's antibodies. The organ may become enlarged and congested with Kupffer cell hyperplasia and periportal mononuclear cell infiltration (Murray et al., 1980). Dogs with pernicious anaemia may have centrolobular necrosis of the liver. The main changes in the bone marrow are a reduction in the cellular components affecting the red blood cells, lymphocytes and platelets (Eloy and Lucheis, 2009).

The heart is consistently damaged in dogs infected with *T. b. brucei*, *T. cruzi* or *T. congolense* producing distinct lesions (Katherine and Edith, 2004; Mario et al., 1997). Pathogenesis of American canine trypanosomosis starts immediately after contamination of feeding site of trito-

mine. These vectors pass out *T. cruzi* in their faeces during blood meal which accidentally penetrates the feeding site on the dog. The bite causes itching and the act of scratching facilitates the penetration of parasites into the tissues. Acute Chagas disease is usually seen in young dogs between 5 to 6 months old (Caliari, 1996; Hunt, 2010). Such dogs may just die suddenly due to severe inflammatory reactions in the heart often confused with more common causes of heart disease (Eloy and Lucheis, 2009). Though this condition is rare expect in cases of invasion of large numbers of parasites into the heart (Ettinger et al., 1997). Dogs with acute experimental infections with *T. cruzi* showed alterations in the neurons of the Auerbach's plexus and myositis in the lower third of the esophagus (Mario et al., 1997).

Chronic Chagas disease is characterized by myocarditis, as in man associated with remodeling of cardiac structure resulting to right-sided cardiac dysfunction and unusual conduction disturbances such as arrhythmias (Meurs et al., 1998). These conditions are easily detected with electrocardiographic and echocardiography examination of the heart (Meurs et al., 1998). Such dogs may have alterations in the brain and the peripheral nerves during the acute and chronic phases of the disease (Eloy and Lucheis, 2009). Cardiac lesions associated with African *T. b. brucei* infections shows a marked cellular infiltration within the perivascular and interstitial locations. Such infiltrates are composed mainly of lymphoid cells, plasma cells, macrophages and occasionally eosinophils (Andrade et al., 1997). Cardiac lesions associated with *T. congolense* infection have scanty cellular infiltrate consisting of small lymphocytes and occasionally macrophages and plasma cells (Adah et al., 1992; Murray et al., 1980). Infections with *T. congolense* are mostly vascular with few extravascular parasites (Adah et al., 1992).

Oedema of the perivascular and interstitial spaces is often observed in canine trypanosomosis particularly at the terminal stage. The perivascular oedema of the cardiac musculature possibly reflects increased permeability and extensive degeneration of the heart fibers. This is probably due to anoxia caused by the prolonged anemia and immune mediated pathology. Dogs infected with *T. b. brucei* and *T. cruzi* show a severe meningo-encephalitis similar to those described in fatal cases of human trypanosomosis.

Clinical signs

Knowledge of the clinical/pathological features in response to *Trypanosoma* species infections in dogs has been supplemented by studies in dogs experimentally infected with these pathogens. Clinical manifestations of American canine trypanosomosis differ markedly from that of African canine trypanosomosis. Experimental infection with canine trypanosomes typically follows three

successive stages: acute, sub-acute and chronic forms, though under a natural challenge scenario, it may be more complex (Katherine and Edith, 2004). Dogs are refractory to infection with *T. rangeli* (CVBD, 2010). In dogs *T. b. brucei* is responsible for an acute disease with high parasitemia. The early acute phase of the disease is marked by the continuous presence of trypanosomes in the blood at detectable concentration (10^3 to 10^8 /ml) (OIE, 2008; Nwoha and Nwoha, 2011a). Pyrexia is highest at the first peak of parasitemia, thereafter at parasitemic waves which often corresponds with the development of anemia (Aquino, 1997). Anemia is the most prominent feature of Canine trypanosomosis (Franciscato et al., 2007; Nwoha and Anene, 2011b). This is easily observed clinically as pallor of the mucous membrane. The virulence of the infecting parasite population and the age, nutritional status and breed of the host influence the severity of anemia.

African canine trypanosomosis is marked by infiltration of the subcutaneous tissues with fluid (oedema) swelling of the eyelids, the lips and the skin beneath the lower jaw (Nwoha and Anene, 2011a). Some dogs develop keratitis which may result in unilateral or bilateral cornea opacity with moderate lacrimal discharge (Nwoha and Anene, 2011a). A few cases develop the neurological form of the disease usually in post-therapy. This form is similar to rabies and terminates fatally within few weeks. Emaciation may or may not be seen in dogs with acute infection of the disease. Most dogs show marked weakness and lethargy. In the terminal stage of the disease, animals become extremely weak and are often unable to rise, death of the infected animal may occur in the first few weeks or months after infection as a result of the acute disease (Nwoha and Anene, 2011a).

In contrast to the acute phase of infection, dogs infected with *T. congolense*, *T. evansi*, *T. rangeli*, *T. cruzi* and *T. caninum* often show a chronic form of the disease with ocular signs such as keratitis, uveitis, coagulopathies in *T. evansi* and blepharo conjunctivitis (Amole et al., 1982; Mario et al., 1997). *T. evansi* sometimes produces acute syndrome in dogs manifested as urticarial plaques and ophthalmitis which are transitory and may relapse (Mario et al., 1997). Similarly, less frequently, others cause acute syndrome in imported dogs, with pyrexia, prostration, severe anaemia and death in 2 to 3 weeks post infection (CVBD, 2010; Nwoha and Anene, 2011b). Clinical signs in American canine trypanosomosis often present asymptomatic to chronic disease states in dogs and cats (Teixeira et al., 1990). The acute phase often is seen in young dogs characterized by generalized myocarditis and extensive degeneration of the central nervous system. Such dogs exhibit signs of lethargy, splenomegally, enlarged lymphnodes, diarrhea, myocarditis and sudden death. Diseased hearts may slowly deteriorate in function and resultant symptoms may be confused with those of other heart diseases (Kirchhoff, 2011).

Chronic form of the disease is commonly seen in adult dogs after several months of initial infection and is characterized by ventricular arrhythmias and myocardial dilation. The cardiac insufficiency is initially detected on the right side and later progressed to left ventricular insufficiency (Ettinger et al., 1997). Cats may have pyrexia, convulsions and paralysis of the hind limbs (Kirchhoff, 2011).

Anemia in the chronic phase is not strictly associated with the presence of parasite in the blood, but is as a result of exhaustion of the limited pluripotent stem cells of the bone marrow from constant assaults by waves of parasitemia (Manson-Bahr, 1931). Dogs may be intermittently parasitemic at this period. Dogs having chronic canine trypanosomosis are weak, cachexic debilitated towards the terminal stage of the disease. Regardless of their weakened condition, some dogs continue to eat, and this may last for months before their death (Nwoha and Anene, 2011a).

Pathology

There are no pathognomonic lesions in trypanosome infected dogs. General lesions are congestive, inflammatory, coagulopathies, oedematous, degenerative and sometimes haemorrhagic changes in various organs such as the heart, central nervous system, (CNS), eyes, testes, ovaries and the pituitary gland (Eloy and Lucheis, 2009). There is usually oedema of the head, thorax and forelimbs. The carcass shows muscle wasting and gelatinous appearance of cutaneous fat (Nwoha and Anene, 2011a).

Congestive heart failure is an important cause of death in chronic cases and is related to the combined effects of prolonged anemia, myocardial damage and increased vascular permeability (Katherine and Edith, 2004). The superficial lymph nodes are slightly enlarged, oedematous on cut surface, the liver and spleen are swollen and congested, while the kidneys are pale and on the cut surface shows hemorrhages especially along the corticomedullary junction (Nwoha and Anene, 2011a).

In chronic cases, lymphnodes and spleen frequently return to normal size and in some cases, they eventually become atrophy and sclerose (Katherine and Edith, 2004). There is hydrothorax and hydropericardium containing straw coloured fibrin flaked fluid (Nwoha and Anene, 2011a). The pericardial fats are gelatinous and the lungs are emphysematous with haemorrhages in the trachea.

The meninges of the brain are haemorrhagic. The urine shows deviation from the normal amber colour with a pH of 6.0 with evidence of an increased number of leukocytes in urine; this could be the reason for the slight change in specific gravity and increased turbidity of the urine (Katherine and Edith, 2004; Nwoha and Anene, 2011a).

Diagnosis

The diagnosis of canine trypanosomosis is dependent on a combination of detailed clinical examination, proper sample selection/collection, sample size, appropriate diagnostic tests, and proper conduction of tests and logical interpretation of results. In canine trypanosomosis where disease prevalence is high, some tests of low diagnostic sensitivity may suffice (OIE, 2008). Parasitological diagnosis could be made by microscopic examination of either the lymph node aspirates of blood, or cerebrospinal fluid (CSF) of infected dogs (François et al., 2005). Blood samples should be examined as soon as possible to avoid immobilization and subsequent lysis of trypanosomes in the sample. Often blood samples collected at the tip of the ear yield a larger quantity of parasites when compared to venepuncture (Uche, 2010). The collected blood sample should be preserved in an ice pack container away from sunlight because trypanosomes are rapidly destroyed by sunlight (OIE, 2008). In preparation of wet blood films, a drop (about 2 µl) of blood is placed on a clean slide and covered with a cover slip to eliminate air bubbles. It is then examined microscopically (magnification, 400x) with condenser aperture, phase-contrast or interference contrast for proper visualization (22 x 22 mm). A detailed procedure of this test is seen in WHO Trypanosomosis Control Manual (1983).

Although, this technique has a very low detection power of 10,000 parasites in 200 microscopic fields, it is the most commonly used test in trypanosomosis (François et al. 2005). Microscopic examination enhances detection of trypanosomes darting across the microscopic field in positive *T. brucei* while *T. congolense* parasites move sluggishly and thus allows a definite diagnosis. The movement of the surrounding erythrocytes often attracts attention to the presence of trypanosomes in the blood. Due to fluctuations in parasitemia, blood samples should be collected every other day to check for a peak in parasitemia when the parasites will be easily detected. The sensitivity of this technique may be significantly improved by lysis of the RBCs before examination using a haemolytic agent such as sodium dodecyl sulfate (SDS) (OIE, 2008).

Lymph node aspirate

Examination of lymph aspirates from prescapular lymph nodes detects up to 80% of the infection (Robson and Ashkar, 1972). Lymph is aspirated from enlarged cervical lymph nodes and one to two drops of the fresh aspirate is expelled onto a slide, and a cover slip is applied to spread the sample and prepare a smear. The wet preparation is mounted immediately and viewed under the microscope (magnification, 400x) for the presence of motile trypanosomes (François et al., 2005). The sensitivity of

this procedure varies between 40 and 80% depending on the parasite strain, the stage of the disease (sensitivity is higher during the acute stage), and concurrent infection with pathogen that causes lymphadenopathy (Simarro et al., 2003; Van Meirvenne, 1999).

Thin and thick blood smears

Thin/thick blood smear is another parasitological technique that can be used in the diagnosis of trypanosomosis. This technique is not tedious and can be carried out easily by an experienced technician. Giemsa- or Field's-stained thin blood films are made by placing a drop of blood (about 5 μ l and 5 to 10 μ l for thick blood) film at one end of a slide, the edge of another slide is placed just close enough to the drop of blood for it to spread along the edge. Then, with a swift movement blood is spread on the slide. Ideally, thin films should be prepared so that the RBCs are fairly closer to each other but with no overlapping. The slide is air dried and then fixed in methanol. The fixed slide is later stained with Giemsa stain in phosphate buffered saline at a pH of 7.2. A more detailed technique can be found in WHO trypanosomosis control manual. After preparation, the stained slide is allowed to dry and then examined under a phase contrast microscope. This technique helps in the identification of the particular infecting trypanosome species and is often used where there is no centrifuge (Lumsden et al., 1979). Sensitivity of this test may be improved by increasing the thickness of stained slides. A fixed smear should be kept dry and protected from dust, heat, flies and other insects that may feed on them (OIE, 2008).

Microhematocrit centrifugation technique (mHCT):

This technique sometimes referred to as the capillary tube centrifugation technique or as the Woo test, was developed more than 30 years ago and is still used in the diagnosis of trypanosomosis in man and animals (Woo, 1970; 1971). In this procedure, heparinized capillary tubes are three-quarter filled with the suspected blood sample containing anticoagulant. The dry ends of the capillary tubes are sealed with plasticine or heat (OIE, 2008). The capillary tubes are centrifuged at 3000 rpm for 6 to 8 min. Trypanosomes becomes concentrated at the level of the white blood cells, between the plasma and the erythrocytes. The centrifuged capillary tubes can then be examined under the microscope at low magnification of x100 or x200 for motile parasites. mHCT is a more sensitive technique than wet mount, and the sensitivity of mHCT is increased with the number of tubes examined (OIE, 2008).

Quantitative buffy coat (QBC): The quantitative buffy coat or Murray method (QBC; Beckton-Dickinson) was initially developed for the rapid assessment of differential cell counts, but is now being applied to the diagnosis of

hemoparasites including trypanosomes (Levine et al., 1989; Bailey and Smith, 1992). It is a widely used improved method of diagnosis of trypanosomes involving the staining of trypanosome kinetoplasts and nuclei with acridine orange for easy differentiation from the white blood cells at the buffy coat level (François et al., 2005). About 1500 to 2000 μ l of blood in heparinized capillary tubes containing acridine orange is centrifuged at 3000 rpm to allow separation. The buffy coat is aspirated into a microhaematocrit capillary tube and re-centrifuged. Motile trypanosomes can be identified by their fluorescing kinetoplasts and nuclei in the expanded buffy coat. The flourescented trypanosomes are best appreciated in a dark room using ultraviolet light generated by a cold light source connected by a glass fiber to a special objective containing the appropriate filter. The QBC has about 95% sensitivity and can detect positive cases of low parasitemia (François et al., 2005).

Mini-anion-exchange centrifugation technique (mAECT):

The mAECT was introduced by Lumsden et al. (1979) based on a technique developed by Lanham and Godfrey (1970). An updated version has been described by Zillmann et al. (1996). The technique is based on the ability of the negatively charged RBCs to be held back in the anion column, and the less negatively charged trypanosomes to pass through with the solution. The trypanosomes are concentrated in the solution by low-speed centrifugation (François et al., 2005). The concentrate is then examined in a special holder under the microscope for the presence of trypanosomes. This technique is highly sensitive than most of the other described techniques because of large blood volume (300 μ l) used, which enables the detection of less than 100 trypanosomes/ml (OIE, 2008).

In vitro cultivation

In vitro cultivation of *T. brucei* has been described over the years but with varying degrees of success (McNamara et al. 1995; OIE, 2008). About 5 to 10 ml of blood is cultured in the laboratory and blood stream forms of trypanosome transforms into large proliferating procyclic forms detectable within three to four weeks (François et al., 2005). The technique requires sophisticated equipment, it is time consuming and not suitable for large scale or routine diagnosis. KIVI kit can be used *in vitro* in the isolation and amplification of all species of *T. brucei* in humans, domestic and game animals (Truc et al., 1992). However, its effectiveness in the isolation of *T. congolense* and *T. brucei brucei* in dogs is yet to be determined.

Animal inoculation

Mouse inoculation may be used for the detection of posi-

tive cases with sub-clinical infections by the inoculation of specific pathogen free (SPF) mouse with blood sample from animals suspected to be infected with trypanosomosis, and allowing for establishment of infection and screening them for parasitemia (WHO, 1998). The immunity of the test mouse can be suppressed by administration of corticosteroids or by irradiation in order to increase their chances of developing parasitemia and isolating the parasite. The SPF mice are bled thrice a week for at least two months until detection of parasitemia. Factors such as chronic infections of low parasitemia, is the fact that some strains of *T. congolense* do not replicate in the mice and the animal welfare regulations may influence the use of this technique (OIE, 2008).

DNA AMPLIFICATION TESTS

A polymerase chain reaction (PCR) technique could be used as a diagnostic tool in cases of canine trypanosomosis as it can be applied on any patient sample that contains trypanosomes DNA (OIE, 2008). The technique involves the amplification of specific DNA of different trypanosome species. Samples to be analyzed should be protected from sunlight to avoid DNA degradation (François et al., 2005). Currently, the technique has been applied on *T. brucei* for detection of its three species and three types of *T. congolense* with success. Other species of trypanosome that affect dogs can undoubtedly be tested with it. This test is important in the detection of possible new strains of trypanosomes that may affect dogs. The primer sets available for different *T. brucei* subgenus, species and types are referred to as follows: Trypanozoon subgenus - TBR1 and TBR2; *T. congolense* (savannah type) - TCN1 and TCN2; *T. congolense* (forest type) - TCF1 and TCF2; *T. congolense* (Kenya Coast type) - TCK1 and TCK2. Due to the multiplicity of these taxon-specific primers, a full trypanosome species identification requires that five PCR test can be carried out per sample, and therefore cannot be used as a routine diagnostic technique in dogs (OIE, 2008).

Serological techniques

Antibody-detection enzyme-linked immunosorbent assay (indirect assay)

The technique of antibody ELISA has recently been developed for use in the diagnosis of trypanosomosis in animals (Lumsden, 1977) and has been used in large-scale surveys of bovine trypanosomosis (Desquesnes, 1997; Hopkins et al., 1998), though it could also be employed in canine trypanosomosis. The standard antigen for trypanosomosis antibody tests is derived from

purified bloodstream-forms of trypanosomes and the procedure can be obtained from OIE Terrestrial manual (OIE, 2008).

In bovine trypanosomosis, ELISA, using *T. congolense* or *T. vivax* precoated microtitre plates, has been developed for diagnosis of bovine trypanosomosis (OIE, 2008). Similar precoated microtitre plates can also be produced for diagnosis of canine trypanosomosis especially as it has an advantage of providing a standardized denatured antigen that can be preserved for a long time at room temperature. The suspected test serum is reacted with trypanosomal antigens present in the ELISA microtitre plate, after which the resulting antigen/antibody complex is then incubated with an enzyme-conjugated antiglobulin to IgG fraction of the suspected dog. The reaction is then visualized by the addition of enzyme substrate and chromogen, with the resulting colour change allowing a photometric interpretation (Luckins, 1973).

The absorbances of each ELISA-sample tested is expressed as a percentage (percentage positivity: PP) of the strong positive reference standard or the positive and negative reference standard results (OIE, 2008). The cut-off value is determined using known positive and negative field or experimental samples. Both antibody-detection tests have high sensitivity and genus specificity. Their species specificity is generally low, but may be improved by using a standardized set of the three species-specific tests (Desquesnes, 2004) or by fractionation of test crude trypanosomal antigen extract which will enable discrimination between infecting species (Ijagbone et al., 1989).

Laboratory assays essential for confirmation of trypanosomosis

Most of the immunohistochemical techniques are of high sensitivity and little specificity such as (Ag, Ab) ELISA test described above which often detects the presence of IgM during acute infection and IgG in chronic cases. The reduced specificity encountered in the use of these techniques is because of cross reactivity between trypanosomal species and with concurrent infections such as microfilaria and *Leishmania*. Recently, the invention of modified ELISA technique (Cellabs Elisa *T. cruzi* and Hemagen Chagas kit) used in diagnosis of *T. cruzi* infection in humans gives a 100% sensitivity and specificity (Annette et al., 2006). This modified ELISA technique may also give similar result in the diagnosis of canine trypanosomosis. Indirect fluorescent antibody test (IFAT) has been used extensively in the diagnosis of typanosomosis in both man and animals. The original method for this test has been replaced by a new technique for the preparation of trypanosomal antigens. This involves fixation of live trypanosomes using a mixture of 80% cold acetone and 0.25% formalin in normal

saline. The use of IFAT in the diagnosis of bovine trypanosomosis has proven to be both specific and sensitive in detecting trypanosomal antibodies in infected cattle (Wilson, 1969; Luckins and Mehlitz, 1978) and camels (Luckins et al., 1979).

Thus, it may show similar sensitivity and specificity in the diagnosis of canine trypanosomosis. The technique involves the preparation of a thin smear from a suspected blood sample which is allowed to dry and is later fixed in acetone for few minutes. About 5 mm diameter circles are marked on glass slides using nail varnish. A 1: 40 diluted test sample's serum is pipetted into each circle, ensuring that the area in each circle is completely covered. The antigen/test serum preparation is incubated at 37°C for 30 min in a humid chamber. Afterwards, the preparations are washed thrice in PBS for 5 min each time at 4°C with gentle agitation and then air-dried. Rabbit or goat anti-bovine IgG conjugate that have been conjugated to fluorescein isothiocyanate is added and then slide washed and incubated as aforementioned. A clear detailed procedure could be seen in OIE Terrestrial manual (OIE, 2008).

The slides are further rinsed in distilled water and air dried. The dried slides are mounted in PBS or buffered glycerol and examined for fluorescence. However, this technique has some limitations which include high cost of the technique involving sophisticated microscope and cross reactivity between trypanosomal species. Therefore, IFAT cannot be used for routine test diagnosis of canine trypanosomosis.

Biochemistry analysis

Though there is so much inconsistency in the biochemical changes observed in canine trypanosomosis, there are still some parameters that are somewhat consistent from the literature. Barr et al. (1991) recorded elevated serum liver enzymes alanine transferase ALT, aspartate amino phosphotase ASP and LDH in the acute phase of Chagas disease in dogs. Eloy and Linchein (2009) observed hyperproteinaemia which contradicts the findings of Barr et al. (1991) on TP. The hyperproteinaemia was attributed to high antigenic stimulation associated with trypanosomosis (Aquino, 2002). African canine trypanosomosis caused by *T. brucei brucei*, *T. congolense* and *T. evansi* are mostly characterized by elevated liver enzymes, blood urea nitrogen BUN, creatinine and bilirubin concentrations (Aquinos, 2002; Nwoha et al., 2013).

However under field infection ASP has been the only liver enzyme found above the normal range in the serum and has been attributed to either hepatic or muscular damage (Franciscato et al., 2007). Several workers have recorded decreases in TP in experimental African canine trypanosomosis and attributed it to loss of albumin in urine (Franciscato et al., 2007; Nwoha et al., 2013). The

discrepancies in biochemistry changes in canine trypanosomosis are function of the diagnostic technique, expertise and physicochemical dynamics in the dog.

Treatment

Treatment of African canine trypanosomosis is an area that has been under a lot of challenges especially as regards to the availability of effective trypanocides in the market. There have been development of several compounds with efficacy against canine trypanosomosis, however none of these products have been produced in a large commercial scale or even available in the market. The apparent unavailability of new trypanocides in the market have remained a great challenge to the treatment of the disease. Diminazene aceturate have shown efficacy when used to treat canine trypanosomosis used at the dose of 3.5 mg/kg in *T. congolense* infection; 7 mg/kg in *T. brucei brucei* and *T. evansi* (Aquinos, 2007). Usually parasitaemia disappears after 48 h post treatment. The constant use of diminazene aceturate over time has led to the development of resistant strains of canine trypanosomes. There are abundant strains of canine trypanosomes especially *T. brucei brucei*, *T. congolense* and *T. evansi* which are refractory to diminazene thus results to repeat treatment of infected dogs and constant relapses (Doyle, 2009; Nwoha and Anene, 2013; Chigozie et al., 2012). Treatment of American trypanosomosis is equally as difficult, as infected dogs often develop remodeling of the heart which gradually leads to heart failure.

Hence, treatment does not provide complete recovery but only sustains the life of the dog for some reasonable period (Amoro, 2004; Desquenes et al., 2001). The use of beta adrenergic blockers such as carvedolol, propranolol and atenolol could be beneficial to reduce the blood volume and cardiac out. This helps to reduce the stress on an ailing heart, low doses of angiotensin conversion enzymes inhibitors (ACEIs) and in particular enalapril, veno- or iono-dilators like prazosin or pimo-bendan, calcium transport and utilization of modifiers singly or in various combinations may be useful in attenuating the progression of HDs to HF in infected dogs (Sisson, 1994; Wolley et al., 2007) and therefore could be of some clinical benefit in cases of Chagas disease in dogs.

Vaccination

Attempts have been made by some workers to produce a protective vaccine against trypanosomosis both in humans and animals. One which seems to provide hope in this direction is the administration of anti-idiotypic (anti-id) antibodies to infected animals. Anti-id induces lymphocytes and antibodies of complementary specificity under

certain experimental conditions (Benca et al., 1980, Miller et al., 1981). Injection of minute amounts of anti-id antibodies induces antigen-specific helper T-cells and enhances the expression of the corresponding id in subsequent antibody response (Kelsoe et al., 1980). Administration of the anti-id produces antigen-binding id positive molecules in the absence of exposure to antigen, and therefore may be used to regulate the immune system of dogs by its expansion of B-cells clones bearing the appropriate id without specific antigen stimulation. Mice immunized against trypanosomosis with anti-id antibodies gave a partial to complete immunity to infection (David et al., 1982) and this may be tried in dogs.

Recently, dogs were vaccinated with a fixed *T. rangeli* against canine trypanosomosis (Basso et al., 2007). Experimental infections of the vaccinated dog produced disease of low parasitaemia apparently from vaccine induced immunity. Furthermore, feeding of the vaccinated dogs with the nymph stage of triatomine reduced the rate of infection in the bugs. Since dogs are the reservoir of Chagas disease in man, advances in this area could reduce the rate of infection of kissing bug which will in turn aid in the control of the disease in man (Basso et al., 2007).

Differential diagnosis

Some diseases can be confused with clinical cases of trypanosomosis in dogs and these include:

1. Acute trypanosomosis with pyrexia: canine babesiosis, canine anthrax, canine anaplasmosis, canine haemorrhagic and septicaemia.
2. Chronic trypanosomosis with anaemia and emaciation: canine ancylostomosis, canine ascariasis, malnutrition and other haemoparasitosis.

REFERENCES

- Abubakar A, Iliyasu B, Yusuf AB, Igweh AC, Onyekwelu NA, Shamaki BA, Afolayan DO, Ogbadoyi EO (2005). Antitrypanosomal and haematological effects of selected Nigerian medicinal plants in Wistar rats. *Biokemistri*, 17:95-99.
- Adah MI, Otesile EB, Joshua RA (1992). Changes in levels of transaminases in goats experimentally infected with *T. congolense*. *Revue Elev. Med. Vet. Pays. Trop.* 45(3-4):284-286.
- Ahmed AB (2007). High trypanosome infections in *Glossina palpalis palpalis* Robineau-Desvoidy 1830 in Southern Kaduna State, Nigeria. *Sci. World J.* 2-2.
- Akpa PO, Ezeokonkwo RC, Eze CA, Anene BM (2008). Comparative efficacy assessment of pentamidine isethionate and diminazene aceturate in the chemotherapy of *Trypanosoma brucei brucei* infection in dogs. *Vet. Parasitol.* 151:139-149.
- Amole BO, Clarkson JR, Shear HL (1982). Pathogenesis of anaemia in trypanosome *brucei* infected mice. *Infect. Immun.* 36(3):1060-1068.
- Andrade ZA, Andrade SG, Sadigurski M, Wenthold RJ, Hilbert S, Law Ferrans VJ (1997). The Intermediate phase of chagas disease: ultrastructural characterization of cardiac changes in the canine model. *Am. J. Trop. Med. Hyg.* 57:328-336.
- Amora SSA (2004). Epidemiologia da leishmaniose e tripanossomiase canina no municipio de Mossoro (Master's thesis). Fortaleza: faculdade de Veterinaria da Universidade Estadual do Ceara, 90p.
- Anene BM, Chukwu CC, Anika SM (1989). Immunosuppression of humoral immune response in canine trypanosomosis. *Microbios Let.* 40:37-46.
- Anene BM, Ifebigh AO, Igwilo IA, Umeakuana PU (2010).¹Prevalence and haemato-biochemical parameters of trypanosome infected pigs at Nsukka, Nigeria. Springer London.
- Annette KM, Erick A, Sheldon EL, Harry RH, Christiane ML (2006). Serological Diagnosis of *Trypanosoma cruzi*; evaluation of three enzyme immunoassays and an indirect immunofluorescent assay. *J. Med. Microbiol.* 55, 171-178.
- Aqinos LPCT (2007). Importancia da infeccao por trypanosomaa evansi em caes no Brasil; <http://www.fav.br/programasinstreristas/revistas2007/veterinaria/imp-ortancia-da-infeccao.pdf>.
- Aquino LPCT (1997). Aspectos clinicos, imunologicos patologicos da infeccao experimental em caes por trypanosome evansi.(Master's thesis). Jaboticabal: faculdade de ciencias Agrariase Veterinarians da Universidade Estadual Paulista. 93 p.
- Aquino LPCT, Machado RZ, Alessi AC, Santana AE, Castro MB, Marques LC, Malheirus EB (2002). Haematological biochemical and anatomopathological aspects of experimental infection with trypanosome evansi in dogs. *Arg Bras Med Vet Zootec.* 55(1):8-18.
- Ashkar T, Ochilo M (1972). The application of the indirect fluorescent antibody .test to samples of dried sera and blood from cattle in the Lambwe Valley, South Nyanza, Kenya. *Bull. Wild Hlth Organ.* 47:769-772.
- Bailey JW, Smith DH (1992). The use of the acridine orange QBC technique in the diagnosis of African trypanosomiasis. *Trans. R. Soc. Trop. Med. Hyg.* 86:630.
- Barr SC, Gossett KA, Klei TR (1991). Clinical, clinicopathologic and parasitologic observations of trypanosomiasis in dogs infected with North American *Trypanosoma cruzi* isolates. *Am J. Vet. Res.* 52(6):954-960.
- Barros JHS, Almeida ABPF, Figueiredo FB, Sousa VRF, Fagundes A, Pinto AGS, Baptista C, Madeira MF (2012). Occurrence of *Trypanosoma caninum* in areas overlapping with leishmaniasis in Brazil: what is the real impact of canine leishmaniasis control?. *Trans. R. Soc. Trop. Med. Hyg.* 106(7):419-423.
- Basso B, Castro I, Introini V, Gil P, Truyens C, Moretti E (2007). Vaccination with *Trypanosoma rangeli* reduces the infectiousness of dogs experimentally infected with *Trypanosoma cruzi*. *Vaccine* 25(19): 3855-3858.
- Benca RJJ, Quintas JF, Kearney PMF, Schreiber H (1980). Studies on phosphorylcholine-specific T cell idiotypes and idotype specific immunity. *Mol. Immunol.* 17:823.
- Bengaly Z, Sidibe I, Ganaba R, Desquesnes M, Boly H, Sawadogo L (2002). Comparative pathogenicity of three genetically distinct types of *Trypanosoma congolense* in cattle: clinical observations and haematological changes. *Vet. Parasitol.* 108(1):1-19.
- Caliali ER, Caliali MV, De Lana M, Tafuri WL (1996). Quantitative and Qualitative studies of the Auerbach and Meissner plexuses of the esophagus in dogs inoculated with *Trypanosoma cruzi*. *Rev. Soc. Bras. Med. Trop.* 29:17-20 (in Portuguese).
- Cohen JE, Gurtler RE (2001). Modelling household transmission of American trypanosomosis. *Science.*
- CVBD (2010). Canine Vector Born Diseases. Trypanosomosis. 4th Internal Symposium.
- De Souza IWA (1999). Short Review on the morphology of *T. cruzi*. *Mem. Inst. Osw. Cruz.* 99(1):17-36.
- Desquesnes M, Beangaly Z, Millogo L, Meme Y, Sakande H (2001). The analysis of the cross- reactions occurring in antibody ELISA for the detection of trypanosomes can improve identification of the parasite
- Desquesnes M (1997). Standardisation internationale et régionale des épreuves immuno-enzymatiques: méthode, intérêts et limites. *Rev. Sci. Tech. Off. Int. Epiz.*, 16: 809-823.
- Desquesnes M (2004). Livestock Trypanosomosis and their Vectors in Latin America. OIE and CIRAD (Centre de coopération internationale en recherche agronomique pour le développement). World Organisation for Animal Health (OIE), Paris, France. 192.

- species involved. *Ann Trop. Med. Parasitol.* 95(2):141-155.
- Doyle RL (2006). Eficacia de tres medicamentos no controle da infecção experimental por trypanosome evansi em ratos (*Rattus norvegicus*) linhagem wistar. (Master's thesis). Santa Maria; Centro de Ciências Rurais da Universidade Federal desanta maria. P 40.
- Eloy LJ, Lucheis SB (2009). Canine trypanosomiasis: etiology of infection and implications for public health. *J. Venom. Anim. Toxins incl. Trop. Dis.* 15(4):589-611.
- Ettinger SJ, Feldman EC (1997). *Tratado de medicina Veterinaria internamolestias do cao e do gato.* 4th ed. Sao Paulo; manole. 2-2930.
- Finelle P (1973). African animal trypanosomiasis., *Animal Production and Health Division, FAO, Rome.*
- François C, Louis L, Pere S, Veerle L, Philippe B (2005). Options for Field Diagnosis of Human African Trypanosomiasis. *Ame. Soc. Micro.* 18(1):133-146
- Franciscato C, Lopes STA, Teixeira MMG, Monteiro SG, Wolkmer P, Gramatz BC (2007). Cao naturalmente infectado por trypanosome evansi em santa maria, RS, Brasil. *Cienc. Rur.* 37(1):288-291.
- Gow AH, Simpson JW, Picozzi K (2007). First report of canine African trypanosomiasis in the UK. *J. Small Anim. Pract.* 48(11):658-661.
- Hopkins JS, Chitambo H, Machila N, Luckins AG, Rae PF, Van den Bossche P, Eisler MC (1998). Adaptation and validation of the antibody trapping ELISA using dried blood spots on filter paper, for epidemiological surveys of tsetse transmitted trypanosomiasis in cattle. *Prev. Vet. Med.* 37:91-99.
- Hunt RC (2010). Molecular parasitology: trypanosomes eucaryotic cells with a different way of doing things. *Microbio. Immunol. on line.*
- Ijagbone IF, Staak C, Reinhard R (1989). Fractionation of trypanosome antigens for species specific diagnosis. *Vet. Parasitol.* 32:293-299.
- Jiménez-Coello M, Guzmán-Marín E, Ortega-Pacheco A, Acosta-Viana KY (2010). Serological Survey of American Trypanosomiasis in Dogs and Their Owners From an Urban Area of Mérida Yucatán, México. *Trans. Emerg. Dis.* 57 (1-2):33-36.
- Jones TC, Hunt RD, King NW (2000). Molestias causadas por protozoarios. In: Jone TC, Hunt D, King NW, editors. *Patologia Veterinaria.* 6th ed. Sao Paulo: manole: 559-610.
- Katherine T, Edith ML (2004). Pathogenesis of animal Trypanosomiasis. *CAB Int.* 331-350.
- Kelsoe G, Reth M, Rajewsky K (1980). Control of idiotype expression by monoclonal anti-idiotope antibodies. *Immunol. Rev.* 52:75.
- Kirchhoff L (2011). Chagas disease (American Trypanosomiasis), WebMD LLC.
- Lanham SM, Godfrey DG (1970). Isolation of salivarian trypanosomes from man and other mammals using DEAE-cellulose. *Exp. Parasitol.* 28:521- 534.
- Levine RA, Wardlaw SC, Patton CL (1989). Detection of haematoparasites using quantitative buffy coat analysis tubes. *Parasitol. Today* 5:132-134.
- Losos GJ (1986). *Infectious Tropical diseases of domestic animals,* Churchill Livingstone Inc., New York.
- Luckins AG (1973). Methods for diagnosis of Trypanosomiasis in livestock. *FAO Corp. Doc. Repos.* 5, 79-87.
- Luckins AG, Boid R, Rae P, Mahmoud MM, El Malik KH, Gray AR (1979). Serodiagnosis of infection with *Trypanosoma evansi* in camels in the Sudan. *Trop. Anim. Hlth Prod.* 11:1-12.
- Luckins AG, Mehlitz D (1978). Evaluation of an indirect fluorescent antibody test, enzyme-linked immunosorbent assay and quantification of immunoglobulins in the diagnosis of bovine trypanosomiasis. *Trop. Anim. Health Prod.*, 10:149-159.
- Lumsden WH, Kimber CD, Evans DA, Doig SJ (1979). *Trypanosoma brucei*: miniature anion-exchange centrifugation technique for detection of low parasitaemias: adaptation for field use. *Trans. R. Soc. Trop. Med. Hyg.* 73:312-317.
- Madeira MF, Sousa MA, Barros JH, Figueiredo FB, Fagundes A, Schubach A, DE Paula CC, Faissal BN, Fonseca TS, Thoma HK, Marzochi M (2009). *Trypanosoma caninum* n. sp.(Protozoa: Kinetoplastida) isolated from intact skin of a domestic dog (*Canis familiaris*) captured in Rio de Janeiro, Braz. *Parasitol.*, 136, 411-423.
- Manson-Bahr P (1931). The Epidemiology of Human Trypanosomiasis at the Present Time. *Proc. Roy. Soc. Med.*, 24(7):837-845.
- Mario L, De La Rue RAS, Geraldo ADC (1997). Coagulopathy in dogs infected with trypanosome (trypanozoon) evansi (steel, 1885) Balbiani, 1888. *Parasitol. Dia.* Vol 21 (3-4).
- Masumu T, Marcotty D, Geysen Vansnick E, Geerts S, Vanden Bossche PA (2006). Modified AFLP for *Trypanosoma congolense* isolate characterization, *J. Biotechnol.* 125, 22-26.
- Maywald PG, Machado MI, Costa-cruz JM, Gonçalves-pires MRF (1996). Leishmaniose tegumentar, visceral e doença de chagas caninas em municípios do triangulo mineiro e Alto parariba, minas Gerais, Brasil. *Cad. Saude Publica.* 12(3):321-328.
- McNamara JJ, Bailey JW, Smith DH, Wakholi S, Godfrey DG (1995). Isolation of *Trypanosoma brucei gambiense* from northern Uganda: evaluation of the kit for in vitro isolation (KIVI) in an epidemic focus. *Trans. R. Soc. Trop. Med. Hyg.* 89:388-389.
- Meurs KM, Anthony MA, Slater M, Miller MW (1998). Chronic trypanosomiasis cruzi infection in dog: 11 cases (1987-1996). *J. Am. Vet. Med. Ass.* 213(4):497-500.
- Miller GG, Nadler PI, Asano Y, Hodes RJ, Sachs DH (1981). Induction of idiotype-bearing nuclease-specific helper T cells by in vivo treatment with anti-idiotypic. *J. Exp. Med.* 154: 24.
- Molyneux DH (1997). Current public status of the Trypanosomiasis and Leishmaniasis. In Hide G. Mottram, JC, Coombs, GH, Holmes, PH (Eds), *Trypanosomiasis and .Leishmaniasis: Biology and control.* CAB International, Wallingford, UK. 12:39-50.
- Murray M, Dexter TM (1998). Anemia in bovine African trypanosomiasis. *Acta Trop.* 45, 389-432.
- Nwoha RIO, Anene BN (2011a). Clinical signs and pathological changes in dogs with single and conjunct experimental infections of *Trypanosoma brucei brucei* and *Ancylostoma caninum*. *J. Vet. Parasitol.* 24(2):91-102.
- Nwoha RIO, Anene BN (2011b). Changes in packed cell volume and haemoglobin concentration in dogs with single and conjunct experimental infections of *T. brucei* and *A. caninum*. *Ohillip. J. Anim. Sci.* 37(2):151-158.
- Nwoha RIO, Eze IO, Anene BM (2013). Serum biochemical and liver enzymes changes in dogs with single and conjunct experimental infections of *Trypanosoma brucei* and *Ancylostoma caninum*. *Afr. J. Biotech.* 12(6):618-624.
- OIE (2008). Trypanosomiasis. *OIE Terrestrial Manual.* Chapter 4, 18.
- OIE (2010). Trypanosomiasis, Tsetse Transmitted. Scientific.dept@oie.int, 2009.
- Raina AK, Kumar R, Rajora VS, Sridhar SRP (1985). Oral transmission of *Trypanosoma evansi* infection in dogs and mice. *Vet. Parasitol.* 18(1):67-69.
- Robson J, Ashkar TS (1972). Trypanosomiasis in domestic livestock in the Lambwe Valley area and a field evaluation of various diagnostic techniques. *Bull. Wld Hlth Organ.*, 47:727-734.
- Rosypal AL, Cortes-Vecino JA, Gennari SM, Dubey IP, Tidwell RR, Lindsay PD (2007). Serological survey of leishmania infantum and trypanosome cruzi in dogs from urban areas of Brazil and Colombia. *Vet. Parasitol.* 149(3-4):172-177.
- Samdi SM, Abeng JN, Kalgo AM (2006). Trypanosomiasis in hunting dogs in Kaduna, North Central Nigeria: implications on the disease in humans. *J. biomed. Invest.* 4(1):15-18.
- Serap A, Wendy CG, Michael JL (2003). Interactions between tsetse and trypanosomes with implications for the control of trypanosomiasis. *Adv. Parasitol.* 53:1-83.
- Simarro PP, Louis FJ, Jannin J (2003). Sleeping sickness, forgotten illness: what are the consequences in the field? *Med. Trop.* 63, 231-235.
- Sisson DD (1994). On behalf of the Intensive Multi-center Prospective Randomised Veterinary Enalapril (Improve) Study Group: Hemodynamic, Echocardiographic, Radiographic and Clinical Effects of Enalapril in dogs with chronic heart failure. *Momentum Farm and Companion Animals*, 3.
- Steverding D (2008). The history of African trypanosomiasis. *Parasit. Vect.* 1:3.
- Teixeira ARL, Cunha Neto E, Rizzo LV, Silva R (1990). Trypanocidal nitroarene treatment of experimental trypanosome cruzi infection does not prevent progression of chronic phase heart lesion in rabbits. *J. Infec. Dis.* 162(6):1420.
- Tola RW, Muniz A (2010). Trypanosomiasis. *eMedicine Specialties > Pediatrics: General Medicine > Parasitology.* Complete this reference

- Uche UE (2010). Diagnosis of Canine Trypanosomosis. Three sites of blood collection compared. *J. Small Ani. Prac.* 26(6):349-352.
- Uilenberg G (1998). A field guide for the diagnosis, treatment and prevention of African animal. Chapter 1 African animal trypanosomes FAO Corporate Document Repository.
- Van Meirvenne N (1999). Biological diagnosis of human African trypanosomiasis, In Dumas M, Bouteille B, Buguet A (ed.), *Progress in human African trypanosomiasis, sleeping sickness*. Spri.Ver. Par., Fra. 235-252.
- WHO (1983). *Trypanosomiasis control manual* World Health Organization, Geneva, Switzerland.
- WHO (1998). *Control and surveillance of African trypanosomiasis: report of a WHO expert committee*. World Health Organization, Geneva, Switzerland.
- WHO (2006). *African Trypanosomosis (sleeping sickness) Fact sheet 259*. Geneva: WHO.
- WHO (2007). *Trypanosomosis. A division of control of Tropical diseases*© Microbiol. Bytes
- WHO (2010). *African Trypanosomosis (sleeping sickness)*. E. mail mediainquiries@who.int.
- WHO (2013). *Trypanosomiasis: world health organization. WHO facts sheets*.
- William SR, Deborah AW (1997). *Canine trypanosomosis*. *Southwest. Vet.* Vol 30(2).
- Wilson AJ (1969). Value of the indirect fluorescent antibody test as a serological aid to diagnosis of Glossina-transmitted bovine trypanosomiasis. *Trop. Anim. Health Prod.* 1:89-93.
- Wolley R, Smith P, Munro E, Smith S, Swift S, Devine G, Corcoran D, French A (2007). Effects of Treatment types on the vertebral heart size (VHS) in dog with myxodermatous mitral valve disease. *Intern. Journal Applied Research in Veterinary Medicine*, 5(1):43-47.
- Woo PT (1970). The haematocrit centrifuge technique for the diagnosis of African trypanosomiasis. *Acta Trop.* 27:384-386.
- Woo PT (1971). Evaluation of the haematocrit centrifuge and other techniques for the field diagnosis of human trypanosomiasis and filariasis. *Acta Trop.* 28, 298-303.
- Zillmann U, Konstantinov SM, Berger MR, Braun R (1996). Improved performance of the anion-exchange centrifugation technique for studies with human infective African trypanosomes. *Acta Trop.* 62:183-187.

Full Length Research Paper

Micropropagation of *Helianthemum lippii* L. var *Sessiliflorum* (Cistaceae) an important pastoral plant of North African arid areas

Amina Hamza^{1*}, Maher Gtari² and Neffati Mohamed¹

¹Range Ecology Laboratory, Arid Lands Institute of Médenine, 4119 Médenine, Tunisia.

²Laboratory of the Microbiology and Actives Biomolecules, Faculty of Sciences of Tunis, Campus 1060 Tunisia.

Accepted 27 September, 2013

Helianthemum lippii L. is a perennial, little brush distributed in sandy regions of southern Tunisia as well as of Mediterranean basin. It presents an important ecological, economical and pastoral interest. All these characteristics encourage us to try the *in vitro* propagation because it is a feasible alternative for the rapid multiplication and the preservation of plants. A very high frequency of sprouting and shoot differentiation were observed in the primary cultures of nodal explants of *H. lippii* on MS medium, without growth regulators or with a lower concentration of 6-benzylaminopurine (BAP, 0.5 mg L⁻¹ or 1.0 mg L⁻¹ BAP). *In vitro* proliferated shoots were multiplied rapidly by culture of shoot tips on MS medium free or with BAP (0.5 to 2.0 mg L⁻¹) which produced the greatest multiple shoot formation. BAP had a positive effect on multiplication and on growth, but a concentration that exceeds 2.0 mg L⁻¹ decreased the growth. A high frequency of rooting (71%) with development of healthy roots was observed with shoots cultured on MS/8 medium hormone-free. After *in vitro* rooting, and transfer to soil, a number of plantlets suitable for reintroduction in nature were produced.

Key words: *Helianthemum lippii*, axillary buds, micropropagation, plant conservation.

INTRODUCTION

Helianthemum lippii L. (*H. lippii*) is a perennial little brush, which occurs on sandy soils and sandy limestone gypsum low-lying in North African (Raynaud, 1987; Escudero et al., 2007). It is distributed in the bioclimatic semi-arid (Perez-Garcia and Gonzalez-Benito, 2006; Belghith, 2007) where it is well suited to severe climatic conditions in Southern Tunisia. It presents an important ecological, economical and pastoral interest and plays a pivotal role of struggle against desertification and the stabilization of vulnerable sites (Diez et al., 2002). Besides, it has a medicinal interest because the powder or the compress of the aerial part is used to treat

coetaneous lesion. This plant is known to be usually associated to desert truffles. *H. lippii* is the host plant for several species of desert truffles, which have interest for food, medicinal and economic purposes, development of the rural and local populations, (Plenchette and Duponnois, 2004; Slama et al., 2006; Mandeel et al., 2007), and in particular *Terfezia boudieri* Chatin and *Tirmania nivea* (Desf) (Slama et al., 2006). The presence of *H. lippii* is in a continuous regression. This plant is highly affected by the change in their floristic composition under the effect of overgrazing, land clearing and increased pastoral care (Aidoud et al., 2006). *H. lippii*

*Corresponding author. E-mail: hamza.amina82@yahoo.fr. Tel: +21623963566.

Abbreviations: BAP, 6-Benzyladenine (cytokinine); IAA, indole-3-acetic acid (auxin); IBA, indole-3-butyric acid (auxin); Kin, kinetin (cytokinine); MS, Murashige and Skoog medium; NAA, 2-naphthalene acetic acid (auxin); 2iP, 2-isopentenyladenine (cytokinine).

becomes endangered, rare and endemic flora of the western basin of the Mediterranean Sea (Escudero, 2007). All these characteristics encourage us to try the *in vitro* propagation. *In vitro* culture seems to be a very interesting alternative to preserve *H. lippii* against the scourge of extinction under effect of overgrazing, land clearing and increased pastoral care. This work is the multiplication of the species and this was through the micropropagation by axillary budding.

MATERIALS AND METHODS

Plant material

Young shoots were collected from 4 to 5-years-old trees of *H. lippii* growing in the garden of Arid Lands Institute of Medenine (IRA) (Medenine: latitude 32° 57' 09" N, longitude 11° 38' 26" E, with arid climate characterized by a mean rainfall of 150 mm/year). The types of explants that were used were the nodal explants (1 cm) from bud to ensure multiplication by axillary budding.

Disinfection

Stem cuttings, 8 to 10 cm length, were collected from plants growing in southern Tunisia (Medenine: latitude 32° 57' 09" N, longitude 11° 38' 26" E, with arid climate characterized by a mean rainfall of 150 mm/year), defoliated and surface-sterilized and washed with fungicide (Benlate 1 g/l) for 20 min to remove all surface adherents and then left in running tap water for 10 min. Subsequently, the defoliated twigs were surface decontaminated with 0.01% (w/v) mercuric chloride for 15 min. The treated twigs were washed several times with sterile distilled water and finally the use of a solution of sodium hypochlorite (NaOCl 50%) at different immersion times (5 to 20 min). Prior to inoculation, the twigs were trimmed into 1-cm-long pieces each having one or two nodes.

Micropropagation of *Helianthemum lippii*

Initiation

Explants were individually placed in test tubes (25 to 160 mm) containing 10 ml of MS (Murashige and Skoog 1962), supplemented with hormones. The hormones used were 6-benzylaminopurine (BAP: 0.25 to 2.0 mg L⁻¹) and α -naphthalene acetic acid (NAA: 0.5 and 1.0 mg L⁻¹). Sucrose (2%, w/v) was used as a carbon source and media were solidified with 0.8 % (w/v) agar. Media pH was adjusted to 5.8 before autoclaving at 121°C for 20 min. Cultures were incubated at 25 ± 2°C under a 16 h photoperiod at 60 μ mol m⁻² s⁻¹ provided by cool-white fluorescent lights for 10 weeks (Iriondi, 1995).

Multiplication

Shoots (1 ± 0.3 cm) obtained from the first step of micropropagation were transferred to MS medium with different concentrations of BAP added (0.25 to 2.0 mg L⁻¹).

Rooting

The root induction was performed on MS media diluted (MS/2, MS/4, MS/6 and MS/8) and MS medium containing different concentrations of acetic acid α -naphthalene (NAA: 0.25 to 1.5 mg L⁻¹

¹). All cultures were maintained in growth room for 3 months at 25 ± 2°C under a photoperiod (16/8 h) at a photosynthetic flux of 12, and 6 μ mol m⁻² s⁻¹ provided by cool daylight fluorescent lamps.

Acclimatization

Plantlets with at least five well-developed roots were transferred to plastic pots (150 ml) containing a mixture of peat and perlite (2:1, v/v). Potted plantlets were placed in a growth chamber set at 100% relative humidity, 25 ± 2°C, with a 16 h photoperiod (100 μ mol m⁻² s⁻¹), for three months, and then transferred to a glasshouse under natural daylight conditions at 25°C temperature. Plantlets were watered twice a week for 3 months with a diluted solution of 1/2MS.

Statistic analysis

The response data was arcsine transformed before analysis. All data were subjected to one-way analysis of variance (ANOVA). For each treatment, 20 explants were cultured.

RESULTS

Disinfection of explants

The appropriate protocol of disinfection is to soaking explants in mercuric chloride (0,01%) for 15 min, then immersion in bleach (NaOCl 50%) for 20 min; this protocol gives a low rate of contamination. The treatment of explants with the solution of HgCl₂ (1 g L⁻¹) for 20 min gave satisfactory results. In fact, this treatment ensures complete sterilization without damaging the explants compared to 10 and 15 min soaking. In the literature, several types of disinfectants are used for the sterilization of plant material such as alcohol, petroleum ether, calcium hypochlorite, sodium hypochlorite, acetone, etc at concentrations and durations of dips that vary depending on the species (Phatak and Heble, 2002; Moran et al., 2003; Tan et al., 2004).

Induction of auxiliary bud

The effect of the concentration and nature of growth regulators on bud explants is reported in Table 1. In the absence of growth regulators, the rate of axillary bud was very high (almost 100%) than it was for a complete basal medium (MS) or in medium supplemented with 0.5 or 1.0 mg L⁻¹ of BAP (M₂ and M₃). The addition of a cytokinin (BAP); however, favored the development of axillary buds.

Explants that have ridden the first giving a percentage of bud highest (~100%) are those that have been grown in a medium supplemented with 0.5 mg L⁻¹ of BAP. The combination of BAP and NAA at a dose of 0.25 mg L⁻¹ was accompanied by a decrease in the percentage of bud break (30%) and a decrease in shoot length (0.56 cm) (Figure 1a, b). Endogenous hormones of tissue *H. lippii* are able alone to stimulate axillary bud.

Table 1. Effect of growth regulators on induction of axillary bud tissues *Helianthemum lippii* L.

Medium	Composition (mg.L ⁻¹)	Bud (%)	Means long shoots (cm)
M ₁	MS	100	1.08 ^c
M ₂	MS + 0.5 BAP	100	2.28 ^a
M ₃	MS + 1.0 BAP	100	1.2 ^b
M ₄	MS + 2.0 BAP	60.0	0.58 ^d
M ₅	MS + 1.0 BAP + 0.25 NAA	30.0	0.56 ^d
M ₆	MS + 1.0 BAP + 0.5 NAA	27.4	0.8 ^{bc}
M ₇	MS + 1.0 BAP + 1.0 NAA	21.7	0.58 ^d

Means with different letters are significantly different at level $p < 0.05$ (Duncan test).

The addition of growth substances also increased to an excessive concentration of growth regulators making them toxic.

The combination of 1.0 mg L⁻¹ of BAP and 0.25 mg L⁻¹ NAA (M₅) however positively influenced callus thus the addition of NAA to the culture medium promotes callus formation at the expense of bud development. This can be explained by the fact that the addition of NAA in the culture medium decreased bud development at the expense of callus (Figure 1c). At a higher dose of NAA (1.0 mg L⁻¹) (M₇), the percentage of bud and shoot length decreased (21%, 0.58 cm). Also, the leaves begin to wrap and the shoots begin to be less vigorous. However, other authors such as Figueiredo et al. (2001) showed that auxin-cytokinin combination decreased the proliferation of shoots in *Periploca laevigata* and *Rollinia mucosa* respectively.

Proliferation of axillary shoots

The results (Table 2) show that the rate of axillary bud varied between 60 and 90%. Indeed, in all walks of multiplication, bud occurred after two weeks of culture; whereas it is three weeks for primary buds. This result can be explained by the fact that explants from the establishment phase of culture have acquired juvenile characters that can be considered as a response to *in vitro* culture.

The results showed that the rate of increase is positively correlated with the concentration of BAP. But arriving at a concentration of 2 mg L⁻¹ of BAP (M₆), the number of nodes/shoot decreases and the BAP beginning to have an inhibitory effect; this is justified by the low percentage of multiplication as well as the loss of shoot vigor (Figure 1c, d).

The effect of cytokinins on axillary buds has been studied by several authors. Thus, Souayah et al. (2003) found that the addition of BAP improves the rate of bud in *Atriplex*, but the increase in the concentration of BAP induced a decrease in the number of axillary shoots strong and no vitrified.

Rooting

Adventitious root formation is a phenomenon of great importance for the success of propagation. This is very difficult to achieve in wood. Rooting was obtained on media without growth regulators. The rate of rooting and number of roots per explants differ according to the environment (medium). On media supplemented with different concentrations of auxin (NAA), no evidence of rooting was observed. The findings highlight the appearance of callus at the base of the shoots. The rooting rate was from 32 to 71.8% for MS to MS/8. Rooting was observed after 3 to 4 weeks of culture. After this period, there was a gradual increase in the number of roots. Concerning the effect of dilution of minerals on rooting, we noted that the media MS/4, MS/6 and MS/8 gave the best rooting rate (54.2, 66.06 and 71.8%, respectively). This factor clearly affects the rate and number of roots/explant which considerably increased with the dilution of mineral medium (Figure 1f).

In examining these results, we noted that rooting was obtained on media without growth regulators. Uploading of auxin in the culture medium strongly inhibits rooting. This is due to the wealth of *H. lippii* in endogenous growth regulators. Rooting was obtained on media without growth regulators, on media supplemented with different concentrations of auxin (NAA), no evidence of rooting was observed in *H. lippii* (Table 3).

Acclimatization

The *in vitro* regenerated plantlets were acclimatized in a growth chamber at high relative humidity (90 to 95%) for two months and then they were transferred to the glasshouse. Results show that after six months of acclimatization, the percentage survival was 60%. This increase could be due to a more developed and efficient rooting system. Micropropagated plants showed good growth and uniformity *ex vitro* and exhibited normal development. When reintroduced into their natural habitat during 8 months, these plants showed 45% of survival (Figure 1f, h).

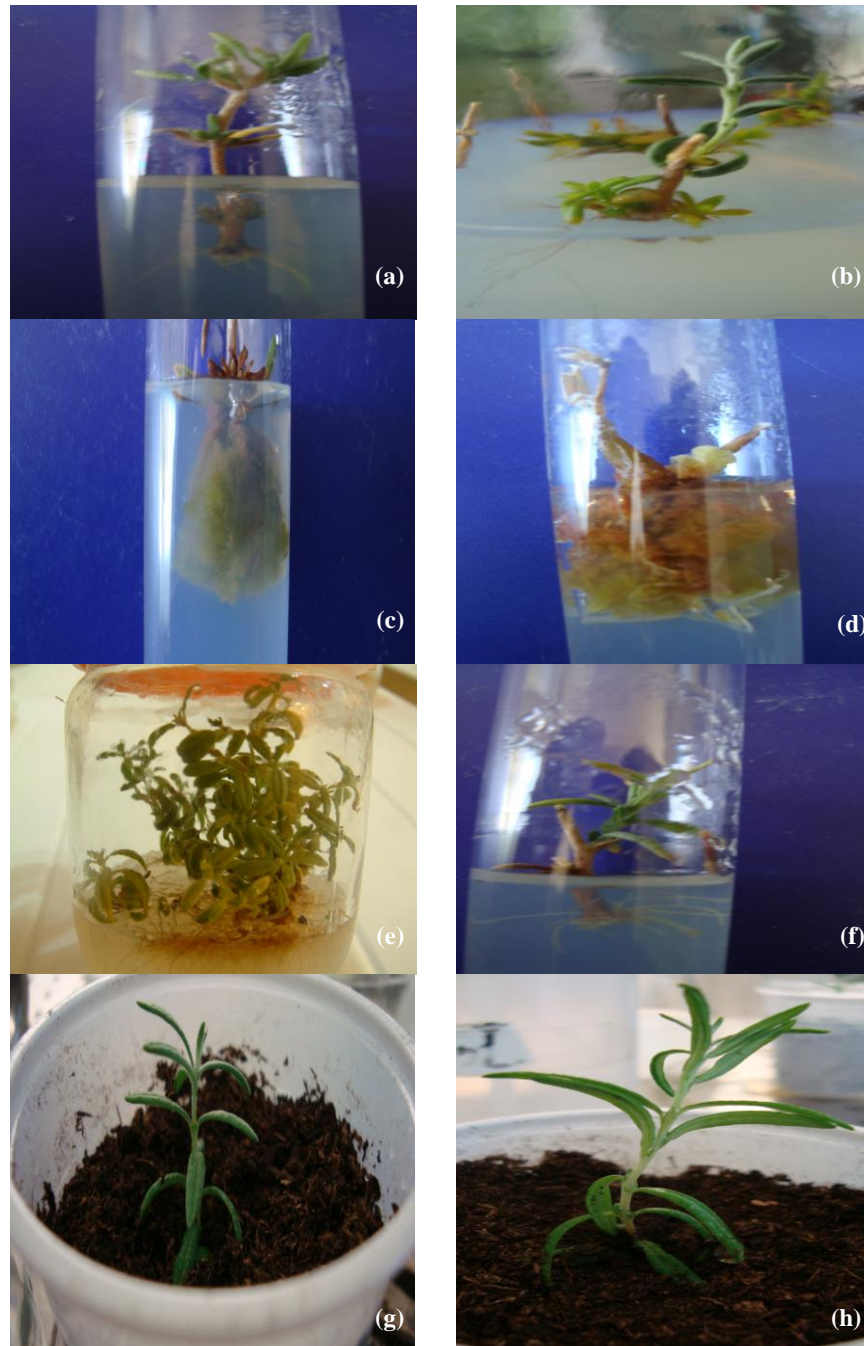


Figure 1. Proliferation of auxillary shoots of *Helianthemum lippii* on (a) MS + 1.0 mg.L⁻¹ BAP + 0.25 mg.L⁻¹ NAA; (b) MS medium; (c) Callus induction from nodal segment on MS + 0.50 mg.L⁻¹ BAP + 0.25 NAA mg.L⁻¹; (d) MS + 1.0 mg.L⁻¹ BAP + 1.0 NAA mg.L⁻¹; (e) MS + 1.0 mg.L⁻¹ BAP; (f) F, rooting on MS/8; (g + h) regenerated plantlets transferred in micropots (100%).

DISCUSSION

Explants of *H. lippii* presents a good disinfection in mercuric chloride solution (1%) after immersion in bleach (50%) for 20 min; some plants such as *Carissa carandas*

have been disinfected by dichloride of mercury (HgCl₂) at different concentrations and different soaking time. Other species such as *Cyrtontus* were sterilized by hypochlorite of sodium (NaOCl) (Moran et al., 2003). The addition of a cytokinin with low concentrations (BAP: 0.5 to 1.0 mg L⁻¹),

Table 2. Effect of BAP concentration on the multiplication phase of *Helianthemum lippii* vitro pousses.

Medium	Composition (mg.L ⁻¹)	Bud (%)	Means fibre of nodes/shoot	Means long shoots (cm)
M ₁	MS + 0.25 BAP	68.6	2.4 ^{ab}	1.32 ^d
M ₂	MS + 0.5 BAP	80	3.6 ^a	2.01 ^c
M ₃	MS + 0.75 BAP	60	3.46 ^b	2.20 ^b
M ₄	MS + 1.0 BAP	90	3.4 ^b	2.88 ^a
M ₅	MS + 1.5 BAP	80	3b ^c	2.98 ^a
M ₆	MS + 2.0 BAP	80	1.8 ^d	2.36 ^b

Means with different letters are significantly different at level $p < 0.05$ (Duncan test).

Table 3. Effect of dilution and NAA (auxin) on the induction of rooting vitro pousses of *Helianthemum lippii*.

Medium	Composition (mg.L ⁻¹)	Rooting rate (%)	Number of roots/explant	Long means roots (cm)
M ₁	MS	32 ^e	5,6 ^c	1.85 ^b
M ₂	MS/2	48.6 ^d	5.6 ^c	2 ^c
M ₃	MS/4	54.7 ^c	6.6 ^b	2.4 ^d
M ₄	MS/6	66.06 ^b	7.8 ^a	3.08 ^e
M ₅	MS/8	71.80 ^a	7.4 ^a	4.48 ^f
M ₆	MS + 0.25 NAA	0	0	0
M ₇	MS + 0.5 NAA	0	0	0
M ₈	MS + 1 NAA	0	0	0
M ₉	MS + 1.5 NAA	0	0	0

Means with different letters are significantly different at threshold $p < 0.05$ (Duncan test).

avored the development of auxiliary buds; however, the increase in concentration had a negative effect. The work of Armstrong et al. (2001) on *Ceratopetalum gummiferum* showed that the number of shoots per explants increased with increasing concentration of BAP, Kinetin, Zeatin and 2iP. However, the use of excessive concentrations cytokinin caused a decrease in the number of shoots per explant, the shoot length and shoots weight. These doses also cause browning of explants bases, yellowing shoots, deformation and stunting of the leaves. The addition of auxin had a negative effect on the induction of auxiliary bud. Figueiredo et al. (2001) have shown that increasing the concentration of NAA decreases the proliferation of axillary shoots *R. mucosa*. The effect of auxin-cytokinin combination was also studied by Fracaro et al. (2001) for *Cunila galioides*. These studies show that the addition of different auxins: NAA, IAA or IBA to a multiplication medium (MS + BAP) significantly reduced the number of shoots per explants. However, Souayah et al. (2003) showed that in *A. halimus*, adding NAA enhances the rate of multiplication but with increased callus.

The addition of BAP in *H. lippii*, improves the rate of buds, but the increase in the concentration of BAP induced a decrease in growth of shoots. The toxic effect of high concentrations of growth regulators was observed when explants of hypocotyl and epicotyl of *R. mucosa* are

grown in culture media containing high doses of kinetin, BAP or 2,4-D (Fingueiredo et al., 2001). Similarly, Zhijun and al. (2001) explained the toxic effect of the use of high concentrations of BAP for axillary bud by the fact that the addition of exogenous BAP causes an imbalance in the concentration of growth regulators causing toxicity to cultured buds. The rooting of shoots in culture medium without growth regulators was also obtained for *Vaccinium macrocarpon* (Debnath et al., 2001). Similarly, Iriondo et al. (1995) working on *Helianthemum polygonoides* showed that the rooting of this species can be done even on MS media without auxin. Also, Morte and Honrubia (1992) showed that the roots of *Helianthemum almeriense* occur after dilution of the culture medium. Similarly, for *Atriplex halimus* (Souayah et al., 2003), for which rooting is obtained on media without growth regulators indeed increased with diluting the mineral medium.

Conclusion

In vitro propagation is a feasible alternative for the rapid multiplication and the preservation of germoplasm. With the development of biotechnology and its contribution axis at all levels (genetic resources conservation, sanita-

tion plants, creating new genetic combinations, etc.), *in vitro* propagation methods are essential for plant genetic resources management and are becoming increasingly important for conservation of rare and endemic plant species (Sudha et al., 1998; Iankova et al., 2001). Similarly, these techniques facilitate the application of genetic manipulation procedures and long-term storage (Hawkes et al., 2000). Micropropagation of *H. lippii* was successfully implemented using the explants from specimens of the Medenine region (Tunisia, IRA plot: Institute of Arid Regions). To obtain this result, it was necessary to develop an adequate disinfection system in order to have sterile cultures. This disinfection protocol requires the use of a fungicide and then of a solution of mercuric chloride (1 g L^{-1}) for a period of 20 to 30 min. The rate of axillary bud is almost 100% on MS or MS supplemented with low concentration of BAP (does not exceed 0.5 mg L^{-1} of BAP), while the combination of BAP and NAA caused a decrease in the percentage of bud and shoot length growth.

Results show that the rate of multiplication is positively correlated with the concentration of BAP, at a high concentration; the BAP has an inhibitory effect; this is justified by the low percentage of multiplication as well as the loss of shoot vigor and decreases of number nodes per shoot. Rooting was obtained on media without growth regulators. The rate of rooting and number of roots per shoot differ according to the medium composition minerals. On media supplemented with different concentrations of auxin (NAA), no evidence of rooting was observed. Endogenous hormones of tissue *H. lippii* are able alone to stimulate axillary bud. The addition of growth substances also increased to an excessive concentration of growth regulators making them toxic.

ACKNOWLEDGEMENTS

The authors acknowledge the technical assistance rendered by all members of Range Ecology Laboratory in Arid Land Institute Tunisia, and Professor Pacioni Giovanni of the University dell'Aquila Italy.

REFERENCES

- Aidoud A, Le Floc'h E, Le Houérou HN (2006). Les steppes arides du nord de l'Afrique. *Sécheresse*. 17(1-2):19-30.
- Armstrong G, Johnson K (2001). Micropropagation of *Ceratopetalum gummiferum*, an important Australian cut flower crop. *In vitro Cell. Dev. Biol. Plant* 37:137-177.
- Belghith A (2007). Les indicateurs radiométriques pour l'étude des dynamiques écosystèmes arides (région de Zougrata, Sud-est tunisien). *Science et changement planétaires, Sécheresse*. 14(4):267-74.
- Debnath S, Kenneth B (2001). An efficient *in vitro* shoot propagation of cranberry (*Vaccinium macrocarpon* Ait.) by axillary bud proliferation. *In vitro Cell. Dev. Biol.-Plant* 37:243-249
- Diez J, Manjon JL, Martin F (2002). Molecular phylogeny of mycorrhizal desert truffles (*Terfezia* and *Tirmania*), host specificity and edaphic tolerance *Mycologia*. 94:247-259.
- Escudero A, Martinez I, Cruz A, Ojalora MAG, Master FT (2007). Soil lichens have species-specific effects on the seedling emergence of three gypsophile plant species. *J. Arid Environ*. 70: 18-28.
- Figueiredo S, Albarello N, Viana V (2001). Micropropagation of *Rollinia mucosa* (Jacq.). *In vitro Cell. Dev. Biol.-Plant*. 37:471-475
- Hawkes JG, Maxted N, Ford-Lloyed BV (2000). *The ex situ conservation of plant genetic resources*. Kluwer Academic Publishers, Dordrecht. The Netherlands.
- Iankova E, Cantos M, Linan J, Robeva P, Troncoso A (2001). *In vitro* propagation of *Angelica panceicii* Vauds, an endangered plant species in Bulgaria. *Seed Sci. Technol*. 29:477-482.
- Iriondo JM, Prieto C, Pérez G (1995). *In vitro* regeneration of *Helianthemum polygonoides*, an endangered salt meadow species. *Botanic Gardens Micropropagation News*, 2 (1).
- Mandeel QA, Al-Laith AAA (2007). Ethnomycological aspects of the desert truffle among native Bahraini and non-Bahraini peoples of the Kingdom of Bahrain. *J. Ethnopharm*. 110:118-129.
- Moran AL, Manahan DT (2003). Energy metabolism during larval development of green and white abalone, *Haliotis fulgens* and *H. sorenseni*. *Biol. Bull*. 204:270-277.
- Morte MA, Honrubia M (1992). *In vitro* propagation of *Helianthemum almeriense* Pau (Cistaceae). *Agronomie* 12:807-809.
- Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol. Plant*. 15:473-497.
- Perez-Garcia F, Gonzalez-Benito ME (2006). Seed germination of five species: Effect of temperature and presowing treatments. *J. Arid Environ*. 65:688-693.
- Phatak SV, Heble M.R (2002). Organogenesis and terpenoid synthesis in *Mentha arvensis*. *Fitoterapia* 73:32-39.
- Plenchette C, Duponnois R (2004). Growth response of the saltbush *Atriplex nummularia* L. to inoculation with the arbuscular mycorrhizal symbiosis: Data and hypothesis. *Experientia* 61:535-540.
- Slama A, Fortas Z, Neffati M, Khabar L, Boudabous A (2006). Etude taxonomique de quelques *Acsoomycota* hypogés (*Terfeziaceae*) de la Tunisie méridionale. *Bull. Soc. mycol. Fr*. 122(2-3):187-195.
- Souayah N, Khouja ML, Rejeb MN, Bouzid S (2003). Micropropagation d'un arbuste sylvo-pastoral, *Atriplex halimus* L. (Chénopodiacees). *Cahiers Options Méditerranéennes*.
- Sudhat CG, Krishnan PN, Pushpahagan P (1998). *In vitro* propagation of *Holostemma Annulare* (Roxb.) K. Schum, a rare medicinal plant. *In Vitro Cell. Dev. Biol. - Plant* 34:57-63.
- Tan L, Fung S, Shao X (2004). Observation of magnetospheric relativistic electrons accelerated by Pc-5 ULF waves. *Geophysical Research Letters* 31(14): doi: 10.1029/2004GL019459. issn: 0094-8276.
- Zhijun L, Zhanhai L (2001). Micropropagation of *Camptotheca acuminata* Decaisne from axillary buds, shoot tips and seed embryos in a tissues culture system. *In Vitro Cell. Dev. Biol.-Plant* 37:84-88.

Full Length Research Paper

Partial dehydration of 'Niagara Rosada' GRAPES (*Vitis labrusca* L.) targeting increased concentration of phenolic compounds and soluble solids

Wesley Esdras Santiago, Rodolpho César Dos Reis Tinini, Rafael Augustos De Oliveira and Barbara Janet Teruel

Postgraduation Program, Phd Course of Agricultural Engineering. Av. Cândido Rondon, 501, CEP: 13.083-875 Barão Geraldo, Campinas, São Paulo, Brazil.

Accepted 24 September, 2013

The partial dehydration of grapes after harvest and aimed wine-making, has been shown to be a process that brings increased concentration of sugar and phenolic compounds in the must, which results in the quality of the wines produced. However, the works developed so far studied the process for temperatures up to a maximum of 25°C and air velocity less than 1 m.s⁻¹. This study aimed to analyze the physical and chemical changes (concentration of total soluble solids (TSS) and phenolic compounds (CPC)) after partial dehydration of 'Niagara Rosada' grapes at the temperature subjected to two treatments combining two temperatures and one air velocity (T₁= 22.9°C/1.79 m.s⁻¹ and T₂ = 37.1°C/1.79 m.s⁻¹), and relative humidity of approximately 40%. The loss of water in the grapes was approximately 14% and the drying process lasted between 20 to 50 h for the treatments T₁ and T₂, respectively. We experimentally and statistically verified that the treatments promoted significant increase in TSS and CPC; however, for CPC at the temperature of 37.1°C, the increase accounted for approximately 29%, whereas, for the temperature of 22.9°C, it was only 5%. For TSS, the increase was on average 14.4 ± 3.9% between both treatments.

Key words: Chaptalization, temperature, air velocity, winemaking.

INTRODUCTION

Besides facilitating the transport, storage and microbiological stability, the dehydration process of agricultural products causes physical, chemical and organoleptic changes; therefore, it must be performed in a controlled manner and meet the limits established to not affect the quality (Sampaio et al., 2006). The reduction of the moisture content in fruits for processing, whether for juice, pulp and/or concentrates, causes an increase in the concentration of soluble solids (Azeredo et al., 2006; Dionello et al., 2009). When the grapes are used for winemaking, it is desirable the highest possible concentration of soluble solids and phenolic compounds.

Phenolic compounds, as well as anthocyanins and flavonoids, provide the sensory characteristics of the wine, and they are indicated as beneficial in the prevention of cardiovascular diseases (Bradamante et al., 2004; Freitas et al., 2010; Lasa et al., 2011).

According to the study of Barnabé and Filho (2008), the concentration of soluble solids in the must is the key factor so that, during winemaking, a significant amount of alcohol is produced. When the concentration of soluble solids is low, it becomes difficult, or even impossible, to obtain table wines with alcohol levels according to the required by the Brazilian law, which must be between 8.6

at 14%, according to note published in the Official Gazette ((Diário Oficial da União, 2004).). Studies developed by Guerra et al. (2003) showed that the shriveling of grapes in the vineyards, caused by loss of water, contributed to significant changes in the concentration of total soluble solids (TSS), in addition to the increase in the concentration of phenolic compounds in the skin, also as direct effects of over-ripening caused by rising temperatures in vineyards of grapes at the end of the maturation cycle. Later studies on the application of partial dehydration at temperature below ambient began by using a cold chamber intended for this function, the dehydration process may take up to 45 days (Bellincontro et al., 2004). While in some countries with wine tradition the partial dehydration of grapes is an important methodology for achieving quality table wines (Curvelo et al., 2005), in Brazil it is common in many wineries the addition of sugar to the must, a process called chaptalization, seeking to improve the alcohol content; however, this process results in wines of low quality, lower market value and higher cost of production.

Results obtained from several studies have proven the positive effect on wine produced from grapes that have undergone partial dehydration, supported by the increased concentration of sugar, phenolic compounds and aromatic compounds (Constantini et al., 2006; Moreno et al., 2008; Barbanti et al., 2008; Serratososa et al., 2010). More studies recently on dehydration of grapes for winemaking has evaluated with temperatures below of 25°C and air velocity below 1.5 m.s⁻¹. In this work we have proposed as objectives the analysis of the physical and chemical changes (concentration of soluble solids and phenolic compounds) after partial dehydration of 'Niagara Rosada' grapes at the temperatures of 22.9 and 37.1°C in a forced air system with air velocity of 1.79 m.s⁻¹. With these thermal and fluidic conditions we seek to accelerate the loss of water process without affecting the quality of the fruit to improve the quality of the must.

MATERIALS AND METHODS

Raw material

'Niagara Rosada' grapes (*Vitis labrusca* L.), from the harvest of July to November, 2011, were collected in the city of Jales, northwest of the State of São Paulo, stored in cardboard boxes with a capacity of 7 kg and transported to the Laboratório de Termodinâmica e Energia da Faculdade de Engenharia Agrícola da Universidade de Campinas (Laboratory of Thermodynamics and Energy from the School of Agricultural Engineering, at the University of Campinas). After completion of the pre-cleaning of the grape clusters to remove stems and grapes damaged or compromised by the presence of fungi, we carried out the distribution of samples according to the heat treatment to be applied and the subsequent analyzes of the physicochemical characterization.

Partial dehydration of grapes

The fruits were stored in 50 x 30 x 25 cm plastic package, with 25%

of effective opening area, containing 25 grape clusters, longitudinally arranged. The package was placed inside a cooling system with forced air (air flow rate of 2,900 m³. h⁻¹), which is installed inside a refrigerated chamber (cooling capacity of 4,400 kcal.h⁻¹ at -10°C) (Figure 1a). With the air flow perpendicular to the position of the clusters, the rates of heat and mass transfer are increased, allowing the dehydration process to occur in a shorter period without forced air (Figure 1a). The system is instrumented with sensors for temperature, relative humidity and mass measurement (Figure 1b). The system instrumentation was already achieved in previous works (Santiago et al., 2012b; Silva et al., 2011).

The temperature sensors are of the Pt 100 type, (FM= 0 at 100°C; model TR106; 4 to 20 mA; accuracy = ±0.2%); the one to measure the relative humidity are of the RHT-WM type with compact electronic module and transmitter of values (FM= 0 at 100%UR; 4 to 20 mA; accuracy = ± 1.5%); and to measure mass, a weighing system comprising a load cell, model PW12C3 - IMB (50 N (50 kgf); sensibility of 2±0.1% mV.V⁻¹).

Experimental delimitation

The experimental design was completely randomized with two treatments; the effects of treatments were evaluated in pairs by comparing the values before and after treatment. The treatments were a combination of two temperatures (T₁ = 22.9°C and T₂ = 37.1°C) with an air speed of 1.79 m s⁻¹. These temperature values were defined based on subsidies obtained from previous works (Santiago et al., 2012a; Santiago et al., 2012c) in which a range of temperature between 20 and 50°C were studied, obtaining the best results for the concentration of soluble solids and phenolic compounds with greater weight loss for the temperature values of 22.9 and 37.1°C. The results obtained were submitted to analysis of variance, being the averages compared by the Tukey test at 95% confidence in the statistical software Sisvar 5.3.

Physical-chemical analyzes

To perform the physical-chemical analysis, we randomly took six grapes from each cluster, comprising the area of the base, middle and apex of the cluster, according to the procedure proposed by Araújo et al. (2009). Then, the selected grapes were macerated for the preparation of the must for the analysis. The characterization of the review was based on specific methodologies standardized by the Adolfo Lutz Institute (2005) for the analysis of TSS in °Brix. The moisture content, in dry basis, was determined by drying a sample (100 g) in an oven (model MA035/1, manufacturer Marconi) with forced air circulation at 60°C, until reaching the constant weight of the sample. Concentration of phenolic compounds (CPC) was quantified in mg of gallic acid per 100 g of must according to the methodology described by Vargas et al. (2008).

RESULTS AND DISCUSSION

The results of the physicochemical analyses of grapes, before and after treatment are shown in Table 1. Figure 2 shows the variation in response to partial dehydration treatments (%). The results show that it is possible, by modulating the psychometric and thermal parameters of the dehydration process, to adjust the loss of water of grapes, with the consequent increase in the concentration of soluble solids and polyphenols. The use of the forced air system provided considerable decrease in time

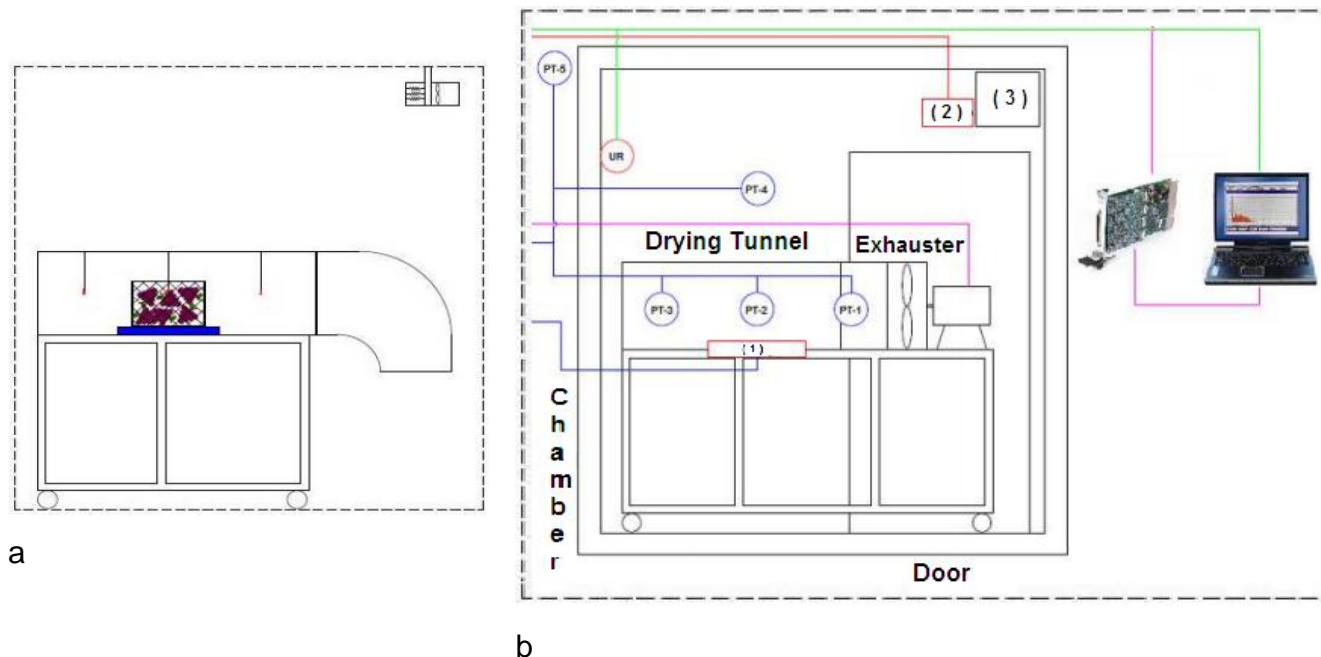


Figure 1. a, Schematic of the structure of forced-air drying; b, schematic of the system instrumentation. 1, Scale; 2, resistance; 3, evaporator.

Table 1. Physical-chemical characterization and analysis of variance for the concentration of total soluble solids (TSS) and concentration of phenolic compounds (CCF) in the must.

Test ($^{\circ}\text{C}/\text{m}\cdot\text{s}^{-1}$)	TSS ($^{\circ}\text{Brix}$)		CPC ($\text{mg gallic acid}\cdot 100\text{g}^{-1}$ must)		Dbm	
	Before	After	Before	After	Before	After
T_1 (22.9/1.79)	17.57	20.07*	927.33	974.00*	3.54	3.04*
T_2 (37.1/1.79)	17.23	20.60*	772.00	1096.00*	3.76	2.66*
Average	17.40	20.34	849.67	1035.00	3.65	2.85

*Parameters with significant change at 95% confidence level compared to initial value.

to process the loss of water up to the level of control established which was 14%, lasting 20 h (T_1) and 50 h (T_2), with relative humidity of approximately 40%. Partial dehydration caused a significant increase in concentration of soluble solids and phenolic compounds. However, for CPC at the temperature of 37.1 $^{\circ}\text{C}$, the increase accounted for approximately 29%, whereas, for the temperature of 22.9 $^{\circ}\text{C}$, it was only 5%. For TSS, the increase was on average $14.4 \pm 3.9\%$ between both treatments.

The results indicate that partial dehydration of grapes for winemaking can bring satisfactory results in the parameters of soluble solids and polyphenols, not only for the temperature below room temperature but as well as above. In works carried out in Europe, dehydration has been performed only at temperatures between 10 and 25 $^{\circ}\text{C}$ (Bellincontro et al., 2004; Barbanti et al., 2008). According to Barbanti et al. (2008), usually the loss of

water in grapes at psychrometric ambient conditions and without control may last from 90 to 120 days to reach the optimum vinification, and the grapes may still lose up to 40% of mass, exceeding the 20% limit recommended by the International Code of Oenological Practices (2006).

In an experiment carried out by Bellincontro et al. (2009) maximum loss of water was 20%, at the temperature of 10 and 20 $^{\circ}\text{C}$ and air velocity of 1.5 $\text{m}\cdot\text{s}^{-1}$. Dehydration lasted 26 days (624 h) and 16 days (384 h), respectively. However, the increase of $^{\circ}\text{Brix}$ went from 21 to only 24. In the present study, the dehydration time was not more than two days, providing a loss of water of 14% and increased concentration TSS from 17 to 20 $^{\circ}\text{Brix}$, that is, for the same increase in TSS, which was 3 $^{\circ}\text{Brix}$ in both cases, there was a decrease of time of 14-22 days, which would improve the logistics and cost-effectiveness of the processes involved, as well as for the chain of wine production.

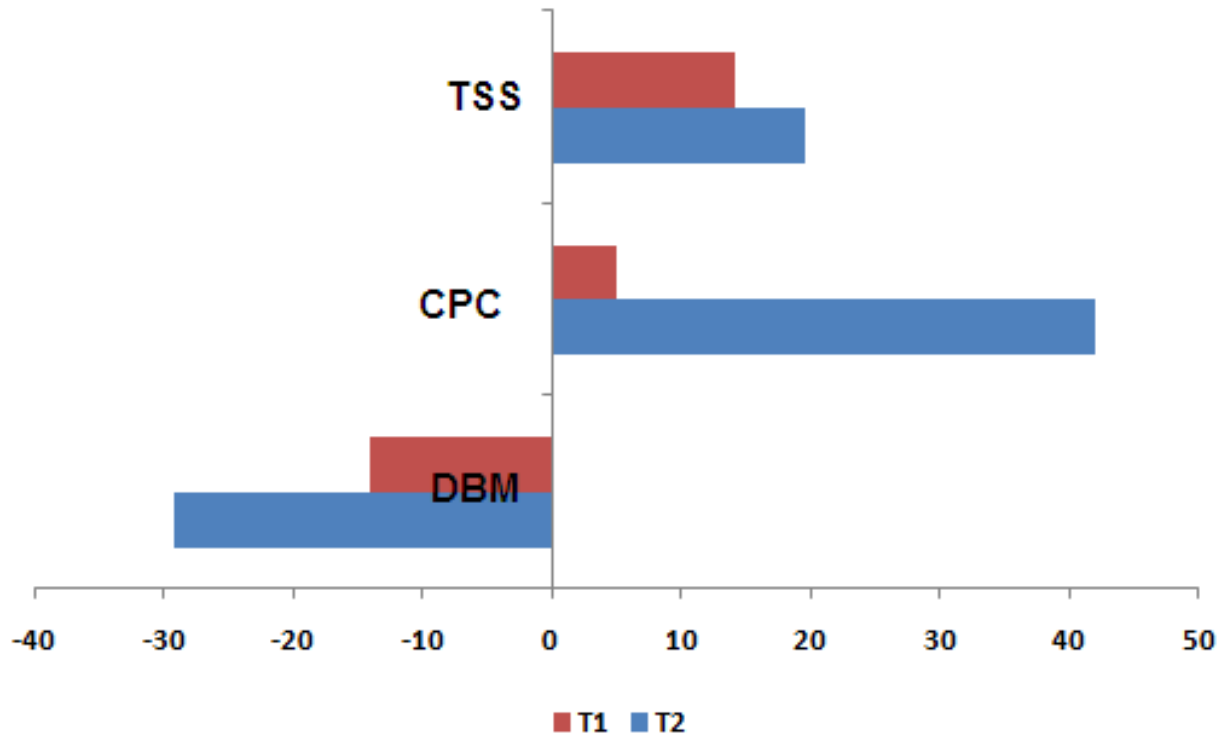


Figure 2. Variation of the physicochemical response to the treatments of dehydration. TSS, Concentration of total soluble solids; CPC, concentration of phenolic compounds; DBM, moisture in dry basis.

Concentration of total soluble solids (TSS)

The average values of TSS significantly varied in both treatments. Treatment “T₂” showed the greatest value of TSS (20.6 °Brix). Although it is just above the value of TSS obtained by treatment “T₁”, this result may be associated with the moisture content of the clusters, since according to Dionello et al. (2009) and Serratosa et al. (2010) the reduction of moisture of grape bundles results in musts with increased value of total soluble solids. By lowering the water content of the grapes, which was approximately 14%, there was a positive impact on the concentration of soluble solids and polyphenols. Studies developed by Moreno et al. (2008) and Bellincontro et al. (2009) indicate loss of water of the order of 14 to 20% with equal positive impact on the concentration of soluble solids. The concentration of total soluble solids, besides acting as an important indicator of the maturity and influencing the chemical and enzymatic modifications occurring in other components of the grapes, serves as the basis of calculation for obtaining the alcoholic potential of the must, since the fructose present in solid soluble is responsible for most of the fermentation.

Varieties of European grapes, such as Malvasia, Trebbiano and Sangiovese, were partially dehydrated, with an increase of up to 34% of total soluble solids in the must, but at the temperature of 21°C, air velocity from 1

to 1.5 m.s⁻¹ and humidity of 42% (Bellincontro et al., 2004). Between 10 and 25°C, Barbanti et al. (2008) observed a similar increase to the value of total soluble solids in the must.

The partial dehydration of grapes above room temperature accelerated the process of mass transfer, and as a consequence the loss of water, which was in total of 14%, still within the norms established. According to the International Code of Oenological Practices, (2006), in grapes for winemaking, the total loss of water in grapes during dehydration cannot be above 20%, as it can cause physiological damages to the product, compromising the quality of the winemaking process and the wine produced. The controlled loss of water in the grapes can be an alternative to produce wines with appropriate alcohol content, since the fruit does not always reach the desired ripeness, and the concentration of soluble solids in the must is essential for the production of alcohol. In Brazil, specifically, the legislation requires the alcohol content to be between 8.6 at 14%. When this content is not met, then it is allowed the addition of exogenous sugar to correct the alcohol content (chaptalization); however, the law limits this correction as a sufficient quantity to produce 3 degrees GL of alcohol, which in wines most often does not meet this limit imposed.

The amount of soluble solids obtained in the study demonstrates the high fermentation potential; even

though the 'Niagara Rosada' Grape cultivar is not used in Brazil to produce wines (Manfroi et al., 2006; Rizzon and Miele, 2004; Chavarria et al., 2008), this value of TSS equates to the value of TSS of the must of some European grape varieties (*V. vinifera* L.).

Concentration of phenolic compounds (CPC)

Treatment "T₁" showed no significant response to the polyphenol content and we believe that the significant effect obtained by the second treatment was due to the fact that, at the temperature of 37.1°C, there is the disintegration or breaking of the pectin molecules of the skin, allowing phenolic compounds present there to be released, as discussed by Vedana et al. (2008). The increase in the concentration of polyphenols obtained in this study also becomes promising when aiming practical application, as phenolic compounds are of great importance in Oenology, since they are directly or indirectly related to the quality of wine and are antioxidants beneficial to the human health.

Conclusions

The results obtained so far open a perspective of practical application of the technology of partial dehydration at the temperature of 37.1°C to the sector of Viticulture. With the combination of forced air and temperature of 37.1°C, it is possible to accelerate the process of loss of water of grapes, providing increased concentration of phenolic compounds and total soluble solids, thus reaching the alcohol content in must required for the manufacture of wines without the need for the chaptalization process.

The significant reduction in the time required to remove the amount of water recommended from the grapes before vinification, with the consequent increase in the chemical properties evaluated, could contribute to the cost-effectiveness of the processes involved in the production chain of Viticulture.

REFERENCES

- Araújo EG, Piedade SMS, Conceição MAF, Pereira JC (2009). Métodos de amostragem e tamanho de amostra para avaliar o estado de maturação da uva Niagara rosada. *Rev. Bras. Biom.*, São Paulo. 27(4): 501-518.
- Azeredo HMC, Jardine JG (2006). Desidratação osmótica de abacaxi aplicada à tecnologia de métodos combinados. *Rev. Bras. Prod. Agroind.*, Campina Grande. 8(2): 153-162.
- Barbanti D, Mora B, Ferrarini R, Torielli GB, Cipriani M (2008). Effect of various thermo-hygrometric conditions on the withering kinetics of grapes used for the production of "Amarone" and "Recioto" wines. *J. Food Eng.* 85(3):350-358.
- Barnabé D, Filho WGV (2008). Recuperação de etanol a partir do bagaço de uva. *Rev. Ener. Agric. Botucatu.* 23(4):1-12.
- Bellincontro A, De Santis D, Botondi R, Villa I, Mencarelli F (2004). Different postharvest dehydration rates affect quality characteristics and volatile compounds of Malvasia, Trebbiano and Sangiovese grapes for wine production. *J. Sci. Food Agric.* 84:1791-1800.
- Bellincontro A; Nicoletti I, Valentini M, Tomas A, Santis D, Corradini D, Mencarelli F (2009). Integration of Nondestructive Techniques with Destructive Analyses to Study Postharvest Water Stress of Winegrapes. *Am. J. Enol. Vitic.* 60(1):57-65.
- Bradamente S, Barenghi L, Villa A (2004). Cardiovascular Protective Effects of Resveratrol. *Cardiovasc. Drug. Rev.* 22:3.
- Chavarria G, Santos HP, Zanús MC, Zorzan C, Marodin GAB (2008). Caracterização físico-química do mosto e do vinho Moscato Giallo em videiras cultivadas sob cobertura de plástico. *Pesq. agropec. bras.*, Brasília, 43(7):911-916.
- Código Internacional De Práticas Enológicas (2011). O.I.V. Rue D'Aguesseau, Paris, 2006. Available in: <http://news.reseauconcept.net/images/oiv_it/Client/Code_Ed_2006_FR.pdf > Access in: Feb 09, 2011.
- Constantini V, Bellincontro A, De Santis D, Botondi R, Mencarelli F (2006). Metabolic changes of Malvasia grapes for wine production during postharvest drying. *J. Agr. Food Chem.* 54: 3334-3340.
- Curvelo-Garcia AS (2005). Práticas enológicas internacionalmente reconhecidas. *Ciênc. Téc. Vitiv.* v. 20, p.105-130.
- Diário Oficial Da União (2011). Law No. 10.970, of November 12, 2004. Available in: <http://www.receita.fazenda.gov.br/legislacao/Leis/2004/lei10970.htm> Access in: Oct 24, 2011.
- Dionello RG, Berbert PA, Molina MAB, Pereira RC, Viana AP, Carlesso VO (2009). Secagem de fatias de abacaxi in natura e pré-desidratadas por imersão-impregnação: cinética e avaliação de modelos. *Tecnol. Aliment.*, Campinas, 29(1):232-240.
- Freitas AA, Detoni AM, Clemente E, Oliveira CC (2010). Determinação de resveratrol e características químicas em sucos de uvas produzidas em sistemas orgânico e convencional. *Rev. Ceres, Viçosa*, v. 57(1):001-005.
- Guerra CC, Zanús MC (2003). Uvas viníferas para processamento em regiões de clima temperado. *EMBRAPA - Sistema de Produção*, 4, ISSN 1678-8761.
- Instituto Adolfo Lutz (2005). Normas analíticas do Instituto Adolfo Lutz: Métodos químicos e físicos para análises de alimentos. V. 1, 4 ed. Brasília. 1018 p.
- International Code of Oenological Practices (2006). Organización Internacional de La Viña Y El Vino. Rue D'Aguesseau, Paris.
- Iasa MJ, Churruga I, Simón E, Arias N, Milagro F, Martínez JA, Portillo MP (2011). The combination of resveratrol and CLA does not increase the delipidating effect of each molecule in 3T3-L1 adipocytes. *Nutr. Hosp.* 26:5 Madrid.
- Manfroi L, Miele A, Rizzon LA, Barradas CIN (2006). Composição química do mosto da uva Cabernet Franc conduzida no sistema lira aberta. *Ciênc. agropec.*, Lavras. 30(4):787-792.
- Moreno JB, Cerpa-Caldrón F, Cohen SD, Fang Y, Qian M, Kennedy JA (2008). Effect of postharvest dehydration on the composition of pinot noir grapes (*Vitis vinifera* L.) and wine. *Food Chem.* 109:755-762.
- Rizzon LA, Miele A (2004). Avaliação da cv. Tannat para elaboração de vinho tinto. *Ciênc. Tecnol. Aliment.*, Campinas, 24(2): 223-229.
- Sampaio SM, Queiroz MR (2006). Influência do processo de secagem na qualidade do cogumelo shiitake. *Eng. Agríc. Jaboticabal.* 26(2):570-577.
- Santiago WE, Silva JTR, Teruel BJ, Oliveira RA (2012a). Mudanças físico-químicas de uvas Niagara Rosada após secagem parcial. *Rev. Bras. Energ. Ren.* 1:239-252.
- Santiago WE, Silva JTR, Teruel BJ, Oliveira RA (2012b). Sistema de pesagem dinâmica para determinação da perda de massa de produtos hortícolas durante secagem. *Anais do X Congresso Latino Americano y del Caribe de Ingeniería Agrícola e XLI Congresso Brasileiro de Engenharia Agrícola (CLIA-CONBEA).*

- Santiago WE, Tinini RCR, Teruel BJ (2012c). Qualidade de uvas niágara rosada (*Vitis labrusca*) submetidas a desidratação em diferentes temperaturas. In: XXII Congresso Brasileiro de Fruticultura, 2012, Bento Gonçalves. Anais do XXII Congresso Brasileiro de Fruticultura.
- Serratos MP, Marquez A, Lopez-Toledano A, Merida J, Medina M (2010). Compostos fenólicos obtidos de uvas secas em câmara com temperatura controlada da casta Pedro Ximénez. Livro das Actas 30/04/2010. 17: 24. 8: 123-131. Simpósio de Vitivinicultura do Alentejo. Evento Alentejo das Gastronômias Mediterrânicas - Festival Internacional.
- Silva JCTR, Teruel BJM (2011). Control system for forced-air cooling of horticultural products. Eng. Agríc. (Impresso). 31:621-630.
- Vargas PN, Hoelzel SC, Rosa CS (2008). Determinação do teor de polifenóis totais e atividade antioxidante em sucos de uva comerciais. Alim. Nutr. Araraquara ISSN 0103-4235.19(1):11-15.
- Vedana MIS, Ziemer C, Miguel OG, Portella AC, Candido IMB (2008). Efeito do processamento na atividade antioxidante de uva. Alim. Nutr. Araraquara. 19(2):159-165.

Full Length Research Paper

Preliminary screening of plant essential oils against larvae of *Culex quinquefasciatus* Say (Diptera: Culicidae)

M. Ramar^{1*}, M. Gabriel Paulraj² and S. Ignacimuthu²

¹Department of Zoology, APSA, College, Thiruppattur, Tamil Nadu, India- 626141.

²Entomology Research Institute, Loyola College, Chennai, Tamil Nadu, India -600034.

Accepted 27 August, 2013

Preliminary screenings of 22 plant essential oils were tested for mortality of the mosquito larvae *Culex quinquefasciatus* under laboratory conditions. Percent (%) mortality of the mosquito larvae were obtained for each essential oil. At different exposure periods, viz. 1, 3, 6, 12 and 24 h among the 22 plant oils tested, eight oils viz., aniseed, calamus, cinnamon, clove, lemon, orange, thyme, and tulsi oils gave promising results on larvicidal activity. For larvicidal screening bioassay, the mortality was recorded at different exposure periods viz., 1, 3, 6, 12 and 24 h, and it was found that larval mortality increased when exposure time increased. The clove oil was found to be the most effective treatment. In the preliminary screening, clove oil gave 100% mortality at all exposure periods. Vetiver oil recorded 36.2, 61.2, 76.2, 87.5 and 100% mortality in 1, 3, 6, 12 and 24 h, respectively. Results of this study show that the essential oils may be a potent source of natural larvicides.

Key words: Screening, essential oils, *Culex quinquefasciatus*, Larvicidal.

INTRODUCTION

Culex quinquefasciatus, a domestic mosquito mainly found in urban areas is a vector of human filariasis in India. *C. quinquefasciatus* acts as a vector of *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori*, which are responsible for lymphatic filariasis, a prevalent disease in India. Filariasis is an endemic disease in many parts of India especially in Kerala, Mysore, Tamil Nadu, Andhra Pradesh and Maharashtra states. Lymphatic filariasis infects 80 million people annually, of which 30 million cases exist in chronic infection. In India, 45 million cases of lymphatic filariasis have been recorded (Bowers et al., 1995).

The plant world comprises a rich storehouse of phytochemicals which are widely used as alternatives to synthetic insecticides. Secondary metabolites obtained from plants have shown proven mosquito control poten-

tial. *Chloroxylons wietenia* DC. (Rutaceae) is a medicinal as well as an aromatic tree of dry deciduous forests. It is commonly known as East Indian Satin Wood. The potential mosquitocidal activity of *C. wietenia*, is widely known in tribal areas of South India, where it is common for people in these forest areas to hang leaf garlands in their houses to eradicate mosquitoes and other insects (Ansari and Razdan, 1995).

Pyrethrin based products have been widely used to protect people from mosquito bites through their repellent and killing effects. Many other material products of botanical origin especially, essential oils hold significant promise in insect vector management (Skinner and Johnson, 1980). Kiso and Hikino (1991) have reported that the compounds vinblastine and vincristine obtained from *Catharanthus roseus* inhibited cell division in

*Corresponding author. Email:ramareri@gmail.com.

Table 1. Plant essential oils selected for the bioassay experiments.

Botanical name	Family	Common name
<i>Pimpinella anisum</i>	Apiaceae	Aniseed
<i>Citrus bergamia</i>	Rutaceae	Bergamot
<i>Acorus calamus</i>	Aeraceae	Calamus
<i>Cinnamomum camphora</i>	Lauraceae	Camphor
<i>Cedrus atlantica</i>	Pinaceae	Cedarwood
<i>Cinnamomum veerum</i>	Lauraceae	Cinnamon
<i>Cymbopogon nardus</i>	Poaceae	Citronella
<i>Myrtus caryophyllus</i>	Myrtaceae	Clove
<i>Eucalyptus globules</i>	Myrtaceae	Eucalyptus
<i>Pelargonium graveollens</i>	Geraniaceae	Geranium
<i>Citrus limon</i>	Rutaceae	Lemon
<i>Cymbopogon flexuosus</i>	Poaceae	Lemongrass
<i>Citrus aurantifolia</i>	Rutaceae	Lime
<i>Gaultheria frgagrantiissima</i>	Acanthaceae	Luchi
<i>Myrtica fragrans</i>	Myristiceae	Nutmeg
<i>Citrus sinensis</i>	Rutaceae	Orange
<i>Cymbopogon martini</i>	Lamiaceae	Palmarosa
<i>Pinus radiata</i>	Pinaceae	Pine
<i>Rosmarinus officinalis</i>	Lamiaceae	Rose mary
<i>Thymus vulgaris</i>	Labiatae	Thyme
<i>Ocimum sanctum</i>	Lamiaceae	Tulsi
<i>Vetiveria zizanioides</i>	Poaceae	Vetiver

fertilized mosquito eggs. Tulsi (*Ocimum sanctum*) is known to have various insectidal properties (Saxena et al., 1979). Extracts of *Vitex* species and *Ocimum* species leaves have been traditionally known as natural insecticides. Indian farmers have been using various plant extracts as insecticides and pesticides (Ghosh, 2000).

Many studies have shown the bioactivity of essential oils against mosquitoes as growth inhibitors and/or larvicides, adulticides, repellents, or oviposition deterrents (Sukumar et al., 1991; Carvalho et al., 2003; Cavalcanti et al., 2004; Ansari et al., 2005). Essential oils from a large number of plants, including *Ocimum* spp, *Cymbopogon* spp), *Eucalyptus maculate citriodon* *Pelargonium citrosum*, *Artemisia vulgaris*, *Lantana camara*, *Mentha piperita*, *Vitex rotundifolia*, *Curcuma* spp. (Pitasawat et al., 2003), *Conyza newii*, *Plectranthus marrubioides*, *Tetradenia riparia*, *Tarhonianthus camphoratus*, *Lippia javanica* and *Lippia ukambensis* (Omolo et al., 2004), have been demonstrated to exhibit good repellent properties against vector mosquitoes. Paula et al. (2003) reported the chemical composition, toxicity and mosquito repellency of *Ocimum selloi* oil. *Ocimum* essential oils have been traditionally used to kill or repel insects, and also to flavour food products.

In the present investigation, the essential oils were selected in order to find out a new mosquitocidal compound against filarial vector mosquito *C.*

quinquefasciatus. The plant essential oil is largely cultivated throughout India and in all tropical countries.

MATERIALS AND METHODS

Plant essential oils

The plant essential oils were obtained from Tegraj and Co (P) Ltd, Chennai, Tamil Nadu. Based on various biological effects on larvae, the 22 essential oils were selected (Table 1).

Maintenance of larvae

The larvae of laboratory stock culture were reared in plastic troughs (size 18 cm diameter x 19 cm height) and were maintained at 27 ± 1°C, 75 to 85% RH, under 14:10 L/D photoperiod cycles. The larvae were fed with dog biscuits and yeast at 3:1 ratio (per weight). Water was changed alternate days. The breeding medium was regularly checked and dead larvae were removed at sight and the troughs in which larvae were maintained were kept closed with muslin cloth to prevent contamination through foreign mosquitoes.

Screening assay

The selected plant oils were screened for larval toxicity by following standard procedure as follows: In the preliminary screening a single dose (500 ppm) of each oil was tested. Twenty fourth instar larvae of *C. quinquefasciatus* were kept in a 500 ml glass beaker containing 249 ml of dechlorinated water and 1.0 ml of desired plant

Table 2. Preliminary screening of plant essential oils against fourth instar larvae of *Culex quinquefasciatus* at 500 ppm in different exposure periods.

Essential oil	Larval mortality (%)				
	1 h	3 h	6 h	12 h	24 h
Aniseed	91.2 ± 0.6 ^g	100 ± 0.0 ^j	100 ± 0.0 ^m	100 ± 0.0 ^k	100 ± 0.0 ^j
Bergamot	11.2 ± 0.2 ^{bc}	18.7 ± 0.7 ^{bc}	30.0 ± 0.5 ^{gh}	45.0 ± 0.4 ^e	61.2 ± 0.8 ^{efg}
Calamus	21.2 ± 0.2 ^{de}	32.5 ± 0.2 ^e	43.7 ± 0.3 ^j	66.2 ± 0.4 ^f	100 ± 0.0 ^j
Camphor	0 ^a	2.25 ± 0.0 ^a	7.5 ± 0.2 ^{bc}	20.0 ± 0.9 ^{bc}	38.7 ± 0.4 ^c
Cedarwood	18.7 ± 0.4 ^c	30.0 ± 0.2 ^e	40.0 ± 0.3 ^j	58.7 ± 0.6 ^f	68.7 ± 0.8 ^{hi}
Cinnamon	47.5 ± 0.8 ^g	83.7 ± 0.5 ^a	100 ± 0.0 ^m	100 ± 0.0 ^k	100 ± 0.0 ^j
Citronella	8.75 ± 0.3 ^c	20.0 ± 0.5 ^{cd}	35.0 ± 0.4 ^{hi}	52.5 ± 0.6 ^f	93.7 ± 0.4 ⁱ
Clove	100 ± 0.0 ^h	100 ± 0.0 ⁱ	100 ± 0.0 ^m	100 ± 0.0 ^k	100 ± 0.0 ^j
Eucalyptus	0 ^a	3.7 ± 0.2 ^a	12.5 ± 0.5 ^{cd}	26.2 ± 0.4 ^c	38.8 ± 0.4 ^c
Geranium	0 ^a	2.5 ± 0.4 ^a	15.0 ± 0.5 ^{de}	25.0 ± 0.4 ^c	36.2 ± 0.7 ^c
Lemon	23.7 ± 0.2 ^c	40.0 ± 0.5 ^f	53.7 ± 0.7 ^k	86.2 ± 0.8 ⁱ	100 ± 0.0 ^j
Lemongrass	0 ^a	1.2 ± 0.3 ^a	3.75 ± 0.4 ^{ab}	16.2 ± 0.6 ^b	28.7 ± 0.5 ^b
Lime	13.7 ± 0.4 ^{bc}	22.5 ± 0.2 ^b	38.7 ± 0.6 ^{ij}	57.5 ± 0.6 ^f	67. ± 0.6 ^g
Luchi	5.0 ± 0.4 ^c	21.2 ± 0.4 ^d	33.7 ± 0.7 ^{hi}	58.75 ± 0.6 ^f	73.7 ± 1.1 ⁱ
Nutmeg	10 ± 0.8 ^b	15.0 ± 0.9 ^{bcd}	30.0 ± 0.9 ^{gh}	40.0 ± 0.8 ^d	56.2 ± 0.2 ^{def}
Orange	28.7 ± 0.2 ^{cd}	43.7 ± 0.8 ^f	58.7 ± 0.2 ^k	75.0 ± 0.4 ^h	100 ± 0.0 ^j
Palmarosa	5.0 ± 0.8 ^{bc}	12.5 ± 0.9 ^{bcd}	28.7 ± 0.9 ^{gh}	37.5 ± 0.8 ^d	52.5 ± 0.2 ^d
Pine	2.7 ± 0.2 ^{ab}	11.2 ± 0.2 ^b	21.2 ± 0.4 ^{ef}	35.0 ± 0.7 ^d	55.0 ± 0.7 ^{de}
Rose mary	1.2 ± 0.4 ^b	13.7 ± 0.7 ^c	25.0 ± 0.4 ^{f^g}	36.2 ± 0.7 ^d	82.5 ± 0.5 ^{fg}
Thyme	42.5 ± 0.2 ^f	57.5 ± 0.6 ^g	68.7 ± 0.2 ^l	80.0 ± 0.4 ^{hi}	100 ± 0 ^j
Tulsi	47.5 ± 0.2 ^{ef}	68.7 ± 0.6 ⁱ	92.5 ± 0.6 ^m	100 ± 0.0 ^k	100 ± 0 ^j
Vetiver	36.2 ± 0.4 ^{ef}	61.2 ± 0.6 ^g	76.2 ± 0.8 ^m	87.5 ± 0.6 ^j	100 ± 0 ^j
Reference pesticide (temephos 1 ppm)	40.0 ± 0.8 ^{ef}	65.0 ± 0.4 ^{hi}	100 ± 0.0 ^m	100 ± 0 ^k	100 ± 0 ^j
Control (Tween 80 0.01%)	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a

Each value (mean ± S.E.) represents mean of four replicates, Mean values followed by same letters in a column are not significantly different at $P < 0.05$ level (Tukey's test).

essential oil concentrations. The larvae were collected by dropper, placed onto filter paper strips and immediately transferred to the beaker containing test solutions. Five replicates for each concentration were maintained. In the control, 1.0 ml of Tween 80 (0.01%) in 249 ml of dechlorinated water was added. Mortality of larvae was recorded after 1, 3, 6, 12 and 24 h of treatment. While recording the percentage mortalities for each concentration, the moribund and dead larvae in five replicates were combined. It has been described that dead larvae are those that cannot be induced to move when they are probed with a needle in the siphon or the cervical region; moribund larvae are those incapable of rising to the surface (Hasan and Deo, 1994).

Statistical analysis

The larvicidal mortality was corrected by Abbott's formula (Abbott, 1925). Percentages of mortality were determined and transformed to arcsine square root values for analysis of variance (ANOVA). Tukey's test ($P < 0.05$) was used to analyze for significant differences among the test essential oils against mosquitoes.

RESULTS AND DISCUSSION

All the 22 plant oils were screened against the larvae of

C. quinquefasciatus for larvicidal activity. The results are given in Table 2. Among the 22 plant oils tested, eight oils viz., aniseed, calamus, cinnamon, clove, lemon, orange, thyme, and tulsi oils gave promising results on larvicidal activity. For the larvicidal screening bioassay, the mortality was recorded at different exposure periods viz., 1, 3, 6, 12 and 24 h, and it was found that larval mortality increased when exposure time increased. The clove oil was found to be the most effective treatment. In the preliminary screening, clove oil gave 100 percent mortality at all exposure periods.

Vetiver oil recorded 36.2, 61.2, 76.2, 87.5 and 100% mortality in 1, 3, 6, 12 and 24 h, respectively. The lowest mortality of 28.7% was recorded at 500 ppm concentration in lemongrass oil after 24 h exposure. In the temephos treatment, larval mortality of 40, 65 and 100% was recorded at 1 ppm concentration in 1, 3 and 6 h treatments.

Vector control by means of chemicals is creating many unwanted effects including emergence of pesticide resistance in vector mosquitoes (Chandra et al., 1998). Botanical insecticides may serve as suitable alternatives

to synthetic insecticides in future as they are relatively safe, biodegradable and are readily available in many areas of the world (Prabakar and Jebanesan, 2004). Interest in the development of natural products has been revived during the last two decades. Plants are considered as rich source of bioactive chemicals and they may be an alternative source of mosquito control agents. Natural products of plant origin with insecticidal properties have been tried in the recent past for control of variety of insect pests and vectors (Wink, 1993). More than 2000 plant species have been reported to possess chemicals with pest control properties (Ahmed et al., 1984) and among them about 344 species of plants have been known to possess some degree of activity against the mosquitoes (Sukumar et al., 1991).

Though several plants from different families have been reported for insecticidal activity only a few botanicals like neem based insecticides have moved from the laboratory to field use, which might be due to the light and heat stability of neem compounds compared to synthetic insecticides (Green et al., 1991). In the present study, 22 essential oils registered larvicidal, pupicidal, adulticidal, ovicidal, oviposition deterrent, growth regulator regulating and histopathological effects. Plenty of literature is available with regard to bioefficacy of volatile oils against vector mosquitoes.

The most successful method to minimize the incidence of mosquito borne diseases is by means of eradicating the mosquito vector through systematic treatment of their breeding places with the help of larvicides (Cetin et al., 2004). Chemical pesticides that are applied in water bodies are not only killing mosquito larvae but also killing non-target organisms. In addition chemical pesticides select for resistance in mosquitoes. As an alternative to chemical pesticides volatile oils are largely studied against *C. quinquefasciatus* mosquito. In the present study, the larvicidal activity was tested at 500 ppm concentration. Among the 22 essential oils, eight oils registered high larvicidal activity. The larval mortality was found to be directly proportional to the exposure period. Clove oil was found to be the most toxic to the larvae since it registered 100% mortality from 1 h treatment period onwards.

The present investigation revealed that the essential oils possess remarkable larvicidal activity against the tested mosquito *C. quinquefasciatus*. The control of selected mosquito by affecting their survival by essential oils was found evident. Further purification and characterization of the bioactive compound of effective oils are underway in our laboratory.

ACKNOWLEDGEMENTS

We are deeply indebted to the Director, Entomology Research Institute, Loyola College, Chennai, for his interest to promote research activities.

REFERENCES

- Abbott WS (1925). A method of computing the effectiveness of an insecticide. *J. Econ. Entomol.* 18:265-266.
- Ahmed S, Graivge M, Hylin JW, Mitchell WC, Listering JA (1984). Some promising plant species for use as pest control agents under traditional farming system. In: Schmutterer, H. and K.R.S. Ascher (eds.) Proc. 2nd International Neem Conference, Rauscholzhausen, Germany. pp. 565-580.
- Ansari MA, Mittal PK, Razdan RK, Sreehari U (2005). Larvicidal and mosquito repellent activities of Pine (*Pinus longifolia*, Family: Pinaceae) oil. *J. Vect. Borne Dis.* 42:95-99.
- Ansari MA, Razdan RK (1995). Relative efficacy of various oils in repelling mosquitoes. *Ind. J. Malariol.* 32:104-11.
- Barnard, D.R., 1999. Repellency of essential oils to mosquitoes (Diptera: Culicidae). *J. Med. Entomol.* 36:625-629.
- Bowers WS, Sener B, Evans H, Bingal F, Erodogan I (1995). Activity of Turkish medicinal plants against mosquitoes *Aedes aegypti* and *Anopheles gambiae*. *Insect Sci. Appl.* 16:339 - 342.
- Carvalho AFU, Melo VMM, Craveiro AA, Machado MILL, Bantim B, Rabelo EF (2003). In : *Memorias Do Instituto-Oswaldo Cruz.* 11 Fortaleza, CE, Brazil. 98:569-571.
- Cavalcanti ESB, de Moraes CC, Lima MAA, Santana EWP (2004). Larvicidal activity of essential oils from Brazilian plants against *Aedes aegypti* L. *Mem. I. Oswaldo Cruz* 99:541-544.
- Cetin B, Ozer H, Kuscu H (2004). Economics of drip irrigation for apple (*Malus domestica*) orchards in Turkey. *N. Z. J. Crop Hortic. Sci.* 32:349-354.
- Chandra F, Darrit F, Darder M, Cuany A, Doannio JMC, Pasteur N, Guillot P (1998). Prethroid resistance in *Culex quinquefasciatus* form West Africa. *Med. Vet. Entomol.* 12:359-366.
- Ghosh GK (2000). In: *Biopesticide and Integrated pest Management* A.P.H. publishing corporation, New Delhi. pp. 400-446.
- Green MM, Singer JM, Sutherland DJ, Hibben CR (1991). Larvicidal activity of *Tagetes minuta* (Mari gold) towards *Aedes aegypti*. *J. Am. Mosq. Control Assoc.* 7:283-286.
- Hasan SB, Deo PG (1994). *Ocimum sanctum* seeds for mosquito control. *Int. Pest Control* 36:20-21.
- Kiso Y, Hikino H (1991). In: *Plant Biochemistry and it's Bioactivity.* Academic Press, London. pp. 219-233.
- Omolo MO, Okinyo D, Ndiege IO, Lwande W, Hassanali A (2004). Repellency of essential oils of some Kenyan plants against *Anopheles gambiae*. *Phytochemistry* 65:2797-2802.
- Paula JP, Gomes Carneiro MR, Paumgarten FJR, De Paula JP (2003). Studies on effect of *Ocimum selloi* oil against the adult mosquitoes. *J. Ethnopharmacol.* 24(88):253-260.
- Pitasawat B, Choochote W, Tuetun B, Tippawangkosol P, Kanjanapothi D, Jitpakdi A, Riyong D (2003). Repellency of aromatic turmeric *Curcuma aromatica* under laboratory and field conditions. *J. Vect. Ecol.* 28:234-240.
- Prabakar K, Jebanesan A (2004). Larvicidal efficacy of some Cucurbitaceous plant leaf extracts against *Culex quinquefasciatus* (Say). *Bioresour. Technol.* 95(1):113-114.
- Saxena BP, Koul O, Tikku A, Atal CK, Suri, OP, Suri KA (1979). Aristolochic - acid an insect chemosterilant from *Aristolochia bracteata* retz. *Ind. J. Expt. Biol.* 17:354.
- Skinner WA, Johnson HL (1980). The design of insect repellents. In: Ariens, E.T. (Ed.), *Drug Design*, vol. 10. Academic Press, New York. pp. 277-305.
- Sukumar K, Perich MJ, Boobar LR (1991). Botanical derivatives in mosquito control: A review. *J. Am. Mosq. Control Assoc.* 7:210-237.
- Wink M (1993). Production and application of phytochemicals from an agricultural perspective. In van Beek, T. A. and H. Breteleur, editors. eds. *Phytochemistry and agriculture.* Oxford, United Kingdom Clarendon Press. pp. 171-213.

Full Length Research Paper

The role of seed priming in improving seed germination and seedling growth of maize (*Zea mays* L.) under salt stress at laboratory conditions

Gebremedhn Yohannes¹ and Berhanu Abraha^{2*}

¹Department of Biology, Wachamo University, Ethiopia.

²Department of Biology, Bahir Dar University, Ethiopia.

Accepted 23 October, 2013

Salinity is considered as a major abiotic stress affecting germination, seedling growth and crop production in arid and semi-arid regions. Many techniques are used to improve tolerance to salinity. Priming is believed to be an effective technique that increases germination, plant growth and improve yield of several vegetables and crops under saline soil condition. The objective of this study was to see the effect of seed priming with 5 g/L NaCl on germination and seedling growth of maize (*Zea mays* L.) exposed to five salinity levels under laboratory conditions. Maize seeds were soaked in solutions of NaCl (5 g/L) for 12 h at room temperature. Primed and unprimed seeds were sown in Petri dishes and were irrigated with saline solutions of five concentrations (0, 2, 4, 6 and 8 g/l NaCl). Priming seeds with NaCl significantly improved ($P < 0.05$) germination and growth of maize. As a result, fresh and dry weights of shoot and root were improved. Seed priming alleviated the inhibitory effect of salt stress on germination and seedling growth of maize under salt stress. Thus, seed priming with 5 g/l NaCl could be used to improve germination and early growth. Further, this study needs to be continued if performance of mature maize plants could also be improved and yield increased by sowing primed seeds in saline soils.

Key words: Maize, salinity, seed germination, seed priming, seedling growth.

INTRODUCTION

Salinity affects 6% of the world's total land area which is approximately 800 million hectares (FAO, 2008). Salinization is more spreading in irrigated lands because of inappropriate management of irrigation and drainage. Rain, cyclones and wind also add NaCl into coastal agricultural lands (FAO, 2008). Salinity has an adverse effect on seed germination of many crops, by creating an osmotic potential outside the seed inhibiting the absorption of water, or by the toxic effect of Na^+ and Cl^- (Khajeh-Hosseini et al., 2003). Osmotic and saline stresses are responsible for the inhibition and hindrance of germination and plant growth (Almansouri et al., 2001).

Water uptake during the imbibitions stage decreases and salinity induces an excessive absorption of toxic ions by the seed.

Seed priming or osmoconditioning is one of the physiological methods which improves seed performance and provides faster and synchronized germination. It is an easy, low cost and low risk technique, which is recently being used to overcome the salinity problem in agricultural lands (Neto and Tabosa, 2000). It entails the partial germination of seed by soaking in either water or in a solution of salts for a specified period of time, and then re-drying them just before the radicle emerges

*Corresponding author. Email: berhanu.tsegay@yahoo.com.

(Neto and Tabosa, 2000). Seed priming stimulates many of the metabolic processes involved in the early phases of germination, and it has been noted that seedlings from primed seeds emerge faster, grow more vigorously, and perform better in adverse conditions (Cramer, 2002). Some of the factors that affect seed priming response are solution composition and osmotic potential (Chinnusamy et al., 2005). It has been shown that NaCl seed priming could be used as a solution to improve salt tolerance of seeds (Cayuela et al., 1996; Elouaer and Hannachi, 2012).

Ethiopia is reported to possess over 11 million hectares of unproductive naturally salt affected wasteland (PGRC, 1996). These areas are normally found in the arid and semi-arid lowlands and in rift valley and other areas that are characterized by higher evapotranspiration rates in relation to low precipitation. According to the study of Hawando (1994) salt-affected lands are increased from 6 to 16% of the total land area of Ethiopia in the last years of the 20th century and 9% of the population lived in these areas. About 44 million ha (36% of the country's total land areas) are potentially susceptible to salinity problems. Reclaiming back these salt affected areas and using them for agricultural production is very costly and time consuming.

Maize (*Zea mays*) production in Ethiopia ranges from large-scale commercial farms to smallholders and subsistence farmers. It is consumed as main food, with its crop residues and by-products commonly fed to Livestock and used as source of fuel. Hence, priming of maize seeds with NaCl could be important in improving the growth and yield of maize in areas that are potentially susceptible to salinity problems and totally unproductive salt affected lands. Such studies were not done before with maize in the Ethiopian agricultural landscapes. Therefore, this study investigated the effect of priming on improving seedling growth of maize under different salinity levels at laboratory conditions.

MATERIALS AND METHODS

The study was conducted in Bahir Dar city at Bahir Dar University (Peda campus). Bahir Dar city is the capital of the Amhara National Regional State in the Federal Democratic Republic of Ethiopia. It is located at 11°37'30"N and 37°27'30"E on the southern side of Lake Tana with an altitude of 1801 m above the mean sea level. The annual temperature of Bahir Dar city ranges from a minimum average of 12.6°C to a maximum average of about 27.0°C, and the mean annual rainfall is about 1406.98 mm (NMABDBO, 2013).

Seed materials and priming

Maize seeds (Melkasa 4) were obtained from the certified seed supplier of Ethiopian seed enterpriser (ESE) store located in Bahir Dar, Ethiopia. The seeds were surface sterilized (disinfected) with 5% sodium hypochlorite (Na₂HCl) solution for 3 min and then thoroughly washed for 5 min with distilled water. Subsequently, the seeds were primed by soaking with 5 g/L NaCl solution for 12 h at room temperature under shade and the ratio of seed weight to solu-

tion volume was 1/5 (g/ml). After priming, seeds were removed and washed with tap water and then rinsed three times in distilled water. Finally, seeds were left in air between two filter papers to re-dry to their original moisture level (Afzal et al., 2008).

Experimental design

The study was carried out from October 20, 2012 to March 16, 2013. A complete random design (CRD) study was used to study the effect of seed priming on germination and seedling establishment of maize under salt stress. This part of the experiment was carried out in the laboratory in 40 Petri dishes that is, 20 for NaCl primed seeds and 20 for unprimed (control) seeds. The experiment was replicated four times. All petri dishes were sterilized and labeled for various treatments (0, 2, 4, 6 and 8 g/L) of NaCl.

Primed and unprimed seeds were placed in 15 cm diameter Petri dishes on a layer of filter paper (Whatman # 1). Thirty seeds were placed in each Petri dish. Petri dishes were irrigated with five different saline solutions (0, 2, 4, 6 and 8 g/L concentrations of NaCl) 10 ml per Petri dish daily. The Petri dishes were placed at room temperature. The experiment was a factorial with two factors which are salinity at 5 levels (0, 2, 4, 6 and 8) g/L and priming with 2 levels (unprimed and NaCl primed seeds), arranged in a completely randomized design with four replications and thirty seeds per replicate. Seed germination was recorded daily up to day 14 after the start of the experiment. A seed was considered as germinated when the radicle emerges by about 2 mm in length (Ahmadvand et al., 2012).

Physiological parameters such as seed germination percentages, mean germination time, germination index and coefficient velocity of germination were determined. Shoot and root length of five randomly selected seedlings was measured using transparent ruler from each petri dish after the seedlings were separated carefully from their roots. Then, shoot and root fresh weight was measured immediately. To determine the dry weight of these seedlings, the separated shoots and roots were dried to 70°C for 12 h (Ahmadvand et al., 2012) and then weighed.

Computing measurements of physiological parameters

Total germination (TG) was measured in the 14th day after sowing using the formula

$$TG(\%) = \frac{n}{N} \times 100$$

Where, n is the total germinated seeds and N is the total seeds sowed. Mean germination time (MGT) was calculated according to the formula used by Fuller et al. (2012).

$$MGT = \frac{\sum(T_i n_i)}{\sum n_i}$$

Where, n_i is the number of germinated seeds on the ith day and T_i is the rank order of day i (number of days counted from the beginning of germination). Vigour index (VI) was measured using the formula of Elouaer and Hannachi (2012) and it is calculated as follows.

$$VI = \frac{TG(\%) \times \text{Seedling Length}(cm)}{100}$$

Table 1. Analyses of Variance of seed germination of Maize seedlings under laboratory conditions.

Source	TG (%)	MGT	SL	RL	R/S	VI	SFW	SDW	RFW	RDW
Priming	3180.319*	92.045*	44.796*	21.433*	0.206*	38.796*	1.197*	0.316*	0.632*	0.060*
Salinity level	9142.315*	75.312*	433.215*	362.430*	0.928*	416.314*	27.341*	1.810*	9.269*	0.277*
Priming× salinity level	260.137*	8.229*	3.624*	7.142*	0.030*	5.175*	0.138	0.033	0.084	0.014
Error	59.352	0.107	1.084	0.55	0.003	0.907	0.157	0.027	0.066	0.006

*Significant at 0.05 according to Post Hoc LSD test.

Data analysis

All the data obtained from the experiments were subjected to an analysis of variance (Two way ANOVA) using the Statistical Package for the Social Sciences (SPSS) (version20.0) software and the difference between means were compared by Fisher's Post Hoc LSD tests ($P < 0.05$).

RESULTS AND DISCUSSION

Examination of variance showed that both salinity level and priming have significant effects on the studied parameters. Moreover, the interaction of salinity level and priming with salt solution had significant effect on all parameters tested at 5% significant level (Table 1). Priming with NaCl increases the germination parameters (TG (%), MGT) and growth parameters (RL, SL, SFW, SDW, RFW, RDW, R/S and VI) of maize, as compared to unprimed seeds, under different levels of saline conditions.

Total germination percentage (TG (%)) and mean germination time (MGT)

Figure 1a and 1b shows the effect of NaCl priming on maize TG (%) and MGT at different salinity concentrations, respectively in 14 days. TG (%) from both primed and unprimed seeds decreased significantly ($P < 0.05$) with increasing NaCl salinity level (Figure 1a). However, this reduction in TG (%) was significantly higher for unprimed seeds compared to primed seeds. The data in Table 2 indicated a reduction of 83.75% on TG (%) due to an increase in salinity level from 0 to 8 g/L. Although salinity stress significantly ($P < 0.05$) increase MGT for both primed and unprimed maize seeds, the primed seeds had significantly lower MGT compared to unprimed seeds. Such variation is shown in Figure 1b. Generally, increasing salinity causes a decrease in maize germination; this might be due to the toxic effects of Na^+ and Cl^- in the process of germination (Khajeh-Hosseini et al., 2003). It alters the imbibitions of water by seeds due to lower osmotic potential of germination media, causes toxicity which changes the activity of enzymes of nucleic acid metabolism, changes protein metabolism, interrupts

hormonal balance, and reduces the utilization of seed reserve food (Gomes-Filho et al., 2002).

Primed seeds of maize might have better competency for water absorption from the growing media that enabled metabolic activities in seeds during germination process of a start much earlier than radicle and plumule appearance (Elouaer and Hannachi, 2012). Similarly increased solubilization of seed storage proteins like the beta subunit of the globulin and reduction in lipid peroxidation and enhanced antioxidative activity in primed seeds facilitated germination. This faster germination was due to the synthesis of DNA, RNA and protein during priming (Afzal et al., 2008).

Vigor index

Based on the analysis of variances, salinity level, seed priming and their interaction have significant ($P < 0.05$) effect on vigor index (Table 1). Increasing salinity causes a significant decrease ($P < 0.05$) in maize vigor index for both unprimed and primed seeds (Table 2 and Figure 2). This decrease was from 17.684 at 0 g/L to 0.184 at 8 g/L (Table 2). However, the relative decrease for primed seeds was significantly ($P < 0.05$) different when compared to seedlings from unprimed seeds. Vigor index was increased in primed compared to unprimed maize seeds (Figure 2). Similar results were found by Ruan et al. (2002a) working on rice seeds, priming showed higher vigour index than non-primed ones. The significant improvement in seedling length and total germination percentage may be a result of earlier induced germination by primed seeds over unprimed seeds (Farooq et al., 2005), which resulted in vigorous seedlings with more root and shoot length than the seedlings from unprimed seeds.

Shoot and radicle length

Salinity had a significant inhibitory effect on shoot length for both primed and un-primed seeds. However, this effect was significantly ($P < 0.05$) less pronounced in seedlings from NaCl primed seeds compared to the unprimed seeds (Figure 3a). Similarly, salinity affected

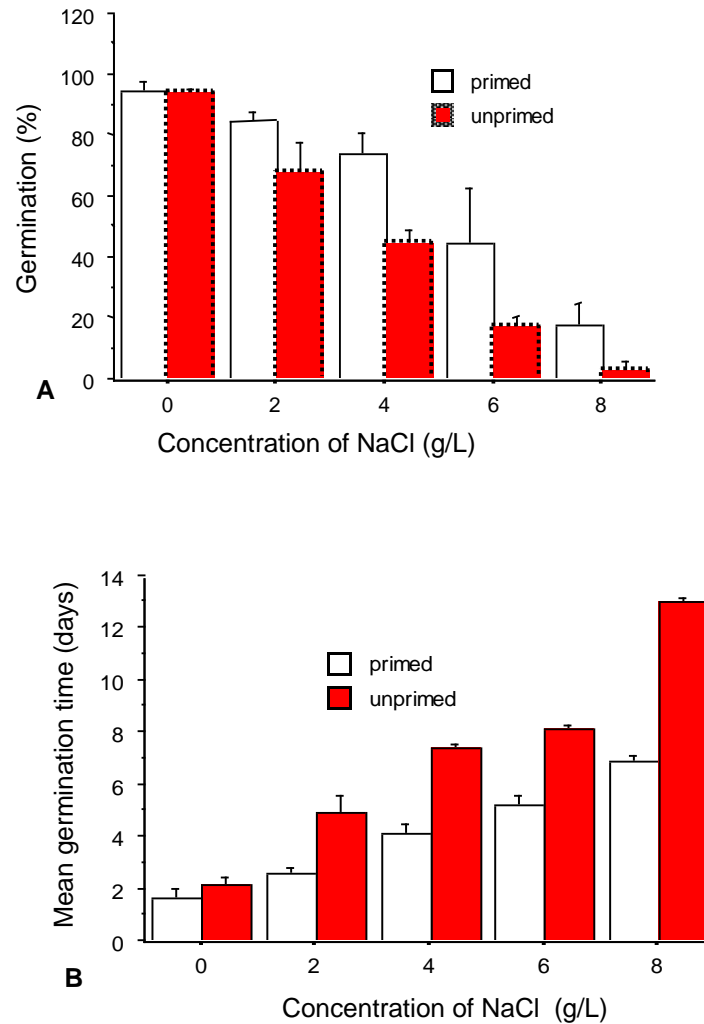


Figure 1. Effect of different salinity levels on germination percentage (a) and mean germination time (b) of primed (NaCl) and unprimed seeds of maize under laboratory conditions in 14 days. (Error bars ± 1 standard deviation).

Table 2. Means comparison of the traits under different salinity levels after 14 days of germination time.

Salinity (g/L)	TG (%)	MGT (Day)	SL (cm)	RL (cm)	R/S	VI	SFW (g)	SDW (g)	RFW (g)	RDW (g)
0	94.583 ^a	1.900 ^a	18.700 ^a	16.350 ^a	0.896 ^a	17.684 ^a	4.675 ^a	1.185 ^e	2.604 ^a	0.451 ^a
2	76.667 ^b	3.811 ^b	7.362 ^b	4.285 ^b	0.536 ^b	5.813 ^b	2.325 ^b	0.413 ^a	0.812 ^b	0.108 ^b
4	59.583 ^c	5.780 ^c	4.262 ^c	1.965 ^c	0.255 ^c	2.750 ^c	1.325 ^c	0.288 ^b	0.249 ^c	0.046 ^c
6	31.250 ^d	6.705 ^d	1.586 ^d	0.667 ^d	0.153 ^d	0.619 ^d	0.420 ^d	0.044 ^c	0.114 ^c	0.024 ^c
8	10.833 ^e	10.01 ^e	0.345 ^e	0.054 ^e	0.053 ^e	0.184 ^e	0.066 ^d	0.014 ^c	0.015 ^c	0.003 ^c

*Means with the same letters in each column are not significantly different at 0.05 according to LSD test.

seedlings' radicle length of both primed and unprimed seeds of maize with increasing salinity level. But this influence was highly prominent in shoot (Figure 3a) and radicle (Figure 3b) lengths from unprimed seeds when compared to the primed ones. When the concentration of

salinity increase, the growth of root and shoot becomes very slow and mostly the roots died after some days.

Salinity has both osmotic and specific ionic effects on seedlings growth. Likewise, toxic ion accumulation (Na^+ and Cl^-) negatively affect plant metabolism. It has also

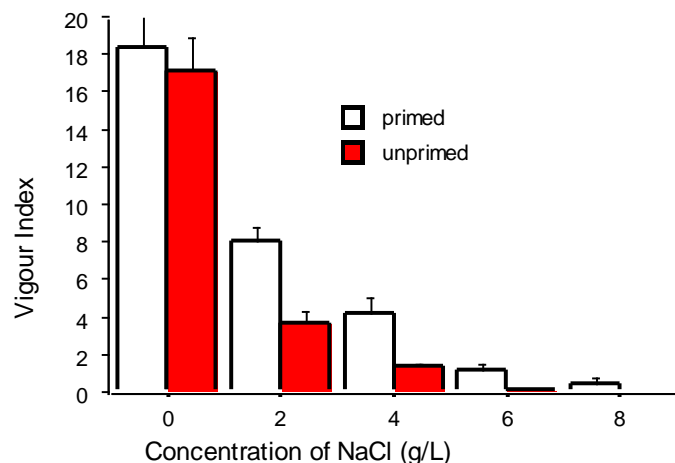
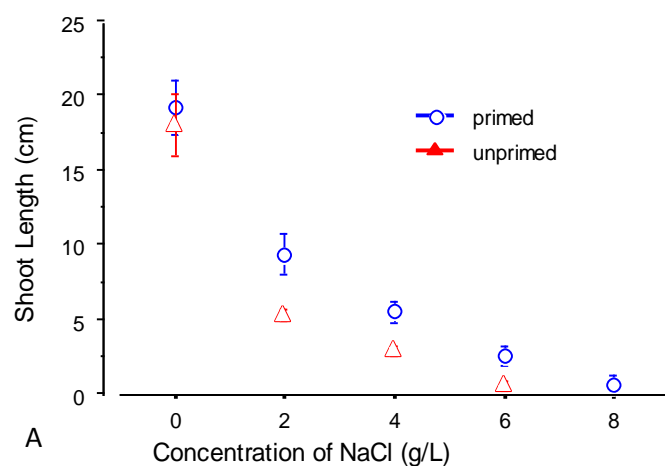
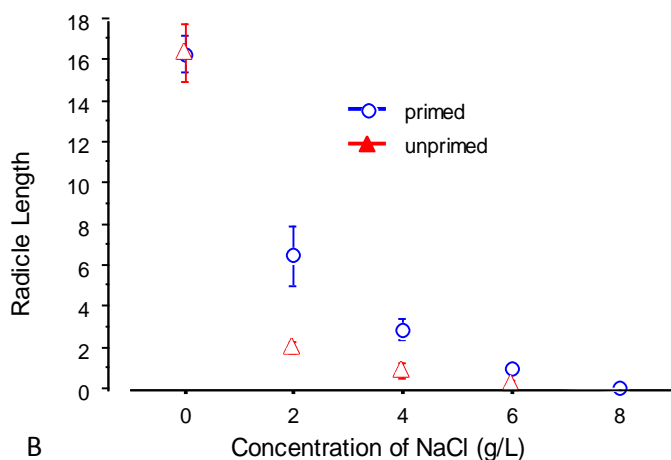


Figure 2. Effect of different salinity levels on Vigor Index of primed and unprimed Maize seeds under laboratory conditions. Error bars ± 1 standard deviation.



A



B

Figure 3. Effect of different salinity levels on shoot length (A) and radicle length (B) of primed and unprimed maize seeds under laboratory conditions. Error bars ± 1 standard deviation.

been reported that salinity suppresses the uptake of essential nutrients like P and K which could adversely affect seedlings growth (Nasim et al., 2008). In this study, seed priming significantly improved maize seedling growth at different salinity levels. Significant improvement in root and shoot length in the primed seeds may be attributed to earlier germination induced by priming (Farooq et al., 2005). During priming, the embryo expands and compacts the endosperm. The compaction force of the embryo and hydrolytic activities on the endosperm cell walls may change the tissues to have their flexibility upon dehydration, producing free space and facilitating root and seedling fast projection after rehydration (Mohammadi, 2009). This resulted in vigorous seedlings with more roots and shoot length than the seedlings from unprimed seeds. The present results confirm the findings of Nawaz et al. (2012) in tomato (*Lycopersicon esculentum* Mill.) and in Canola (*Brassica napus* L.) where priming of the seeds significantly improved shoot and radicle length.

Root length to shoot length ratio (R/S)

Analysis of variance indicated that salinity level, priming and their interaction have significant effects on root to shoot ratio (Table 1). It was decreased significantly with increasing salinity level from 0 g/L (0.896) to 8 g/L (0.053) (Table 2), but the effect was more pronounced in unprimed seeds (Figure 4). This study showed that salt stress inhibits radicle growth more than primary shoot growth. Decreased R/S with increasing salinity level could be due to the first exposition of the root to salinity, rapidly affected before the shoots (Akram et al., 2010). In saline conditions, NaCl priming increased root to shoot ratio of maize seedlings. This may in turn have the advantage of increased ratio of water up take by seedlings. High root to shoot ratio in primed seeds under salt stress may be considered important for salt tolerance of growth attributes (Alian et al., 2000). Thus, high root to shoot ratio induced by maize seeds primed with NaCl appears to be an adaptation (solution) to salinity.

Shoot and root fresh and dry weights

Increasing salinity significantly ($P < 0.05$) decreased maize seedlings fresh and dry weight for both primed and unprimed seeds. In actual fact, the increase in salt concentration in culture medium reduced shoot (Figure 5a) and root (Figure 5c) fresh weight from 4.675 g at 0 g/L to 0.066 g at 8 g/L and from 2.604 g at 0 g/L to 0.015 g at 8 g/L, respectively (Table 2). However, primed seeds showed better performance than unprimed seeds. The difference in performance was significant at 95% confidence level. In the same way seed priming with NaCl significantly ($P < 0.05$) enhanced maize shoot dry weight (Figure 5b) and root dry weight (Figure 5d) as

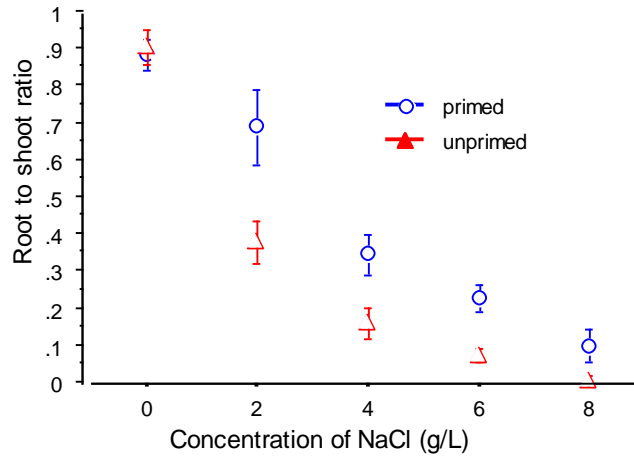


Figure 4. Effect of different salinity levels on root to shoot ratio of primed and unprimed maize seeds under laboratory conditions. Error bars ± 1 standard deviation.

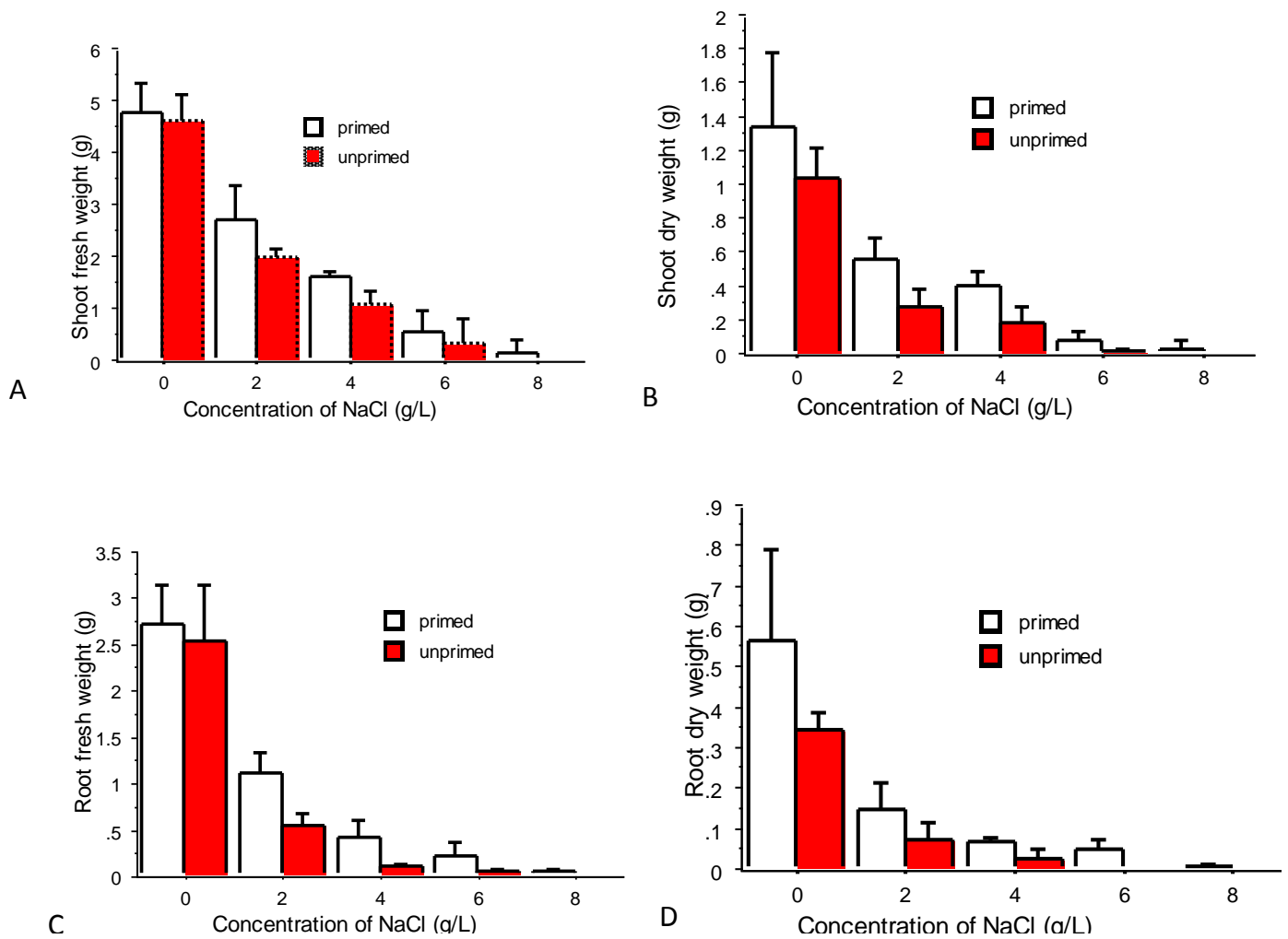


Figure 5. Effect of different salinity levels on shoot fresh weight (A), shoot dry weight (B), root fresh weight (C) and root dry weight (D) of primed (NaCl) and unprimed maize seeds under laboratory conditions. Error bars ± 1 standard deviation.

compared to the unprimed seeds.

The results in this study are similar to that found by Achakzai et al. (2010) where they found that salinity can rapidly inhibit seedling and root growth and this might be due to the ability of inhibiting uptake of water and essential mineral nutrients from culture media. Also increment of the toxic effect of sodium at high salt level caused physiological effects that resulted in strong reduction in photosynthesis, enzymatic process and protein synthesis, which resulted in limited growth and poor leaf area development. Increment of shoot and root fresh and dry weights were observed in primed seeds in contrast to the unprimed maize seeds. This might be as a result of improvement of plant water status and minimizing the toxic effects of Na⁺ resulted in maximum fresh and dry weights (Tahir et al., 2006).

Conclusion

Generally, salinity inhibits germination and early seedling growth of maize seeds. Roots were affected more than shoots as evidenced from the root to shoot ratio. Priming is an effective method to meet the demands of farmers during the installation of the culture in conditions of salt stress. Seed priming with NaCl improved germination and early growth of maize seeds. Primed seeds should be sown in saline soils to increase germination and early growth of maize seeds. Further study is required to investigate the effects of seed priming on later growth and yield stages of this plant. Additional molecular research is needed to explore priming induced alteration in physiological and biochemical attributes both at seed and whole plant levels in maize.

REFERENCES

- Achakzai AK, Kayani SA, Hanif Z (2010). Effect of salinity on uptake of micronutrients in sunflower at early growth stage. *Pakistan J. Bot.* 42:129-139.
- Afzal I, Rauf S, Basra SMA, Murtaza G (2008). Halopriming improves vigor, metabolism of reserves and ionic contents in wheat seedlings under salt stress. *Plant Soil Environ.* 54:382-388.
- Ahmadvand G, Soleimani F, Bijan Saadatian B, Pouya M (2012). Effects of seed priming on germination and emergence traits of two soybean cultivars under salinity stress. *J. Basic Appl. Sci. Res.* 3:234-241.
- Akram M, Ashraf MY, Ahmad R, Waraich EA, Iqbal J, Mohsan M (2010). Screening for salt tolerance in maize (*Zea mays* L.) hybrids at an early stage. *Pakistan J. Bot.* 42:141-151.
- Alian A, Altman A, Hever B (2000). Genotypic difference in salinity and water stress tolerance of fresh market tomato cultivars. *Plant Science.* 152:59-65.
- Almansouri M, Kinet JM, Lutts S. (2001). Effect of salt and osmotic stresses on germination in durum wheat (*Triticum durum*). *Plant and Soil.* 231:243-254.
- Cayuela E, Perez-Alfocea F, Caro M, Bolarin MC (1996). Priming of seeds with NaCl induces physiological changes in tomato plants grown under salt stress. *Physiol. Plant.* 96:231-236.
- Chinnusamy V, Jagendorf A, Zhu JK (2005). Understanding and improving salt tolerance in plants. *Crop Science.* 45:437-448.
- Cramer GR (2002). Sodium-calcium interactions under salinity stress in Lauchli A, Luttge salinity. *Environment-plants.* 4:205-227.
- Elouaer MA, Hannachi C (2012). Seed priming to improve germination and seedling growth of safflower (*Carthamus tinctorius*) under salt stress. *Eurasian J. Biosci.* 6:76-84.
- FAO (2008). Land and Plant Nutrition Management Service. <http://www.fao.org/ag/agl/agll/>. Accessed on November/ 15/2012.
- Farooq M, Basra S, Saleem B, Nafees M, Chishti S (2005). Enhancement of tomato seed germination and seedling vigor by osmopriming. *Pakistan J. Agric.Sci.* 42:52-58.
- Fuller M P, Hamza JH, Rihan H Z, Al-Issawi M (20012). Germination of Primed Seed under NaCl Stress in wheat. *Int. Scholarly Res. Network Bot.* 2:1-5.
- Gomes-Filho E, Machado Lima CRF, Costa JH, Da Silva AC, Da Guia Silva Lima M, Gupta NK, Meena SK, Gupta S, Khandelwal SK (2002). Gas exchange, membrane permeability and ion uptake in two species of Indian jujube differing in salt tolerance. *Photosynthetica.* 40:535-539.
- Hawando T (1994). Desertification in Ethiopian highlands, Norwegian Church Regional Senior Consultant on Environment and Natural Resource Management, Report No. 200. Addis Ababa, Ethiopia.
- Khajeh-Hosseini M, Powell AA, Bingham IJ (2003). The interaction between salinity stress and seed vigor during germination of soybean seeds. *Seed Sci. Technol.* 31:715-725.
- Mohammadi GR (2009). The Influence of NaCl priming on seed germination and seedling growth of canola (*Brassica napus* L.) under salinity conditions. *J. Agric. Environ. Sci.* 5:696-700.
- Nasim M, Qureshi R, Aziz T, Saqib M, Nawaz S, Sahi ST, Pervaiz S (2008). Growth and ionic composition of salt stressed *Eucalyptus camaldulensis* and *Eucalyptus teretecornis*. *Pakistan J. Bot.* 40:799-805.
- National Metrological Agency Bahir Dar Branch Office (NMABDBO) (2013). Annual Metrology report, Bahir Dar, Ethiopia.
- Nawaz A, Amjad M, Jahangir MM, Khan MS, Huawei, Cui H, Jin Hu (2012). Induction of salt tolerance in tomato (*Lycopersicon esculentum* Mill.) seeds through sand priming. *Afr. J. Crop Sci.* 6:1199-1203.
- Neto AD, Tabosa JN (2000). Salt stress in maize seedlings: I. Growth analysis. *Revista Brasileira de Engenharia agrıcola e Ambiental.* 4:159-164.
- PGRC (1996). Ethiopia: country report to the FAO international technical conference on plant genetic resources, Addis Ababa, Ethiopia.
- Ruan S, Xue Q, Tytkowska K (2002a). Effects of seed priming on germination and health of rice (*Oryza sativa* L.) seeds. *Seed Sci. Technol.* 30:451-458.
- Tahir MA, Rahmatullah T, Aziz M, Ashraf S, Kanwal, Maqsood MA (2006). Beneficial effects of silicon in wheat (*Triticum aestivum* L.) under salinity stress. *Pakistan J. Bot.* 38:1715-1722.

Full Length Research Paper

Pathogenicity of *Beauveria bassiana* and production of cuticle-degrading enzymes in the presence of *Diatraea saccharalis* cuticle

Virgínia Michelle Svedese^{1*}, Patricia Vieira Tiago¹, Jadson Diogo Pereira Bezerra¹, Laura Mesquita Paiva¹, Elza Áurea de Luna Alves Lima¹, Ana Lúcia Figueiredo Porto²

¹Departamento de Micologia, Laboratório de Controle Biológico, Universidade Federal de Pernambuco, Avenida Professor Nelson Chaves, s/n, Cidade Universitária, CEP: 50670-420, Recife, Pernambuco, Brazil.

²Departamento de Morfologia e Fisiologia Animal, Laboratório de Tecnologia de Bioativos, Universidade Federal Rural de Pernambuco - Rua Dom Manoel de Medeiros, s/n, Dois Irmãos - CEP: 52171-900 - Recife, Pernambuco, Brazil.

Accepted 17 October, 2013

The sugarcane borer, *Diatraea saccharalis*, is one of the worst pests in Brazilian sugarcane crop, causing high levels of financial losses every year. *Beauveria bassiana* is an entomopathogenic fungus widely used in the biological control of several agricultural pests. The aims of this study were to: (1) evaluate the pathogenicity of *B. bassiana* strains against *D. saccharalis* (2) investigate the production of proteases and chitinase by *B. bassiana* in the presence of the cuticle of sugarcane borer; and, (3) evaluate the relation between the production of enzymes and pathogenicity of the strains. All isolates tested were pathogenic to *D. saccharalis* and the mortality ranged from 36 to 88%. The production of enzymes was higher in the medium containing cuticle, showing that the process is stimulated by specific components found in the cuticle of the host. Pr1 activity was higher than Pr2 and both were produced at 24 h. The highest production of chitinase was obtained at 96 h of culture for all strains tested. Levels of specific cuticle-degrading enzymes such as proteases correlated positively with specific virulence parameters. *B. bassiana* URM2915 showed promising features to be used in a biological control program of *D. saccharalis*.

Key words: Biological control, sugarcane, subtilisin-like protease, trypsin-like protease, chitinase.

INTRODUCTION

Sugarcane (*Saccharum officinarum* L.) is an important source of sugar and alcohol in the Brazilian economy. However, part of the crop is lost due to the action of a number of insect pests, of which the borer *Diatraea saccharalis* Fabricius (Lepidoptera: Crambidae) is one of the most important (Gallo et al., 2002; Oliveira et al., 2008). Due to its cryptic lifestyle, conventional control measures by deploying chemical insecticides targeted at the larvae are ineffective.

An alternative to chemical control is the use of entomo-

pathogenic fungi, such as *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* (Metschnikoff) Sorokin. These fungi are widely used as biocontrol agents for a number of insect pests (Lacey, 2001; Hajek and Delalibera, 2010) and the efficiency of *B. bassiana* has already been proven against a number of Lepidoptera, such as *Castnia licus* Drury (Alves et al., 2002), *Ostrinia nubilalis* Hübner (Lewis et al., 2002), *Plutella xylostella* L. (Silva et al., 2003), *Spodoptera frugiperda* Smith (Vijayavani et al., 2009) and

*Corresponding author Email: vsvedese@hotmail.com. Fax: +55 81 2126-8479.

Table 1. Mortality (%) of *Diatraea saccharalis* larvae at seven days after application of different strains of *Beauveria bassiana*.

Access number	Insect host of origin	Site of origin	Mortality ^a (%) ± SD
URM2912	<i>Cyclonella sanguinea</i> (Coleoptera)	Paraná/BR	42 ± 3.42c
URM2915	<i>Nezara viridula</i> (Hemiptera)	Paraná/BR	88 ± 3.8a
URM2916	Coleoptera	Brasília/BR	76 ± 4.2ab
URM2920	<i>Anticarsia gemmatalis</i> (Lepidoptera)	Brasília/BR	68 ± 4.5b
URM2921	<i>Lebia concinna</i> (Coleoptera)	Paraná/BR	84 ± 5a
URM2923	<i>Deois flavopicta</i> (Homoptera)	Paraná/BR	76 ± 4.7ab
URM2924	<i>Deois flavopicta</i> (Homoptera)	Brasília/BR	36 ± 3.5d
URM2930	<i>Euschistus heros</i> (Hemiptera)	Paraná/BR	79 ± 5 ab
URM3447	<i>Castnia licus</i> (Lepidoptera)	Pernambuco/BR	86 ± 4.5 a
URM4548	<i>Diabrotica speciosa</i> (Coleoptera)	Buenos Aires/ARG	46 ± 5.1 c
Control			5.4±1e

^aMean followed by the same letter are not significantly different in the Tukey test at 5% probability. Original data, for statistical analysis were transformed into arcsine (X); SD: standard deviation.

Thaumetopoea pityocampa Den. & Schiff (Sevim et al., 2010). Entomopathogenic fungi have a number of determinants of pathogenicity, including production of cuticle-degrading enzymes, such as proteases, chitinases and lipases (Bidochka and Khachatourians, 1987). These enzymes are pointed out as important in the infection process, since they have already hydrolyzed polymer protein and chitin complexes, the major components of the insect's cuticle (St. Leger et al., 1986).

The best model to determining the level of pathogenicity in entomopathogenic fungi is based on a protease of the subtilisin-like, called Pr1, first studied in *M. anisopliae* by St. Leger et al. (1988). A trypsin-like enzyme (Pr2) belonging to the serine protease group also occurs during the early stages of cuticle colonization suggesting that it plays a role in degrading extracellular proteins complementary to that of Pr1 (St. Leger et al., 1996).

Gupta et al. (1994) have shown a correlation between the production of high levels of chitinases and proteases and the virulence of *B. bassiana* against *Galleria mellonella* L. and *Trichoplusia ni* Hubner. In addition, Fang et al. (2005) proved that an overexpression of a chitinase gene (Bbchit1) enhanced the virulence of *B. bassiana* to aphids (*Myzus persicae* Sulzer), compared with a wild-type strain.

Only a few papers have focused on the production of these enzymes by *B. bassiana* strains on the presence of host's cuticle. Campos et al. (2005) have detected the presence of chitinases and proteases in the cuticle of *Boophilus microplus* Canestrini. In addition, Dias et al. (2008) and Montesinos-Matías et al. (2011) evaluated the production of Pr1 after growing the fungus in the presence of coffee-borer cuticle *Hypothenemus hampei* Ferrari and *Tenebrio molitor* L., respectively. These works suggest that the enzymes from *B. bassiana* were expressed differently according to the type of insect cuticle and there are not reports about the production of

Pr1, Pr2 and chitinases in medium supplemented with *D. saccharalis* cuticle, important pest in sugarcane. The pathogenicity of *B. bassiana* strains were studied in order to verify if there is a correlation between the production of these enzymes and the virulence, contributing to the understanding of the parasite-host relation and to the selection of *B. bassiana* strains for biological control of *D. saccharalis*.

The objectives of this study were: (1) evaluate the pathogenicity of *B. bassiana* strains against *D. saccharalis* (2) investigate the production of proteases and chitinase by *B. bassiana* in the presence of the cuticle of sugarcane borer; and, (3) evaluate the relationship between the production of enzymes and pathogenicity of the strains.

MATERIAL AND METHODS

Diatraea saccharalis larvae

The second instar larvae of *D. saccharalis* were obtained from the Sugarcane Experimental Station of Carpina/Federal Rural University of Pernambuco/Brazil and were maintained with artificial diet, which basically consists in a solution of vitamins, sugar, soy meal, wheat germ, ascorbic acid and water, according to the protocol of Hensley and Hammond (1968). However, 24 h before the bioassays, each larva was confined individually in plastic containers (17 x 21 x 25 cm) with sugarcane stalks as the food source.

Reactivated inoculum

The *B. bassiana sensu lato* strains isolated originally from different hosts were supplied by Micoteca URM (University Recife Micology/UFPE) (Table 1). To reactivate the strains, they were inoculated in Petri dishes containing potato dextrose agar plus chloramphenicol (0.05% v/v), supplemented with 0.5% of yeast extract (PDAY) and incubated at 26°C for 12 days for conidiation. Following incubation, conidia harvest were prepared in 0.01% v/v Tween 80 in sterilized distilled water and were sprayed through the

use of micro-atomizer brand Paasche "VL" on ten *D. saccharalis* until insect death. Newly emerged conidia from the insect were subcultured not more than four times, at ten days intervals in PDAY and used to prepare the reactivated inoculum suspension containing 10^8 conidia/ml (Ito et al., 2007). To confirm viability, the conidia were spread on PDAY and incubated for 16 h at 26°C. Germination rates were scored at 400 × magnification by observing under microscope, at random, 100 conidia for the presence of germ tubes. Germination was at least 90% throughout the study.

Screening bioassay protocol

The basic measure of virulence generated in this study was the mortality recorded seven days post-inoculation. To assess infection, *D. saccharalis* larvae were placed in a sterile Petri dish (9 cm in diameter) and then sprayed with 1 ml of the reactivated inoculum suspension, as per as per reported in item Reactivated inoculum. The control group was sprayed, with sterile water containing 100 µl of Tween 80 (Tefera and Pringle, 2003). The design was completely randomized, with 11 treatments (10 strains of *B. bassiana* + Control).

For each treatment, five containers were used, each containing 10 larvae. The observations were taken daily for a period of seven days and larvae died were removed daily. To confirm the mortality by the fungus, the dead larvae were immediately surface sterilized with 70% alcohol for 10 s, followed in 1% sodium hypochlorite for 3 min and three rinses with sterile distilled water, placed on sterile wet filter paper in sterile Petri dishes. Mortality due the fungus was confirmed by microscopic examination of hyphae and spores on the surface of the cadaver.

Enzyme assays

B. bassiana strains that showed higher mortality against *D. saccharalis* (Table 1) and higher conidia production were selected for testing to enzyme production. A suspension containing 1×10^6 conidia/ml was inoculated in 30 ml of the minimal liquid medium (MM) (Pontecorvo et al., 1953) as control and was inoculated in MM+ cuticle (MM + CUT): MM lacking a source of nitrogen and of glucose but with the addition of cuticle from larvae of *D. saccharalis* (0.5%) w/v. The larvae were dissected with a scalpel under an ocular for removal of the viscera and the cuticle was then oven-dried at 65°C for 1 h. After that, the cuticle was prepared using a solution of 0.2 M potassium tetra-borate and sterilized with water vapor without pressure. After, it was added to previously sterilized MM (121°C for 15 min) and again sterilized with water vapor without pressure for 5 min (Dias et al., 2008).

Cultures were incubated at 28°C and maintained under agitation (180 rpm) out to 24, 48, 72 and 96 h. After incubation, each culture was centrifugated at 8000 g for 15 min at 4°C to separate the mycelium and the supernatant obtained was stored at -20°C for enzyme assays. The design was completely randomized, in a factorial scheme 3 × 4 (3 strains × 4 incubation times), for a total of 12 treatments, with two repetitions. The subtilisin-like (Pr1) and trypsin-like (Pr2) activities were assayed using N-Suc-(Ala)₂-Pro-Phe-p-nitroanilide and N-benzoyl-Phe-Val-Arg-p-nitroanilide (Sigma, St. Louis, MO) as substrates, respectively. Each assay consisted of 0.05 ml 1 mM substrate, 0.1 ml enzyme sample, and 0.85 ml 15 mM Tris-HCl, pH 8.0. The mixture was incubated for 30 min at 28°C and the reaction was stopped by adding 0.25 ml of 30% acetic acid and left to stand for 15 min in ice, after which samples were centrifuged at 1250 g for 5 min at 4°C. The supernatants were compared at 410 nm. One unit of enzyme activity (U) was defined as the amount of the enzyme able to release 1 µm of nitroanilide (NA) per milliliter per second at 28°C (Donatti et al., 2008).

Chitinase activity was assayed by the method of Nahar et al. (2004). The reaction mixture containing 1 ml of 1% colloidal chitin and 0.5ml of supernatant solution was incubated for 1 h at 50°C and the reaction was stopped by immersion in a bath of boiling water for 1 min. After centrifugation, 250 µL from the supernatant fluid was incubated with 50 µl of 0.8M sodium tetra-borate, pH 8.0 at 100°C for 3 min. After the mixture was cooled, 1.5 ml of p-dimethyl aminobenzaldehyde (DMAB) solution (1 g of DMAB dissolved in 100 ml of glacial acetic acid containing 1% v/v hydrochloric acid) was added and the mixture was incubated for 20 min at 37°C. Absorbance at 585 nm was measured against water as a blank. One unit of chitinase activity was defined as the amount of enzyme which produced sugars equivalent to 1 µmol of *N*-acetylglucosamine per min under the conditions described above.

Statistical analysis

Percent mortality was corrected for control mortality (Schneider-Orelli, 1947) and normalized by arcsine (X) transformation before being subjected to analysis of variance (ANOVA). The Tukey test analysis was used to separate the means as a post-ANOVA procedure ($p < 0.05$). Enzyme production data were subjected to the analysis of variance (ANOVA). The averages of the characteristics evaluated in response to the qualitative factor (strains) were subjected to Tukey test at 5% probability. These statistical analyses were performed using the ASSISTAT 7.5 beta software (Silva and Azevedo, 2002). Pearson's Correlation Coefficient was used to check the level of correlation between mortality caused by each strains and enzyme activity and it was calculated using enzyme's production at 96 h and mortality rate at 96 h post-inoculation. The analysis was performed using BioEstat software system, version 5.3.

RESULTS

Screening bioassay

Results of the screening assays are presented in Table 1. Mean mortality in the control was 5.4% seven days after the treatment. All the fungal isolates were pathogenic to larvae of *D. saccharalis* and there were significant differences in mortality between fungal isolates ($F=75.26$; $df: 10$; $p < 0.01$). The mortality ranged from 36 to 88% (Table 1). Three strains caused mortality above 80% (URM2915, URM2921 and URM3447), while the URM2924, URM2912 and URM4548 caused below 50%. *B. bassiana* URM2915 is most indicated for the control of *D. saccharalis* because besides causing high mortality also showed good conidial production, fundamental characteristics to be used in programs for biological pest control.

Production of subtilisin-like and trypsin-like enzymes and chitinase in the presence of the cuticle of sugarcane borer

Based on the mortality rate (Table 1) and characteristics, like the production of conidia (data not shown), three strains were selected for production testing of Pr1, Pr2 and chitinase. The production of these enzymes was determined after the growth of the strains (URM2915,

Table 2. Factorial analysis of the strains, culture media and incubation times in the production of proteases (Pr1 and Pr2) and chitinases by *Beauveria bassiana* in the presence of *Diatraea saccharalis* cuticle.

Factor	Pr1			Pr2			Chitinase		
	df ^a	Mean square	F	df	Mean square	F	df	Mean square	F
Strains (S)	2	78.77	2.55*	2	0.357	0.45*	2	46.68	27.81*
Culture media (CM)	1	2020.5	654.82*	1	6441.5	8257.4*	1	2062.5	1228.7*
Time (T)	3	3881.5	125.82*	3	556.5	713.45*	3	318.55	189.77*
S × CM	2	238.8	7.74*	2	0.35	0.45*	2	52.27	31.13*
S × T	6	13.97	0.45	6	4.75	6.09*	6	2.89	1.72
CM × T	3	2490.9	80.74*	3	556.55	713.4*	3	287.60	171.3*
S × CM × T	6	35.27	1.14	6	4.75	6.09	6	2.29	1.36

*Significant to the 99% level of probability ($p < 0.01$); ^adf. degree of freedom.

Table 3. Subtilisin-like (Pr1) activity (U/ml) in supernatant cultures of *Beauveria bassiana* grown in minimal medium (MM) and minimal medium plus *Diatraea saccharalis* cuticle (MM+CUT)^a

Strain	24 h		48 h		72 h		96 h	
	MM	MM+CUT	MM	MM+CUT	MM	MM+CUT	MM	MM+CUT
URM2915	0	1.53±0.04	0	46.83±0.08	0.83±0.02	68.83±0.19	2.92±0	70.79±0.19
URM2930	0	0.55±0.01	0	45.16±0.2	0	73.74±0.12	3.4±0.01	70.46±0.04
URM4548	0	1.04±0.02	15.26±0.24	40.70±0.57	15.89±0.17	68.70±0.40	17±0.26	66.70±0.25

^aThe Pr1 activities are means standard errors of the means based on three replicates.

Table 4. Trypsin-like (Pr2) activity (U/ml) in supernatant cultures of *Beauveria bassiana* grown in minimal medium (MM) and minimal medium plus *Diatraea saccharalis* cuticle (MM+CUT)^a.

Strain	24 h		48 h		72 h		96 h	
	MM	MM+CUT	MM	MM+CUT	MM	MM+CUT	MM	MM+CUT
URM2915	0	1.95±0.01	0	26.83±0.06	0	30.04±0.01	0	35.12±0.04
URM2930	0	1.46±0.04	0	25.71±0.08	0	30.59±0.07	0	34.71±0.04
URM4548	0	6.41±0.01	0	25.16±0.14	0	29.34±0.04	0	30.66±0.00

^aThe Pr2 activities are means standard errors of the means based on three replicates.

URM2930 and URM4548) in liquid medium in the presence and absence of cuticle of *D. saccharalis*. All strains of *B. bassiana* produced Pr1 and Pr2 in MM+CUT and there were significant differences between the strains (Table 2). For each of the three strains, Pr1 activity was detected at 24 h of growth. For URM2930 and URM4548, the peak of activity was observed at 72 h of growth. In the MM, Pr1 activity was detected at 48 h for URM4548, at 72 h for URM2915 and at 96 h of incubation only for the *B. bassiana* URM2930 (Table 3). Similarly, PR2 activity was detected at 24 h of incubation in the MM + CUT, and the peak of the activity was observed at 96 h. It was not possible to detect activity in the medium containing nitrate as the sole nitrogen source

(MM) (Table 4). As shown in Tables 3 and 4, the strains studied showed higher levels of production of Pr1 than Pr2, highlighting URM2930 in production of Pr1 (73.74 U/ml) and URM2915 in production of PR2 (35.12 U/ml).

Chitinase activity was detected at 24 h of incubation on MM+CUT and at 72 h on MM for URM2915 and URM4548. The amount of secreted enzymes varied between the stains and the highest activity was observed for *B. bassiana* URM2915 strain (28.93U/ml) at 96 h. Enzyme activity was investigated for a period of 96 h and the highest chitinase activity was observed on the last time in the two culture media (Table 5). However, the chitinase activity was higher in MM + CUT at all incubation times compared to activities on MM.

Table 5. Chitinolytic activity (U/ml) in supernatant cultures of *Beauveria bassiana* grown in minimal medium (MM) and minimal medium plus *Diatraea saccharalis* cuticle (MM+CUT)^a.

Strain	24 h		48 h		72 h		96 h	
	MM	MM+CUT	MM	MM+CUT	MM	MM+CUT	MM	MM+CUT
URM2915	0	3.70±0.2	0	14.21±0.4	0.17±0	22.2±0.7	0.59±0.0	28.93±0.1
URM2930	0	0.17±0.0	0	10.01±0.4	0	16.73±0.1	0	22.2±0.2
URM4548	0	0.17±0.0	0	4.54±0.1	1.01±0.02	15.05±0.1	1.01±0.01	22.2±0.2

^a The chitinase activities are means standard errors of the means based on three replicates.

There was significant interaction among the strains and culture media in the production of the enzymes and there were also interaction between culture media and incubation time in the production of proteases and chitinase (Table 2). These interactions were caused by the fact that enzyme activity was highest in medium containing cuticle and increased with the length of incubation. Correlation analysis between the percentage of mortality by three *B. bassiana* strains and Pr1 ($r = 0.8207$; $P = 0.0453$) and Pr2 ($r = 0.8133$; $P = 0.049$) activity suggests a positive correlation of these variables in the medium containing cuticle. However there was no correlation with the chitinase activity ($r = -0.0008$; $P = 0.9987$).

DISCUSSION

Although all the 10 fungal isolates tested were pathogenic to *D. saccharalis*, there were significant variations amongst the isolates. These variations have been reported in many arthropod pests (Bugeme et al., 2009; Godonou et al., 2009; Abood et al., 2010; Sevim et al., 2010) and emphasizes the need of screening for strain selection. The results were similar to those of Wraight et al. (2010). These authors evaluated virulence of *B. bassiana* against different species of Lepidoptera and observed that mortality was high and varied positively among the 43 isolates used, thus showing the potential of the fungus against different pests. Kaur and Padmaja (2008) evaluated the action of 23 *B. bassiana* isolates, obtained from different hosts and regions, against *Spodoptera litura* Fabricius and verified that pathogenicity varied among them, but there was no correlation between the variability and the host's geographic origin, as observed in this work.

The insect cuticle forms an effective barrier against organisms lacking an active cuticle penetration mechanism (virus, bacteria and protozoa). Only entomo-pathogenic fungus can penetrate their hosts through the cuticle, using physical and/or enzymatic mechanisms (St. Leger, 1995). In this paper was studied the production of the enzymes involved in the process of infection, in three strains of *B. bassiana* in the presence of *D. saccharalis* cuticle. We observed that when cuticle was added to the medium, there was an increase in production of Pr1 and

Pr2, indicating that this was stimulated by cuticle components. By contrast, Tiago et al. (2002) demonstrated that *M. flavoviride* CG423 (syn. *M. anisopliae* var. *acridum*) has high levels of Pr2 only in minimal liquid medium without cuticle, with other negative effects being found in the presence of cuticle of *Schistocerca pallens* Thunberg.

In this study, production of Pr1 was higher as compared to Pr2, however the peaks of activity were the same (85 h), suggesting that there are different regulatory systems for these enzymes. Donatti et al. (2008) emphasized that the differences in the production of enzymes may reflect different functions in the infection process. In addition, both proteases were produced at 24 h of incubation in medium containing cuticle, suggesting their expression is not coordinated in this fungus. Similar data was observed in *B. bassiana* grown in *H. hampei* cuticle (Dias et al., 2008). Differently, Paterson et al. (1994) reported that Pr2 occurs before and would be involved in the activation or induction of Pr1 in *M. anisopliae* var. *anisopliae*. Differences between fungus species reveal that a complex mechanism is involved in the production of cuticle-degrading proteases.

Chitinase production by *B. bassiana* URM2915, URM2930 and URM4548 was higher during the last days of incubation in the medium containing cuticle as the sole source of carbon. The enhancing effect of cuticle on chitinase production suggests that this enzyme may be specifically induced by a cuticular component.

Studies have evaluated the effects of different sources of carbon in the production of this enzyme and observed that when the fungus was grown in medium with cuticle and glucose, production was lower than in medium containing only cuticle (Campos et al., 2005), and that there was repression of the enzyme when a easily available carbon source, glucose, was added to the medium (Dhar and Kaur, 2010). Chitinase production was lower than Pr1 and PR2 production, which can be explained by the composition of the insect's cuticle, a composite material consisting of arrangements of highly crystalline chitin nanofibers embedded in a matrix of protein (Vincent and Wegst, 2004). As reported by Fang et al. (2009), *B. bassiana* transformants secreting the fusion protein (protease and chitinase gene) penetrated the cuticle significantly faster than the wild type or transformants overexpressing either chitinase or protease

gene.

As reported in the literature, the production of cuticle-degrading enzymes is one of the pre-requisites for fungal infection (Mustafa and Khaur, 2010), and demonstrates the relation between production and virulence that has been targeted by several investigations. The current study has provided evidence for the relationship between enzyme production and virulence of *B. bassiana* against *D. saccharalis*. According to our results, Gupta et al. (1994) showed that levels of production of Pr1, Pr2 and NAGase (chitinase) have been related to virulence parameters in *B. bassiana*.

Moreover, Kim et al. (2010) showed that from the bioassay with the enzyme-inhibited supernatants processed by substrate inhibition, decreased aphicidal activities were observed for all three enzyme-inhibited treatments. This finding provides evidence that the enzymes (Pr1, Pr2 and most particularly the chitinase) in the supernatant of *B. bassiana* were strongly involved in the aphicidal activity.

Pelizza et al. (2011) screened 28 isolates of *B. bassiana* and nine isolates of *M. anisopliae* for chitinase production in solid medium and the results suggest a direct relationship between a high chitinolytic activity and an efficient virulence of the fungal strain against the tested insect pest (*Tropidacris collaris* Stoll). On the other hand, Silva et al. (2005) tested the larvicidal effect of *M. anisopliae* isolates against *Aedes aegypti* L. and the isolates showed a high variability of total protein production and NAGase activity after 48 and 72 h incubation in MM, but no relationship between enzyme levels and insecticidal activity could be detected, suggesting that other factors may be involved in the process.

The results presented in this study reveal that laboratory bioassays are a relevant stage in selecting the most efficient strains for controlling pests. The pathogenicity of *B. bassiana* can be influenced by strain used. These studies confirm that the production of cuticle-degrading enzymes by *B. bassiana* is influenced by specific components of the cuticle of *D. saccharalis*. This paper showed that there was a relationship between enzyme production and pathogenicity of *B. bassiana*, but many factors interfere directly in this process and more research is needed about this complex mechanism.

ACKNOWLEDGMENTS

The authors thank Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for financial support, Dr. David Bousfield for reviewing the manuscript and Venézio Santos for the statistical analysis.

REFERENCES

Aboud F, Bajwa GA, Ibrahim YB, Sajap AS (2010). Pathogenicity of

- Beauveria bassiana* against the Tiger Moth, *Atteva sciodoxa* (Lepidoptera: Yponomeutidae). *J. Entomol.* 7:19-32.
- Alves SB, Rossi LS, Lopes RB, Tamai MA, Pereira RM (2002). *Beauveria bassiana* yeast phase on agar medium and its pathogenicity against *Diatraea saccharalis* (Lepidoptera: Crambidae) and *Tetranychus urticae* (Acari: Tetranychidae). *J. Invertebr. Pathol.* 81:70-77.
- Bidochka MJ, Khachatourians GG (1987). Purification and properties of an extracellular protease produced by *Beauveria bassiana*. *Appl. Environ. Microbiol.* 53:1679-1684.
- Bugeme DM, Knapp M, Boga HI, Wanjoya AK, Maniana NK (2009). Influence of Temperature on Virulence of Fungal Isolates of *Metarhizium anisopliae* and *Beauveria bassiana* to the Two-Spotted Spider Mite *Tetranychus urticae*. *Mycopathologia* 167:221-227.
- Campos RA, Arruda W, Boldo JT, Silva MV, Barros NM, Azevedo JL, Schrank A, Vainstein, MH (2005). *Boophilus microplus* Infection by *Beauveria amorpha* and *Beauveria bassiana*: SEM Analysis and regulation of Subtilisin-like Proteases and Chitinases. *Curr. Microbiol.* 50:257-261.
- Dhar P, Kaur G (2010). Effects of carbon and nitrogen sources on the induction and repression of chitinase enzyme from *Beauveria bassiana* isolates. *Afr. J. Biotechnol.* 9:8092-8099.
- Dias BA, Neves PMOJ, Furlaneto-Maia L, Furlaneto MC (2008). Cuticle-degrading proteases produced by the entomopathogenic fungus *Beauveria bassiana* in the presence of coffee berry borer cuticle. *Braz. J. Microbiol.* 39:301-306.
- Donatti AC, Furlaneto-Maia L, Fungaro MHP, Furlaneto MC (2008). Production and regulation of cuticle-degrading proteases from *Beauveria bassiana* in the presence of *Rhammatocerus schistocercoides* cuticle. *Curr. Microbiol.* 56:256-260.
- Fang W, Feng J, Fan Y, Zhang Y, Bidochka MJ, St. Leger RJ, Pei Y (2009). Expressing a fusion protein with protease and chitinase activities increases the virulence of the insect pathogen *Beauveria bassiana*. *J. Invertebr. Pathol.* 102:155-159.
- Fang W, Leng B, Xiao Y, Jin K, Ma J, Fan Y, Feng J, Yang X, Zhang Y, Pei Y (2005). Cloning of *Beauveria bassiana* chitinase gene Bbchit1 and its application to improve fungal strain virulence. *Appl. Environ. Microbiol.* 71:363-370.
- Gallo D, Nakano O, Silveira Neto S, Carvalho RPL, Batista GC de, Berti Filho, Parra JRP, Zucchi RA, Alves SB, Vendramim JD, Marchini LC, Lopes JRS, Omoto C (2002). Manual de Entomologia Agrícola. 3th edition. Piracicaba, FEALQ.
- Godonou BJ, Atcha-Ahove´ C, Vodouhe´ S, Kooyman C, Ahanche´ de´A, Korie S (2009). Potential of *Beauveria bassiana* and *Metarhizium anisopliae* isolates from Benin to control *Plutella xylostella* L. (Lepidoptera: Plutellidae). *Crop. Prot.* 28:220-224.
- Gupta SC, Leathers TD, El-Sayed GN, Ignoffo CM (1994). Relationships among enzyme activities and virulence parameters in *Beauveria basiana* infections of *Galleria mellonella* and *Trichoplusia ni*. *J. Invertebr. Pathol.* 64:13-17.
- Hajek AE, Delalibera Jr I (2010). Fungal pathogens as classical biological control agents against arthropods. *BioControl* 55:147-158.
- Hensley SD, Hammond AH (1968). Laboratory techniques for rearing the sugarcane borer on an artificial diet. *J. Econ. Entomol.* 61:1742-1743.
- Ito ET, Varéa-Pereira G, Miyagui DT, Pinotti MHP, Neves PMOJ (2007). Production of Extracellular Protease by a Brazilian Strain of *Beauveria bassiana* Reactivated on Coffee Berry Borer, *Hypothenemus hampei*. *Braz. Arch. Biol. Technol.* 50:217-223.
- Kaur G, Padmaja V (2008). Evaluation of *Beauveria bassiana* isolates for virulence against *Spodoptera litura* (Fab.) (Lepidoptera: Noctuidae) and their characterization by RAPD-PCR. *Afr. J. Microbiol. Res.* 2:299-307.
- Kim JS, Roh JY, Choi JY, Wang Y, Shim HJ, Je YH (2010). Correlation of the aphicidal activity of *Beauveria bassiana* SFB-205 supernatant with enzymes. *Fungal. Biol.* 114:120-128.
- Lacey LA, Frutos R, Kaya HR, Vail P (2001). Insect Pathogens as Biological Control Agents: Do They Have a Future? *Biol. Control* 21:230-248.
- Lewis LC, Bruck DJ, Gunnarson RD (2002). Gunnarson, R.D. On-farm evaluation of *Beauveria bassiana* for control of *Ostrinia nubilalis* in Iowa, USA. *BioControl.* 47:167-176.

- Montesinos-Matías R, Viniegra-González G, Alatorre-Rosas R, Loera O (2011). Relationship between virulence and enzymatic profiles in the cuticle of *Tenebrio molitor* by 2-deoxy-D-glucose-resistant mutants of *Beauveria bassiana* (Bals.) Vuill. World J. Microbiol. Biotechnol. 27:2095-2102.
- Mustafa U, Kaur G (2010). Studies on Extracellular Enzyme Production in *Beauveria Bassiana* Isolates. Int. J. Biotech. Biochem. 6:701-713.
- Nahar P, Ghormade V, Deshpande MV (2004). The extracellular constitutive production of chitin deacetylase in *Metarhizium anisopliae*: possible edge to entomopathogenic fungi in the biological control of insect pests. J. Invertebr. Pathol. 85:80-88.
- Oliveira, MAP, Marques, EJ, Wanderley-Teixeira, Barros R (2008). Efeito de *Beauveria bassiana* (Bals.) Vuill. e *Metarhizium anisopliae* (Metsch.) Sorok. sobre características biológicas de *Diatraea saccharalis* F. (Lepidoptera: Crambidae). Acta. Scient. Biol. Sci. 30:220-224.
- Paterson IC, Charnley AK, Cooper RM, Clarkson M (1994). Partial characterization of specific inducers of a cuticle-degrading protease from the insect pathogenic fungus *Metarhizium anisopliae*. Microbiology 140:3153-3159.
- Pelizza SA, Eliades LA, Saparrat MCN, Cabello MN, Scorsetti AC, Lange CE (2011). Screening of Argentine native fungal strains for biocontrol between fungal pathogenicity and chitinolytic enzyme activity. World J. Microbiol. Biotechnol. 28(4):1359-1366.
- Pontecorvo G, Oper JA, Hemmons LM, MacDonald KD, Bufton AWJ (1953). The genetics of *Aspergillus nidulans*. Adv. Genet. 5:141-238.
- Schneider-Orelli O (1947). Entomologisches praktikum. Aarau: Sauerlander, 1947. pp.149.
- Sevim A, Demir I, Demirbag E (2010). Molecular characterization and virulence of *Beauveria* spp. from the pine processionary moth, *Thaumetopoea pityocampa* (Lepidoptera: Thaumetopoeidae). Mycopathologia 170:269-277
- Silva ASF de, Azevedo CAV de (2002). Versão do programa computacional Assistat para o sistema operacional Windows. Ver. Bras. Prod. Agro. 4:71-78.
- Silva RO, Silva HHG, Ulhoa CJ, Luz C (2005). Is there a relationship between *N*-acetyl- β -D-glucosaminidase activity of *Metarhizium anisopliae* (Metschn.) Sorokin (Hyphomycetes) isolates from peridomestic areas in Central Brazil and larvicidal effect on *Aedes aegypti* (L.) (Diptera, Culicidae)? J. Appl. Entomol. 129:158-164.
- Silva VCA, Barros R, Marques EJ, Torres JB (2003). Suscetibilidade de *Plutella xylostella* (L.) (Lepidoptera: Plutellidae) aos fungos *Beauveria bassiana* (Bals.) Vuill. e *Metarhizium anisopliae* (Metsch.) Sorok. Neotrop. Entomol. 32:653-658.
- St Leger RJ, Joshi L, Bidochka MJ, Rizzo NW, Roberts DW (1996). Biochemical characterization and ultrastructural localization of two extracellular trypsins produced by *Metarhizium anisopliae* in infected insect cuticles. Appl. Environ. Microbiol. 62:1257-1264.
- St. Leger RJ (1995). The role of cuticle-degrading proteases in fungal pathogenesis of insects. Can. J. Bot. 73:1119-1125.
- St. Leger RJ, Cooper RM, Charnley AK (1986). Cuticle-degrading enzymes of entomopathogenic fungi: Cuticle degradation in vitro by enzymes from entomopathogens. J. Invertebr. Pathol. 47:167-177.
- St. Leger RJ, Durrands PK, Cooper RM, Charnley AK (1988). Regulation of production of proteolytic enzymes by entomopathogenic fungus *Metarhizium anisopliae*. Arch. Microbiol. 150:413-416.
- Tefera T, Pringle KL (2003). Effect of exposure method to *Beauveria bassiana* and conidia concentration on mortality, mycosis, and sporulation in cadavers of *Chilo partellus* (Lepidoptera: Pyralidae). J. Invertebr. Pathol. 84:90-95.
- Tiago PV, Fungaro MHP, Furlaneto MC (2002). Cuticle degrading proteases from the entomopathogen *Metarhizium flavoviride* and their distribution in secreted and intracellular fractions. Lett. Appl. Microbiol. 34:91-94.
- Vijayavani S, Reddy KRK, Murthy GBVN (2009). Pathogenicity of *Beauveria bassiana* (Deuteromycotina: Euteromycotina: Hyphomycetes) strains on *Spodoptera litura* (Fab.) J. Biopest. 2:205-207.
- Vincent JFV, Wegst UGK (2004). Design and mechanical properties of insect cuticle. Arth. Struct. Dev. 33:187-199.
- Wraight SP, Ramos ME, Avery PB, Jaronski ST, Vandenberg JD (2010). Comparative virulence of *Beauveria bassiana* isolates against lepidopteran pests of vegetable crops. J. Invertebr. Pathol. 103:186-199.

Full Length Research Paper

A comparative study of pectinolytic enzyme production by *Bacillus* species

Torimiro N.^{1*} and Okonji, R. E.²

¹Department of Microbiology, Obafemi Awolowo University, Ile-Ife, 220005, Nigeria.

²Department of Biochemistry, Obafemi Awolowo University, Ile-Ife, 220005, Nigeria.

Accepted 17 October, 2013

The economic and ecological function of pectinase enzymes in industries is gaining much attention with the need of highly productive strains of microorganisms to reduce production cost. The present investigation is a comparative evaluation of *Bacillus* sp. pectinases. The three isolates isolated from agro-waste, *Bacillus stearothermophilus*, *Bacillus cereus* and *Bacillus subtilis*, showed very high pectinase activities with pectin as the substrate. The three isolates also showed varying degree of preference for banana peels and wheat bran but their activity towards orange peel was very low compared to other substrate. The effect of temperature on the pectinase of the three isolates showed that *B. stearothermophilus* had optimum temperature at 60°C while *B. cereus* and *B. subtilis* both showed optimum activity at 50°C. On the effect of pH, *B. stearothermophilus*, *B. cereus* and *B. subtilis* showed optimum pectinase activities at pH 7.5, 8.0 and 9.0, respectively. Metal ions enhanced the activity of pectinase produced by the three isolates. The study exhibited that *B. stearothermophilus* gave the most pectinase activity, optimum temperature and a moderate alkaline pH, possibly making it a better source for industrial purpose.

Key words: Pectinase, enzymes, pectin, bacteria, *Bacillus* species.

INTRODUCTION

Pectin, a major component of plant cell wall occurs primarily in the non-woody parts of plants which are parts that are most likely to be consumed. It also contributes to the firmness and structure of plant tissues (Sathyanarayana and Panda, 2003). During ripening process, plants generally use pectinase enzymes to hydrolyse some of the pectin in and between cell wall making the cell weaker and therefore soft and edible (Sakai, 1992). Pectinases are a complex heterogenous group of different enzymes that act specifically on pectic substances by decreasing intracellular adhesivity and tissue rigidity (Tatiana da Costa and Flevo, 2005). They have also been reported to be responsible for spoilage of fruits and vegetables (Collmer and Keen, 1986; Whitaker,

1990). However, pectinase has varied important uses in the industries especially in the juice and food industry. Reports have shown that the treatment of fruit pulps with pectinases increase juice volume from fruits (Kashyap et al., 2001; Kaur et al., 2004) with a 50% reduction in filtration time (Blanco, 1999). Pectinase has been reported to be useful in the paper and pulp industry (Beg et al., 2001; Viikari et al., 2001) as well as in the decomposition and recycling of plant wastes (Jayani et al., 2005).

Pectinolytic enzymes can be derived from different sources such as plants, animals and microorganisms (Whitaker, 1990; Banu et al., 2010; Namasivayam et al., 2011). Different types of microorganisms have been

exploited for the production of pectinase because microorganisms are not influenced by climatic and seasonal factors and can be subjected to genetic and environmental manipulations to increase yield (Vibha and Neelam, 2010). Microbial pectinases has been reported to account for 25% of the global food enzymes sales and majority of these are from fungal sources (Jayani et al., 2005). Pectinases from fungal sources produce best under acidic pH and low temperature conditions and can therefore not be used in varied industrial processes where neutral to alkaline pH with high temperatures exceeding 45°C are required. It has been shown that bacteria require high pH and temperature to produce pectinase (Chesson and Cordner, 1978; Silley, 1986). *Bacillus spp* have been reported to produce as high as 20-25 g/L of pectinase as compared to other bacterial isolates (Soares et al., 1999; Schallemeijer et al., 2001; Namasivavam et al., 2011). Based on the economic and ecological roles pectinase enzymes play, highly productive strains are required in the industries to reduce production cost. The present investigation is aimed at comparing *Bacillus spp* that produce high level pectinase under various physico-chemical conditions.

MATERIALS AND METHODS

Microorganisms

In 100 ml of sterile distilled water, 1 g of soil collected from agro-waste dumpsite was added. Isolation was carried out using 10 fold serial dilution and pour plate technique in triplicate. The cultures were incubated at 35°C for 48 h. Thereafter, the distinct bacteria colonies were streaked on nutrient agar (LAB M USA, UK) and incubated at 35°C for 24 h. When Gram-stained films were examined, the isolates that appeared as Gram-positive rods were identified as *Bacillus subtilis*, *Bacillus cereus* and *Bacillus steareothermophilus* based on result from biochemical test as enumerated (Breeds et al., 1957; Olutiola et al., 1991). The *Bacillus* isolates were maintained in nutrient agar slants at 4°C.

Chemicals and reagents

Pectin, ammonium chloride, sodium dihydrogen phosphate and potassium chloride were obtained from BDH Chemical Limited, Poole, England. Glucose, 3, 5- dinitrosalicylic acid (DNSA), sodium potassium tartarate, sodium borate, boric acid, sodium citrate, and citric acid were obtained from Sigma Chemical Company, St. Louis, Mo., USA. Sodium hydroxide, Trizma base, Trizma-HCl, ethylenediamine tetraacetic acid (EDTA) were purchased from Sigma Chemical Company, St. Louis, Mo., USA. Other reagents were either purchased from Sigma Chemical or BDH Chemical.

Pectinase production by submerged fermentation

Pectinase production by submerged fermentation was conducted as described (Kumar and Sharma, 2012). Conical flasks containing 100 ml pectin broth containing (0.5 g pectin, 0.1 g yeast extract, 0.715 g NH₄Cl, 0.45 g Na₂HPO₄.12H₂O, 0.63 g KH₂PO₄, 0.075 g KCl,

and 0.025 g MgSO₄.7H₂O) were inoculated with a colony of the test isolates in triplicate. The cultures were incubated in a rotary shaker incubator at 30°C for 36 h at 120 rpm. The cultures were thereafter centrifuged at 4,000 rpm for 10 min and the cell free supernatants were stored at -4°C.

Determination of total protein concentration

The total protein content of the supernatants was determined according to the method described by Bradford (1976) using bovine serum albumin (BSA) as standard protein.

Pectinase assay

The crude pectinase enzymes produced by the three isolates were assayed using the colorimetric method as outlined by Miller (1959). Five milliliter (5 ml) of cell free supernatant was incubated with 2% pectin in 0.1 M acetate buffer at pH 6.0 and the reaction mixture was incubated at 40°C for 10 min. After adding 1.0 ml of DNSA reagent (without sodium potassium tartarate), the mixture was boiled for 5 min at 90°C. The reaction was stopped by adding 1 ml of Rochelle's salt (sodium potassium tartarate- Sigma, USA). The mixture was further diluted by adding 2 ml of distilled water and later followed by reading the absorbance at 595 nm to estimate the reducing sugars released. A standard graph was generated using standard glucose solution. One unit of pectinase activity was defined as the amount of enzyme which liberated 1 µm glucose per min (Karthik et al., 2011).

Effect of substrate concentration

The effect of varying the concentrations of different substrates (banana peel, orange peel, sugarcane bagasse and wheat bran) was studied. The substrates were obtained by drying the peels of the various fruits in an oven maintained at 50°C till they were completely dried except for the wheat which was purchase dried. The dried peels and the wheat were then grinded to powder. The powder was used in the preparation of 2% solution of each of the substrate in 0.1 M acetate buffer, pH 6.0. The concentrations were varied between 0.05 and 0.25 mM for each substrate. The effect of varying the concentration of substrates followed the assay procedure earlier described (Miller, 1959). In place of the pectin in the assay, the other substrates were used.

Effect of temperature

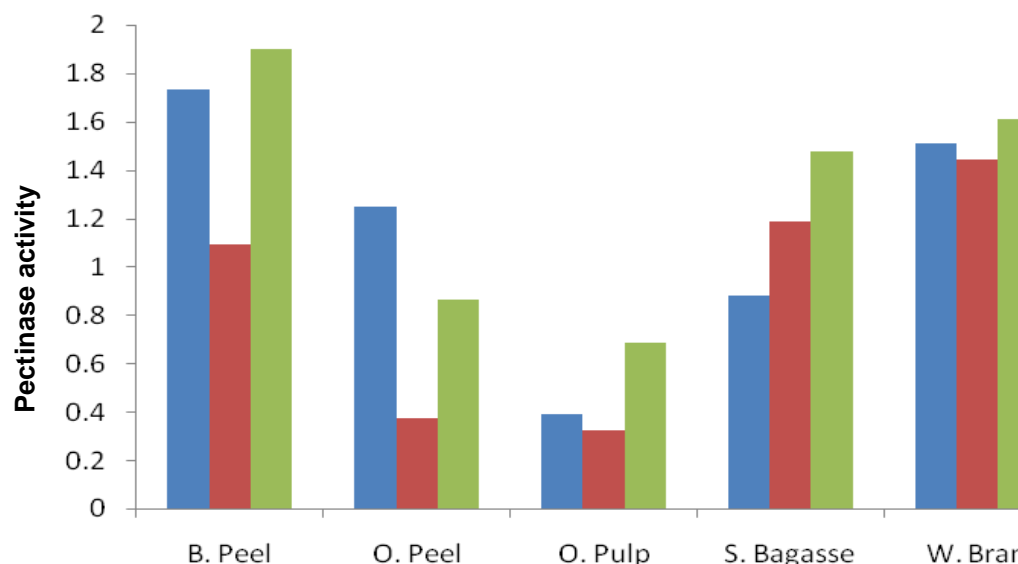
The effect of temperature on the activity of the enzyme was carried out for each isolate. 0.25 ml of 2% pectin in 0.1 ml acetate buffer was added to the crude enzymes and assayed at varying temperatures between 40 and 70°C for the different isolates.

Effect of pH

The effect of pH optimum on pectinase activity was measured for each isolate at a fixed assay temperature of 40°C at various pH values ranging from 3.0 - 9.0. In each case, the following pH buffer solutions were used following the assay method described (Miller, 1959) 0.1 M citrate buffer (pH 3.0-5.0), 0.1 M phosphate buffer (pH 6.0-8.0) and 0.1 M borate buffer (pH 9.0). 0.5 ml of 2% pectin was added into each extract and 0.5 ml of different buffer was added in each case and assayed.

Table 1. Pectinase activities and Total protein concentration in the crude extracts of each test isolate.

Enzyme source	Activity ($\mu\text{mol glucose/min/ml}$)	Protein conc. (mg/ml)	Specific activity
<i>B. cereus</i>	6.589	35.27	0.184
<i>B. subtilis</i>	6.198	33.26	0.186
<i>B. stearothermophilus</i>	6.751	34.74	0.194

**Figure 1.** Effect of substrate (banana peel, orange peel, orange pulp, sugarcane bagasse and wheat bran) on pectinase activity. The blue bar: *B. cereus*; red bar: *B. subtilis*; green bar: *B. stearothermophilus*.

Effect of metal ion

The pectinase activity of each extract was determined with different metal ions using the chloride salt solutions of the following metals Na^+ , K^+ , Ni^{2+} , Mn^{2+} and Zn^{2+} . 0.1 ml of each metal ion was added into 0.25 ml of 2% pectin in 0.1 M acetate buffer, pH 6.0. The mixture was incubated at 40°C for 10 min and assayed.

RESULTS

All the experiments were conducted in triplicate and the result is the mean of the data derived. The results of the activity, protein concentration and specific activities of the different isolates are shown in Table 1. Figure 1 shows the effect of varying concentrations of substrates on the three isolates. The three isolates showed varying degree of preference for banana peels and wheat bran but activity towards orange peel was very small. The effect of temperature on pectinase activities of the three isolates is presented in Figure 2. *B. stearothermophilus* had optimum temperature at 60°C while both *B. cereus* and *B. subtilis* showed optimum activity at 50°C. *B.*

stearothermophilus, *B. cereus* and *B. subtilis* showed optimum pectinase activities at pH 7.5, 8 and 9 respectively (Figure 3). There was no significant pectinase inhibition by the metal ions amongst the isolates (Table 2)

DISCUSSION

Pectinolytic enzyme can be derived from different sources (Whitaker, 1990; Banu et al., 2010; Namasivayam et al., 2011). However, pectinase producing microorganisms have due advantage over other sources because they can be subjected to genetic and environmental manipulations to increase yield (Vibha and Neelam, 2010). It has been reported that most *Bacillus spp.* enhances the production of pectinase (Hirose et al., 1999). In this study, pectinase production by *B. cereus*, *B. subtilis* and *B. stearothermophilus* were compared. Our results in Table 1 show the three *Bacillus spp.* were able to produce pectinase. The results is in good agreement that selected *Bacillus sp.* can produce

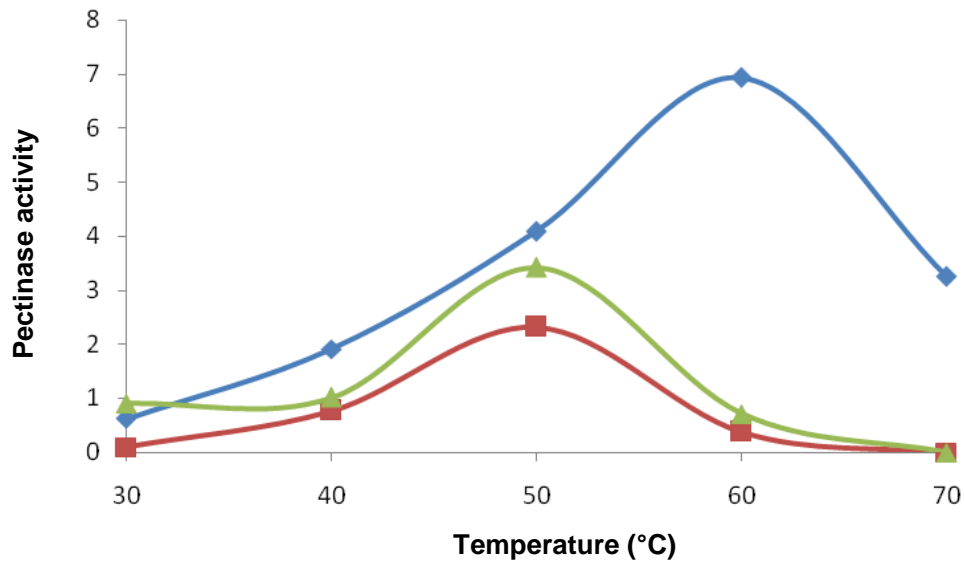


Figure 2. Effect of temperature on the pectinase activities of the isolates. Blue colour, *B. stearothermophilus*; red colour, *B. subtilis*; green colour, *B. cereus*.

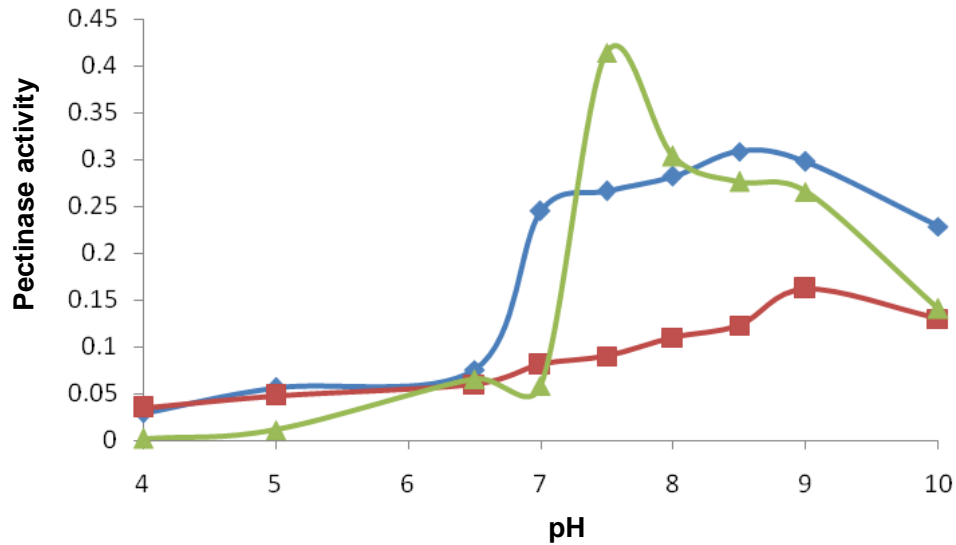


Figure 3. Effect of pH on the pectinase activities of the isolates. Blue, *B. cereus*; red, *B. subtilis*; green, *B. stearothermophilus*.

Table 2. Effect of metal ion on pectinase.

Metal ions (10 mM)	Residual Activiy of pectinase (%)		
	<i>B. cereus</i>	<i>B. subtilis</i>	<i>B. stearothermophilus</i>
Na ⁺	96.56	99.14	99.87
K ⁺	98.68	105.31	100.0
Ni ²⁺	109.00	104.93	100.0
Mn ²⁺	106.83	103.57	103.60
Zn ²⁺	105.81	105.30	103.84

and secrete large quantities (about 20-25 g/L) of extracellular pectinase enzyme (Soares et al., 1999; Schallemeijer et al., 2001; Namasivayam et al., 2011). High pectinase activity was recorded for the three isolates using industrial pectin. The use of other substrates (wheat bran, orange peel, sugar cane peel and banana peel) revealed the preference of banana peels and wheat bran by the isolates for pectinase production. Namasivayam et al. (2011) working on *B. cereus* isolated from market solid waste reported that pectinase production was enhanced by wheat bran. Also, Phutela et al. (2005) reported the enhancement of pectinase by wheat bran and industrial pectin on the production of pectinase from thermophilic *Aspergillus fumigatus* isolated from decomposing orange peels.

In our study, optimum temperature between 50 and 60°C was obtained for the three *Bacillus spp.*, with *B. stearothermophilus* showing highest activity at 60°C while *B. cereus* and *B. subtilis* both showed highest activities of pectinase at 50°C. Different optimum activities have been reported for different pectinases from different sources. In the presence of Ca²⁺, the enzyme from strain of *Bacillus spp.* KSM-P15 degraded polygalacturonic acid with an optimal activity around pH 10.5 and 50-55°C (Kobayashi et al., 1999). Namasivayam et al. (2011) reported an optimum temperature for maximum activity of pectinase from *B. cereus* to be 37°C. Similarly, 35°C was reported for pectinase from *Penicillin chrysogenum* (Banu et al., 2010). Phutela et al. (2005) reported an optimum temperature of 60°C for thermophilic *A. fumigatus* pectinase. Other thermophilic fungi have been reported to optimally produce enzymes at 50°C (Rubinder et al., 2002).

The effect of pH on *B. stearothermophilus*, *B. cereus* and *B. subtilis* showed optimum pectinase activities at pH 7.5, 8 and 9, respectively. Reports have shown the pectinase activity to be highest around alkaline pH (Namasivayam et al., 2011; Kumar and Sharma, 2012) but a slightly acidic pH of 6.5 was obtained for pectinase from *P. chrysogenum* (Banu et al., 2010). In our study, metal ions K⁺, Ni²⁺, Mn²⁺ and Zn²⁺, enhanced the activity of pectinases from the three isolates, while N⁺ had a slight inhibitory effect on the enzyme. Similarly, Ca⁺, Mg²⁺, Mn²⁺ and Zn²⁺ were reported to activate pectinase from *Penicillin italicum* but was inhibited by Cu²⁺ and Fe²⁺ (Alana et al., 1990). Banu et al. (2010) observed little effect of Mg²⁺ and Ca²⁺ on pectinase from *P. chrysogenum* while Ca²⁺ enhanced the production of pectinase by *B. cereus* obtained from market solid waste (Namasivayam et al., 2011).

Conclusion

The physicochemical characteristics of pectinase from these isolates make it an attractive tool for various indus-

trial uses. However, *B. stearothermophilus* gave the most activity, optimum temperature and a moderate alkaline pH, possibly making it a better source for industrial purpose.

REFERENCES

- Alana A, Alkorta I, Dominguez JB, Llama MJ, Serra JL (1990). Pectin lyase activity in a *Penicillium italicum* strain. Appl. Environ. Microbiol. 56:3755-3759.
- Banu RA, Devi KM, Gnanaprabhal GR, Pradeep BV, Palaniswamy (2010). Production and characterization of pectinase enzyme from *Penicillin chrysogenum*. Ind. J. Sci. Technol. 3(4):377-381.
- Beg QK, Kapoor M, Tiwari RP, Hoondal GS (2001). Bleach-boosting of eucalyptus kraft pulp using combination of xylanase and pectinase from *Streptomyces sp.* QG-11-3. Res Bull. Punjab University 57:71-8.
- Blanco P, Sieiro C, Villa TG (1999). Production of pectic enzymes in yeasts. FEMS Microbiol Lett. 175:1-9.
- Bradford MM (1976). A rapid and sensitive method for the quantification of microgram quantities of protein, utilizing the principle of protein dye binding. Anal. Biochem. 72:248-254.
- Breed R, Murray EGD, Smith NR (1957). Bergey's manual of Determinative Bacteriology 7th Edition, The Williams and Wilkins company Baltimore USA
- Chesson A, Codner RC (1978). The maceration of vegetable tissue by a strain of *Bacillus subtilis*. J. Appl. Bacteriol. 44:347-364
- Collmer AC, Keen NT (1986). The role of pectic enzymes in plant pathogenesis. Annu Rev. Phytopathol. 24:383-409.
- Hirose N, Kishida M, Kawasaki H, Sakai T (1999). Purification and Characterization of an Endo-Polygalacturonase from a Mutant of *Saccharomyces cerevisiae*. Biosci. Biotechnol. Biochem. 63:1100-1103.
- Jayani RS, Saxena S, Gupta R (2005). Microbial pectinolytic enzymes: A review. Process Biochem. 40:2931-2944
- Karthik LJ, Gaurav K, Bhaskara Rao KV (2011). Screening of Pectinase producing microorganisms from agricultural waste dump soil. Asian J. Biochem. Pharm. Res. 1 (2):329-337.
- Kashyap DR, Vohra PK, Chopra S, Tewari R (2001). Applications of pectinases in commercial sector: a review. Bioresour. Technol. 77:215-227.
- Kaur G, Kumar S, Satyanarayana T (2004). Production, characterization and application of a thermostable polygalacturonase of a thermophilic mould *Sporotrichum thermophile* Apinis. Bioresour. Technol. 94:239-243.
- Kobayashi T, Koike K, Yoshimatsu T, Higaki N, Suzumatsu A, et al. (1999). Purification and properties of a low-molecular-weight, high-alkaline pectate lyase from an alkaliphilic strain of *Bacillus*. Basic Biotechnol. Biochem. 63:65-72.
- Kumar A, Sharma R (2012). Production of alkaline pectinase by Bacteria (Cocci species) isolated from decomposing fruit materials. J. Phytol. 4(1):1-5.
- Miller GL (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugars. Anal. Chem. 31:426-428.
- Namasivayam E, Ravindar JD, Mariappan K, jiji A, Kumar M, et al (2011). Production of extracellular pectinase by *Bacillus cereus* isolated from market solid waste. J. Bioanal. Biomed 3:070-075.
- Olutiola PO, Famurewa O, Sonntag HG (1991). An Introduction to General Microbiology: A practical Approach. Heidelberg, Germany 1st edition. p. 267.
- Phutela U, Dhuma V, Sandhu S, Chadha BS (2005). Pectinase and polygalacturonase production by a thermophilic *Aspergillus fumigatus* isolated from decomposing orange peels. Braz. J. Microbiol. 36(1):63-69.
- Rubinder K, Chadha BS, Singh N, Singh S, Saini HS (2002). Amylase hyper production by deregulated mutants of the thermophilic fungus *Thermomyces lanuginosus*. J. Ind. Microbiol. Biotechnol. 29:70-74.
- Sakai T (1992). Degradation of pectins. In: Winkelmann G, editor. Microbial degradation of natural products. Weinheim: VCH. pp. 57-81.

- Sathyanarayana NG, Panda T (2003). Purification and biochemical properties of microbial pectinases- A review. *Process Biochem.* 38:987-96.
- Schallemeij, M., Ajay Singh, Owen P (2001). *Microbial Identification System References*. MIDI Inc.
- Silley P (1986). The production and properties of a crude pectin lyase from *Lachnospira multiparus*. *Lett. Appl. Microbiol.* 2:29-31.
- Soares MMCN, da Silva R, Gomes E (1999). Screening of bacterial strains for pectinolytic activity: Characterization of the polygalacturonase produced by *Bacillus sp.* *Rev. Microb.* 30(4):299-303.
- Tatiana da Costa RP and Flevo F (2005). Extraction and assay of pectic enzymes from Peruvian carrot (*Arracacia xanthorriza* Bancroft). *Food Chem.* 89:85-92.
- Vibha B, Neelam G (2010). Exploitation of microorganisms for screening of pectinase from environment. 8th International Conference. Globelics
- Viikari L, Tenakanen M, Suurnakki A (2001). Biotechnology in the pulp and paper industry. In: Rehm HJ, editor. *Biotechnology*. VCH-Wiley. pp. 523-546.
- Whitaker JR (1990). Microbial pectinolytic enzymes. In: Fogarty WM, Kelly CT, editors. *Microbial enzymes and biotechnology*. 2nd ed. London: Elsevier Science Ltd. pp. 133-176.

Full Length Research Paper

Bio-corrosion of water pipeline by sulphate-reducing bacteria in a mining environment

Z. Manafi¹, M. Hashemi¹, H. Abdollahi^{2*} and Gregory. J. Olson³

¹Sarcheshmeh Copper Mine, Research and Development Centre, Rafsanjan, Iran.

²Faculty of Mining Engineering, University of Tehran, Iran.

³2193 S. Juniper St., Lakewood, CO 80228, USA.

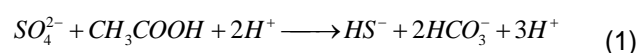
Accepted 5 July, 2012

Detrimental effects of corrosion in conjunction with bacterial activity in water pipeline systems have led to an increase in research in how to prevent such occurrences. In particular, sulphate-reducing bacteria (SRB), found in anaerobic conditions underneath the main corrosion shell, are noteworthy for their effects in promoting localized corrosion. This study investigates the presence of SRB in water, in a water pipeline and in the soil near the pipeline at a mining operation, under conditions that would be expected to be stable toward corrosion. Samples of water in pipes showed a high frequency of SRB. Cast iron coupons placed in pipes gave positive results for SRB colonization after only one month. Corrosion rates of the coupons increased in the presence of SRB, indicating microbially induced corrosion. Application of various biocides to control SRB was evaluated.

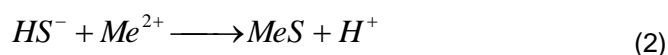
Key words: Sulphate-reducing bacteria, corrosion, water pipeline, biocide.

INTRODUCTION

Sulphate-reducing bacteria (SRB) are one group of anaerobic prokaryotes. The main genus of SRB is *Desulfovibrio*. *Desulfovibrio desulfuricans* is often used to immobilize dissolved heavy metals as metallic sulfides. Beijerinck (1895) showed that living matter could reduce sulphate to sulphide in sediments under anaerobic conditions. Although many bacteria can produce sulphide, only a few do so at a sufficient rate for application in high-rate processes. SRB are nonpathogenic (that is, they are not capable of causing disease) and they are anaerobic bacteria (that is, they require an oxygen free aqueous environment). These rapid sulphide-generating bacteria are able to conserve energy by the reduction of sulphur oxyanions (Widdel and Hansen, 1992). A typical overall conversion is shown in equation 1 (neglecting the small amount of organic material required to produce biomass):



Eight electrons are transferred from the energy source acetic acid to the electron acceptor sulphate to produce sulphide. The reaction equation shows that in the same process, alkalinity is also produced. This leads to an increase in the pH of the water, often to a near neutral value. Typically, a certain amount of metals is present together with the sulphate. These metals will react with the dissolved sulphide to form highly insoluble metal sulphides (Equation 2).



Me²⁺ can, for example, be copper, zinc etc. Combining the action of sulphate reduction by SRB and

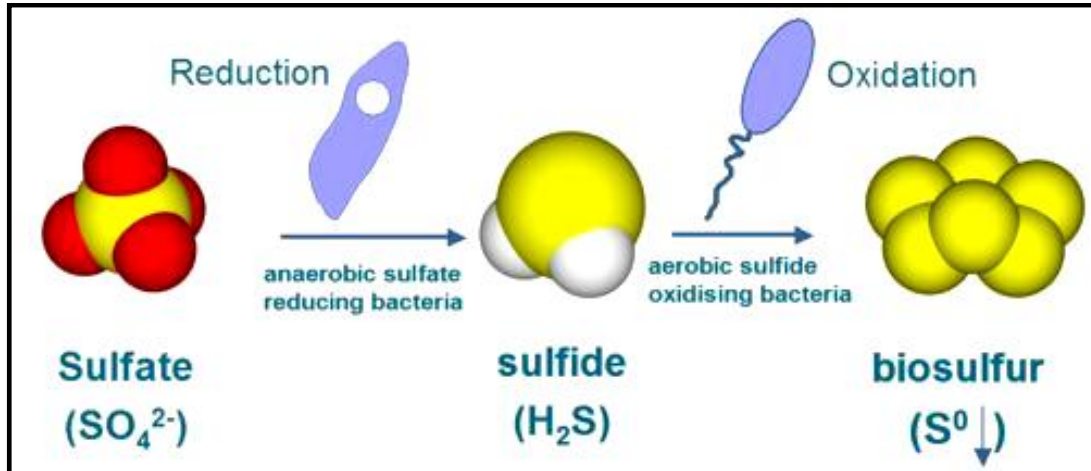


Figure 1. Combining the action of SRBs and sulfide oxidizing microbes (to the left - one SRB bacterium with elemental sulfur particles on the cell membrane)

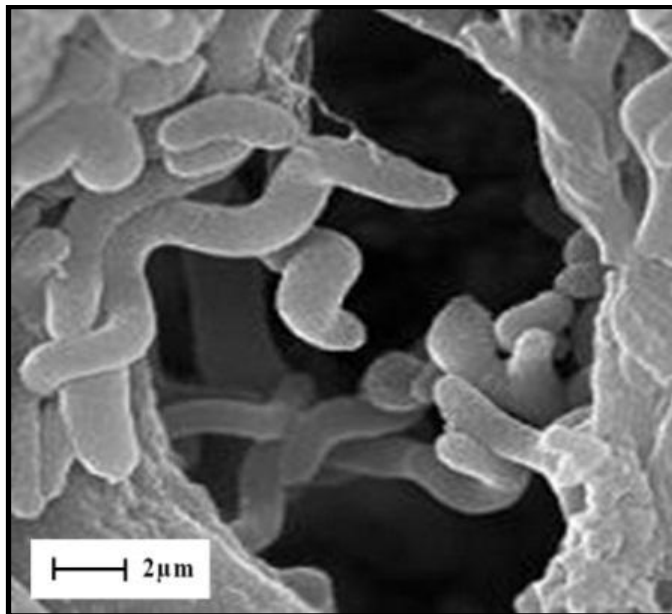


Figure 2. SRB culture with a carbonate precipitate (the bacteria are about 6 to 8 μm long and 2 μm in diameter).

sulphide oxidation by sulphur-oxidising microbes is presented in Figure 1. Carbonate precipitation by SRB is shown in Figure 2.

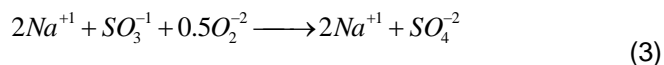
The effluents produced in sulphide ore mines, defined as acid mine drainage (AMD), also contain large amounts of heavy metals. Mining and industrial drainage containing sulphate and heavy metal negatively affects terrestrial and aquatic ecosystems in several countries around the world. SRB can be used to biologically treat metal- and sulphate-rich waste water, reducing sulphate to sulphide to precipitate metal sulphides. In AMD treatment processes, this chemically stabilizes the toxic metal ions

as solid metal sulphides (Zagury et al., 2006). Furthermore, SRB produce carbonate (equation 1) which increases the pH.

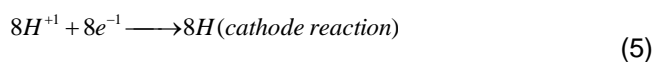
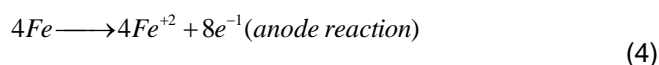
Conversely, a detrimental aspect of the activity of SRB is metal corrosion. The incidence of SRB colonizing ferrous water mains has been reported for many years (Butlin, 1949; Tuovinen et al., 1980; Beech et al., 1994). These bacteria are generally associated with the area underneath the main corrosion shell since they require anaerobic conditions (Butlin, 1949; Beech et al., 1994). However, anaerobic conditions may also occur on the corrosion surface due to the presence of other aerobic bacteria producing anoxic pockets (Spencer, 1971; Gaylarde, 1989; Jain, 1995). Once colonization has begun, bacterial growth is further promoted due to the spread of reducing conditions (Starkey, 1958). SRB are particularly unwanted in ferrous mains because of their notable effect in promoting corrosion by reducing sulphate to sulphide, which may in turn oxidize hydrogen to hydrogen sulphide, and this may react further to produce ferrous sulphides or sulphuric acid, which also contributes acid corrosion (Starkey, 1958; Gaylarde, 1992). In addition, the undesirable "rotten egg" smells of hydrogen sulphide, and the possibility of sloughing of the black slime, can lead to consumer complaints and increased health risks. Microbial corrosion by SRB results in severe graphitization of cast iron, leaving a soft surface liable to collapse (Butlin, 1949; Starkey, 1958; Seth and Edyvean, 2006).

SRB are capable of causing severe corrosion of iron material in a water system because they produce enzymes which have the power to accelerate the reduction of sulphate compounds to the corrosive hydrogen sulphide, thus SRB act as a catalyst in the reduction reaction. However, in order for this reduction to occur, four components must be present. That is, SRB must be present, sulphates must be present, an external energy

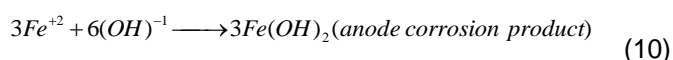
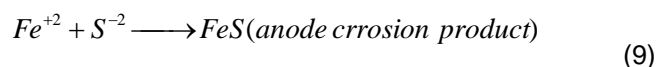
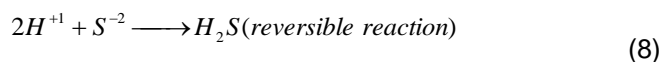
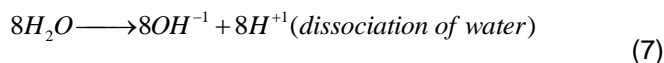
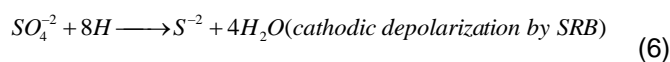
source in the form of free electrons must be present, and the temperature of the water must be less than approximately 65°C (150°F). A water system may naturally contain sulphate based compounds. Another source of sulphate originates from sulphite added to a closed water system as an oxygen scavenger and corrosion inhibitor. The sodium sulphite is oxidized to sodium sulphate as indicated in reaction 3 below.



Excess electrons occur in a water system as a result of iron corrosion at the anode and cathode cells as indicated in reactions 4 and 5 below.



The resultant accelerated corrosion mechanism of iron by SRB is illustrated in equations 6 to 10 below.



Microbiologically influenced corrosion (MIC) is the damage caused or accelerated by the presence of bacteria and other microorganisms and their metabolic activities on metals and alloys. Many types of bacteria, including sulphate-, iron- and CO₂⁻ reducing bacteria, sulphur-, iron- and manganese-oxidizing bacteria are associated with the MIC of metals and alloys (Beech and Sunner, 2004; Anandkumar et al., 2009). Among them, SRB are recognized as a major group involved in anaerobic corrosion. These latter microorganisms can coexist in naturally occurring biofilms with a wide bacterial community, including fermentative bacteria, often forming synergistic communities (consortia) that are able to influence electrochemical processes through cooperative metabolism (Beech and Gaylarde, 1999). The sulphate reductive activity of SRB is thought to account for >75% of the corrosion in productive oil wells, and for >50% of the failures of buried pipelines and cables (Walch, 1992).

SRB are strictly anaerobic microorganisms responsible for the terminal mineralization of organic matter in anoxic environments. They are a diverse group of prokaryotes that may be divided into four groups based on r-RNA sequence analysis: Gram-negative mesophilic SRB, Gram-positive spore-forming SRB, thermophilic bacterial SRB and thermophilic archaeans (Castro et al., 2000). A majority of studies on SRB in oil-field environments have concentrated on the ecology and physiology of mesophilic microorganisms, which grow optimally between 20 and 40°C (Anandkumar et al., 2009).

One of the important practical problems is the control of SRB growth in economically important situations in the petroleum sector. Consequently, considerable research has been devoted to testing various potential micro-biocides (Kumaraswamy et al., 2010).

SRB, which generate large amounts of toxic hydrogen sulfide in aquatic ecosystems, are important not only for ecological reasons but also economically. The activities of SRB in natural and manmade systems are of great concern to engineers in many different industrial operations (Gibson, 1990; Odom, 1990; Odom and Singleton, 1992).

Oil, gas and shipping industries are seriously affected by the sulfide generated by SRB (Battersby, 1988; Hamilton, 1994; Peng et al., 1994; Okabe et al., 1995; Cullimore, 2000). In the oil industry most monitoring of micro-biologically influenced corrosion (MIC) has been conducted by cultivation based techniques (Ghazy et al., 2011). The production of H₂S often indicates the activity and presence of sulphate-reducing microorganisms in natural habitats. The presence of H₂S is obvious by its characteristic smell, black precipitation of ferrous sulfide when iron minerals are present. As a result of SRB metabolism, reduced sulphur compounds can impair water quality, making for unpleasant odour, and also can contribute to biological contamination of well filters (Wargin et al., 2007).

The aim of this study was to evaluate the activity of SRB on the corrosion of the water pipelines located in the Sarcheshmeh copper mine. Also, application of various biocides to decrease numbers of SRB and the corrosion resulting from their activity was investigated.

MATERIALS AND METHODS

Sampling and analysis of corrosion products

To study microbial corrosion potential of water inside the pipeline and surrounding soil, samples were taken from 16 water wells inside and around the Sarcheshmeh copper mine. A total of 96 water samples were analysed. Also, 10 soil samples were collected near the water pipes from various depths between 1 to 2.5 m.

A qualitative test was performed to detect the presence of FeS, an indicator of SRB activity. The test involved addition of a few drops of hydrochloric acid to corrosion products on pipe samples. When hydrochloric acid contacts FeS, H₂S is produced and is readily identified from its distinctive, unpleasant odor.

Mineralogical determinations were made using x-ray diffraction (XRD) while atomic absorption spectroscopy (AAS) and induced

Table 1. The composition of Postgate API medium.

Composition	Value (g/l)
MgSO ₄ . 7 H ₂ O	0.2
KH ₂ PO ₄ (anhydrous)	0.01
Fe(SO ₄) ₂ (NH ₄) ₂ .6H ₂ O	0.2
NaCl	10
Sodium lactate	3.5
Yeast extract	1
Ascorbic acid	0.1
Thioglycolic acid	0.1
FeSO ₄ .7H ₂ O	0.004
Agar	15
Distilled water	1000 ml

Couple plasma (ICP) was used for qualitative analysis.

Table 2. Classification of corrosive environment using TBC factor (cells/mL).

Corrosive environment	TBC
Low level corrosive	10 ³
Medium level corrosive	10 ³ -10 ⁵
High level corrosive	>10 ⁵

Sulphate-reducing bacteria (SRB) and total bacteria counts (TBC) tests

Sulphate-reducing bacteria (SRB) culturing procedure

The bacterial culture medium used in these experiments was Postgate API which is widely used by the oil and gas industry (Table 1).

Also, for proper cultivation of SRB, the process in four stages was carried out as follows: A, A 10cc aliquot of the sample was taken using a syringe; B, the sample inside the syringe was injected into sterile anoxic culture medium; C, the container with the inoculated culture medium was maintained at room temperature for 21 days; D, all the samples were observed at a fixed time every day. Changes in the clarity of the medium, or development of black spots (FeS) which confirmed the presence of SRB bacteria in the sample, were noted.

Procedure for total sulphate-reducing bacteria (SRB) count total bacteria counts (TBC)

Standard culture medium API was used in the following process:

A, the lid of container of the culture medium was removed; B, water sample was added to culture glass containing the medium; C, after 1 min, water content of the glass was discharged and the lid was closed; D, container including culture medium was inoculated at room temperature; E, after 48 h, the total number of bacteria was counted; F, if the total bacteria counts (TBC) in the environmental sample was higher than 10⁵/ml, the effect on corrosion was judged to be high (Table 2).

Tests with biocides

The amount of biocide injected into the water was set at 100 mg/L. In all tests, 10 g of biocide was used in 100 L of water. Coupon (carbon steel) dimensions used in these experiments were 0.16 × 1.27 × 7.63 cm with 22 cm² area. The pH was measured using a pH meter (MP120 model, Mettler Toledo Company, Switzerland) and Eh meter was used for measuring the oxidation reduction potential (826pH mobile model, Metrohm Company, Switzerland). The Eh meter consists of a platinum electrode and a silver/ silver chloride reference electrode in KCl solution.

Polarization tests

The steel coupons used in the biocide tests (Corrosion rate measurements section) were immersed in water for 30 days for polarization tests. Polarization tests were carried out using potentiodynamic method with potentiostat EG and G, Model 263A and FRA EG and G Model 1025 with Easy Corr and Excel programs. Scan rate and potential were set to 1 mV/s and ± 400 mV respectively and then the electrode was put inside the water sample containing 100 mg/L biocide for a week. The working electrode was prepared from the same carbon steel as the water pipeline (1 × 1 cm).

Corrosion rate measurements

The corrosion rate inside the water pipeline was determined using the following two methods: A) the coupon setting method; B) corrater device.

The coupon setting method in water and soil

This method is based on the measured weight loss of metal samples. Dimensions of steel coupons were as follows: A) 0.16 × 1.27 × 7.63 cm (area: 22.2 cm²); B) 0.12 × 0.9 × 7.5 cm (area: 15.5 cm²)

The steel coupon (A) was used in the experiments. To determine the corrosion rate of the samples the following formula was used:

$$mpy = ([M_1 - M_2] \times 3650 \times 1000) / (P \times A \times D \times 25.4) \quad (11)$$

Where, M₁, is the initial sample weight (g), M₂ is the secondary sample weight (g), P is the sample density (gr/cm³), D is the number of days and A is the sample surface (cm²).

To estimate the corrosion rate, coupon setting method was carried out inside the water pipeline using steel coupon with ASTM standard dimensions (0.16 × 1.27 × 7.63 cm) for 30 days and then the coupon was fixed at a special place using a Teflon holder parallel to the water flow direction. The corrosion rate was calculated using the weight loss method according to the mentioned formula/equation (11). For determination of the corrosion rate of the soil around the pipe, two coupons with dimensions (0.1 × 2 × 2 cm and area 8.8 cm²) were used for one month. These coupons were set at a depth of 40 cm from ground level and 50 cm from each other (interval distance). One of the coupons was covered by plastic as control test and the other was placed in soil around the pipe directly and without any coverage. After removal of the coupons from water or soil, they were washed by distilled water and hydrochloric acid solution for one minute and washed with distilled water again and then finally dried and weighted. Corrosion rate can be estimated with amount of weight loss of coupons during exposure in the environment.

Table 3. Average six months analysis of the water in pipeline in the Sarcheshmeh mine.

Component	Value
TDS	208 ppm
pH	7.6
O ₂	>100 ppm
SO ₄ ²⁻	77ppm
SiO ₂	85 ppm
Cl ⁻	35 ppm
Conductivity	(μ s/cm)501

Table 4. Chemical analysis of the pipeline (steel) in the Sarcheshmeh mine.

Component	Value
S	0.05
P	0.04
Mn	1.15
C	0.27

Table 5. XRD analysis of the corrosion sediment in different location.

Sample location	Mineral/phase	Chemical formula
Upper level of sediment	Limonite	FeOOH. nH ₂ O
	Magnetite	Fe ₃ O ₄
Middle of the sediment	Limonite	FeOOH. nH ₂ O
	Magnetite	Fe ₃ O ₄
Lower level of sediment (Near of the pipeline)	Magnetite	Fe ₃ O ₄
	Limonite	FeOOH. nH ₂ O

Corrater device

Corrater is an electronic device for detecting corrosion in water systems which can be used to measure the corrosion rate directly. The corrosion measurement in corrater is based on the linear polarization resistance. In this method, the oxidation reaction occurs at the anode and the cationic reduction reaction takes place at the interface of fluid and the pipe at the cathode area.

RESULTS AND DISCUSSION

Pipeline water chemistry

The water in the pipeline at Sarcheshmeh mine was slightly alkaline, low in chlorides and contained sulphate,

which could serve as an electron acceptor for SRB and therefore is potentially reducible to H₂S (Table 3).

Characterization of corrosion product

The water pipeline at Sarcheshmeh mine was composed of mild steel. The minor components of the pipe included S, P, Mn and C (Table 4).

The corrosion product formed on the internal surface of the pipeline consisted of limonite and magnetite as characterized by X-ray diffraction (XRD) and chemical analysis methods (Table 5 and Figures 3 and 4).

As shown in Table 3, all trivalent iron oxide is formed on the surface and bivalent iron oxide occurred below the rust layer. FeS often is an important phase in corrosion products, but in this case, the dominance of other minerals (Table 5) resulted in FeS not being detected by XRD. Nonetheless, the high amount of sulfur in the corrosion product (16.15%) (Figure 4), and liberation of hydrogen sulfide by hydrochloric acid (hydrochloric acid test and optical microscopic study section), indicates the presence of FeS and that corrosion in the water pipeline could be caused by SRB. The presence of phosphorus in the corrosion products (Figure 4) also has been reported previously; are active, phosphorus compound produced by SRB may be involved in bio-corrosion of iron and steel (Iverson and Olson, 1983).

Hydrochloric acid test and optical microscopic study

A corroded part of the pipeline was examined using a hydrochloric acid test and light microscopy analysis to determine if SRB may be involved in corrosion of the pipe. After polishing of the pipe, a metallographic study was carried out under an optical microscope and several holes and cavities were found on the surface which is illustrated in Figure 5.

Microbial corrosion is always local, (that is, the type is a gap or hole in the pipe) and after addition of a few drops of hydrochloric acid on the black parts of specimens identified in Figure 6, a strong odor of H₂S was detected.

Direct counts of sulphate-reducing bacteria (SRB)

Numbers of SRB in water well no.6 and 16 which were located in the Sarcheshmeh region and also the water main pipeline in the copper mine and the soil around the pipe are given in Table 6. The number of SRB in a soil environmental sample was 4.5×10^6 /ml. It can be concluded that due to higher number of soil SRB compare to other environments, highest corrosion process was observed. No SRB were detected in water samples containing various biocides. It showed that all biocides can destroy SRB effectively, but the efficiency of their operation is different. The SRB bacteria was observed by micro-biological microscope which its shape

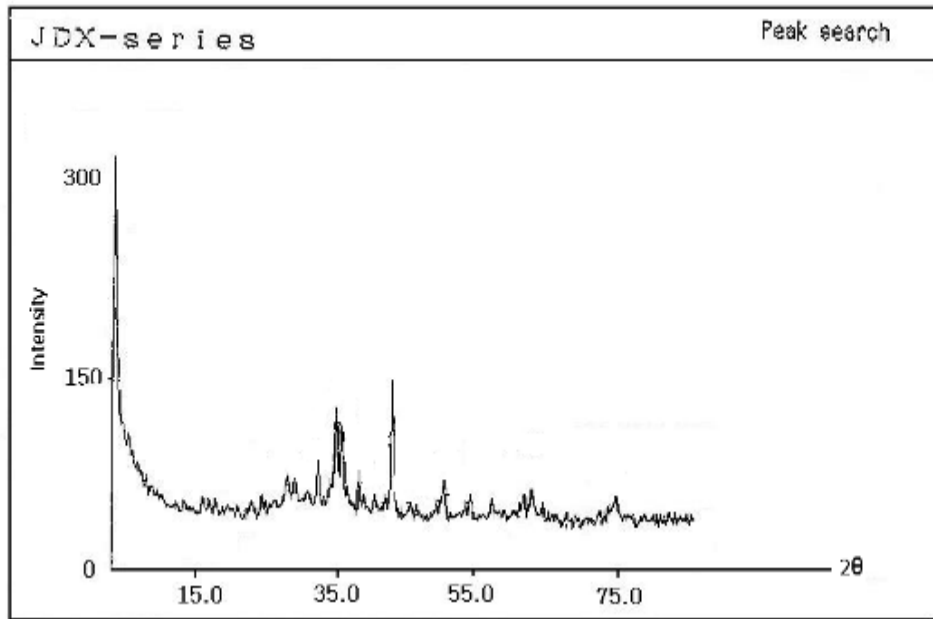


Figure 3. XRD pattern of the corrosion product.

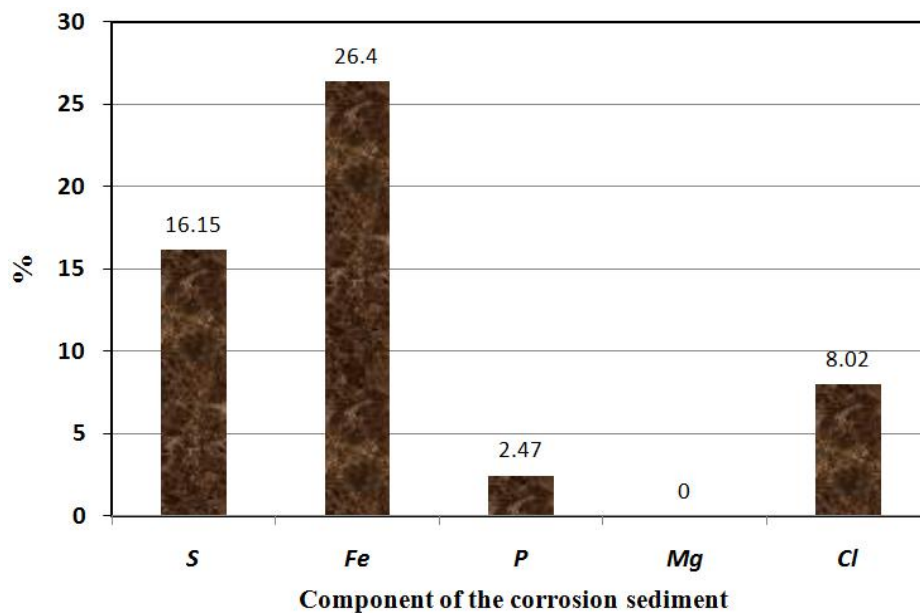


Figure 4. Component of the corrosion product in element forms

and colony in the culture medium are presented in Figure 7.

Corrosion measurement methods (coupon setting and Corratere device)

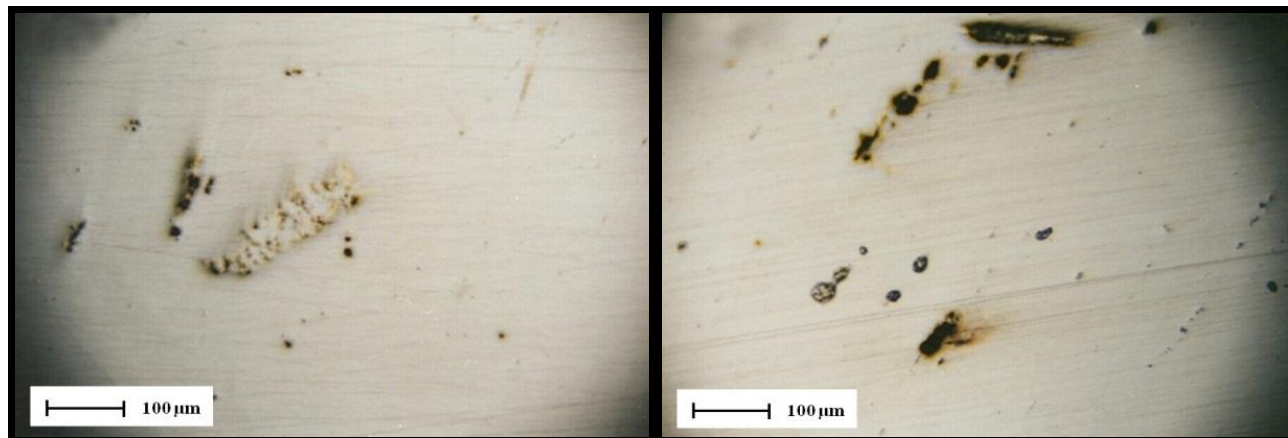
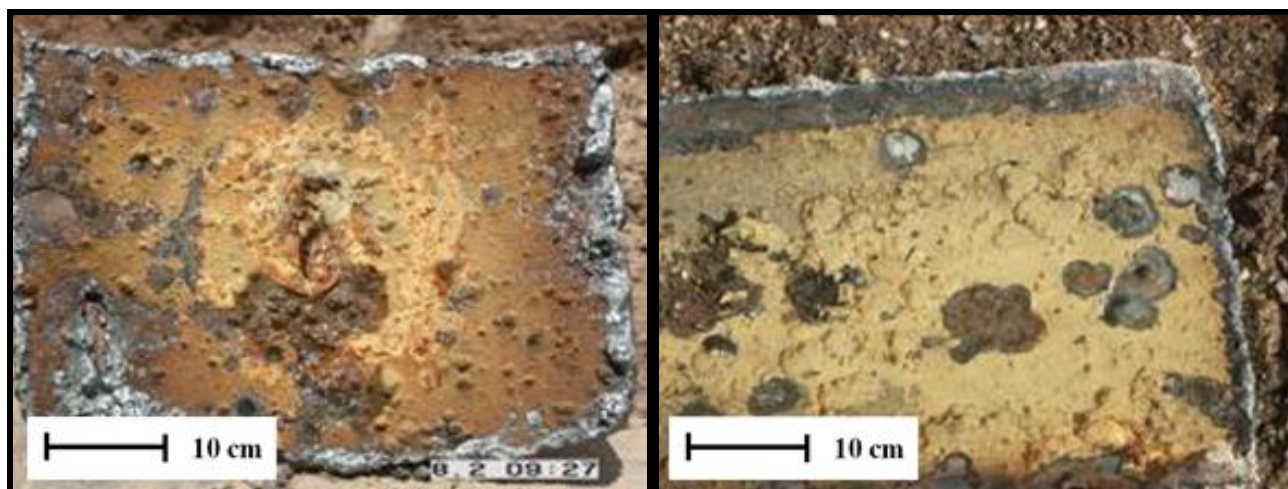
Corrosion rate of water in water pipe using Corratere

device and coupons setting and soil around the pipeline by coupons setting method were investigated (Table 7). It should be mentioned that for measurement of the corrosion rate, weight loss method (mpy) was used.

Results from the Table 7 show that corrosion rate of coupons in soil around the pipe was two times higher than water inside the water pipe, therefore cathodic protection of pipe in soil and application of the appropriate

Table 6. Number of SRB bacteria in different places with corrosion level of coupons

Environment	Count of SRB (in ml.)	Corrosion level
Soil	$10^6 \times 4.5 \cong$	Very severe
Water, well No.16	$10^6 \times 1.5 \cong$	High
Water, well No.6	$10^6 \times 1.2 \cong$	High
Water, Main pipeline	$10^5 \times 1.5 \cong$	Medium
Water with biocides	Not seen	None

**Figure 5.** Presence of cavity on the surface of water pipeline (Optical microscopy).**Figure 6.** Hydrochloric acid test on the dark color corrosion product.

coating is necessary and important. Corrosion rate of water was detected using above two methods (coupon setting and Corraters devices), but due to based works (principle) of the Corraters which is linear polarization resistance, the corrosion rate was measured by more accuracy.

Inhibitors performance on the water pipes

Biocide CWT110 had best performance with lowest corrosion rate (0.743 mpy) and Biocide MBA4517 had weakest efficiency with highest corrosion rate (2.445 mpy) (Table 8). The visual appearance of the coupons

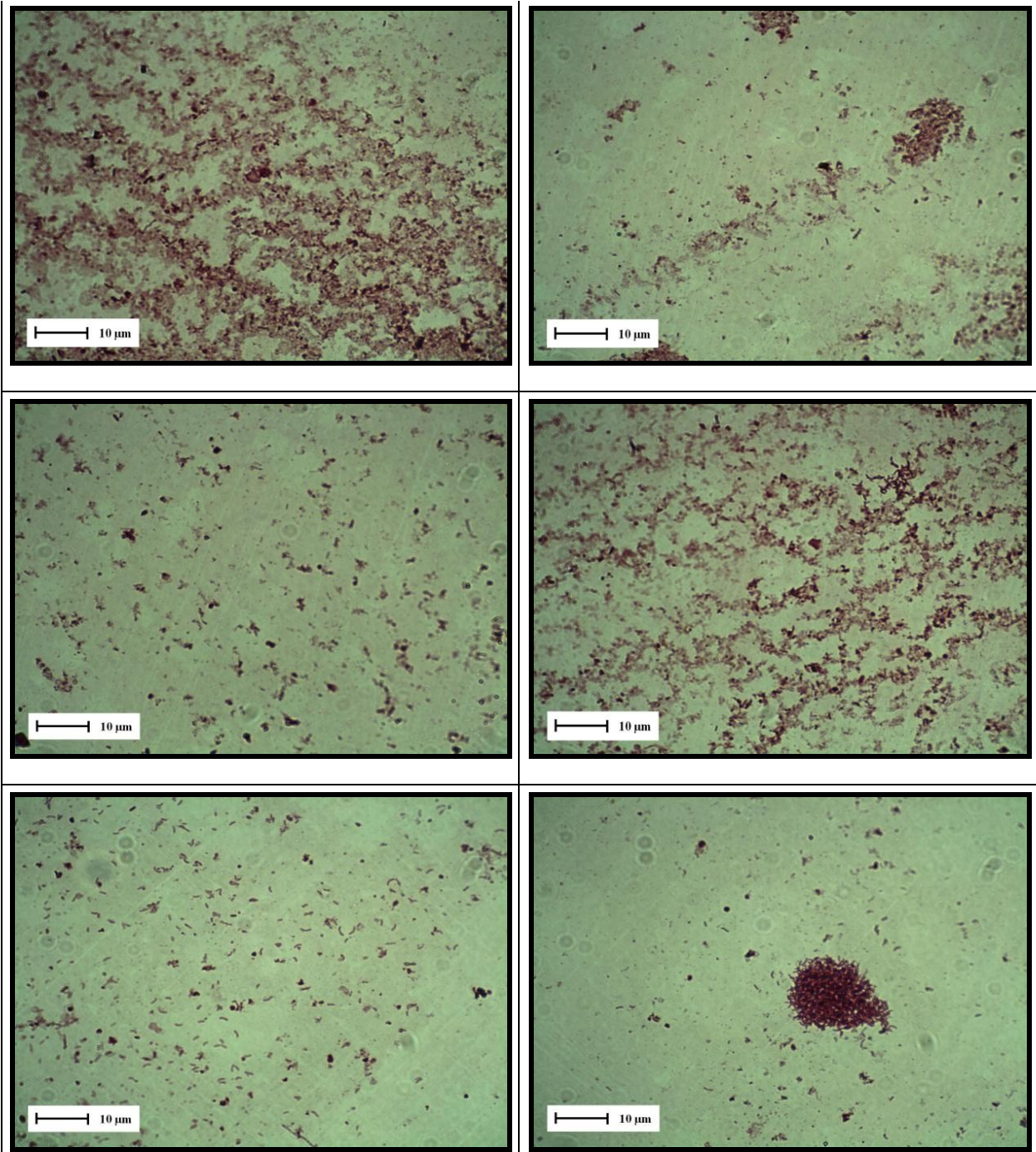


Figure 7. SRB bacteria and its colony (inoculated in the culture medium).

after one month was consistent with their extent of corrosion; the coupons with biocide CWT110 had best appearance after this period of time (Figure 8). The order of different biocide performance is as below:

ISOcwt5> MBA8115> MBA4517>CWT15> MBA8120>
MBA8110>CWT110

pH and Eh of water with different biocides

In this experiment, 0.5 L of water containing various biocides was taken and Eh and pH values were measured (Table 9). The effect of water on iron corrosion in pH = 4.3 to 10 was low. Due to reduction of sulfate to sulfide, corrosive water containing SRB bacteria has

Table 7. Corrosion rate of water and soil around the pipeline.

Environment	Coupon characteristic				Coupon setting period (day)	Density (g/cm ³)	Corrosion rate	
	Area (cm ²)	Primary weight (g)	Secondary weight (g)	Weight loss (g)			Coupon method (mpy)	Corrater device (mpy)
Soil	8.8	37.6312	37.5110	0.1201	30	7.86	8.32	-
Water	22	10.6037	10.4481	0.1555	30	7.86	4.31	4.82

Table 8. Biocide performance on the water pipes.

Biocide	Primary weight (g)	Secondary weight (g)	Weight loss (g)	Density (g/cm ³)	Coupon setting method	
					Period (day)	Corrosion rate (mpy)
CWT110	10.5572	10.5304	0.026	7.86	30	0.743
MBA8110	10.5904	10.5576	0.032	7.86	30	0.906
MBA8120	10.5422	10.4938	0.048	7.86	30	1.338
CWT15	10.5829	10.5298	0.053	7.86	30	1.481
ISOcwt5	10.5802	10.5179	0.062	7.86	30	1.737
MBA8115	10.5802	10.5055	0.074	7.86	30	2.069
MBA4517	10.5222	10.4339	0.083	7.86	30	2.445

Table 9. Eh (mV) and pH values for water containing various biocides.

Biocide	pH	E _h	Oxidizer or non-oxidizer	Component
CWT110	8.9	173	Non-oxidizer	<i>Isothiazolin</i>
MBA8110	8.72	178	Non-oxidizer	<i>Isothiazolin</i>
MBA8120	8.71	182	Non-oxidizer	<i>Quaternary ammonium compounds</i>
CWT15	8.57	194	Non-oxidizer	<i>Metronidazol</i>
ISO CWT5	8.49	196	Non-oxidizer	<i>Metronidazol</i>
MBA8115	8.23	201	Non-oxidizer	<i>Quaternary ammonium compounds</i>
MBA4517	8.12	205	Non-oxidizer	<i>Carbamate</i>
Corrosive water	8.2	350	-	<i>SRB</i>

maximum Eh (350 mV) with lowest pH value (8.2). In water containing different biocides, biocides prevent setting of the reaction and consequently, Eh of the environment was maintained at lower value and pH reached to maximum its value. Biocide with best prevention performance had lowest Eh and highest pH, therefore biocide CWT110 which is classified in isothiazolin group had best efficiency to eliminate SRB bacteria from the water in the water pipeline.

Scanning electron microscopy (SEM) study

Scanning electron microscopy (SEM) was used to investigate the surface of the steel coupons that had been placed under the various corrosive conditions. The surface of the normal steel showed no holes or cavities or

corrosion products (Figure 9a). The surface of a coupon which was set in 1.5 m depth of the soil near the pipeline for 30 days had more holes and corrosion products on the surface (indicated as dark and light spots) indicating corrosive conditions (Figure 9b). The corrosion rate of this coupon was 8.32 mpy (Table 7). There was also a corrosive environment in water of the water pipeline (Figure 9c) but less than the soil. The corrosion rate reached 4.31 mpy. In water containing different biocides (Figure 9d, 9e and 9f), the surfaces of coupons contained fewer pits and corrosion products. These observations were consistent with corrosion rates (Table 8) which were 2.44 mpy for biocide MBA4517, 1.338 mpy for biocide MBA8120 and 0.743 mpy for biocide CWT110. The corrosion rate data and SEM images suggest biocide CWT110 could substantially reduce biocorrosion in the pipeline.

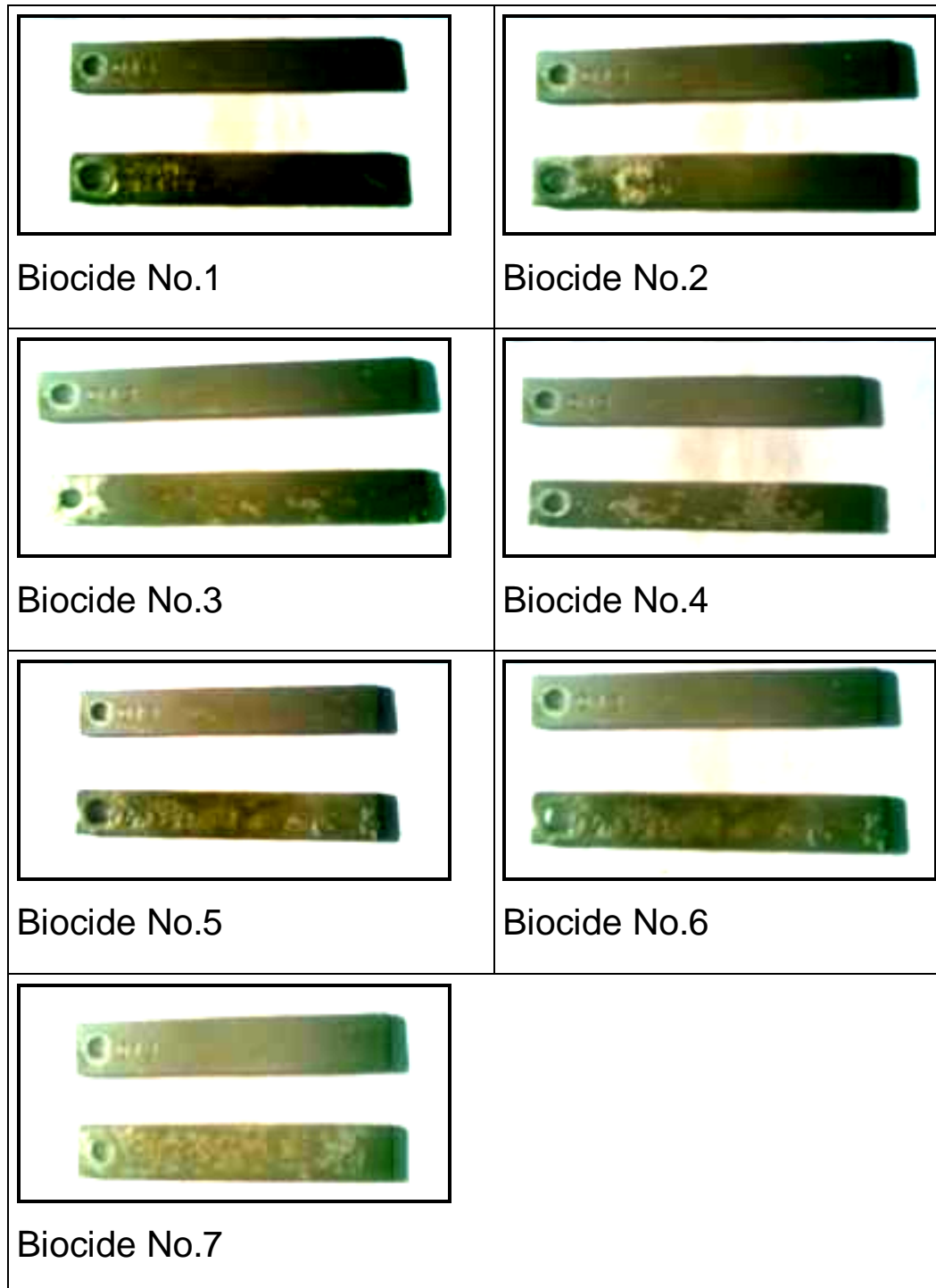


Figure 8. The corroded coupons in the path of water pipes with different biocide.

Polarization test

The polarization curves of steel in water samples without and also containing various biocides are illustrated in Figure 10 (a to h). The amount of biocide value added to

raw water was 10 mg/L. The reference electrode in these experiments was saturated calomel electrode (SCE).

In a polarization curve, whenever the potential is more negative and the current is more positive, the environment is more corrosive. The polarization curves of steel

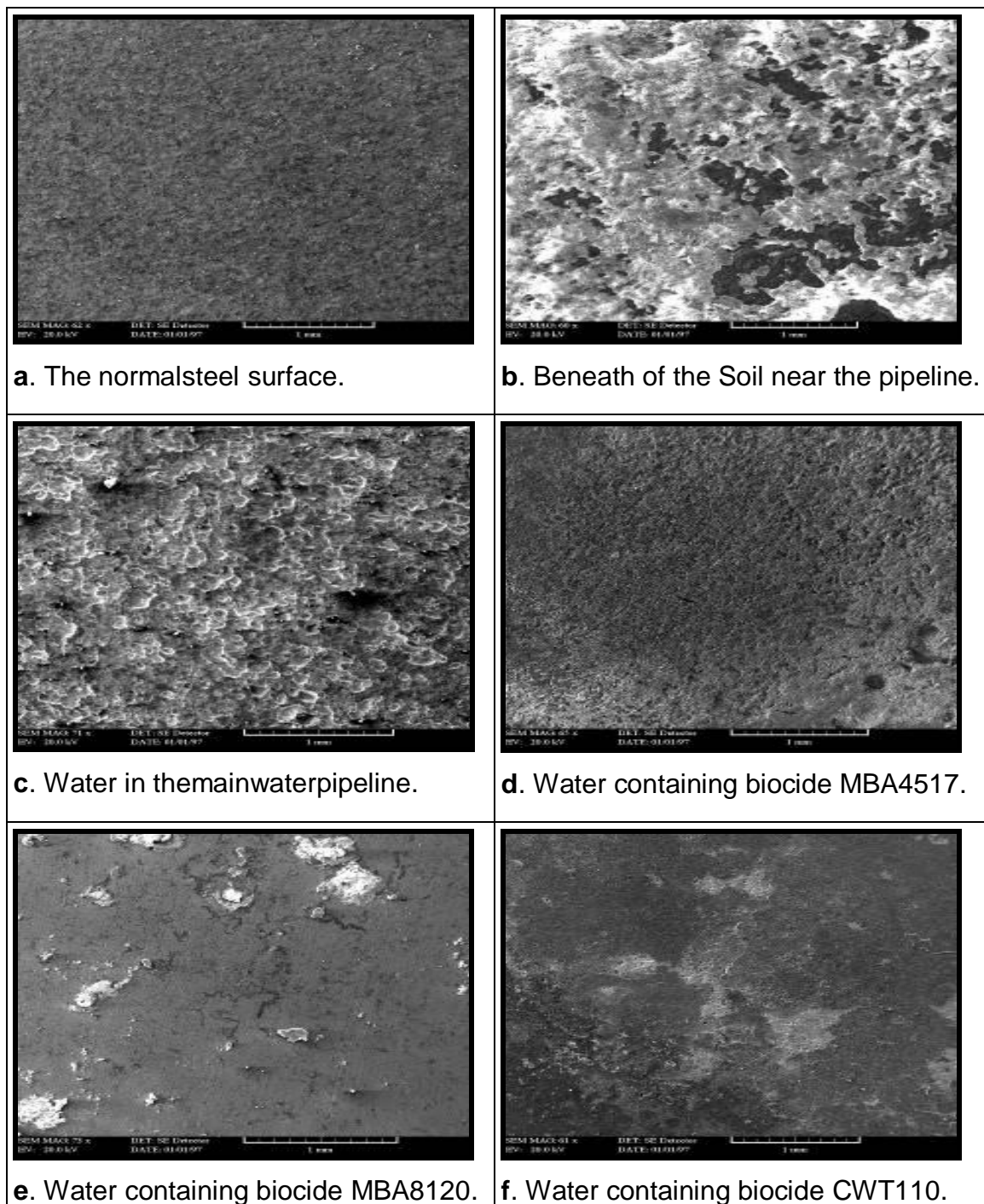


Figure 9. SEM image from the surface of carbon bearing steel coupon.

steel in Figure 10 (a to h), show raw water produced a more negative potential and more positive current than water that contained biocides. The numerical values of parameters obtained from the polarization curves in different situations are given in Table 10. As can be seen, for biocide CWT110, I_{corr} (corrosion current) is minimum and E_{corr} (the potential) is maximum. These results are

consistent with results of corrosion rate determinations and SEM observations indicating this biocide can be used to prevent biocorrosion. Raw water is more corrosive with $9.765 \mu A$ and -690 mV for I_{corr} and E_{corr} , respectively. Also, the anodic and cathodic slopes of the polarization curve are shown by β_a and β_c symbols respectively.

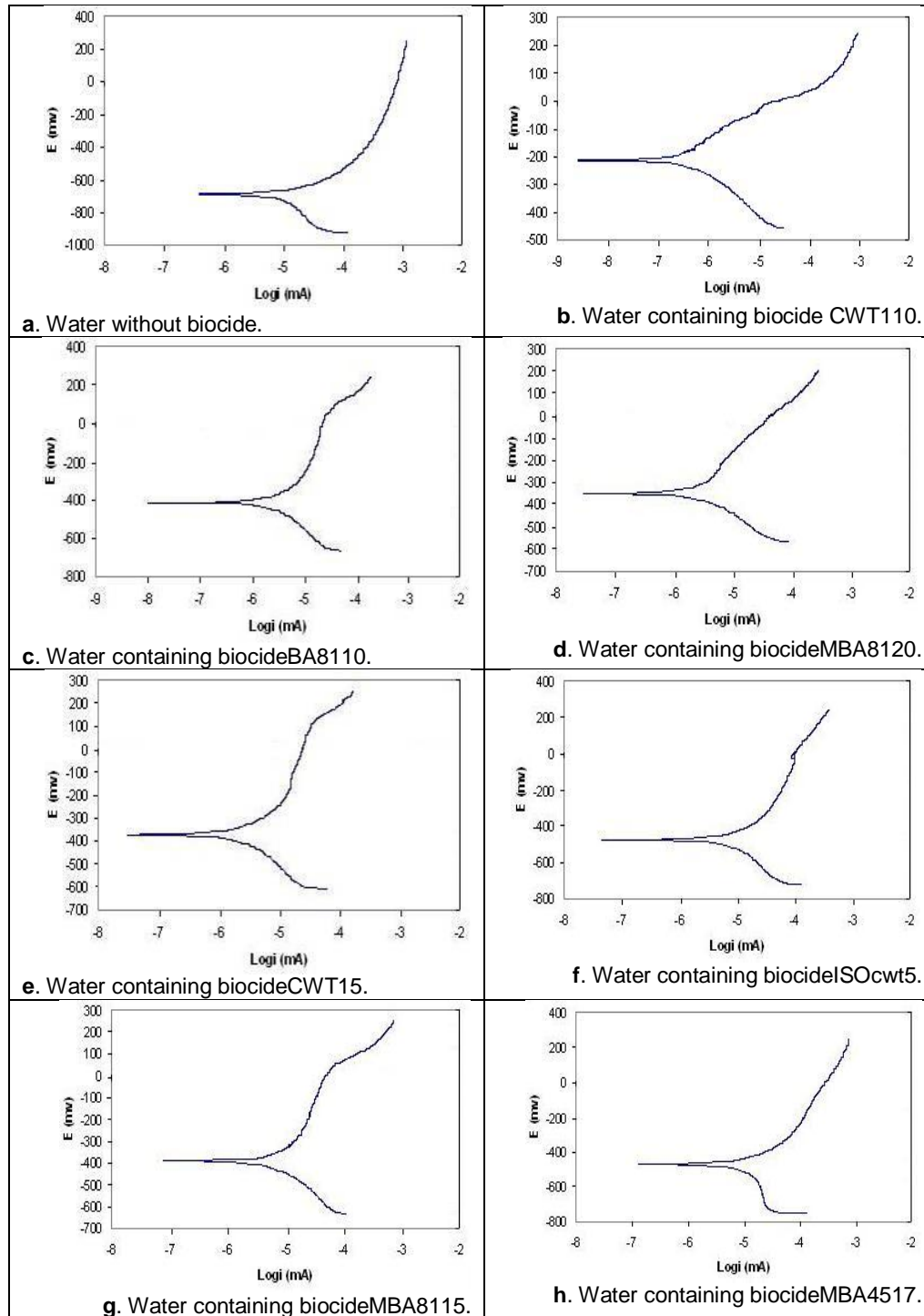


Figure 10. Polarization curve of steel in water samples without and with various biocides.

Conclusion

High amounts of sulphur and the presence of FeS and P in corrosion products indicate that microbial corrosion by SRB occurred in water pipelines at Sarcheshmeh mine. Also, according to the metallographic observations of the

sample and presence of cavities, microbial corrosion of water pipes was confirmed. In addition, the total bacteria count (TBC) and SRB tests in the water samples from 16 wells showed that water in wells No.6 and 16 have the highest amount of SRB bacteria. In view of the presence of SRB in the surrounding soil of water pipeline, cathodic

Table 10. Results of polarization test for raw water and water containing different biocides.

Biocide	I_{corr} (μA)	E_{corr} (mV)	β_a	β_c
CWT110	1.823	-213.2	219×10^{-3}	131×10^{-3}
MBA8110	2.231	-393.8	619.8×10^{-3}	469×10^{-3}
MBA8120	3.292	-358.4	561.8×10^{-3}	219.3×10^{-3}
CWT15	3.312	-371.5	536.1×10^{-3}	456.6×10^{-3}
ISOcwt5	4.124	-473.8	797.7×10^{-3}	1.001
MBA8115	4.672	-390.6	4.649	635.1×10^{-3}
MBA4517	5.392	-479.5	426.8×10^{-3}	2.67×10^3
Raw water	9.765	-690	305.3×10^{-3}	9.987×10^{-3}

protection potential applied to the pipeline (-0.85 V) cannot appreciably protect from pipeline against corrosion by SRB. Results of the Corrater device and coupons setting showed that corrosion potential in soil surrounding of the water pipeline was higher than raw water. Results of corrosion tests, SEM observations and polarization studies with steel coupons immersed in water in the pipeline at Sarcheshmeh mine showed biocide CWT110 to be most effective in reducing biocorrosion. The use of this biocide in pipeline water at the mine has promise for preventing pipe biocorrosion.

ACKNOWLEDGMENTS

This work was supported by the National Iranian Copper Industry Co. We also wish to thank our honorable partners on the project for their contributions to the work reported in this paper. We thank S.A. Fazeli for his helpful comments and suggestions on the manuscript.

REFERENCES

- Anandkumar B, Rajasekar A, Venkatachari G, Maruthamuthu S (2009). Effect of thermophilic sulphate-reducing bacteria (*Desulfotomaculum geothermicum*) isolated from Indian petroleum refinery on the corrosion of mild steel. *Current Sci.*, 97(3):342-348.
- Battersby PA (1988). Sulfate reducing bacteria. In *Methods in Aquatic Bacteriology*. B Austin (ed), New York, John Wiley & Sons: 269-299.
- Beech IB, Edyvean RGJ, Cheung CWS, Turner A (1994). Bacteria and corrosion in potable water mains. *Conference Proceedings of European Federation of Corrosion (15)*, Portugal. 15:328-337.
- Beech IB, Gaylarde CC (1999). Recent advances in the study of biocorrosion - an overview. *Rev. Microbiol.* 30:177-190.
- Beech IB, Sunner J (2004). Biocorrosion towards understanding interactions between biofilms and metals. *Curr. Opin. Biotechnol* 15:181-186.
- Beijerinck MW (1895). *Zentralbl. Bacteriol. Abt.* 2(1):49-59.
- Butlin KR (1949). Sulphate-reducing bacteria and internal corrosion of ferrous pipes conveying water. *Nature* 163:26-27.
- Castro HF, Williams NH, Ogram A (2000). Phylogeny of sulphate-reducing bacteria. *FEMS Microbiol. Ecol.* 31:1-9.
- Cullimore R (2000). *Practical atlas for bacterial identification*. Boca Raton: CRC press.
- Gaylarde CC (1989). Microbial corrosion of metals. *Environ. Engr* 4:30-32.
- Gaylarde CC (1992). Sulfate-reducing bacteria which do not induce accelerated corrosion. *Int. Biodeter. Biodegrad.* 30:331-338.
- Ghazy EA, Mahmoud MG, Asker MS, Mahmoud MN, Abo Elsoud MM, Abdel Sami ME (2011). Cultivation and detection of sulfate reducing bacteria (SRB) in Sea Water. *J. Am. Sci.* 7 (2):604-608.
- Gibson GR (1990). Physiology and ecology of the sulfate reducing bacteria. *J. Appl. Bacteriol.* 69:769-797.
- Hamilton WA (1994). A biocorrosion: the action of sulfate-reducing bacteria. *Biochemistry of Microbial degradation*, C. Ratledge. Dordrecht: Kluwer academic.
- Iverson WP, Olson GJ (1983). Anaerobic Conversion of Iron and Steel: A Novel Mechanism. *Current Perspectives in Microbial Ecology*, M.J. Klug and C.A. Reddy, eds., American Society for Microbiology, Washington, DC. 623-627
- Jain DK (1995). Microbial colonization of the surface of stainless steel coupons in a deionized water system. *Water Res.* 29:1869-1876.
- Kumaraswamy R, Ebert S, Gray MR, Fedorak PM, Foght JM (2010). Molecular- and cultivation-based analyses of microbial communities in oil field water and in microcosms amended with nitrate to control H₂S production. *Appl. Microbiol. Biotechnol.* DOI: 10.1007/s00253-010-2974-2978.
- Odom JM (1990). Industrial and environmental concerns with sulfate reducing bacteria. *ASM News* 56:473-476.
- Odom JM, Singleton R (1992). The sulfate-reducing bacteria: contemporary perspective. New York Springer-Verlag.
- Okabe S, Nielsen PH, Jones WJ, Characklis WG (1995). Sulfide product inhibition of *Desulfovibrio desulfuricans* in batch and continuous cultures. *Water Res.* 29:175-187.
- Peng CG, Park CG, Park JK, Patenaude RW (1994). Statistics base of classification of microbial influenced corrosion in fresh water system. *Wat. Res.* 28:951-959.
- Seth AD, Edyvean RGJ (2006). The function of sulfate-reducing bacteria in corrosion of potable water mains. *Int. Biodeter. Biodegrad.* 58:108-111.
- Spencer AN (1971). Corrosion by sulfate-reducing bacteria. *Nature* 233:491-492.
- Starkey RL (1958). The general physiology of the sulphate reducing bacteria in relation to corrosion. *Producers Monthly*:12-30.
- Tuovinen OH, Button KS, Vuorinen A, Carlson L, Mair DM, Yut LY (1980). Bacterial, chemical, and mineralogical characteristics of tubercles in distribution pipelines. *J. Am. Water Works Assoc.* 72:626-635.
- Walch M (1992). Microbial Corrosion. *Encyclopedia of Microbiology* (ed. Lederberg, J.), Academic Press, New York. 1:585-591.
- Wargin A, Olanczuk-Neyman K, Skucha M (2007). Sulphate-Reducing Bacteria, Their Properties and Methods of Elimination from Groundwater. *Polish J. of Environ. Stud.* 16(4):639-644.
- Widdel F, Hansen TA (1992). The prokaryotes. In: A. Balows, H. G. Trüper, M. Dworkin, W. Harder and K.-H. Schleifer, (eds.), 2nd. Edn. Springer-Verlag, New York. 583-624.
- Zagury GJ, Kulnieks VI, Neculita CM (2006). Characterization and reactivity assessment of organic substrates for sulphate-reducing bacteria in acid mine drainage treatment. *Chemosphere* 64:994-954.

Full Length Research Paper

An investigation of the bioaccumulation of chromium and uranium metals by *Cynodon dactylon*: A case study of abandoned New Union Gold Mine Tailings, Limpopo, South Africa

K. Nelushi¹, J. R. Gumbo^{1*} and F. A. Dacosta²

¹Department of Hydrology and Water Resources, University of Venda, P/Bag x5050, Thohoyandou, 0950, South Africa.

²Department of Mining and Environmental Geology, University of Venda, P/Bag x5050, Thohoyandou, 0950, South Africa.

Accepted 27 March, 2013

Mine waste, including tailings is generally outlined as one of the largest environmental concern which faces defunct mines in South Africa and New Union Gold Mine is no exception. These tailing contain heavy metal such as chromium (Cr) and uranium (U) which poses enormous threat to the environment even at small quantity. The study focuses mainly on bioaccumulation of Cr and U in soil by *Cynodon dactylon*, an indigenous grass. The grass and soil sample were collected at New Union Gold Mine and Ka-Madonsi Village at Malamulele, Limpopo Province, South Africa. The concentration of Cr and U were determined with a Thermofischer ICP MS. The research findings indicate that the range in the levels of Cr and U at mine tailings dam A were 152.60 to 196.12 mg/kg and 0.51 to 0.92 µg/gm, respectively. The ranges in the levels of Cr and U at mine tailings dam B were 151.34 to 229.67 mg/kg and 0.85 to 1.06 µg/g, respectively. The levels of Cr and U at the control site were 81.31 mg/kg and 0.73 µg/g. The pH of mine tailing dam A was 3.23 to 3.34 and for tailing dam B were, 3.25 to 3.29 making both tailing acidic while for the control site, it was slightly alkaline at 7.56. The bioconcentration and translation factors of *C. dactylon* were variable but were dependent on pH conditions. Thus, *C. dactylon* was able to bioaccumulate toxic metals Cr and U from the mine tailings making them potential phytoremediation agent for the rehabilitation of exposed mine tailings. This is important in covering the mine tailings since any exposed part of mine tailings is liable to water and wind erosion. Thus, Cr and U may be exported to external environment such as aquatic ecosystem and neighboring rural communities with negative impacts.

Key words: Phytoremediation, chromium, uranium, indigenous grass, dysfunctional mine tailings

INTRODUCTION

Mining is the breaking up and extraction of minerals of economic importance from the earth's crust for human-kind's benefit. It incorporates auxiliary operations like trans-

portation of ore as well as downstream processing of minerals or ore dressing called beneficiation and the disposal of waste (tailings). The last operation results in

*Corresponding author. E-mail: jabulani.gumbo@univen.ac.za or jabulani_gumbo@yahoo.co.uk. Tel: +27 15 962 8563.

serious environmental and health problems since these tailings contain toxic metals and cyanide residues and may affect communities living nearby. The degree of impacts depends on the scale of mining, mining methods employed and chemicals used to extract the important minerals (Ogola, 2002). According to Ma et al. (2001), phytoremediation can be an essential tool because it is cheap, environmental friendly and long lasting for remediation of the contaminated sites. Bioaccumulation of toxic metals by indigenous grass species from the contaminated site can be a remedy for decreasing the environmental hazard posed by toxic metals on the environment (Conesa et al., 2006; Mulugisi et al., 2009). Toxic metals originating from abandoned gold mine tailings may have a huge impact on the environment and human health (Wong et al., 1999).

According to Zayed and Terry (2003), chromium (Cr) is considered the most hazardous to animals and plants due to its high solubility, morbidity, toxicity as well carcinogenic and mutagenic properties. However, among the Cr species that are commonly known, are the trivalent chromium (Cr^{3+}), an essential element to living organisms and hexavalent form Cr^{6+} , which is toxic (Cheunga and Gua, 2007).

In light of the hazardous nature of chromium, WHO (2011) has issued provisional guideline value of 50 $\mu\text{g/L}$ for total Cr in drinking water. Due to unregulated disposal of mine tailings from New Union Gold Mine, Cr may increase the ecological risk to surface water, sediment, soil and groundwater and it is increasingly becoming environmental and health issue. Han et al. (2002) reported that the cumulative Cr production was estimated to be 105.4 million ton globally in 2000 and has been rapidly increasing since the 1950s.

The use of plants as phytoremedial agents has gained a lot of attention worldwide for its ability to clean up contaminated soil. The use of plants can be a remedy of contaminated soil because of its affordability and environmental friendly. The use of plants for potential toxic metal removal from soil is environmentally sound and low in cost (Schnoor, 1997). The uptake of Cr by plants maybe via carriers of essential ions such as sulphate (Oliveira, 2012) and sulphate is a major contributor anion to acid mine drainage (Sam and Beer, 2000). Thus, the soil factors such as pH and electrical conductivity are able to influence the mobility and availability of chromium such that the Cr^{3+} is the dominate form in acidic conditions (pH < 4) experienced at New Union Gold Mine tailings (Mulugisi et al., 2009; Wuana and Okieimen, 2011).

Winde et al. (2004) reported that in South Africa, uranium (U) was recovered from gold ore production and this U production peaked to 7200 tons in 1980 but has since ceased. However, annual gold ore beneficiation has ensured that approximately 6000 tons of U is deposited on mine tailings in South Africa (Winde et al., 2004). The study of Cowart and Burnett (1994) reported high levels of radioactive and heavy metals in mine tailings that origi-

nated from gold and uranium mining activities. Winde et al. (2004) went further to study the U migration from gold mine tailing into the groundwater and contamination of surface water sources. Winde (2010) reported that neutrotoxic effects of U to learners and gold miners had the potential to affect academic performance and lower their performance. Winde et al. (2004) also stated that there was potential high risk since the communities relied on untreated surface water sources for their drinking purposes.

The people who consume water contaminated by U may suffer from kidney problems (failure) and even cancer (Winde et al., 2004). Exposure to such toxic metal may have serious health problems on humans especially children and old people because of their weakened immune system and to the environment (Au et al., 1998). WHO (2011) has promulgated a U provisional guideline of 30 $\mu\text{g/L}$ in drinking water. The mobility and availability of U is dependent on a number of soil factors such as pH, redox potential, soil moisture levels and microbial activity with the uranyl ion being stable in aqueous solutions of pH < 2.5 (Gavrilescu et al., 2009).

According to Mulugisi et al. (2009), five indigenous grass species, *Cynodon dactylon*, *Cyperus esculentus*, *Hyperthermia tamba*, *Hyperthermia hirta* and *Paspalum dilatatum* were analyzed for their absorption of heavy metals in mine tailings at New Union Gold Mine, Malamulele and Limpopo Province, South Africa. Other plants species such as *C. dactylon* which were grown on the soil contaminated by toxic metals have the ability to accumulate huge amount of toxic metals in their tissues without symptoms of toxicity (Padmavathamma and Li, 2007). Soleimani et al. (2009) observed that *C. dactylon* was able to accumulate Mn metal both in roots and shoots. Manganese was found to be accumulated in the root (63 mg/kg) and shoot (36 mg/kg) of *C. dactylon* growing in natural top soil (Maiti and Jaiswal, 2008). According to the studies above, it is shown that *C. dactylon* can be regarded as hyperaccumulator because of its ability to absorb greater than one (>1) mean metal concentration.

Mulugisi et al. (2009) demonstrated that *C. dactylon* grass species was able to bioaccumulate heavy metals, Mn (up to 225.5 mg/kg); Cu (up to 41.5 mg/kg); Zn (41 mg/kg); Pb (up to 4.5 mg/kg); Co (up to 2.5 mg/kg) and accumulated less (1 mg/kg) of Cd on mine tailings soils at New Union Gold Mine, South Africa. A study was done by Saraswat and Rai (2009) to evaluate the phytoextraction potential of six different plant species for Cr, Zn and Cd removal under laboratory conditions. The study show that *C. dactylon* was able to exhibit maximum bioaccumulation for Cr. However there are limited studies on the bioaccumulation potential of Cr and U uptake by *C. dactylon* grass species under field conditions. The general objective of this study was to assess the bioaccumulation of total Cr and U by *C. dactylon*. The specific objectives were: to determine the contribution of

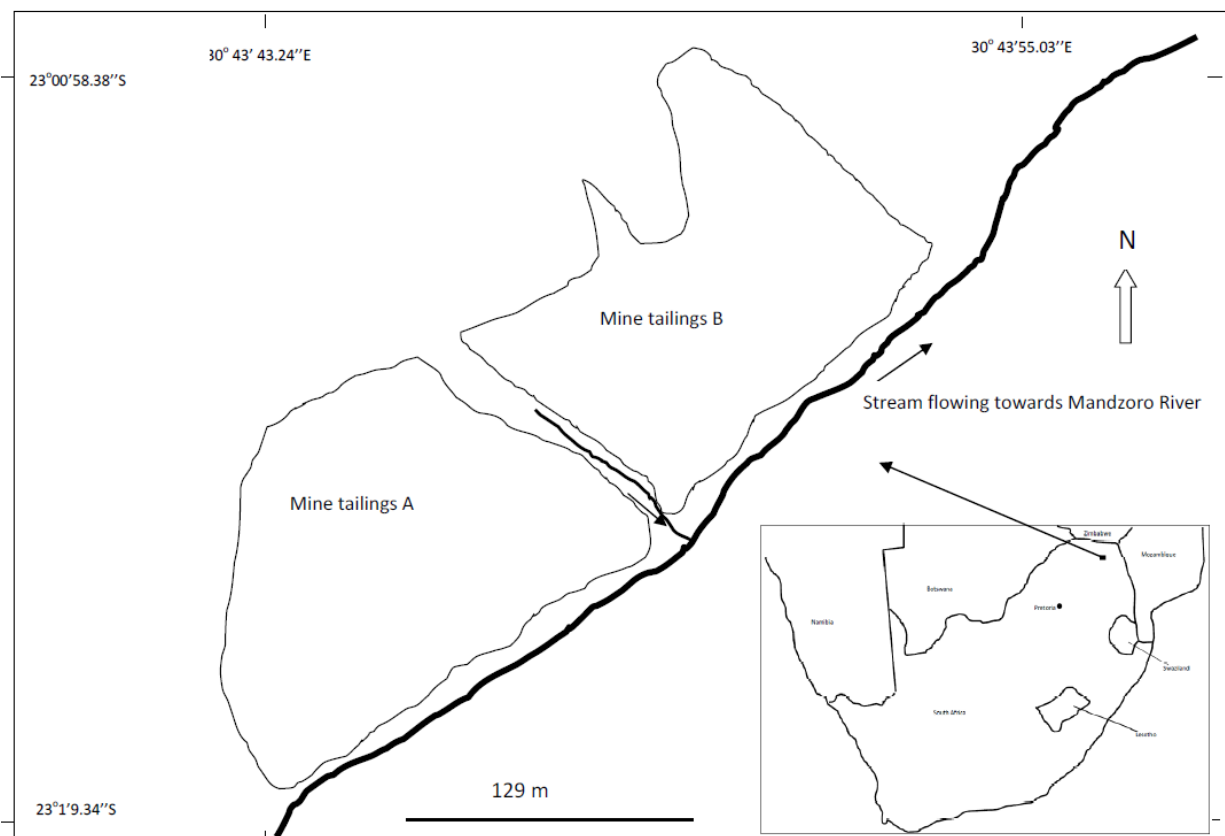


Figure 1. The location of the study area.

pH and electrical conductivity (EC) to the solubility and mobility of total Cr and U metals; to determine the distribution of total Cr and U in different sections of *C. dactylon* grass species (roots, stem, rhizome and leaves) and to determine the distribution of Cr and U in the mine tailings.

MATERIALS AND METHODS

Sample collection and preparation

Mine tailings and grass samples were collected from both tailings Dam A and B. The tailing (soil) and *C. dactylon* grass samples were collected at the following geographical coordinates. A1, A2, B1, B2 and controls were collected at 23°01'05"S and 30°43'50"E, 23°00'59"S and 30°43'53"E, 23°01'06"S and 30°43'47"E, and 23°01'04"S and 30°43'45"E", and 23°00'10.3"S and 30°42'9.9E, respectively (Figure 1). The soil samples were collected from the top profile 0 to 20 cm for both control and mine tailings. The grass and tailing samples were then processed as per procedure of Mulugisi et al. (2009).

Determination of pH and electrical conductivity

The pH and conductivity analysis was based on the procedure of Sampanpanish et al. (2006), where an aliquot of 50 g of mine

tailings/soil was mixed with 50 mL of de-ionized water (1:1 w/w). The contents were stirred for 5 s with a stirring rod and the pH and EC were then simultaneously determined for each sample using the CYBERSCAN CON 500 after calibration as per manufacturer instructions.

The analysis of metal content by ICP MS

The metals total chromium (^{52}Cr) and uranium (^{238}U) in the mine tailings, control samples and grass samples were analysed in duplicate with an Inductively Coupled Plasma Mass Spectrometer (ThermoFischer ICP MS Model, X Series II; ARC Institute of Soil, Climate and Water, Pretoria). The instrument was able to measure more than 40 elements, including ^{52}Cr and ^{238}U , at a detection limit of 0.01 ppb. The instrument was calibrated using the US EPA method 6020A. Analysis was automatic, and data acquisition and processing was controlled by instrument software. The results were expressed as mg per kg and or $\mu\text{g/g}$.

Data analysis

The analytical raw data was processed as per procedure of Mulugisi et al. (2009) and statistical analysis was carried out with Microsoft Excel, single factor ANOVA at a significance level of $p < 0.0$. The bioconcentration factor and translocation factors were calculated as per procedure of Hazrat et al. (2012). The bioconcentration factor (BCF) and translocation factor (TF) data was then log transformed and then analysed for Pearson correlation coef-

Table 1. The levels of total Cr in mine tailings, control site and grass.

Sample site	Parameter	Total Cr content (mg/kg)	<i>Cynodon dactylon</i> * (mg/kg)
Mine tailings A	Range	152.6 to 196.1	379.2 to 519.0
	Mean	174.4 ±30.8	449.1 ± 98.9
Mine tailings B	Range	151.3 to 229.7	314.5 to 518.5
	Mean	190.5 ± 55.4	416.5 ±144.2
Control site	Range	80.1 to 82.6	36.4 to 36.9
	Mean	81.3± 1.3	36.6 ± 0.3

*The sum of total Cr in different plant sections of the grass.

Table 2. The levels of U in mine tailings, control site and grass.

Sample site	Parameter	U content (µg/g)	<i>Cynodon dactylon</i> * (µg/g)
Mine tailings A	Range	0.51 to 0.92	2.92 to 5.41
	Mean	0.72 ± 0.29	4.17 ± 1.76
Mine tailings B	Range	0.85 to 1.06	5.77 to 6.94
	Mean	0.95 ± 0.14	6.36 ± 0.83
Control site	Range	0.68 to 0.78	1.71 to 1.83
	Mean	0.73 ± 0.05	1.77 ± 0.09

*The sum of total Cr in different plant sections of the grass.

Table 3. pH and EC of New Union Gold Mine Tailings and controls.

Parameter	Mine tailing**				Control**
	A1*	A2*	B1*	B2*	
pH	3.34 ± 0.02	3.25 ± 0.07	3.29 ± 0.09	3.29 ± 0.03	7.56 ± 0.01
EC (µS/cm)	1378 ± 59	1568 ± 15	1962 ± 39	1815 ± 6	266 ± 4
Significance difference (p) value	P= 0.00	P = 0.00	P = 0.00	P = 0.00	

*Not significance different ($p > 0.05$); **significance different ($p < 0.05$); Standard error show standard deviation ($n = 2$).

efficient at significance level at $p < 0.05$ and 0.01 using statistical software SPSS 19.0.

RESULTS AND DISCUSSION

The concentration of total Cr and U in mine tailings, soils and grass samples

The levels of total Cr and U were determined in the abandoned New Union Gold Mine tailings, control site and the indigenous grass, *C. dactylon* (Tables 1 and 2). The total Cr levels in the mine tailings were higher than the background levels (controls) by a factor of two. The total Cr in the mine tailings were in excess of the South Africa soil quality standard of 80 mg/kg (Aucamp, 2000). The indigenous grass, *C. dactylon* was able to bioaccumulate more of the total Cr as indicated in Table 1 in comparison with the control grass by a factor of 11 (a

factor of 12.5 for mine tailings A and 11.5 for mine tailings B).

The U levels in the mine tailings were similar to the background levels (controls). The indigenous grass, *C. dactylon* was able to bioaccumulate more of the U as indicated in Table 1 in comparison with the control grass by a factor of 2 (a factor of 2.4 for mine tailings A and 3.6 for mine tailings B). The U content in the New Union Gold Mine tailings was lesser (1.0 µg/g) than the reported U content (2.70 ± 0.03 µg/g) of an abandoned uranium mine tailings in Hungary (Mihucz et al., 2008). The U content in the mine tailings exceeded the South Africa soil quality standard of 80 mg/kg and U has the potential to contaminate the water sources (Aucamp, 2000).

The ability of *C. dactylon* to bioaccumulate more Cr and U might be linked to the mobility and bioavailability of Cr and U as a result of the acidic conditions that were present at the mine tailings (Table 3) (Sampanpanish et al., 2006; Abou-Shanab et al., 2007).

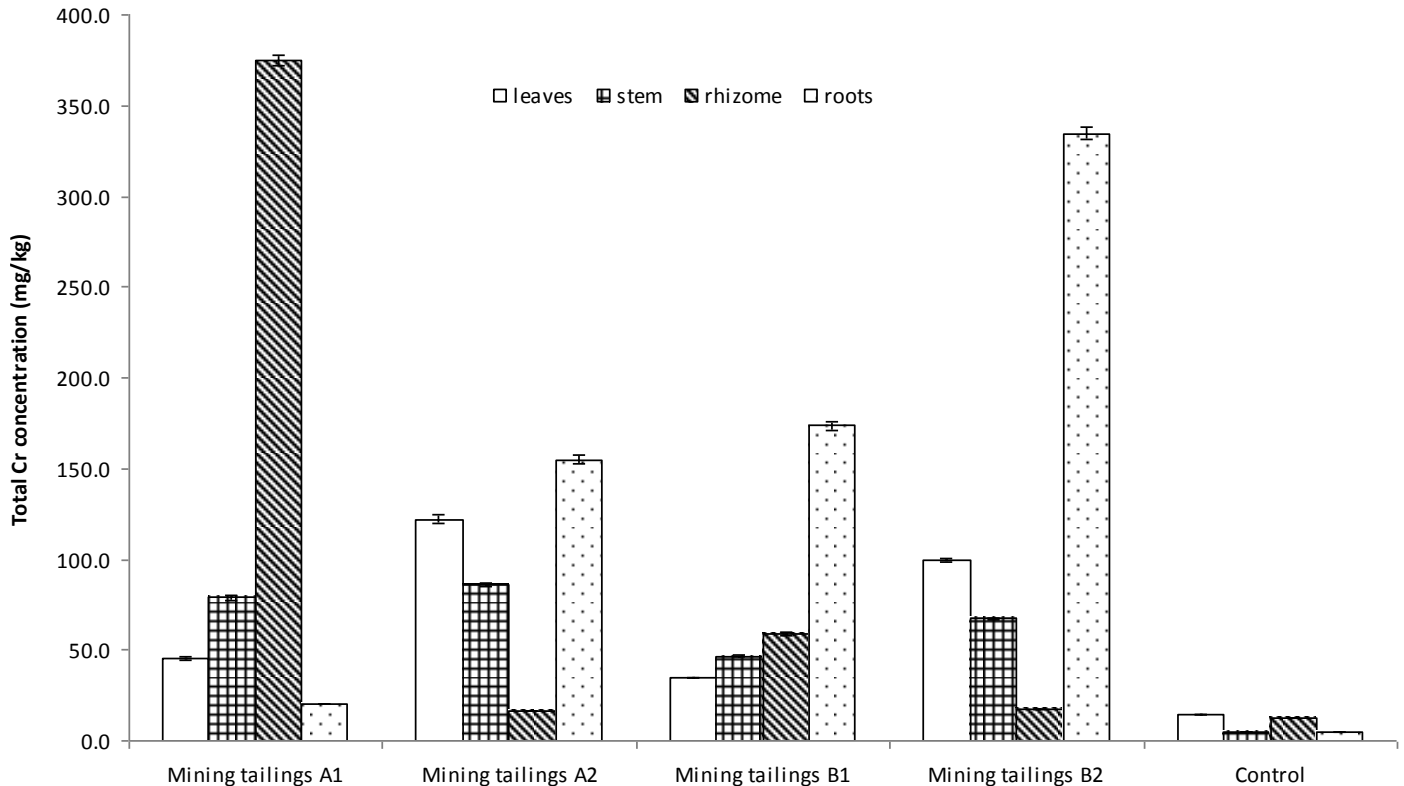


Figure 2. The uptake of total Cr by different tissues of *C. dactylon* grass. Error bars show standard deviation (n = 2).

The uptake of total chromium and its distribution in different tissues of *C. dactylon*

The research findings show that the metals, total chromium (Cr) and uranium (U) were present in the mine tailings, control site and the native grass, *C. dactylon* (Figures 2 and 3). The grass, *C. dactylon* that was growing on the mine tailings accumulated more of total Cr in comparison with the control site (Figure 2) and was significantly different ($p < 0.05$). The reason why grass accumulated more total Cr than the control grass was probably due to low pH of the mine tailings in comparison with the control site where the pH was almost alkaline (Table 1). It is possible that the total Cr content originated from the mine tailings and was taken up by *C. dactylon*. Also, the low pH and high electrical conductivities probably contributed to mobility and bioavailability of total Cr to *C. dactylon*. Abou-Shanab et al. (2007) reported that soil pH plays a significant role in metal uptake by plants.

The accumulation of total Cr in different tissues of *C. dactylon* grass was variable between the mine tailings and the control site (Figure 2). *C. dactylon* grass that grew in mine tailings A1 accumulated the total Cr in different tissues, the accumulation order was rhizome > stem > leaves > roots. The total accumulation of Cr in different tissue of *C. dactylon* was 519.1 mg/kg for mine

tailings A1. For mine tailings A2, the following level of Cr was accumulated in different tissues of *C. dactylon* grass, the highest was in roots > rhizome > stem > leaves. The overall total accumulation of Cr of different tissue of *C. dactylon* grass was 314.6 mg/kg for mine tailings A2. *C. dactylon* grass that grew in mine tailings B1 accumulated the following level of Cr in its different tissues, roots > leaves > stem > rhizome. The total accumulation of Cr for mine tailings B1 in different tissue of *C. dactylon* grass was 379.0 mg/kg. In mine tailings B2, different level of Cr was accumulated in different tissues of *C. dactylon* grass, the highest level was in roots > leaves > stem > rhizome. The total accumulation of Cr for B2 in different tissues of *C. dactylon* grass was 518.6 mg/kg. In the control site, the following level of Cr was accumulated, the highest was in leaves > rhizome > roots > stem. The total accumulation for Cr at the control site for different tissues of *C. dactylon* was 36.7 mg/kg.

The differences in accumulation of total Cr may be attributed to differences in soil pH and electrical conductivity (Table 1) which in turn might influence the mobility and bioavailability of Cr. The differences in total Cr uptake between and within the mine tailings sites may be attributed to the age of the grass shoots. The study of Sampanpanish et al. (2006) in Thailand, at chromium contaminated tannery site, showed that *C. dactylon* young shoots (<30 days) were able to bioaccumulate more

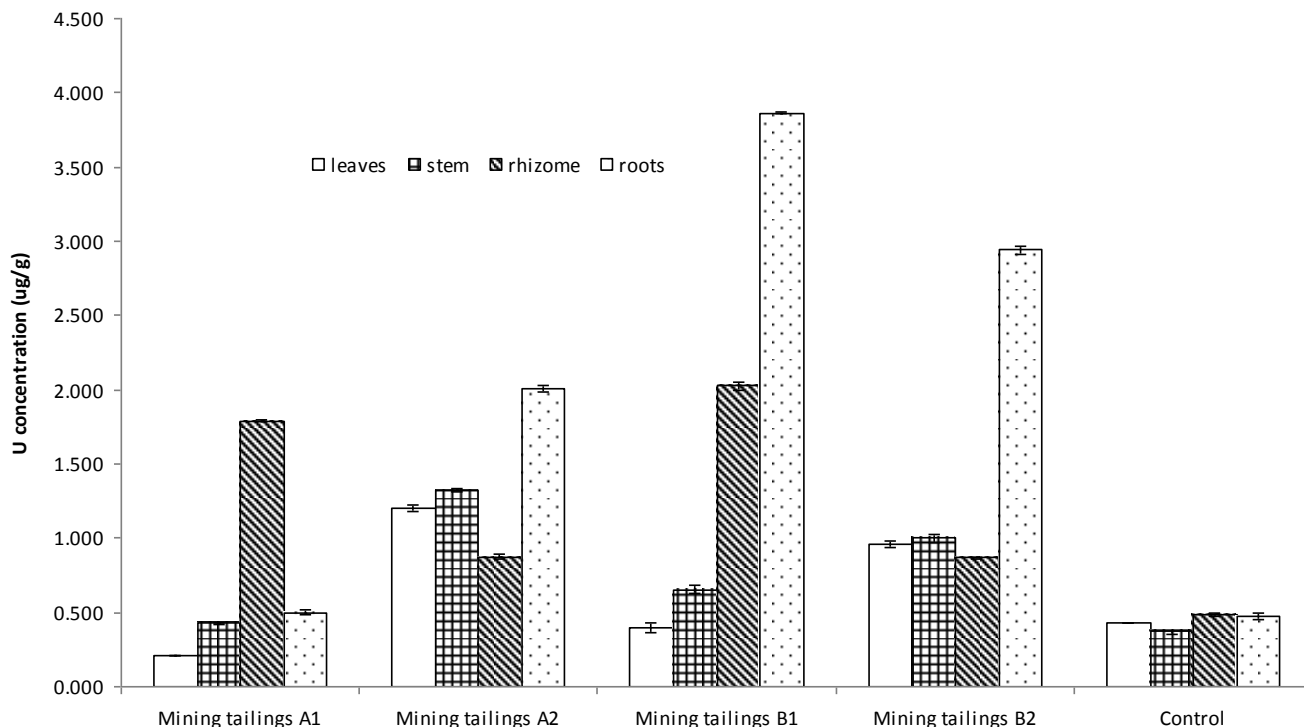


Figure 3. The uptake of U by different tissues of *C. dactylon* grass. Error bars show standard deviation (n = 2).

total Cr in the following accumulation order: roots > stem > leaf. However, for shoots older than (>30 days), the accumulation order was mainly based on the root system as shown by our studies. In our study, the age of the grass was not determined as the grass was collected at random and was found growing at the mine tailings and the control site.

The total Cr content in the mine tailings was less than that of the study of Malarkodi (2007) but was higher than the prescribed Cr standards of developed countries of 150 mg/kg at pH < 7. The research findings of total Cr in the mine tailings (Figure 2) were in excess of the South Africa soil quality standard of 80 mg/kg and Cr has the potential to contaminate the water sources (Aucamp, 2000). But the control sample, the total Cr content (81.3 mg/kg) was slightly above the South Africa soil quality standard of 80 mg/kg.

The bioaccumulation of metals by plants is influenced by factors such as soil pH, electrical conductivity and organic content of the soil and nature and chemical form of the metallic element. The research study shows that the pH of the mine tailings at New Union Gold Mine was highly acidic, whereas at the control site, the pH was slightly alkaline (Table 3). The research findings are similar to the studies of Aucamp (2000) and Naicker et al. (2003) who reported low acidic conditions at gold mine tailings.

There was a significant difference between the pH of the control site and mine tailings ($p < 0.05$) and there was

no significant difference between the mine tailings A and B ($p > 0.05$). The acidic conditions at the mine tailings were probably due to acid mine drainage (AMD) (Sam and Beer, 2000). Thus, during the weathering process (a continuous event), high concentrations of insoluble ferric hydroxide precipitate $\text{Fe}(\text{OH})_3$, dissolved sulphate (SO_4^{2-}) and acid (H^+) are produced. The sulphuric acid produced will seep into adjacent rock or mine tailings and produce secondary reactions that result in heavy metals such as aluminum, manganese, zinc, lead, arsenic and other oxidation products (Sam and Beer, 2000; Mulugisi et al., 2009). In addition to this chemical reaction that produces AMD, the presence of microorganism such as *Thiobacillus ferrooxidans* (Sam and Beer, 2000) accelerates the AMD event. The acidic waters are transported through the subsurface waters by groundwater flow and may become inflows to receiving surface streams. However, if there is sufficient alkalinity, the acidic waters are neutralized but this can be easily overcome when the naturally occurring neutralizing capacity fails and AMD accumulates with pH dropping.

The electrical conductivity (EC) of the tailing samples at New Union Gold Mine tailings was higher than that of the control site (Table 1). There is a significant difference between the EC of the control site and mine tailings ($p < 0.05$). The high EC values of the mine tailings were probably because of the acid mine drainage. Acid mine drainage influences EC to exceed general guidelines of 1200 $\mu\text{S}/\text{cm}$ in the tailing dams due to oxidation process

of pyrite and sulphide minerals upon exposure to atmospheric oxygen and water (James, 1997). The research findings for the mine tailings are similar to that of Aucamp (2000) who reported electrical conductivity range of 1150 to 1711 $\mu\text{S}/\text{cm}$. Mining activities have extremely large global negative impacts on the environment and the greater part of these impacts are from the mine tailings which are typically described by high level of potential toxic metals, low pH, low nutrients, low water retention capacity and also high electrical conductivity (Mulizane et al., 2005; Conesa et al., 2006).

The uptake of uranium and its distribution in different tissues of *C. dactylon*

The results show that the grass, *C. dactylon* absorbed more of the uranium (U) in mine tailings and the control site, and was significantly different, $p < 0.05$ (Figure 3). Furthermore the accumulation of U in different tissues of *C. dactylon* grass varied between the mine tailings and the control site and also within the mine tailings. This might be explained by the mobility and bioavailability of U due to the acidic conditions that were present at the mine tailings. Under alkaline conditions present at the control site, the U was less mobile and therefore not bioavailable. This is supported by the study of Gavrilescu et al. (2009) who showed that the mobility and availability of uranium was dependent on a number of soil factors such as pH, redox potential, soil moisture levels and microbial activity and the uranyl ion was stable in aqueous solutions of $\text{pH} < 2.5$.

The differences in U uptake between and within the mine tailings sites may be attributed to the age of the grass shoots. The study of Sampanpanish et al. (2006) in Thailand, at chromium contaminated tannery site, showed that *C. dactylon* young shoots (<30 days) were able to bioaccumulate more total Cr in the following accumulation in the order roots > stem > leaf. However, for shoots older than (>30 days), the accumulation order was mainly based on the root system as indicated by our results. The presence of U in the leaves of the indigenous grass may be a potential hazard to animal grazers. The study of Winde (2010) showed that the presence of Cd, which was shown in the mine tailings and *C. dactylon* grass (Mulugisi et al., 2009) may lead to higher levels of U toxicity.

The effects of uranium on human health are not immediate and it may take several years before any adverse consequences are recognized. Au et al. (1995, 1998) investigated whether residents residing near uranium mining operations, who were potentially exposed to toxicants from mining waste, had increased genotoxic effects when compared with people residing elsewhere. The authors found that uranium concentrations in soil samples were significantly higher in the target area than those in the control areas. Thus, the mine tailings have

health issue and environmental concern, such as lung cancer, leukemia, stomach cancer and birth defects, to the whole population in the area surrounding the mine (Au et al., 1998).

The negligence of toxic metals resulted in the relocation of toxic metals to the surrounding environment and, contributes to soil contamination, ground and surface water contamination (Liu et al., 2006). Toxic metals contamination in soil is one of the major sources of toxic metals in groundwater and surface water (Fayiga et al., 2004). The contamination of surface and groundwater by toxic metals pose a particular threat to the health of the community and animals around that particular area affected by toxic metals such as radioactive metals, especially to the community that drink this water without any proper treatment (Winde et al., 2004; Bitala and Kweyunga, 2009).

Bioconcentration and translation in *C. dactylon*

The study of Al-Qahtani (2012) shows that the bioaccumulation of metals by plants is influenced by factors such as soil pH, electrical conductivity and organic content of the soil, and oxidation state of the metallic element. The research findings show that the BCF ratio was variable between the *C. dactylon* grass that grew on mine tailings and the control (Table 4). This BCF variability may be attributed to metal mobility and bioavailability as a result of acidic conditions. Al-Qahtani (2012) stated that neutral to alkaline conditions restrained metal mobility and therefore the metal bioavailability in plant uptake and translocation into plant tissues. Our study show that at the acidic mine tailings, the BCF was less than 1 with exception of the root system which was greater than 1 for mine tailings such as A2 and B2 (Table 4). Also, the alkaline conditions probably contributed to low BCF ratios for the control site. There was no significant correlation between $\text{pH} > 3.34$ for mine tailings A1 and control site; Pearson correlation coefficient of 0.456 at $p > 0.05$ (2 tailed). Whereas, at $\text{pH} < 3.29$, there was significant correlation between mine tailings A2 and B1, Pearson correlation coefficient of 0.740 at $p < 0.05$ (2 tailed) and between B1 and B2, Pearson correlation coefficient of 0.861 at $p < 0.01$ (2 tailed). It appears that the bioaccumulation of metals (Cr and U) was influenced by acidic $\text{pH} < 3.29$.

The use of translocation factor (TF) indicates the mobility of metal uptake from soil to root, rhizome, shoot and leaves. According to Al-Qahtani (2012), the TF ratio is a suitable pointer to whether the plant is an accumulator, excluder or indicator. Our study shows that there was considerable variation in the TF ratios between the *C. dactylon* grass that grew within the acidic mine tailings and the alkaline control site (Table 5). The research findings show that at the acidic mine tailings, the TF was less than 1 with exception of the root system which was

Table 4. Bioconcentration factors (BCF) of *C. dactylon* for Cr and U.

Parameter	Mine tailing				Control
	A1	A2	B1	B2	
pH	3.34 ± 0.02	3.25 ± 0.07	3.29 ± 0.09	3.29 ± 0.03	7.56 ± 0.01
Root Cr	0.104	1.014	0.756	2.211	0.063
Rhizome Cr	1.910	0.108	0.258	0.113	0.152
Stem Cr	0.402	0.564	0.204	0.445	0.060
Leaves Cr	0.231	0.798	0.152	0.657	0.175
Root U	0.972	2.176	3.661	3.445	0.659
Rhizome U	3.485	0.951	1.918	1.019	0.667
Stem U	0.831	1.435	0.622	1.173	0.519
Leaves U	0.405	1.306	0.375	1.127	0.589

Table 5. Translocation factors (TF) of *C. dactylon* for Cr and U.

Parameter	Mine tailing				Control
	A1	A2	B1	B2	
pH	3.34 ± 0.02	3.25 ± 0.07	3.29 ± 0.09	3.29 ± 0.03	7.56 ± 0.01
Leaves Cr	2.236	0.787	0.200	0.297	2.784
Stem Cr	3.882	0.556	0.270	0.201	0.961
Rhizome Cr	18.448	0.107	0.342	0.051	2.431
Leaves U	0.417	0.600	0.102	0.135	0.892
Stem U	0.855	0.659	0.170	0.223	0.786
Rhizome U	3.586	0.437	0.524	0.689	1.011

greater than 1 for mine tailings such as A2 and B2 (Table 5).

There was significant correlation between pH > 3.34 for mine tailings A1 and control site; Pearson correlation coefficient of 0.906 at $p < 0.01$ (2 tailed) whereas at pH < 3.29, there was significant correlation between mine tailings A2 and B1, Pearson correlation coefficient of 0.833 at $p < 0.05$ (2 tailed) and between B1 and B2, Pearson correlation coefficient of 0.947 at $p < 0.01$ (2 tailed). It appears that the mobility and metal translocation of metals (Cr and U) was influenced by soil pH. The bioaccumulation of U and total Cr by *C. dactylon* has the potential of transferring these heavy metals to animal grazers (cattle and goats) that visit the mine tailings for lavish grass during summer as shown by the study of Mothetha (2009).

Conclusion

The study shows that total Cr and U was present in mine tailings and in the indigenous grass, *C. dactylon*. This was evident from the BCF and TF values which indicate that the *C. dactylon* was able to uptake the metals within the different tissues. The low pH and high electrical conductivity further enhances the mobility and bio-availability of heavy metals for *C. dactylon* uptake. The

presence of these heavy metals in mine tailings and grass indicates the potential to contaminate the water sources and the environment bearing in mind that U contamination has long term negative impact due to its radioactivity. For animal grazers, the consummation of *C. dactylon* maybe the route for transfer of the heavy metals from mine tailings to humans. Lastly, *C. dactylon* has the potential to be used as vegetative cover for the exposed mine tailings to prevent water and wind erosion.

ACKNOWLEDGEMENTS

The Chief of Madonsi village, community and Triangle cc is appreciated for giving us permission to have access to the New Union Gold Mine. The University of Venda gave financial support for the study project (I431) and travel costs to attend and be present at the IGCP/SIDA PROJECT 606: workshop on Environmental Health Impacts of Major and Abandoned Mines in Sub-Saharan Africa. We acknowledge the funding from NRF (CUPP) grant linked holder scholarship for Mr K. Nelushi.

REFERENCES

Aucamp PJ (2000). Trace element pollution of soils by abandoned gold mine tailings near Potchefstroom, South Africa. Masters thesis.

- Unpublished. University of Pretoria. (date accessed 10/03/2011).
- Abou-Shanab R, Ghanem N, Ghanem K, Al-Kolaibe A (2007). Phytoremediation potential of crop and wild plants for multi-metal contaminated soils. *Res. J. Agric. Biol. Sci.* 3:370-376.
- Al-Qahtani KM (2012). Assessment of Heavy Metals Accumulation in Native Plant Species from Soils Contaminated in Riyadh City, Saudi Arabia. *Life Sci. J.* 9:384-392.
- Au WW, Lane RG, Legator MS (1995). Biomarker monitoring of a population residing near uranium mining activities. *Environmental Health Perspective.* 103:466-470.
- Au WW, McConnell MA, Wilkinson GS, Ramanujam VMS, Alcock N (1998). Population monitoring: experience with residents exposed to uranium mining/milling waste. *Mutation Research/Fundamental and Molecular Mechanisms.* 405:237-245.
- Bitala MF, Kweyunga C (2009). Level of Heavy Metals and Cyanide in Soil, Sediment and Water from Vicinity of North Mara Gold Mine in Tarime District, Tanzania. http://www.olca.cl/oca/tanzania/north_mara_pollution_report.pdf (date accessed 21/06/2012).
- Cheunga K, Gua J (2007). The Mechanism of hexavalent chromium detoxification by Microorganisms and bioremediation application potential: A review. *Int. Biodeter. Biodegrad.* 59:8-15.
- Conesa HM, Faz Á, Arnaldos R (2006). Tolerant Plant Species to Heavy Metals that grow at Mining Tailings in the semiarid Cartagena-La Unión Mining District (SE Spain). *Sci. Total Environ.* 36:1-11.
- Conesa HM, García G, Faz Á, Arnaldos R (2007). Dynamics of metal tolerant plant communities' development in mine tailings from the Cartagena-La Unión Mining District (SE Spain) and their interest for further revegetation purposes. *Chemosphere.* 68:1180-1185.
- Cowart JB, Burnett WC (1994). The distribution of uranium and thorium decay-series radionuclides in the environment- a review. *J. Environ. Quality.* 23:651-662.
- Fayiga AO, Ma LQ, Cao X, Rathinasabapathi B (2004). Effects of heavy metals on growth and arsenic accumulation in the arsenic hyperaccumulator *Pteris vittata* L. *Environ. Pollut.* 132:289-296.
- Gavrilescu M, Vasile L, Cretescu I (2009). Characterization and remediation of soils contaminated with uranium. *J. Hazard. Mater.* 163:475-510.
- Han FX, Banin A, Su Y, Monts DL, Plodinec MJ, Kingery WL, Triplett GB (2002). Industrial age anthropogenic inputs of heavy metal into the pedosphere. *Naturwissenschaften.* 89:497-504.
- Hazrat A, Naseer M, Sajad MA (2012). Phytoremediation of heavy metals by *Trifolium alexandrinum*. *Int. J. Environ. Sci.* 2:1459-1469.
- James AR (1997). The Prediction of Pollution Loads from Coarse Sulphide-Containing Waste Materials. Water Research Commission (WRC) Report No. 559/1/97. Pretoria
- Liu Y, Zhang H, Zeng G, Huang B, Li X (2006). Heavy metal accumulation in plants on Mn mine tailings. *Pedosphere.* 16:131-136.
- Ma L Q, Komar KM, Tu C, Zhang W, Cai Y, Kennelly ED (2001). A fern that hyperaccumulates arsenic. *Nature* 409:579.
- Maiti SK, Jaiswal S (2008). Bioaccumulation and translocation of metals in the natural vegetation growing on fly ash lagoons: A field study from Santaldih thermal power plant, West Bengal, India. *Environ. Monit. Assess.* 136:355-370.
- Malarkodi M (2007). Remediation of metals contaminated soils using plants- A review. *Agric. Rev.* 28:107-117.
- Mihucz VG, Varga Z, Tatár E, Virág I, Van GR, Koleszár Z, Záray G (2008). Redistribution of uranium and thorium by soil/plant interaction in a recultivated mining area. *Microchemistry J.* 90:44-49.
- Mulizane M, Katsvanga CAT, Nyakudya IW, Mupangwa JF (2005). The Growth Performance of Exotic and Indigenous Tree Species in Rehabilitating Active Gold Mine Tailings Dump at Shamva mine in Zimbabwe. *J. Appl. Sci. Environ. Manag.* 9:57-59.
- Limpopo Province. Honours thesis (unpublished). University of Venda, Thohoyandou.
- Mothetha ML (2009). Assessment of environmental impacts from a dysfunctional New Union Gold Mine: A case study from Malamulele, Mulugisi G, Gumbo JR, Dacosta FA, Muzerengi C (2009). The use of indigenous grass species as part of rehabilitation of mine tailings: A case study of New Union Gold Mine. Proceedings of the International Mine Water Conference. Pretoria, South Africa.
- Naicker K, Cukrowska E, McCarthy TS (2003). Acid mine drainage arising from gold mining activity in Johannesburg, South Africa and environs. *Environ. Pollut.* 122:29-40.
- Ogola JS, Mutuilah WV, Omulo MA (2002). Impacts of gold mining on the environment and human health: A case study in the Migori Gold Belt, Kenya. *Environmental Geochemistry and Health.* 24:141-158.
- Oliveira H (2012). Chromium as an Environmental Pollutant: Insights on Induced Plant Toxicity. *J. Bot.* Doi:10.1155/2012/375843.
- Padmavathiamma PK, Li LY (2007). Phytoremediation Technology: Hyper-accumulation metals in plants. *Water, Air Soil Pollut.* 184:105-126.
- Sam III JI, Beer KM (2000). Effects of coal mine drainage on stream water quality in the Allegheny and Monogahela River Basins Sulfate Transport and Trends http://pa.water.usgs.gov/reports/wrir_99-4208.pdf (Date assessed 01/08/2011).
- Sampanpanish P, Pongsapich W, Khaodhiar S, Khan E (2006). Chromium removal from soil by phytoremediation with weed plant species in Thailand. *Water, Air and Soil Pollution: Focus.* 6:191-206.
- Saraswat S, Rai JPN (2009). Phytoextraction potential of six plant species in multimetal contaminated soil. *Chemist. Ecol.* 25:1-11.
- Schnoor JL (1997). Phytoremediation, TE-97-01, Groundwater Remediation Technologies Analysis Center, Pittsburg, PA.
- Soleimani M, Hajabbasi MA, Afyuni M, Charkhabi AH, Shariatmadari H (2009). Bioaccumulation of Nickel and Lead by Bermuda grass (*Cynodon dactylon*) and Tall Fescue (*Festuca arundinacea*) from two contaminated soils. *Caspian J. Environ. Sci.* 7:59-70.
- WHO (World Health Organization) (2011). Water quality guidelines. 4th edition. Geneva. http://whqlibdoc.who.int/publications/2011/9789241548151_eng.pdf (date assessed 22/06/2012)
- Winde F (2010). Uranium pollution of the Wonderfontein spruit, 1997-2008 Part 1: Uranium toxicity, regional background and mine-related sources of uranium pollution. *Water SA.* 36:239-256.
- Winde F, Wade P, Van der Walt IJ (2004). Gold tailings as a source of waterborne uranium contamination of streams-the Koekmoerspruit (Klerksdorp Goldfield, South Africa) as a case study. Part I of III: Uranium migration along the aqueous pathway. *Water SA.* 30:219-239.
- Wong HKT, Gauthier A, Nriagu JO (1999). Dispersion and toxicity of metals from abandoned gold mine tailings at Goldenville, Nova Scotia, Canada. *Sci. Total Environ.* 228:35-47.
- Wuana RA, Okieimen FE (2011). Heavy metals in contaminated soils: a review of sources, chemistry, risks and best available strategies for remediation. *ISRN Ecology.* Doi:10.5402/2011/402647
- Zayed AM, Terry N (2003). Chromium in environment: Factors affecting biological remediation. *Plant Soil* 249:135-156.

Full Length Research Paper

***In vitro* cultivation of *Pleurotus ostreatus* and *Lentinula edodes* in lignocellulosic residues from Amazon**

Ceci Sales-Campos¹, Diego A. Pires¹, Samira R. L. Barbosa¹, Raimunda Liége S. Abreu¹ and Meire C. N. Andrade^{2*}

¹Instituto Nacional de Pesquisas da Amazônia- INPA, Coordenação de Tecnologia e Inovação. Av. André Araújo, 2936 - Aleixo. Caixa Postal 478. CEP 69060-001. Manaus, AM, Brasil.

²Universidade do Sagrado Coração- USC, Centro de Ciências Exatas e Sociais Aplicadas. Rua Irmã Arminda, 10-50, Jardim Brasil. CEP: 17011-160. Bauru, SP, Brasil.

Accepted 23 October, 2013

The mycelial growth speed of *Pleurotus ostreatus* (POS 09/100) and *Lentinula edodes* (LED 96/13) was evaluated in four substrates based on *Simarouba amara* sawdust, *Anacardium giganteum* sawdust, *Euterpe precatoria* seeds and *Musa* sp. AAB stems. The loss of organic matter of the substrates and the mycelial vigor of the strains studied were also evaluated. The greatest mycelial growth for *P. ostreatus* occurred in the substrates formulated with *S. amara* sawdust (29.45 cm³/day) and *A. giganteum* (27.58 cm³/day). The best performance for *L. edodes* occurred in the substrate of *A. giganteum* (13.22 cm³/day), followed by the *S. amara* (11.30 cm³/day). The most intense rates of vigor were presented in the *E. precatoria* substrate which was added with brans. The most significant loss of organic matter for both mushrooms occurred in the substrate formulated with a supplement of *A. giganteum* (54% for *P. ostreatus* and 61% for *L. edodes*). There was no mycelial growth in the formulation prepared with banana stem in both mushrooms tested.

Key words: Edible fungi, mycelial growth, alternative substrates.

INTRODUCTION

The importance of mushrooms cultivation is significant because it represents an efficient choice to enable the use of organic matter for bioconversion into high-value added products, such as edible mushrooms (Sales-Campos et al., 2010). *Pleurotus ostreatus* and *Lentinula edodes* are among the species with a great economic importance for cultivation. *L. edodes* is high quality food, full of proteins, vitamins and minerals, and low fat and calories, thus being widely suggested for cultivation and consumption (Andrade et al., 2008). According to the study of Bononi et al. (1999), its worldwide consumption has increased significantly, and some European and

American countries have been importing this mushroom more and more. There is a potential consumer market in Brazil, although there are only some small producers, despite the presence of a huge Asian colony in the country.

On the other hand, the consumption of *P. ostreatus* has increased significantly in the last years because of its outstanding taste and great availability in the market, occupying the fourth position in the world production of edible mushrooms (Bononi et al., 1999). The *Pleurotus* genus fungi compete with the other edible fungi due to their growth speed and ability to adapt to a series of sub-

*Corresponding author. E-mail: mcnandrade@hotmail.com. Tel: +55 14 2107-7034.

strates (Vieira et al., 2013), which happens because of an enzymatic complex composed of cellulases, ligninases, laccases, xylanases, peroxidases and hemicellulases (Elisashvili et al. 2008). The behavior of the mycelial growth may vary according to a series of factors, such as the fungi species, genetic characteristics of the strain, temperature, type of substrate, supplementation, carbon/nitrogen ratio, and others (Andrade et al., 2008; Sales-Campos et al., 2008). Several steps must be taken in the production of edible mushrooms from the obtention of the inoculum to the sale of the final product, as stated by Donini et al. (2005).

Studying the conditions of the use of wood in industries from Amazonas, Sales-Campos et al. (2000) verified a loss of up to 60% of this raw material due to lack of technology and appropriate handling, thus generating a great amount of waste with an underestimated potential. The residues generated by this sector in the Amazon region have caused environmental pollution, and some alternatives have been found for making good use of these residues (Vianez and Barbosa, 2003). However, little has been done to soothe this matter. Sales-Campos et al. (2010) showed the use of different wood and agro-industrial residues of the region for the cultivation of edible fungi. Nevertheless, the author reports the need of testing the different substrates in relation to the fungus to be cultivated. Therefore, the objective of the present study was to test the viability of regional residues, aiming at their future use in the cultivation of edible mushrooms, by evaluating the mycelial growth speed of *P. ostreatus* and *L. edodes* in substrates formulated with regional lignocellulosic residues.

MATERIALS AND METHODS

The present study was carried out at the Laboratory of Edible Fungi Cultivation of the Coordination of Technology and Innovation (CTI) of the National Institute for Amazon Research (INPA), Manaus, Amazonas, Brazil. The residues were selected according to the production of local residues. *Simarouba amara* Aubl. (marupá) sawdust and *Anacardium giganteum* Hanc. Ex. Engl. (cajuí) sawdust, obtained from researchers at the CTI/INPA, were used as wood residues. The materials were processed at the CTI saw-mill, dried in solar dryer and stored in plastic bags for the formulation of the substrates. The agro-industrial residues were *Musa* sp. AAB cv. Pacova stalks (pacovã banana) and *Euterpe precatoria* Mart. (açai), which were ground in a DPM4 forage grinder, following the same drying and storage processes used for the wood residues.

The preparation of the primary matrix (inoculum) was carried out by transferring small pieces of the mycelium of the fungus (stored inside test tubes) to Petri's dishes containing BDA medium (Bononi et al., 1999). Next, the dishes were incubated inside a biological oxygen demand (BOD) at 25°C for the mycelial growth of the fungus until it reached about three quarter of the dishes. Then, they were kept in cold storage at 4°C to stop the mycelial growth until the experiment was assembled. The *P. ostreatus* strain used was POS 09/100 and the *L. edodes* was LED 96/13, which were obtained from the collection of the School of Agronomic Sciences of the São Paulo State University (FCA/UNESP). The substrates used

in this experiment followed the methodology adopted by Sales-Campos et al. (2010), with the ratios presented in Table 1. CaCO₃ was used for the pH adjustment of the substrates. The substrates were homogenized, moistened for around 75% of humidity and disposed in 500 ml bottles, and each bottle was filled with 200 g of the substrate. Next, they were sterilized at 121°C, under the pressure of 1 atm. Soon after sterilization, they were cooled up to the environmental temperature and taken to a previously sterilized laminar flow chamber in order to avoid contamination and, thus, receive the inoculum from the previously prepared primary matrixes.

The mycelial growth was measured by four millimetric tapes, which were placed equidistantly outside the bottles. The bottles were then stored in a BOD at 25°C, which is considered the optimal temperature for the fungal growth (Bononi et al., 1999). The measurement was performed daily to the point of total colonization of the substrates by the fungus. To compare the growth speed (cm³/day), data from the 15th day was collected, when one of the fungi reached the bottom of the bottle. The averages of the volumetric mycelial growth were obtained by calculating the cylindrical volume of the colonized solid medium ($B \times r^2 \times H$) and the average speed was evaluated by the ratio between the average volume of growth and the time spent for such growth (Gonçalves, 2002). A subjective scale was used to explain the results of the analysis of the mycelial vigor of the fungi in the different substrates: *, weak; **, regular; ***, good; ****, intense. For calculating the percentage of organic matter loss, the colonized substrates were daily dried and weighed until they obtained constant weight in order to be compared with the weights of the substrates that were not colonized by the fungi (control). The following formula was used:

$$\text{PMO (\%)} = \frac{\text{Mass of initial substrate} - \text{mass of residual}}{\text{Mass of initial substrate}} \times 100$$

The design of the present experiment was totally randomized 8 x 2 (types of substrates x strains) with 5 repetitions each, totalizing 80 bottles. The experimental results were submitted to parametric variance analysis by the Tukey test at the level of 5% of significance, using the Sisvar 4.2 software. The Origin 3.0 software by MicroCal was used to compare the parameters of the polynomial regression model.

RESULTS AND DISCUSSION

When comparing the different substrates analyzed, the mycelial growth of POS 09/100 and LED 96/13 showed a significant difference in all the treatments, except in the treatments formulated with banana stalks, in which neither of the strains tested grow (Table 2). The mycelial growth for *P. ostreatus* was reached in the SIA-MA SUPL treatment, although it was not statistically different from the SIA-MA N SUPL, the SIA-CA N SUPL and the SIA-AÇA SUPL.

The best result for *L. edodes* occurred for the substrate SIA-CA SUPL, which showed no statistical difference from the SIA-MA SUPL. It was also observed, in a general way, that the supplementation also favored the growing rate of the fungus in the substrate based on marupá (SAI-MA SUPL), as Sales-Campos et al. (2008) evaluated when they analyzed the mycelial kinetic of *P. ostreatus* grown in *S. amara* sawdust to determine the

Table 1. Mixing ratio of ingredients for each substrate formulation.

Substrate	Ingredients %					
	Marupá sawdust	Cajuí sawdust	Açaí seed	Banana marc	Bran mixture	CaCO ₃
MA-SUPL	80				18	2
CA-SUPL		80			18	2
AÇA-SUPL			80		18	2
BAN-SUPL				80	18	2
MA N SUPL	98					2
CA N SUPL		98				2
AÇA N SUPL			98			2
BAN N SUPL				98		2

Mixture of bran cereal, rice, corn and wheat in proportions of 7:2.5:0.5.
Represents the SUPL supplemented substrates and N SUPL not supplemented.

Table 2. Mean speed of mycelial growth (cm³ / day) strains POS 09/100 and LED 96/13 on different substrates based on regional lignocellulosic residues, after 15 days of incubation.

Lineage	Substrate							
	SIA-MA SUPL	SIA-MA Ñ SUPL	SIA-CA SUPL	SIA-CA Ñ SUPL	SIA-AÇA SUPL	SIA-AÇA Ñ SUPL	SIA-BAN SUPL	SIA-BAN Ñ SUPL
POS 09/100	29.45 Aa	27.30 Aab	24.57 Ab	27.58 Aa	26.75 Aab	19.12 Ac	0.00 Ad	0.00 Ad
LED 96/13	11.30 Ba	1.40 Bcd	13.22 Ba	3.71 Bc	1.47 Bcd	7.43 Bb	0.00 Ad	0.00 Ad

Means followed by the same letters, lowercase and uppercase on each line in each column do not differ by Tukey test at the 5% level of significance ($p < 0.05$).

Table 3. Subjective scale of force mycelial growth on different substrates based on regional lignocellulosic residues.

Treatment	Vigor/substrate	
	POS 09/100	LED 96/13
SIA-MA SUPL	***	**
SIA-MA N SUPL	*	*
SIA-CA SUPL	***	***
SIA-CA N SUPL	**	*
SIA-AÇA SUPL	****	****
SIA-AÇA N SUPL	***	***

*, Weak; **, regular; ***, good; ****, intense.

optimal condition for the mycelial growth. Through the experiment, we verified that there was no growth in the substrates formulated with banana stalks, even with the supplementation of cereal brans. Motato et al. (2006) evaluated the capacity of mycelial growth of *Pleurotus djamor* in banana (*Musa paradisiaca*) agroindustrial residues and abarco (*Cariniana pyriformis*) sawdust under different temperatures and also verified that there was no growth in the substrates formulated with banana

stalks, concluding that the pure banana stalks or their mixture with sawdust was not appropriate for the cultivation of this fungus. The probable cause for the restraint in the fungal growth in substrates based on stalks (BAN SUPL and BAN N SUPL) is the presence of a high amount of hydrolysable tannins.

In the evaluation made by Soffner (2001) about the potential use of stem of *Musa sp* for pulp production, approximately 46.75% of extractives were found in their chemical composition, which are included in this category of hydrolysable tannins. However, when conducting the processes of washing and pre-aqueous extraction of stem for the purpose of reducing the content of the extractives soluble in water, the author found the washing procedure, followed by pre-extraction aqueous, removed 46.45% of the constitution of the material with an extractive removal efficiency of both fungi showing different vigor for each substrate as shown in Table 3. The results show a relationship between the bran supplementation and the mycelial vigor, where the supplementation promotes the intensity of force in both fungal lineages tested. Observing the two lineages (POS 09/100 and LED 96/13), a greater vigor occurred in the SIA-ACA SUPL substrate, however, it showed a lower growth rate compared to other substrates, by approximately

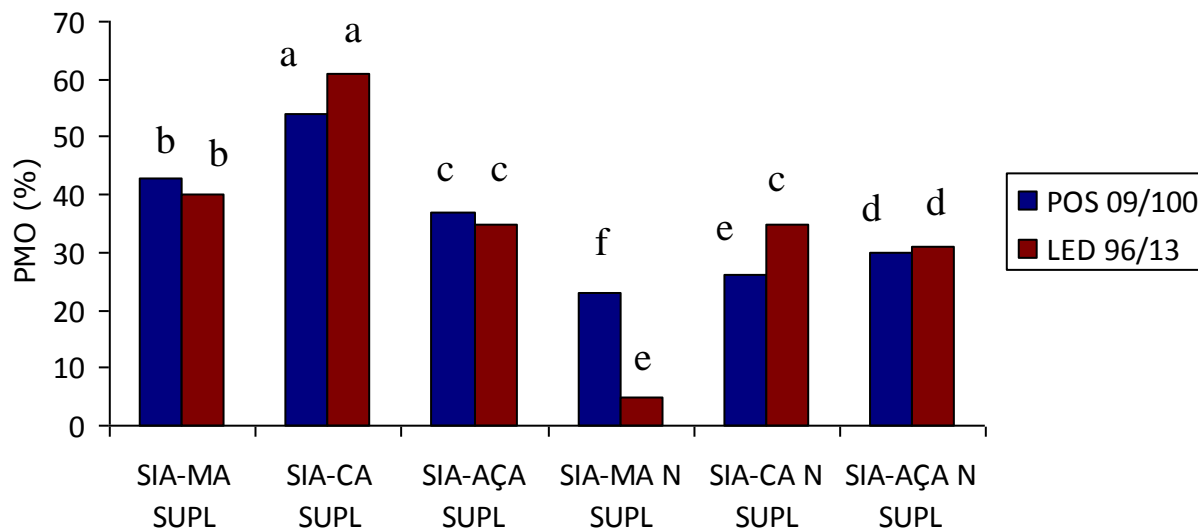


Figure 1. Loss of organic matter (PMO) (%) obtained with *Pleurotus ostreatus* (POS 09/100) and *Lentinula edodes* (LED 96/13) in regional lignocellulosic residues. Means comparison of treatments in each line with the same letter do not differ by Tukey test at 5% significance.

89.7%. Both fungi showed different vigor for each substrate as shown in Table 3. The results show a relationship between the supplementation with bran and mycelial vigor, where the supplementation promotes the intensity of force in both fungal lineages tested. By observing the two lineages (POS 09/100 and LED 96/13), a greater vigor occurs on the SIA-ACA SUPL substrate; however, it showed a lower growth rate compared to other substrates.

Pedra and Marino (2006), during the evaluation of the mycelial growth, induction of primordia precocity and productivity of mushrooms *Pleurotus* spp. in sawdust coconut shell (supplemented with bran) as an alternative substrate for the production of edible mushrooms, found that supplementation with 20 or 40% wheat bran and rice increased the speed and mycelial vigor growth of three strains of *Pleurotus* spp. The present study found similar results, where supplementation improved the rate of mycelial growth and vigor of both lineages.

In the analysis of loss of organic matter (PMO) of the substrates used in the cultivation of POS 09/100, LED 96/13, it was observed that treatment SUPL CIS-CA had the highest amount of loss in both strains (54 and 61%, respectively) differing from the other treatments (Figure 1). The study of Furlan et al. (2000) addressing the adaptation of the production technology of edible mushrooms of the genus *Pleurotus* waste of irrigated rice, supplemented by determining the substrate that best promotes the production, indicated that supplementation had no effect on the rate of PMO where the greatest loss was attributed to treatment Y1 supplemented with a 1% yeast extract (68%), an increase of approximately 9% com-

pared to the control, which received no supplementation. In the present study, the greatest PMO also occurred on substrates that received supplementation, although no significant difference was found between strains. This explains why supplementation in the right dose promotes colonization and subsequent decomposition of the substrate, since the fungus removes greater amounts of nutrients for production.

Figure 2 shows a volumetric growth rate (cm³/day) of edible fungus *P. ostreatus* (POS 09/100) and *L. edodes* (LED 96/13) on different substrates. The results of the polynomial regression curves show a very wide instantaneous daily growth for each species, with different peaks being indicated for each day of incubation (not understood). The mycelial growth on substrates started only from the 3rd day of incubation, and showed an oscillation in its speed over the days.

By analyzing the POS 09/100, polynomial regression curves show a fairly homogeneous growth rate of the fungus in each substrate examined, however, the treatment MA SUPL SIA achieved the best average speed at the outset of colonization, which then remained at a steady speed. The treatment CIS-ACA SUPL grew very slowly on the seventh day of incubation. However, it showed a very high peak on the eighth day and decreased again, re-turning to grow slowly during the day. In the analysis of lineage LED 96/13, there was a variation in speed between the substrates analyzed where we could observe that the highest growth rate occurred with the treatment SIA CA-SUPL. As for the SIA-CA N SUPL, which showed a speed of decreasing colonization, one can observe that, from the fourth day of

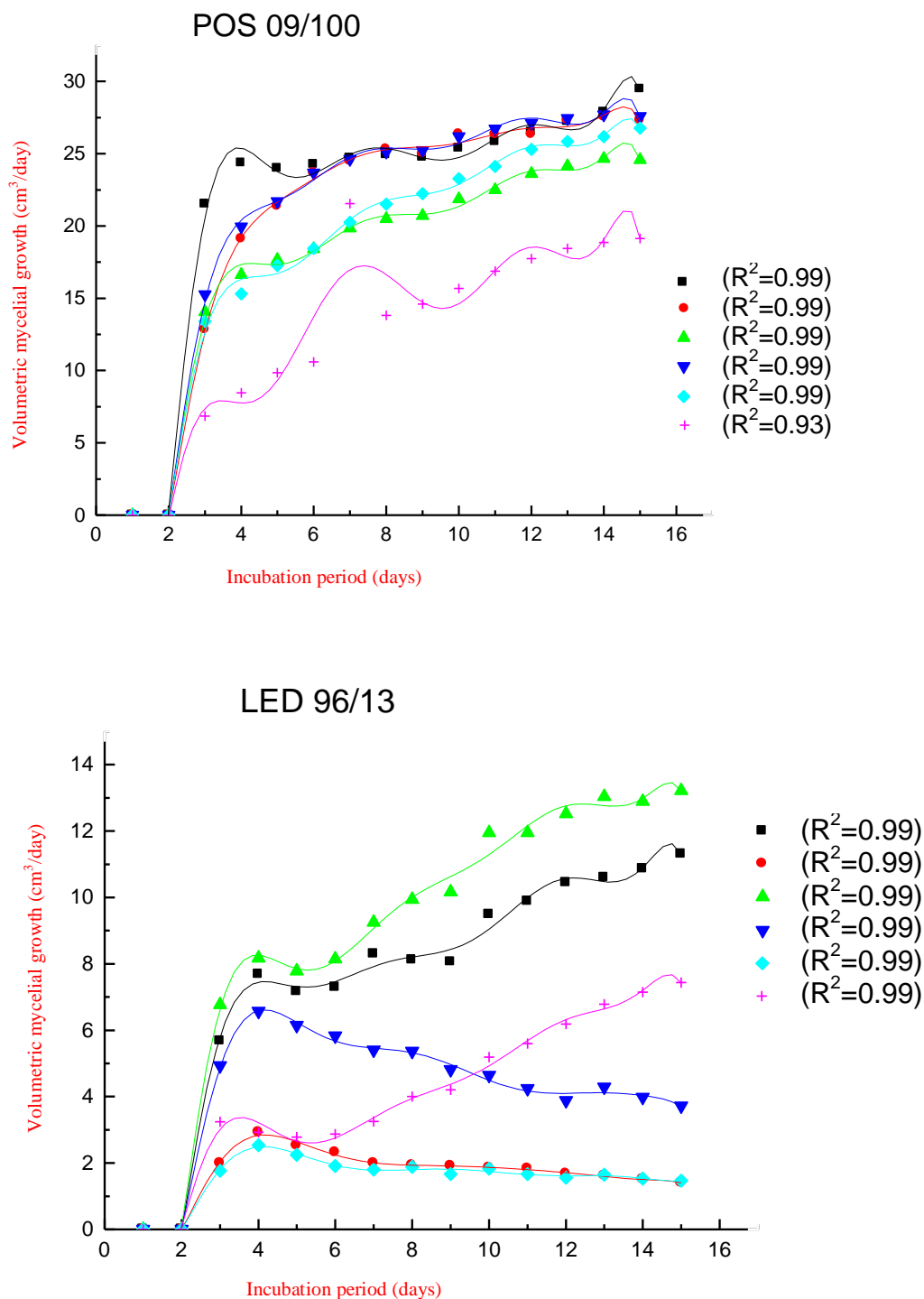


Figure 2. Instant volumetric mycelial growth (cm³/day) from the edible fungus *Pleurotus ostreatus* (POS 09/100) and *Lentinula edodes* (LED 96/13) in different substrates.

incubation, its speed began to decrease gradually. Rossi et al. (2000) argue, in assessing the rate of mycelial growth of *L. edodes*, the effect of the depth of the bottle

used and the substrate supplementation with rice bran and molasses from sugar cane, the mycelium of *L. edodes* grows faster when it is nearer the surface of the

substrate, since higher gas exchange occurs, allowing an increased oxygen supply, lignocelulolítica higher activity and thus a greater growth rate.

Conclusion

There was a positive effect on supplementation of cereal bran in all treatments, which allowed a higher growth rate and vigor of mycelial strains. The substrates formulated based on banana stem were not presented as an efficient alternative for growing mushrooms, but there is need for further study on the use of stem to identify ways to make its use in biotechnology mushrooms viable. The substrates source timber had a higher viability for growing mushrooms of the genera *Pleurotus* and *Lentinula*, since the fungi have provided the best indices to ensure a good yield.

REFERENCES

- Andrade MCN, Minhoni MTA, Zied DC (2008). Caracterização bromatológica de oito linhagens de *Lentinula edodes* (Shiitake) cultivada em toras de *Eucalyptus grandis*. *Ciência e Tecnologia de Alimentos*, 28:793-797.
- Bononi VLR, Capelari M, Maziero R, Trufem SFB (1999). Cultivo de Cogumelos Comestíveis. São Paulo: Ícone Editora, pp 206.
- Donini LP, Bernardi E, Minotto E, Nascimento JS (2005). Desenvolvimento *in vitro* de *Pleurotus spp.* sob a influência de diferentes substratos e Dextrose. *Arquivo do Instituto Biológico*, 72:331-338.
- Elisashvili V, Penninckx M, Kachlishvili E, Tsiklauri N, Metreveli E, Kharziani T, Kvesitadze G (2008). *Lentinus edodes* and *Pleurotus* species lignocelulolytic enzymes activity in submerged and solid-state fermentation of lignocelulosic wastes of different composition. *Bioresour. Technol.* 99:457-462.
- Furlan SA, Mendonça MM, Gern RMM, Santos VMSC, Araújo RP (2000). Influência da suplementação de palha de arroz na produção de *Pleurotus sajor-caju*. *Revista Saúde e Ambiente*, 1:60-63.
- Gonçalves CR (2002). Efeito da fragmentação do micélio visando a obtenção de inoculantes em suspensão para cultivo de Shiitake. Tese (Doutorado) - Universidade Estadual Paulista, Araraquara.
- Motato KE, Mejía AI, León A (2006). Evaluación de los residuos agroindustriales de plátano (*Musa paradisíaca*) y aserrín de Abarco (*Cariniana piriformes*) como sustratos para el cultivo Del hongo *Pleurotus djamor*. *Vitae*, v.13, p.24-29.
- Pedra WN, Marino RH (2006). Cultivo axênico de *Pleurotus spp.* em serragem da casca de Coco (*Cocos nucifera* Linn.) suplementada com farelo de arroz e/ou de trigo. *Arquivo do Instituto Biológico*, 73:219-225.
- Rossi IH, Monteiro AC, Machado JO (2000). Desenvolvimento micelial de *Lentinula edodes* como efeito da profundidade e suplementação do substrato. *Pesquisa Agropecuária Brasileira*, 36:887-891.
- Sales-Campos C, Abreu RLS, Vianez BF (2000). Condições de uso e processamento de madeira nas indústrias madeireiras de Manaus, Amazonas, Brasil. *Acta Amazonica*, 30:319-331.
- Sales-Campos C, Eira AF, Jesus MA, Campagnolli F, Andrade MCN. (2008). Crescimento micelial de *Pleurotus ostreatus* em resíduo de *Simarouba amara*. *Pesquisa Agropecuária Brasileira*, 43:1633-1635.
- Sales-Campos C, Minhoni MTA, Andrade MCN (2010). Produtividade de *Pleurotus ostreatus* em resíduos da Amazônia. *Interciencia*, 35:198-201.
- Soffner MLAP (2001). Produção de polpa celulósica a partir de engaço de bananeira. 70p. Dissertação (Mestrado) - Universidade de São Paulo, Piracicaba.
- Vianez BF, Barbosa AP (2003). Estudo de alternativas de uso dos resíduos gerados pela indústria madeireira em Manaus e Itacoatiara, Estado do Amazonas. Manaus: INPA, pp 50.
- Vieira FR, Andrade MCN, Minhoni MTA (2013). Growth of *Pleurotus ostreatus* in culture media based on formulated straw or grass. *Afri. J. Agric. Res.* 8:2345-2352.

Full Length Research Paper

Second derivative spectrophotometric determination of cyclophosphamide in pharmaceutical formulations

Nabil A. Fakhre^{1*}, Hemn A. Qader² and Alaadin M. Naqishbandi³

¹Chemistry Department, College of Education, Salahaddin University, Erbil, Iraq.

²Pharmaceutical Chemistry Department., College of Pharmacy, Hawler Medical University, Erbil, Iraq.

³Pharmacognosy Department, College of Pharmacy, Hawler Medical University, Erbil, Iraq.

Accepted 10 October, 2013

A new, simple, rapid, wide applicable range and reliable second derivative spectrophotometric method has been developed for determination of cyclophosphamide (CP) in bulk and pharmaceutical dosage forms. Calibration graph is linear in the concentration range of 25 - 200 µg/ml of CP with 10 µg/ml of detection limit and correlation coefficient of 0.9976. The precision and accuracy were acceptable depending upon the values of relative standard deviation and error percentage. Developed second derivative spectrophotometric method can be directly and easily applied for analyzing pure form and commercial pharmaceutical preparations of CP. The method was compared with a standard high performance liquid chromatography (HPLC) method and can be used for the quality control of pharmaceutical preparations in Erbil City.

Key words: Cyclophosphamide, derivative spectrophotometry.

INTRODUCTION

Cyclophosphamide (CP) is widely used in cancer chemotherapy, mostly in combination with other antineoplastic agents, and as an immunosuppressant (Karahalil and Akkoyunlu, 2003). Cyclophosphamide (belongs to the group of alkylating agents) is a pro-drug that is activated via 4-hydroxylation by cytochromeP450s such as CYP2B6 and CYP3A4 to generate alkylating nitrogen mustards (phosphoramidate mustard and the byproduct acrolein). The resultant mustards can alkylate DNA to form DNA-DNA cross-links, leading to inhibition of DNA synthesis and cell apoptosis (Malothu et al., 2009).

Several analytical methods have been reported and published for quantitative determination of CP in biological fluid and environmental samples, such as: High performance liquid chromatography (HPLC) methods

(Malothu et al., 2009; Ahmad et al., 2011), gas chromatography (GC)-mass spectrometry (Sugiura et al., 2010), and after extraction of CP by solid phase extraction techniques, using solid-phase extraction and GC-MS spectrometry (Martins et al., 2004), on-line sample preparation method by micro-extraction packed sorbent (MEPS) followed by LCMS/MS (Kamel et al., 2009), liquid chromatography with diode array detector (Alcântara et al., 2010), and spectrophotometric method (Karen et al., 2009). To our knowledge, there are no derivative spectrophotometric methods concerning the determination of cyclophosphamide in pharmaceutical formulations.

Derivative spectrophotometry is an analytical technique of great utility for extracting both qualitative and quanti

*Corresponding author. E-mail: nabil_fakhri@yahoo.com/ havras@yahoo.com.

Abbreviations: CP, Cyclophosphamide; HPLC, high performance liquid chromatography; GC, gas chromatography; MEPS, micro-extraction packed sorbent.

tative information from spectra composed of unresolved bands, and for eliminating the effect of baseline shifts and baseline tilts. It consists of calculating and plotting one of the mathematical derivatives of a spectral curve. Derivative spectrophotometry is now a reasonably prized standard feature of modern micro-computerized UV spectrophotometry (Patel et al., 2010). Derivative UV spectrophotometry has been widely used as a tool for quantitative and control analysis in agricultural, pharmaceutical and biomedical fields (Stanisz et al., 2009). The aim of this study was to develop a simple, rapid and efficient second derivative spectrophotometric method for the determination of CP in pharmaceutical formulations and compared with HPLC method that can be used for the quality control of pharmaceutical preparations in Erbil City.

MATERIALS AND METHODS

Apparatus

A CECIL CE 3021 UV/Vis scanning spectrophotometer equipped with 10 mm path length quartz cell was used to record normal and second derivative spectra of CP solutions.

Chromatographic conditions

HPLC instrument, Smartline manager 5000, smartline UV detector 2500, smartline column thermostat 4000, smartline HPLC pump 1000. Knauer advanced scientific instruments, Germany with analytical column: C18, 5 μm , 100 x 4.6 mm from Dr. Ing. H. Knauer GmbH, Germany. The mobile phase was acetonitrile: water (30:70) with a flow rate: 1 ml/min and injection volume 20 μl . The eluent were monitored spectrophotometrically at 197 nm at temperature 30°C.

Chemicals

All chemicals used were of analytical reagent grade. Stock CP solution (1000 $\mu\text{g/ml}$) (Sigma-Aldrich) was prepared by dissolving 1000 mg of CP in distilled water and diluting to 1000 ml in a volumetric flask and stored in a refrigerator. Each working standard solution was freshly prepared by diluting the stock solution with distilled water.

Recommended procedure

The standard solutions were prepared by dilution of the stock solution with distilled water in a concentration range of (25 - 200) $\mu\text{g/ml}$ for normal and second derivative spectrophotometric methods. Distilled water was used as a reagent blank. The second derivative spectral recording were carried out using smoothing of 2 and 25 nm of interval at a scanning speed of 10 nm/sec in the wavelength range of 190 - 400 nm.

Sample preparation

Amount of the CP vial content were accurately weighed and dissolved in distilled water and transferred to 25 ml volumetric flask, then diluted to the mark with distilled water. Appropriate dilutions

were made to obtain a solution in the concentration range of the calibration curve, and recommended procedure was applied.

RESULTS AND DISCUSSION

The normal and second derivative spectra of the solutions of CP were recorded at the wavelength range of 190 - 400 nm against reagent blank as shown in Figures 1 and 2 which show maximum absorption at 193 nm for normal spectra and 207.3 nm for second derivative spectra of the solutions, respectively.

Statistical data of the calibration curve

Under the recommended experimental conditions for second derivative spectrophotometric method the calibration graph was found to obey Beer's law in the concentration range of 25 - 200 $\mu\text{g/ml}$, with the detection limit of 10.0 $\mu\text{g/ml}$. Regression analysis was made for the slope, intercept and correlation coefficient values. The regression equation of calibration curve is $y = 0.0456x + 0.0982$ and $R^2 = 0.9976$.

Precision and accuracy

The precision and accuracy of the second derivative spectrophotometric determination of CP were studied depending upon the value of the relative standard deviation percentage (RSD %) and the relative error percentage (error %) for five replicate measurements of three different concentrations, respectively. Table 1 shows the results.

Application of the method

The proposed method was applied to the determination of CP in some vials of CP samples from Baxter Company which are the only vials available in the hospitals of Erbil City. In the present method, from each vial samples (500 and 1000) mg different solutions were prepared and the recommended procedure was applied. The results are summarized in Table 2. The method was in a good agreement in comparison with the results obtained using HPLC for the standard CP (Figure 3) and qualification and determination of CP in the two vial samples as shown in Figures 4 and 5.

Conclusion

The second derivative spectrophotometric methods have been described for quantification of cyclophosphamide in pure form of CP and its ratio in the vial content, at the time there were no any UV and derivative

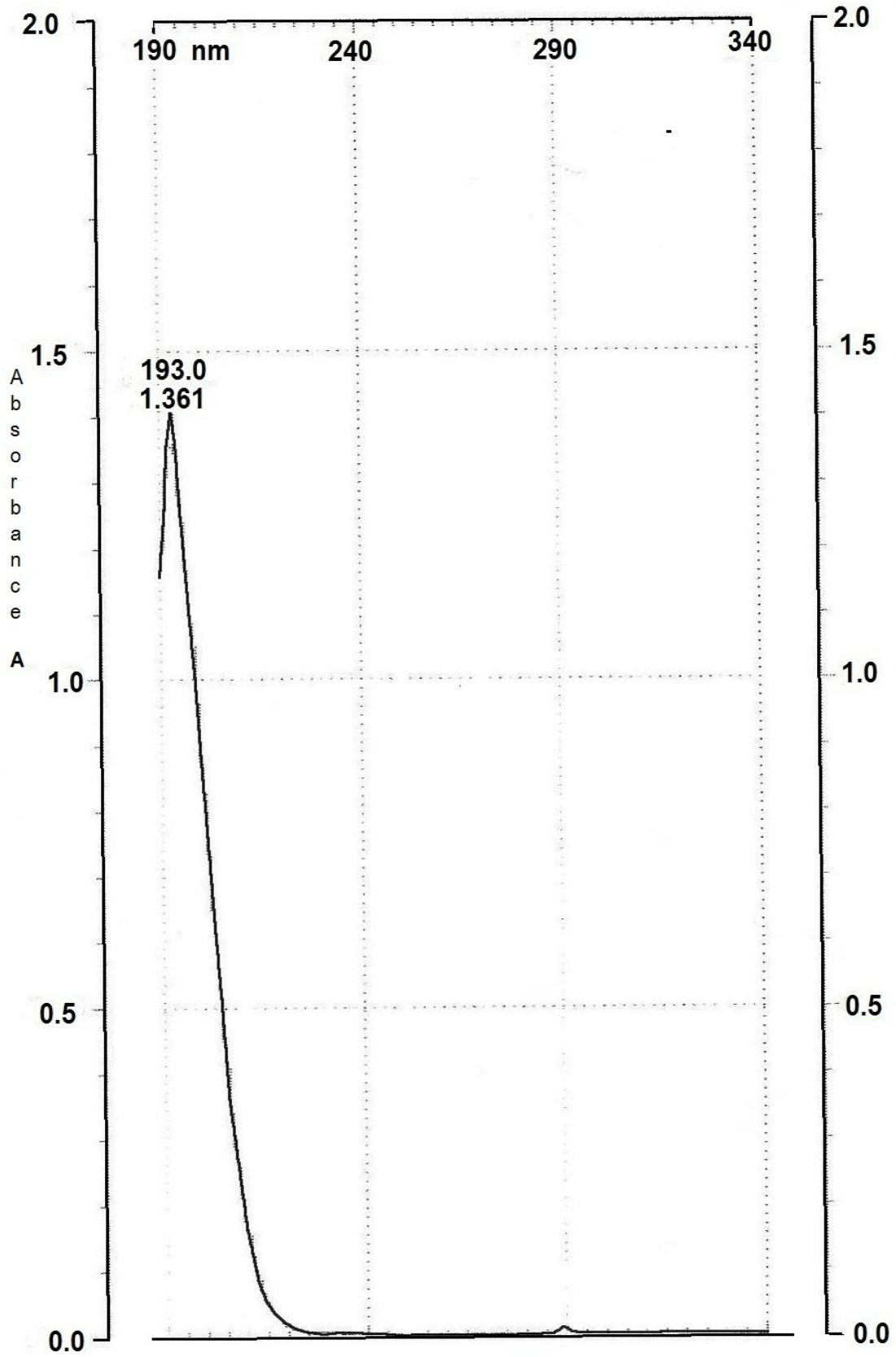


Figure 1. Normal spectrum of CP solution.

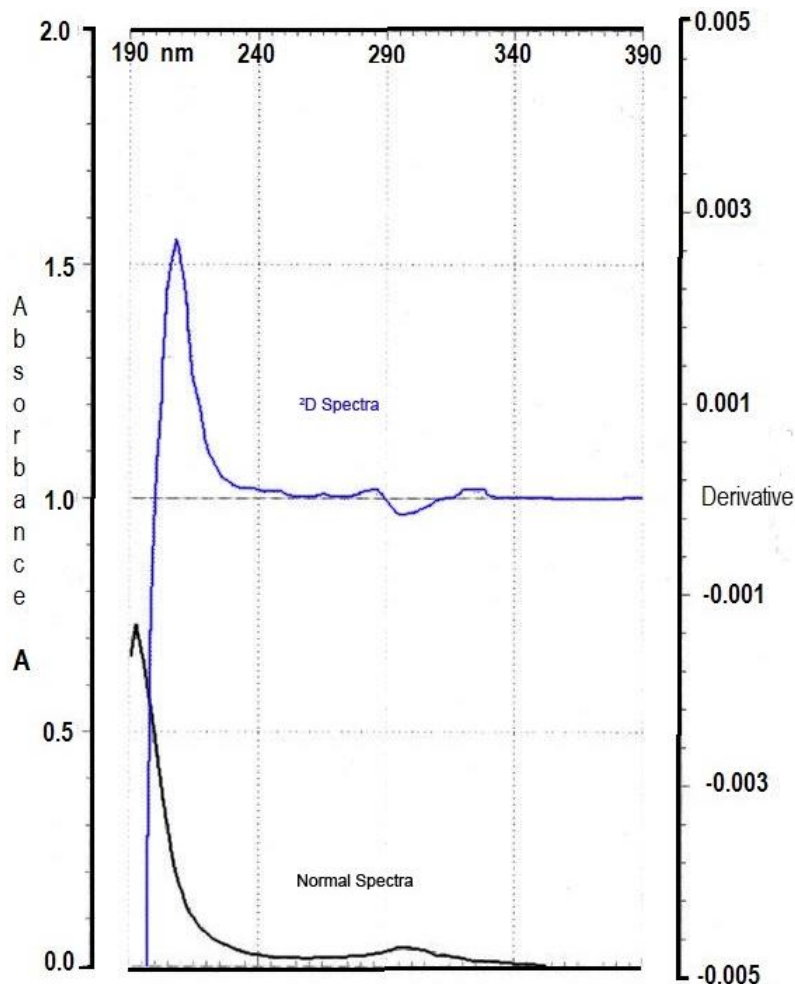


Figure 2. Normal and second derivative spectra of CP solution.

Table 1. Precision and accuracy of the proposed second derivative spectrophotometric method.

Cyclophosphamide concentration ($\mu\text{g/ml}$)	RSD%	Error%
25	2.19	+ 3.67
100	1.19	+ 1.79
200	0.75	+ 1.56

Table 2. Determination of CP in vial samples with the proposed method.

Samples	Amount found with proposed $^{2\text{D}}$ method (mg)	Amount found with modified HPLC method (mg)	Recovery with the proposed $^{2\text{D}}$ method %
500 mg	504.050	501.138	100.81
1000 mg	1009.040	1014.448	101.4

spectrophotometric methods for this aim. The proposed method is simple, rapid, wide applicable range, no requires extraction step and reagents to the determination of CP in the pharmaceutical formulations in

compare with other methods like HPLC. The method shows a good precision and accuracy and it is in a good agreement in comparison with standard HPLC method and can be used for the quality control of pharmaceutical

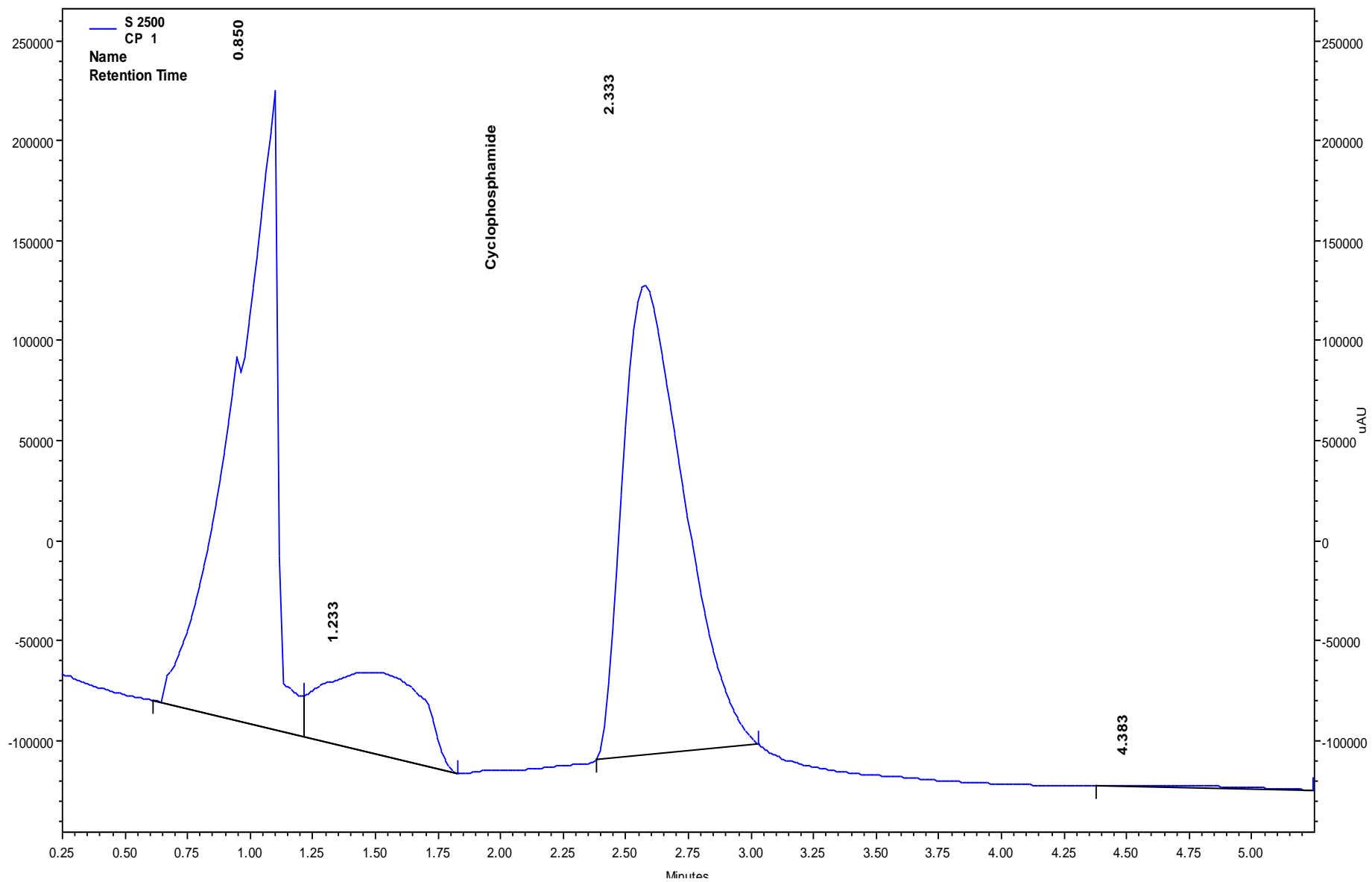


Figure 3. Representative chromatogram of cyclophosphamide.

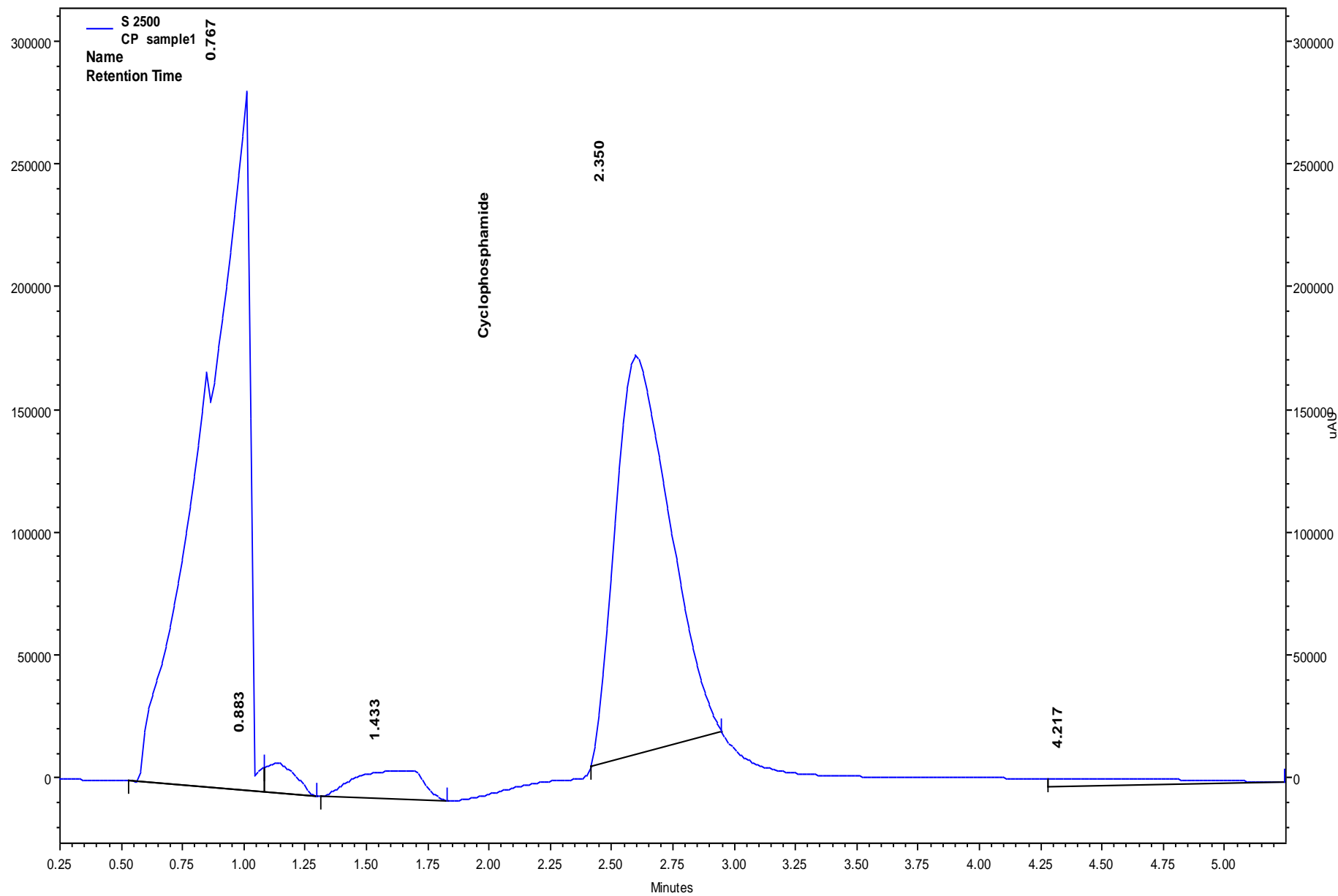


Figure 4. Chromatogram of the first sample.

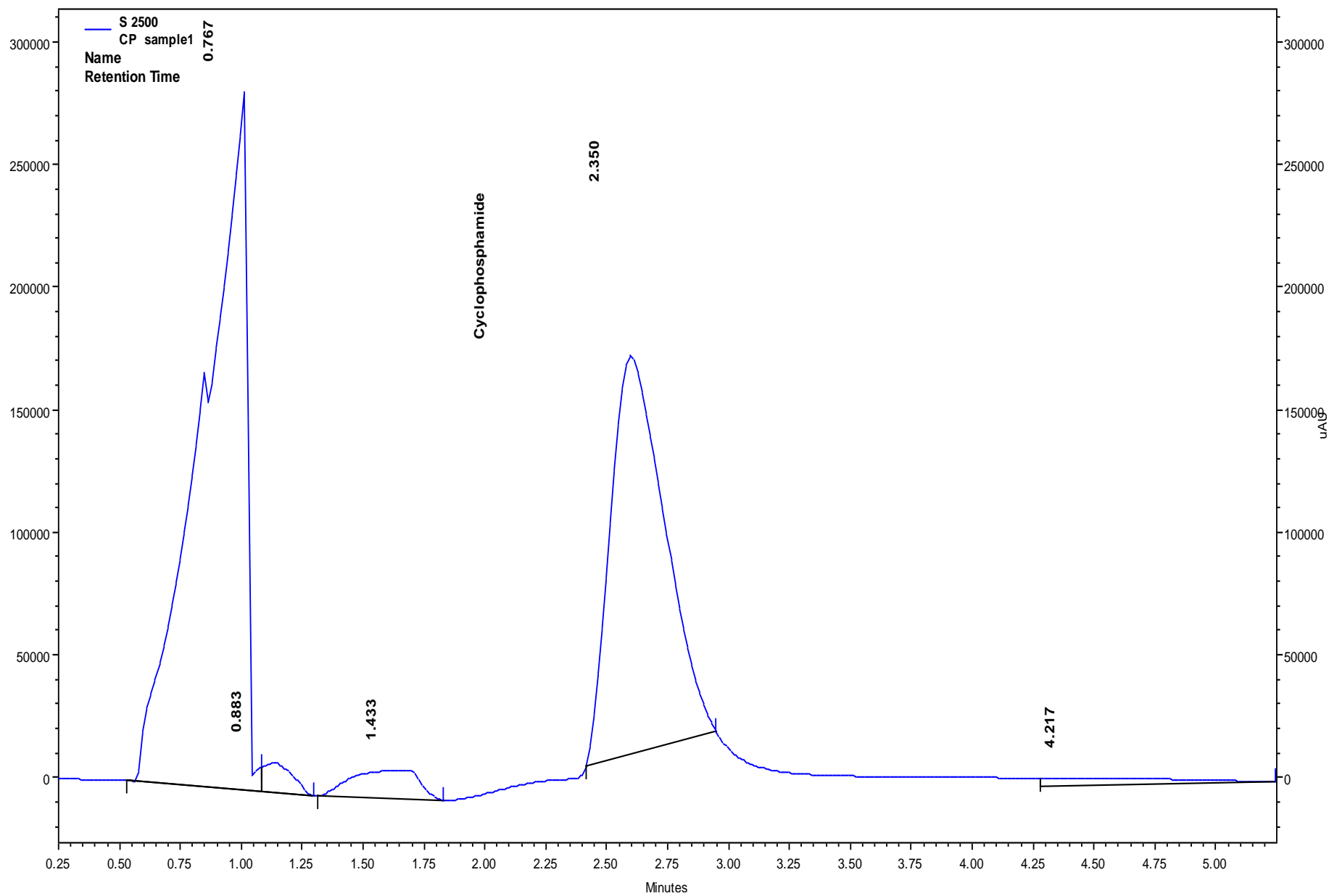


Figure 5. Chromatogram of the second sample.

preparations in Erbil City.

REFERENCES

- Ahmad M, Usman M, Madni A, Zubair M, Zaman Q, Qureshi MS, Munir A, Ahmad A, Mahmood A (2011). A fast and simple HPLC-UV method for simultaneous determination of three anti-cancer agents in plasma of breast cancer patients and its application to clinical pharmacokinetics. *Afr. J. Pharm. Pharmacol.* 5(7):915-922.
- Alcântara AMP, Vitor RV, Vieira EP, Martins I (2010). Simultaneous detection of three antineoplastic drugs on gloves by liquid chromatography with diode array detector. *Brazilian Journal of Pharmaceutical Sciences.* 46(4):731-740.
- Kamel M, Said R, Elbeqqali A, Bassyouni F, Abdel-Rehim M (2009). On-line determination of cyclophosphamide in blood samples utilizing microextraction by packed sorbent and liquid chromatography tandem mass spectrometry (MEPS-LC-MS/MS). *The Open Spectrosc. J.* 3:26-30.
- Malothu N, Veldandi UK, Devarakonda RK (2009). Development of rapid and simple HPLC method for the determination of cyclophosphamide in human serum. *Asian J. Pharm. Clin. Res.* 2(3):34-38.
- Martins I, Della Rosa HV, Apostoli P (2004). Cyclophosphamide identification in wipe test by GC-MS and solid phase extraction. *Brazilian J. Pharm. Sci.* 40(1):67-73.
- Patel VM, Patel JA, Havele SS, Dhaneshwar SR (2010). First and second derivative spectrophotometric methods for determination of olanzapine in pharmaceutical formulation. *Int. J. ChemTech Res.* 2(1):756-761.
- Stanisz B, Paszun S, Lesniak M (2009). Validation of UV derivative spectrophotometric method for determination of benazepril hydrochloride in tablets and evaluation of its stability. *Acta Poloniae Pharmaceutica-Drug Research.* 66(4):343-349.
- Sugiura S, Nakanishi H, Asano M, Hashida T, Tanimura M, Hama T, Nabeshima T (2010). Multicenter study for environmental and biological monitoring of occupational exposure to cyclophosphamide in Japan. *J. Oncol. Pharm Pract.* 17(1):20-28.

Short Communication

Effects of hypoxia on serum hepatic chemistries of Tibet chicken and Shouguang chicken

Wenpeng Han^{1#}, Meiling Song^{1,2#}, Hui Yuan^{1#}, Haigang Bao¹, Chong Liu³, Changxin Wu¹ and Chunjiang Zhao^{1*}

¹College of Animal Science and Technology, China Agricultural University, Beijing 100193, China.

²Dongying technician college, Shandong, 257091, China.

³Institute of Environment and Sustainable Development in Agriculture, Chinese Academy of Agricultural Sciences, Beijing 100081, China.

Accepted 26 June, 2013

Hypoxia is a major factor that affects the subsistence and development of multicellular organisms. Tibet chicken, as a unique native chicken breed in altiplano, shows genetic adaptation to hypoxia comparing with the breeds at the low altitude. In the present study, to explore effects of hypoxia on chicken fetal livers, eggs of Tibet chicken and Shouguang chicken were collected and the samples from each breed were divided into two groups, incubated in hypoxia and in normoxia respectively. The blood of embryos on the 16th day of incubation was collected and the serum chemistry parameters indicating liver metabolism were determined, which included glutamic-pyruvic transaminase (GPT), aspartate aminotransferase (GOT), total bilirubin (TB), direct bilirubin (DB), total bile acid (TBA), gamma glutamyltransferase (GGT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), creatine kinase (CK), glucose and creatinine. The results show that biochemical indices varied significantly between hypoxia and normoxia except for GPT and glucose. Moreover, the concentration of ALP and LDH showed significant differences between the breeds and the incubations. The results suggest that the livers of both Shouguang chicken and Tibet chicken suffered damages in hypoxia, but the former was more serious. The results of this study support the opinion that Tibet chicken had better genetic adaptability on hypoxia, and made a good basis for further study of the genetic mechanism of adaptation to hypoxia.

Key words: Hypoxia adaptation, liver metabolism, serum chemistry, Tibet chicken, chicken embryo.

INTRODUCTION

Hypoxia is a major causative factor of diseases in altiplano, which influences seriously the subsistence and development of multicellular organisms, such as plants, animals, and human beings (Heacock and Sutherland,

1990; Archer et al., 2004). Previous studies have shown that chronic intermittent hypoxia can lead to liver injury and nonalcoholic fatty liver disease, via oxidative stress and excessive glycogen accumulation, and then resulted

*Corresponding author. E-mail: cjzhao@cau.edu.cn. Tel: +86-10-62894888.

#These authors contributed equally to this work.

Abbreviation: GPT, Glutamic-pyruvic transaminase; GOT, aspartate aminotransferase; TB, total bilirubin; DB, direct bilirubin; TBA, total bile acid; GGT, gamma glutamyltransferase; ALP, alkaline phosphatase; LDH, lactate dehydrogenase; CK, creatine kinase.

Table 1. Serum chemistry parameters in Shouguang Chicken and Tibet Chicken under different incubation conditions.

Serum component	Shouguang Chicken		Tibet Chicken	
	Normoxia	Hypoxia	Normoxia	Hypoxia
GPT (IU/L)	3.26±0.41 ^a	3.84±0.69 ^a	3.67±0.36 ^a	4.22±0.35 ^a
GOT (IU/L)	21.83±0.35 ^a	27.66±5.61 ^b	18.61±0.73 ^a	18.84±1.46 ^a
TB (umol/L)	10.38±0.25 ^a	16.34±0.35 ^b	15.26±0.35 ^b	13.1±0.31 ^c
DB (umol/L)	0.56±0.026 ^a	0.92±0.047 ^b	0.80±0.042 ^b	0.66±0.032 ^b
TBA (umol/L)	0.66±0.036 ^a	1.14±0.036 ^b	0.60±0.050 ^a	0.86±0.028 ^c
ALP (IU/L)	622.83±6.66 ^a	1244.65±10.92 ^b	806.09±7.27 ^c	1011.13±10.37 ^d
GGT (IU/L)	1.21±0.14 ^b	1.82±0.14 ^a	1.44±0.17 ^b	1.86±0.14 ^a
CK (IU/L)	106.11±5.15 ^b	119.60±5.92 ^a	129.64±8.05 ^a	116.22±4.75 ^a
Glucose (umol/L)	5.44±0.060 ^a	5.27±0.052 ^a	5.77±0.061 ^a	5.39±0.047 ^a
Creatinine (umol/L)	1.12±0.29 ^a	4.73±0.31 ^b	1.70±0.097 ^a	5.54±0.044 ^b
LDH (IU/L)	207.20±4.54 ^a	394.61±4.55 ^b	199.83±7.27 ^c	237.84±7.92 ^d

Values in the same row with different letters means that the difference between them is significant difference ($p < 0.05$).

changes on levels of some serum content including glutamic-pyruvic transaminase (GPT), aspartate amino-transferase (GOT), bilirubin, gamma glutamyltransferase (GGT), alkaline phosphatase (ALP) (Puhl et al., 2005; Tanne et al., 2005; Savransky et al., 2007a, b, 2009). Therefore, the determination of those enzymes and substances was performed to evaluate the potential pathological changes of organisms (Nakamura et al, 2008).

In this study, we employed Tibet Chicken, a unique native chicken breed inhabiting in Tibet alti-plano with an average altitude above 3 000 m, and aimed to study the changes of fetal liver in hypoxia by determining the serum chemistry, compared with Shouguang chicken, an indigenous Chinese chicken breed in Shouguang county of Shandong province with an altitude less than 100 m, to explore the change patterns of the serum hepatic chemistry of the two chicken breeds in hypoxia.

MATERIALS AND METHODS

Animals and sampling procedures

The fertile eggs of each breeds, Tibet chicken and Shouguang chicken, from the experiment chicken farm of the China Agricultural University (CAU, Beijing, 100m altitude) were divided into two groups, and exposed to sustained normal oxygen partial pressure (21% O₂) and hypoxia (13% O₂) with the same other incubation conditions (such as 37.8°C, 60% relative humidity and so on) respectively, after the eggs and the incubators were sterilized. On the 16th day of incubation, the eggshells were broken at the air-cell and then the blood was taken using injector treated with heparin sodium from the thickest artery on the chorioallantoic membrane pulled out. Serum separated from blood samples after centrifuged at 1000 g for 10 min. Ten (10) blood samples were collected from each studied group to be analyzed separately and totally 40 samples were determined in the present study.

Determination of serum chemistry parameters

The serum chemistry assays were determined using automatic biochemistry analyzer (Hitachi 7 600, Hitachi, Japan) in the 309 Hospital in Beijing, and performed in terms of glutamic-pyruvic transaminase (GPT), aspartate aminotransferase (GOT), total bilirubin (TB), direct bilirubin (DB), total bile acid (TBA), gamma glutamyltransferase (GGT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), creatine kinase (CK), glucose, and creatinine.

Statistical analysis

Data of this study were subjected to variance analysis using SAS software (Version 8.02, SAS Inc, US), and the significant level was fixed to $p < 0.05$ and extreme significance $p < 0.01$. The model using to analyze the data was:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \varepsilon_{ijk}$$

Where, Y_{ijk} , is the parameter of serum hepatic chemistries of embryo k from breed i and incubation condition j ; μ , is the overall mean; α_i is the main effect of breed i ($n = 2$); β_j , is the main effect of incubation condition j ($n = 2$); $(\alpha\beta)_{ij}$, is the interaction between breed and incubation condition; and ε_{ijk} , is the random error term. Values were shown as mean and standard error.

RESULTS AND DISCUSSION

The purpose of the study was to investigate the changes in serum hepatic biochemistry of Tibet Chicken and Shouguang Chicken incubated in hypoxia, and the results are shown in Table 1.

Liver injury, which led to the elevations of serum chemistries such as GPT, GOT, ALP, has been demonstrated in hypoxia in many studies (Tanne et al., 2005; Savransky et al., 2007a, b, 2009). The reports of the effects in chronic intermittent hypoxia showed that chronic

liver injury and nonalcoholic fatty liver disease included isolated fatty liver, nonalcoholic steatohepatitis, fibrosis and liver cirrhosis (Tanne et al., 2005; Kallwitz et al., 2007; Mishra et al., 2008; Norman et al., 2008). However, the levels of these enzymes can be elevated in a variety of liver injury (Savransky et al., 2007a, b, 2009). Previous studies interpreted that both hypoxia alone and cooperating with other factors can lead to the changes of serum hepatic chemistries, but the changes were not all coincident. The ratio of GOT to GPT was sometimes used in clinical diagnosis of hepatic disorders (Kang, et al, 2013). Different ratios were present in a variety of hepatic disorders, and mostly the ratio of GOT/GPT greater than 2 was present in alcoholic hepatitis and some other disorders such as Wilson's disease (Sorbi et al., 1999; Giannini et al., 2003). The GPT in the present study, which was considered to be more specific for hepatic injury because it exists mainly in the cytosol of the liver and in low concentrations elsewhere (Giboney, 2005), was not significantly changed, but the ratios of GOT/GPT (27.6 ± 5.6064 IU/L(GOT), 3.8 ± 0.6855 IU/L (GPT) in Shouguang Chicken and 18.8 ± 1.4560 IU/L (GOT), 4.2 ± 0.3464 IU/L(GPT) in Tibet chicken, respectively) were more than 2-fold in hypoxia in our work, which indicated that the fetal livers of both Shouguang chicken and Tibet chicken were injured in hypoxia and Shouguang chicken suffered more serious damages. However, ratio utility had significant limitations. Except for the hepatic disorders, some evidences showed sex-related differences and physiological changes in metabolism of avian can also induce the elevations of GOT, GPT and bilirubin (Walzem et al., 1999; Scholtz et al., 2009).

In this study, ALP and LDH showed dramatic changes between the breeds and the incubations. Though both of them are less specific than GPT and GOT in diagnosis of hepatic disorders, they were sometimes employed to provide further evidences about the patterns of liver injury (Musana et al., 2004; Shokrzadeh, et al, 2012). Elevation of ALP in serum were derived from the liver and bone predominantly during the third trimester of pregnancy of women and the rapidly growth of adolescents (Wolf, 1978; Pratt and Kaplan, 2000). Therefore, the elevation of ALP in this study may be due to both liver injury and embryonic growth predominantly, which should be verified by other parameters because the damages of kidney, intestine and placenta also elevate the level of ALP slightly. While in hypoxia, the elevation of LDH may be that, there was not enough oxygen to meet the need of organism due to the lack of red blood cells in blood (Garba and Ubom, 2005; Macedo et al., 2009), or the organs of metabolism and respiration were damaged (Granchi et al., 2010), and the expression of both LDH-A mRNA and protein were inducible by hypoxia (Robin et al., 1984; Marti et al., 1994; Firth et al., 1994, 1995). Several other evidences in which the production of LDH increases in hypoxia support this point (Rees et al., 2001;

Koukourakis et al., 2006). In addition, the ratio of GPT/LDH was also used in diagnosis of liver disorders (Kotoh et al., 2008; Balasubramanian et al., 2010). In this study, both ALP and LDH have shown significant differences between the breeds and the incubations, and the changes of Shouguang chicken in hypoxia was greater than that of Tibet chicken, which may suggest that Tibet chicken has a better adaptability to the changes of oxygen partial pressure. But the various trends of other parameters may be due to the decrease of the metabolic levels of Tibet chicken in hypoxia or the limited samples in our study.

In conclusion, our results suggested that though the liver metabolism of both chicken breeds was affected negatively by hypoxia, Tibet chicken suffered less damage than Shouguang chicken in hypoxia. So our study supported the opinion that Tibet chicken has better genetic adaptability in hypoxia.

ACKNOWLEDGEMENTS

This research was supported by the National Program on Key Basic Research Project (Grant No. 2006CB102101) of China.

REFERENCES

- Archer SL, Wu XC, Thebaud B, Moudgil R, Hashimoto K, Michelakis E D (2004). O₂ sensing in the human ductus arteriosus: Redox sensitive K⁺ channels are regulated by mitochondria-derived hydrogen peroxide. *Biol. Chem.* 385(3-4):205-216.
- Balasubramanian S, Kaarthigeyan K, Srinivas S, Rajeswari R (2010). Serum ALT: LDH ratio in typhoid fever and acute viral hepatitis. *Indian Pediatr.* 47 (4):339-341.
- Firth JD, Ebert BL, Pugh CW, Ratcliffe PJ (1994). Oxygen-regulated control elements in the phosphoglycerate kinase 1 and lactate dehydrogenase A genes: similarities with the erythropoietin 3' enhancer. *Proc. Natl. Acad. Sci. USA.* 91(14):6496-6500.
- Firth JD, Ebert BL, Ratcliffe PJ (1995). Hypoxic regulation of lactate dehydrogenase A: Interaction between hypoxia-inducible factor 1 and cAMP response elements. *J. Biol. Chem.* 270(36):21021-21027.
- Garba IH, Ubom GA (2005). Total serum lactate dehydrogenase activity in acute plasmodium falciparum malaria infection. *Singapore Med. J.* 46(11):632-634.
- Giannini E, Risso D, Botta F, Chiarbonello B, Fasoli A, Malfatti F, Romagnoli P, Testa E, Ceppa P, Testa R (2003). Validity and clinical utility of the aspartate aminotransferase-alanine aminotransferase ratio in assessing disease severity and prognosis in patients with hepatitis C virus-related chronic liver disease. *Arch. Intern. Med.* 163 (2):218-224.
- Giboney PT (2005). Mildly elevated liver transaminase levels in the asymptomatic patient. *Review. Am. Fam. Physician* 71(6):1105-1110.
- Granchi C, Bertini S, Macchia M, Minutolo F (2010). Inhibitors of lactate dehydrogenase isoforms and their therapeutic potentials. *Curr. Med. Chem.* 17(7):672-697.
- Heacock CS, Sutherland RM (1990). Enhanced synthesis of stress proteins caused by hypoxia and relation to altered cell growth and metabolism. *Br. J. Cancer* 62(2):217-225.
- Kallwitz ER, Herdegen J, Madura J, Jakate S, Cotter SJ (2007). Liver enzymes and histology in obese patients with obstructive sleep apnea. *J. Clin. Gastroenterol.* 41(10):918-921.
- Kang MC, Kang SM, Ahn G, Kim KN, Kang N, Samarakoon KW, Oh

- MC, Lee JS, Jeon YJ (2013). Protective effect of a marine polyphenol, dieckol against carbon tetrachloride-induced acute liver damage in mouse. *Environ Toxicol Pharmacol.* 35(3):517-523.
- Kotoh K, Enjoji M, Kato M, Kohjima M, Nakamuta M, Takayanagi R (2008). A new parameter using serum lactate dehydrogenase and alanine aminotransferase level is useful for predicting the prognosis of patients at an early stage of acute liver injury: a retrospective study. *Comp. Hepatol.* 7:6
- Koukourakis M I, Pitiakoudis M, Giatromanolaki A, Tsarouha A, Polychronidis A, Sivridis E, Simopoulos C (2006). Oxygen and glucose consumption in gastrointestinal adenocarcinomas: correlation with markers of hypoxia, acidity and anaerobic glycolysis. *Cancer Sci.* 97(10):1056-1060.
- Macedo DV, Lazarim FL, Catanho da Silva FO, Tessuti LS, Hohl R (2009). Is lactate production related to muscular fatigue? A pedagogical proposition using empirical facts. *Adv. Physiol. Educ.* 33(4):302-307.
- Marti HH, Jung HH, Pfeilschifter J, Bauer C (1994). Hypoxia and cobalt stimulate lactate dehydrogenase (LDH) activity in vascular smooth muscle cells. *Pflugers Arch.* 429(2):216-222.
- Mishra P, Nugent C, Afendy A, Bai C, Bhatia P, Afendy M, Fang Y, Elariny H, Goodman Z, Younossi ZM (2008). Apnoeic-hypopnoeic episodes during obstructive sleep apnea are associated with histological nonalcoholic steatohepatitis. *Liver Int.* 28 (8):1080-1086.
- Musana KA, Yale SH, Abdulkarim AS (2004). Tests of liver injury. *Clin. Med. Res.* 2 (2):129-131.
- Nakamura M, Sato S, Shimamoto T, Konishi M, Yoshiike N (2008). Establishment of long-term monitoring system for blood chemistry data by the national health and nutrition survey in Japan. *J. Atheroscler. Thromb.* 15(5):244-249.
- Norman D, Bardwell WA, Arosemena F, Nelesen R, Mills PJ, Loreda J S, Lavine JE, Dimsdale JE (2008). Serum aminotransferase levels are associated with markers of hypoxia in patients with obstructive sleep apnea. *Sleep* 31(1):121-126.
- Pratt DS, Kaplan MM (2000). Evaluation of abnormal liver-enzyme results in asymptomatic patients. *N. Engl. J. Med.* 342:1266-1271.
- Puhl G, Schaser KD, Pust D, Kohler K, Vollmar B, Menger MD, Neuhaus P, Settmacher U (2005). Initial hepatic microcirculation correlates with early graft function in human orthotopic liver transplantation. *Liver Transpl.* 11(5):555-563.
- Rees BB, Bowman JA, Schulte PM (2001). Structure and sequence conservation of a putative hypoxia response element in the lactate dehydrogenase-B gene of *Fundulus*. *Biol. Bull.* 200 (3):247-251.
- Robin ED, Murrphy BJ, Theodore J (1984). Coordinate regulation of glycolysis by hypoxia in mammalian cells. *J. Cell Physiol.* 118(3):287-290.
- Savransky V, Bevans S, Nanayakkara A, Li J, Smith PL, Torbenson MS, Polotsky VY (2007b). Chronic intermittent hypoxia causes hepatitis in a mouse model of diet-induced fatty liver. *Am. J. Physiol. Gastrointest. Liver Physiol.* 293(4):G871-877.
- Savransky V, Nanayakkara A, Vivero A, Li J, Bevans S, Smith PL, Torbenson MS, Polotsky VY (2007a). Chronic intermittent hypoxia predisposes to liver injury. *Hepatology.* 45(4):1007-1013.
- Savransky V, Reinke C, Jun J, Bevans-Fonti S, Navayakkara A, Li J, Myers A C, Torbenson M S, Polotsky V Y. (2009). Chronic intermittent hypoxia and acetaminophen induce synergistic liver injury. *Exp Physiol.* 94(2):228-239.
- Scholtz N, Halle I, Flachowsky G, Sauerwein H (2009). Serum chemistry reference values in adult Japanese quail (*Coturnix coturnix japonica*) including sex-related differences. *Poult. Sci.* 88(6):1186-1190.
- Shokrzadeh M, Shobi S, Attar H, Shayegan S, Payam SS, Ghorbani F (2012). Effect of vitamins A, E and C on liver enzyme activity in rats exposed to organophosphate pesticide diazinon. *Pak J Biol Sci.* 15(19):936-941.
- Sorbi D, Boynton J, Lindor KD (1999). The ratio of aspartate aminotransferase to alanine aminotransferase: potential value in differentiating nonalcoholic steatohepatitis from alcoholic liver disease. *Am. J. Gastroenterol.* 94 (4):1018-1022.
- Tanne F, Gagnadoux F, Chazouilleres O, Fleury B, Wendum D, Lasnier E (2005). Chronic liver injury during obstructive sleep apnea. *Hepatology* 41(6):1290-1296.
- Walzem RL, Hansen RJ, Williams DL, Hamilton RL (1999). Estrogen induction of VLDL assembly in egg-laying hens. *J. Nutr.* 129 (2S Suppl):467S-472S.
- Wolf PL (1978). Clinical significance of an increased or decreased serum alkaline phosphatase level. *Arch. Pathol. Lab. Med.* 102 (10): 497-501.

UPCOMING CONFERENCES

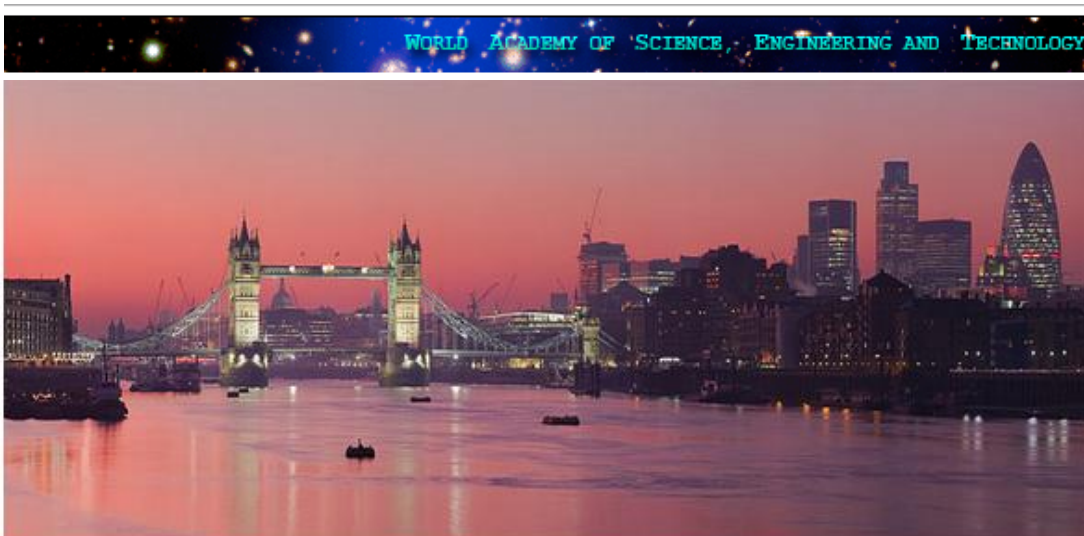
International Conference on Molecular Virology and Microbiology, Bangkok, Thailand, 24 Dec 2013



ICMVM 2013 : International Conference on Molecular Virology and Microbiology

**Bangkok, Thailand
December 24-25, 2013**

International Conference on Biotechnology and Environment Engineering, London, UK, 19 Jan 2014



ICBEE 2014 : International Conference on Biotechnology and Environment Engineering

**GB, London
January 20-21, 2014**

Conferences and Advert

December 2013

International Conference on Molecular Virology and Microbiology, Bangkok, Thailand, 24 Dec 2013

International Conference on Agriculture and Biotechnology, Kuala Lumpur, Malaysia, 29 Dec 2013

January 2014

International Conference on Biotechnology, Chemical and Environmental Engineering, London, UK, 19 Jan 2014

International Conference on Biotechnology and Environment Engineering, London, UK, 19 Jan 2014



Related Journals Published by Academic Journals

- Journal of Cell and Animal Biology
- International Journal of Genetics and Molecular Biology
- 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 Ecology and The Natural Environment
- Journal of Entomology and Nematology
- Journal of Bacteriology Research
- Journal of Bioinformatics and Sequence Analysis
- Journal of General and Molecular Virology
- International Journal of Biodiversity and Conservation
- Journal of Biophysics and Structural Biology
- Journal of Evolutionary Biology Research
- Journal of Yeast and Fungal Research
- International Journal of Plant Physiology and Biochemistry
- Journal of Brewing and Distilling
- Journal of Computational Biology and Bioinformatics Research
- Journal of Developmental Biology and Tissue Engineering
- Journal of Microbiology and Antimicrobials
- International Journal of Biotechnology and Molecular Biology Research