African Journal of Biotechnology

Volume 13 Number 5, 29 January, 2014

ISSN 1684-5315

Academic Journals

ABOUT AJB

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

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

Submission of Manuscript

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

Click here to Submit manuscripts online

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

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

Editor-In-Chief

George Nkem Ude, Ph.D
Plant Breeder & Molecular Biologist
Department of Natural Sciences
Crawford Building, Rm 003A
Bowie State University
14000 Jericho Park Road
Bowie, MD 20715, USA

Editor

N. John Tonukari, Ph.D
Department of Biochemistry
Delta State University
PMB 1
Abraka, Nigeria

Associate Editors

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

Dr. S.K Das
Department of Applied Chemistry
and Biotechnology, University of Fukui,
Japan

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

Dr. Ismail TURKOGLU
Department of Biology Education,
Education Faculty, Fırat University,
Elazığ,
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 Biochemisty,
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 Eczacilik Fakültesi,
Tibbiye cad. No: 49, 34668, Haydarpasa, Istanbul,
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 Histplogy,
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
Revivicor Inc.
100 Technology Drive, Suite 414
Pittsburgh, PA 15219
USA

Dr. Zhongming Zhao
Department of Psychiatry, PO Box 980126,
Virginia Commonwealth University School of Medicine,
Richmond, VA 23298-0126,
USA

Prof. Giuseppe Novelli
Human Genetics,
Department of Biopathology,
Tor Vergata University, Rome,
Italy

Dr. Moji Mohammadi
402-28 Upper Canada Drive
Toronto, ON, M2P 1R9 (416) 512-7795
Canada

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

Dr. Fabian Hoti
PneumoCarr Project
Department of Vaccines
National Public Health Institute
Finland

Prof. Irina-Draga Caruntu
Department of Histology
Gr. T. Popa University of Medicine and Pharmacy
16, Universitatii Street, Iasi,
Romania

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

Dr. Gerardo Armando Aguado-Santacruz
Biotechnology CINVESTAV-Unidad Irapuato
Departamento Biotecnología
Km 9.6 Libramiento norte Carretera Irapuato-
León Irapuato,
Guanajuato 36500
Mexico

Dr. Abdolkaim H. Chehregani
Department of Biology
Faculty of Science
Bu-Ali Sina University
Hamedan,
Iran

Dr. Abir Adel Saad
Molecular oncology
Department of Biotechnology
Institute of graduate Studies and Research
Alexandria University,
Egypt

Dr. Azizul Baten
Department of Statistics
Shah Jalal University of Science and Technology
Sylhet-3114,
Bangladesh

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

Dr. G. Reza Balali
Molecular Mycology and Plant Pthology
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
Departtment of Biotechnology
St. Michael College of Engineering & Technology,
Kalayarkoil,
India.

Dr. Gökhan Aydin
Suleyman Demirel University,
Atabey Vocational School,
Isparta-Türkiye,

Dr. Rajib Roychowdhury
Centre for Biotechnology (CBT),
Visva Bharati,
West-Bengal,
India.

Dr Takuji Ohyama
Faculty of Agriculture, Niigata University

Dr Mehdi Vasfi Marandi
University of Tehran

Dr FÜgen DURLU-ÖZKAYA
Gazi Üniversity, 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: © 2014, 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.

African Journal of Biotechnology

Table of Contents: Volume 13 Number 5, 29 January, 2014

ARTICLES

DNA extraction protocols for *Citrullus lanatus* and *Capsicum frutescens*: Effects of incubation temperatures and ethanol concentrations on DNA purity and quantity
Florence Ifeoma Akaneme, Irene Chinelo Odo and Lynda Afoma Okafor

Mechanism of biological control of *Rhizoctonia* damping-off of cucumber by a non-pathogenic isolate of binucleate *Rhizoctonia*
Mohsen Mohamed Elsharkawy, Naglaa Hassan, Remedios Villajuan-Abgona and Mitsuro Hyakumachi

Assessment of growth and yield of some high-and low-cyanide cassava genotypes in acid ultisols of south eastern Nigeria
Okpara, D. A., Mbah, E. U. and Chukwu, E. I.

Identification of putative candidate gene markers for grain zinc content using recombinant inbred lines (RIL) population of IRRI38 X Jeerigesanna
Naveen Kumar Gande, Pavan J Kundur, Rakhi Soman, Rajeswari Ambati, Ashwathanarayana R, Berhanu Dagnaw Bekele and Shashidhar H.E

Comparsion of bioleaching of copper sulphides by *Acidithiobacillus ferrooxidans*
Kaibin Fu, Hai Lin, Deqiang Luo, Wufei Jiang and Ping Zeng

Characterization of moulds associated with processed garri stored for 40 days at ambient temperature in Makurdi, Nigeria
Aguoru, Celestine U., Onda, Mercy Atoye, Omoni, Victor Taghoghor and Ogbonna, Innocent Okonkwo

Acute toxicity, lipid peroxidation and ameliorative properties of *Alstonia boonei* ethanol leaf extract on the kidney markers of alloxan induced diabetic rats
Enechi, O.C., Oluka, Ikenna H. and Ugwu, Okechukwu P.C.

Table of Contents: Volume 13 Number 5, 29 January, 2014

Antioxidant properties of volatile oils obtained from *Artemisia taurica* Willd. and *Salvia kronenburgii* Rech. Fil. plants and their effects on xanthine oxidase
Mahire BAYRAMOĞLU and Ferda CANDAN

Fractionation and determination of total antioxidant capacity, total phenolic and total flavonoids contents of aqueous, ethanol and n-hexane extracts of *Vitex doniana* leaves
Yakubu, O.E., Nwodo, O.F.C., Joshua, P.E., Ugwu, M.N., Odu, A.D. and Okwo F.

Synergistic effects of ethanolic plant extract mixtures against food-borne pathogen bacteria
Salvador Eduardo Vásquez Rivera, Mayra Alejandra Escobar-Saucedo, Diana Morales, Cristóbal Noé Aguilar and Raúl Rodríguez-Herrera

academicJournals

Vol. 13(5), pp. 634-639, 29 January, 2014
DOI: 10.5897/AJB2013.13510
ISSN 1684-5315 ©2014 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

DNA extraction protocols for *Citrullus lanatus* and *Capsicum frutescens*: Effects of incubation temperatures and ethanol concentrations on DNA purity and quantity

Florence Ifeoma Akaneme[1]*, Irene Chinelo Odo and Lynda Afoma Okafor

Department of Plant Science & Biotechnology, Faculty of Biological Sciences, University of Nigeria, Nsukka, Nigeria

Accepted 20 January, 2014

***Citrullus lanatus* has exceptional levels of oil (42 – 57%) and protein (33.8%) while *Capsicum frutescens* has high quantities of pro-vit A, C, B (B_1, B_2, B_3). In spite of these endowments, no disease resistant variety of *C. lanatus* has been developed in Nigeria through conventional breeding. Likewise, *C. frutescens* improvements has been limited mainly by susceptibility to pepper veinal mottle virus which is incurable and which leads to huge economic losses. Molecular breeding is a tested technique for developing disease-resistant varieties of species. A fundamental step to any molecular biology study is the capability to isolate pure genomic DNA. Many of the published plant DNA extraction protocols are not suitable for all plants due to the presence of secondary metabolites. Since every step of a protocol should be optimized for each species, this study which is the first in a series was, therefore, undertaken to ascertain the best incubation temperature and ethanol concentration that can be suitable for DNA extraction from both species. Results showed that the following combinations were optimal for *C. lanatus*: 100%ethanol/60°C and 95%/60°C. Their A_{260}/A_{280} ratios were 1.950 ± 0.014 and 1.860±0.031, respectively, while their corresponding DNA concentrations were 3.087 and 2.973 µg/µl. For *C. frutescens,* the combinations, 100%/60°C and 95%/60°C were optimal and their A_{260}/A_{280} ratios were 1.963 ± 0.004 and 1.803 ± 0.053 respectively while their corresponding DNA concentrations were 2.973 and 2.820 µg/µl. So it is recommended that any of the combinations for each species could be used for DNA extraction.**

Key words: DNA, extraction, protocol, pepper, egusi.

INTRODUCTION

Citrullus lanatus commonly known as egusi melon belongs to the family Cucurbitaceae. The largest producers are countries in West and Central Africa particularly Nigeria (Van der Vossen et al., 2004). The egusi seed is both protein and oil-rich. Reports have indicated that it contains about 42 – 57% oil (Fokou et al., 2004) and 33.8% protein (Ogbonna, 2013).

Egusi seed has mean total nitrogen of 5.75%. This is higher than those of peanut and cowpea but slightly less than that of soybean (6.65%) (National Academy of Sciences, 2006). The advantage egusi seed, however has over soybean is that it has no antinutrients (NAS, 2006) which make it particularly convenient to be used as a livestock feed (Van der Vossen et al., 2004). The other nutritional compositions of egusi are outstanding. It has exceptional levels of the essential amino acids, arginine,

*Corresponding author. E-mail: ifeoma.akaneme@gmail.com. Tel: (234) 803 6698 201.

methionine and tryptophan. It has good quantities of the vitamins, B1, B2 and niacin as well as the following minerals, phosphorus, potassium, magnesium, manganese, sulphur, calcium, iron and zinc (NAS, 2006).

Due to the potentials of this plant, NAS (2006) reported that it deserves concentrated local, regional and international attention. The authors further listed several areas of research needed on the plant among them is the issue of taxonomic clarification to determine if egusi is an aberrant watermelon. On this issue, NAS (2006) suggested the use of DNA fingerprinting technique and carefully monitored cross-pollination trials to clarify whether egusi is an inedible watermelon or a distinct species. Furthermore, van der Vossen et al. (2004) reported that breeding programmes in egusi will be greatly augumented through the use of molecular marker assisted selection and genetic transformation. These techniques may lead to the development of resistance to diseases and pests which has so far remained unattainable through conventional breeding.

Capsicum frutescens widely known as pepper belongs to the family Solanaceae. Generally it is regarded as the world's second most important crop after tomato (Yoon et al., 1989). It has been noted to be a very popular spice that is used all over the world (Mohammed et al., 2013). The largest producer in Africa is Nigeria which accounts for 50% of total Africa production (Abu et al., 2013). The many uses of pepper have been listed by various authors. These include: The use of pepper as a flavouring and colouring agents in food manufacturing and cosmetics industries (Bosland and Votara, 2000); as a medicine to combat constipation, relieve pain among others (Dagnoko et al., 2013); as a very important component in the preparation of soups, stew and other foods in many Nigerian homes (Onwubuya et al. 2008).

Nutritionally, pepper is highly valued. It contains high quantities of pro-vitamin A, C, B (B1, B2, B3), k, calcium, iron,zinc and fibre. Two species are widely grown in Nigeria and these include *C. frutescens* which is pungent. It has two varieties, bird pepper commonly called 'atawere' in Nigeria and Cayenne pepper or red pepper popularly known as 'Sombo'. The other species is *Capsicum annum* which is less pungent and mild. It also has two varieties, 'atarado' and 'tatase'. Other variations among these are in terms of colour, size and shape (Madu and Uguru, 2006; ICS-Nigeria et al. 2013).

Inspite of the acres of land reserved for pepper production in Nigeria, low yields have been reported by many authors (Adigun, 2000; Jaliya and Sani, 2006). Its production constraints have been limited mainly to different types of pathogenic organisms and insect pests (Madu and Uguru, 2006; Idowu-Agida et al., 2010; Mohammed et al., 2013). Among the disease organisms, the most virulent and of very serious concern to farmers is the pepper veinal mottle virus (PVMV) which is incurable (Madu and Uguru, 2006). The authors further observed that the viral infections can lead to huge economic losses and suggested that the development of resistant varieties is the most likely cost effective means of control of PVMV.

All these point to the fact that the use of molecular breeding techniques is the best option for the improvement of *C. lanatus* and *C. frutescens.* A fundamental step to any molecular biology study is the capability to isolate genomic DNA (Sharma and Purohit, 2012). The authors further noted that the isolated DNA must be of sufficient molecular weight and purity to be suitable for polymerase chain reaction (PCR) and restriction analysis. Successful extraction of such DNA could lead to the development of DNA fingerprinting techniques which could be used as a diagnostic tool for the assessment of genetic variability of a particular species (Vinod, 2004) and in this case for *C. lanatus* and *C. frutescens.*

Porebski et al. (1997) observed that the published DNA extraction protocols are not reproducible for all species judging from the number of species specific protocols being reported. Sangwan et al. (1998) went further to state that the DNA isolation methods need to be adjusted even to each plant tissue due to the presence of secondary metabolites. Vinod (2004) suggested that researchers working with minor crops or unexploited crop species which have no established DNA extraction protocol, should try the various published protocols, do some adjustments in order to derive a suitable protocol for the particular species. This research was, therefore, undertaken to study the effects of three temperature regimes and three ethanol concentrations on the purity and quantity of DNA extracts from *C. lanatus* and *C. frutescens.*

MATERIALS AND METHODS

The seeds of the two species were purchased from Nsukka market Enugu state Nigeria. They were authenticated by Mr Alfred Ozioko of Biodiversity and Conservation Programme/International Centre for Ethnomedicine and Drug Development located at No. 110 Aku Road, Nsukka. The experimental design is as shown in Table 1. The extraction procedure was adapted from the Department of Microbiology, University of Nigeria, Nsukka, Molecular Biology Workshop Manual of 2011 (Ezeonu, 2011). The same steps were used for all the treatment combinations in Table 1.

The seeds of the respective species were separately ground into powder using mortar and pestle. Subsequently, 1 g of the respective powder was transferred into each of 27 Eppendorf tubes into which were added 2 ml of sodium dodecyl sulfate (SDS) homogenization buffer (10mM Tris pH 7.5, 150 mM NaCl, 2mM EDTA, 0.5% SDS). The mixture of powder and SDS was incubated in a water bath for 15 min at the following temperature regimes: 10, 30 and 60°C. Nine tubes from each of the species were respectively incubated at each temperature regime. At the end of the incubation time, the respective tubes were cooled on ice in an ice bath for another 15 min. Subsequently, the homogenate were centrifuged at 12,000 rpm for 5 min. The respective supernatant were collected and transferred into new Eppendorf tubes. Subsequently, 40 µl of chloroform was added to the supernatant by allowing it to run down the side of the respective tubes. The tubes were swirled gently taking care not to mix the two liquids. The denatured proteins formed a white layer on top of the organic chloroform layer and the swirling of the tubes continued for about 2 to 3 min. With the aid of a dropper pipette, the top aqueous layers (homogenate) were transferred carefully into new Eppendorf tubes and the chloroform

Table 1. Experimental Design (Three replications for each treatment combination).

Ethanol concentration (%)	*C. lanatus*			*C. frutescens*		
	Temperature (°C)					
	10	30	60	10	30	60
	1	1	1	1	1	1
70	2	2	2	2	2	2
	3	3	3	3	3	3
	1	1	1	1	1	1
95	2	2	2	2	2	2
	3	3	3	3	3	3
	1	1	1	1	1	1
100	2	2	2	2	2	2
	3	3	3	3	3	3

Figure 1. DNA precipitate.

was poured into a clearly labeled waste container. This procedure was repeated four times for a total of four chloroform extractions. After the last extraction, the aqueous phase was collected leaving a small amount behind to avoid transferring any chloroform to the new tube.

The Eppendorf tubes containing the aqueous layers were allowed to cool on ice for 10 min. Subsequently, 1 ml of respective cold ethanol (70, 95 and 100%) was poured slowly down the side of the tubes. Each concentration of ethanol was added to three tubes from each of the temperature regimes for each of the species. A few minutes later, white stringy precipitate began to form on top of the ethanol in each tube (Figure 1). The DNA precipitate was centrifuged at 12,000 rpm for 8 min. The supernatant was decanted and the pellet was allowed to air-dry. Fifty microliter (50 µl) of TE buffer was added to the DNA precipitate and the tube was stored in a refrigerator.

Subsequently, 5 µl of each DNA extract was diluted in 1 ml of deionized water and was mixed gently. The dilution was transferred to a spectrophotometer cuvette. The spectrophotometer was zeroed at 260 nm with 1 ml of deionized water in an empty quartz cuvette. The cuvettes containing the diluted DNA extracts were then inserted into the spectrophotometer and the absorbance was read at 260 nm. The same procedure was repeated for 280 nm. The assessment of the purity of each of the DNA extracts was done by calculating the ratio of A_{260}:A_{280} (Held, 2001). Pure good quality DNA has A_{260}:A_{280} ratio ≥ 1.8 (Adeel 2008, Oxford Gene Technology, 2011). DNA quantity was obtained using the formular below:

DNA conc (µg/ml) = A_{260} reading x dilution factor x standard value.

The quantities of DNA obtained in this study were compared with the range (2.5 – 5.0 µg) reported by Lipp et al. (2005) for spectrophotometric analyses.

RESULTS

It could be observed from Table 2 that there were highly significant differences in the effects of the ethanol regimes, temperature concentrations and their combinations on the purity of DNA extracted from *C. lanatus* and *C. frutescens.* Table 3 shows that for each of the species, as the temperature and ethanol concentration increased, the DNA purity increased. The F-LSD calculated at 0.05% level of probability, however, revealed that some purity levels were not significantly different from one another while some were. For both species, pure DNA were produced by the following combinations: 100% ethanol / 60°C and 95%/60°C. Thus, 60°C incubation tempera-ture was the best. The purity levels for these various combinations were significantly different from one another and the purest DNA was obtained at 100% / 60°C. The most impure DNA were obtained at 10°C at all the ethanol concentrations and 30°C for 95 and 70% ethanol.

The ANOVA in Table 4 reveals that for each of the species, the quantities of DNA obtained were highly significantly different for the three ethanol concentrations and the three temperature regimes. Table 5 shows that for both species, as the temperature and ethanol concentration increased, the quantity of the DNA extract increased with the exception of quantity obtained at 95%/10°C for *C. frutescens.* The F-LSD calculated at 0.05% level of probability again showed that some DNA quantities were not significantly different from one another while some were. For both species, the quantities produced at 60°C

Table 2. Analysis of variance of the effects of ethanol concentrations and temperature regimes on DNA purity.

Source of Variation	DF	*C. lanatus*		*C. frutescens*	
		MS	VR	MS	VR
Ethanol	2	0.043	64.082**	0.107	47.852**
Temperature	2	0.589	868.443**	0.507	227.832**
Ethanol x Temp	4	0.008	11.992**	0.17	7.594**
Error	18				

**, Significantly different at 1% level of probability.

Table 3. Mean DNA purity levels for the various ethanol/temperature combinations.

Ethanol concentration (%)	*C. lanatus*			*C. frutescens*		
	Temperature (°C)					
	10	30	60	10	30	60
70	1.303± 0.011[f]	1.497± 0.027[e]	1.703±0.015[c]	1.317± 0.018[g]	1.363± 0.004[f]	1.630±0.049[c]
95	1.330±0.019[f]	1.583±0.008[d]	1.860±0.031[b]	1.317±0.016[g]	1.463±0.033[d]	1.803±0.053[b]
100	1.347±0.004[f]	1.617±0.020[d]	1.950±0.014[a]	1.367±0.054[e]	1.630±0.012[c]	1.963±0.004[a]
F-LSD0.05	0.0449					

For each species, means followed by the same lower case letters are not significantly different at 5% level of probability.

Table 4. Analysis of variance of the effects of ethanol concentrations and temperature regimes on DNA quantity.

Source of Variation	DF	*C. lanatus*		*C. frutescens*	
		MS	VR	MS	VR
Ethanol	2	0.077	16.290**	0.113	51.757**
Temperature	2	1.943	408.515**	1.465	672.757**
Ethanol x temp	4	0.012	2.428**	.022	9.900**
Error	18	0.005		0.002	

** = significantly different at 1% level of probability.

Table 5. Mean DNA Quantities obtained from the ethanol/temperature combinations.

Ethanol (%)	*C. lanatus*			*C. frutescens*		
	Temperature (°C)					
	10	**30**	**60**	**10**	**30**	**60**
70	2.030±0.074[f]	2.550±0.046[e]	2.813±0.052[c]	2.003± 0.041[f]	2.350±0.046[e]	2.645±0.011[c]
95	2.037±0.047[f]	2.660±0.032[d]	2.973±0.036[b]	1.997±0.020[f]	2.477±0.025[d]	2.820±0.025[b]
100	2.077±0.062[f]	2.787±0.046[c]	3.087±0.025a	2.033±0.045[f]	2.660±0.014[c]	2.973±0.043[a]
$F\text{-}LSD_{0.05}$	0.087			0.0366		

For each species, means followed by the same lower case letters are not significantly different at 5% level of probability.

for all the ethanol concentrations were significantly differ-rent from one another but the highest quantities were obtained at 100%/60°C. The quantities obtained at 100%/30°C and 70%/60°C were, however, not signifi-cantly different from one another so either of the combi-nations can be used. Moderate DNA quantities were also produced at 95%/30°C and 70%/30°C for only *C. lanatus.* The lowest quantities which were not signifi-cantly differ-rent were obtained at 10°C for all the ethanol concentra-tions.

DISCUSSION

A fundamental step in any molecular biology programme is the capability to extract DNA (Sharma and Purohit, 2012). The extracted DNA must be of high quality so as not to jeopardize the results of subsequent experiments. According to the study of Ginwal and Maurya (2010), 'good quality DNA is essential to achieve good results in experiments just like reagents.' In this study, genomic DNA was successfully isolated from *C. frutescens* and *C. lanatus* using various combinations of ethanol levels and temperature regimes. The purities and quantities of the extracted DNA varied for both species. The purest DNA for the species was obtained at these combinations: 100%/60°C and 95%/60°C. Sufficient quantities of DNA that fell within the range of 2.5 – 5.0 µg stipulated by Lipp et al. (2005) for spectrophotometric analyses were also obtained using the same combinations of ethanol and temperature.

Khan et al. (2007) and Gupta and Preet (2012) noted that incubation temperature is an indispensible criterion for the production of highly pure DNA that is also of good quantity. The earlier authors had to increase their incubation temperature from 65 to 70°C to be able to isolate highly pure and quantifiable DNA. The later authors performed DNA extraction at room temperature, 45, 50 and 55°C. They obtained the highest quantity of DNA at 55°C.

Ezeonu (2011) noted that the temperature of 60°C helps the SDS homogenization buffer to dissolve the cellular proteins. This is in line with the results obtained in this study. It was only 60°C that led to the extraction of DNA of high purity and quantity. The most impure DNA were obtained at 10°C; 30°C for 70% and 95% ethanol. Contaminants of extracted DNA include RNA, proteins, polysaccharides and polyphenol compounds (Pandey et al., 1996; Porebsk et al., 1997). Many authors have reported that A_{260}/A_{280} ratios less than 1.8 indicate protein or residual phenol contamination while ratios approximately 2.0 suggest RNA contamination (Thermo Scientific, 2013; Wang et al., 2013). Fortunately, none of the DNA extracts had a ratio of 2 but many had ratios less than 1.8. Contamination by phenol can affect the ratio calculations as well as contribute to over estimation of DNA concentration (Promega, 2013; Oxford Gene Technology, 2011).

Polyphenols can be removed by using polyrinylpyrrolidone which bind to them or through the use of high concentration of β-mercaptoethanol (Suman et al., 1999). Proteins are generally removed by denaturation and precipitation with chloroform and /or phenol (Vinod, 2004). But to prevent protein contamination *ab initio*, Owyoung (2013) suggested the inclusion of a protease digestion step prior to alcohol precipitation. Fisher (2013) observed that low DNA yield could be due to insufficient lysis, insufficient disruption and DNA being still bound to the membrane. The author proffered solutions as follows: prolonging the incubation time in lysis buffer, thorough homogenization of the sample preferably in liquid nitrogen, eluting the DNA in higher volumes or repeating the elution step up to three times. The elution buffer should also be preheated to 60°C before the elution process.

Gupta and Preet (2012) reported that SDS protocol was the cheapest among the DNA extraction protocols they compared. The other two were commercial kits – Dneasy kit and DNAzol(R). They also reported that the SDS protocol provided purity level comparable to DNeasy R kit and that its yield was 1.4 times higher than the comer-cial kits. This justifies the use of SDS method in this work. In conclusion, incubation temperature of 60°C and ethanol concentrations, 100 and 95% could be used for DNA extraction from *C. lanatus* and *C. frutescens.*

REFERENCES

Abu NE, Uguru MI, Obi IU (2013). Genotypic stability and correlation among quantitative characters in genotypes of aromatic pepper grown over years. Afr. J. Biotechnol. 12(20): 2792 – 2801.

Adeel (2008). Principles of DNA ExtractionChaudhary.kau.edu.sa/Files/0030235/files/19044_2nd YR MY Lab DNA extract_Molecular Genetics Lect 2nd yr MT_1st semester.pdf. Accessed 10th November, 2013.

Adigun JA (2000). Influence of Intra-row Spacing and Chemical weed control on the growth and yield of chilli pepper (*Capsicum frutescens* L) in the Nigerian Northern Guinea Savannah. Nig. J. Hortic. Sci. 5: 67 – 73.

Bosland PW, Votara ET (2000). Pepper, Vegetable and Spices. CABI Publishing Walling Ford, UK. p. 204.

Dagnoko S, Taro-Diarisso N, Sanogo PN, Adetula O, Dolo-Nantoume A, Gamby-Toure K, Traore-Thera A, Katile S, Diallo-Ba D (2013). Overview of pepper (Capsicum spp) breeding in West Africa, Afr. J. Agric. Res. 8(13): 1108 – 1114.

Ezeonu IM (2011). Extraction of DNA from eukaryotic cells (Allium). In: Molecular Biology and Biotechnology Workshop Manual, Department of Microbiology, University of Nigeria, Nsukka. p.30.

Fisher Molecular Biology (2013). Plant Genomic DNA Extraction Maxi Kit. www.fishermolecularbiology.com/uploads/files/DE-042 & DE - 043Plant Genomic MAXI.pdf

Fokou E, Achu MB, Tchouanguep MF (2004). Preliminary nutritional evaluation of five species of egusi seeds in Cameroon. Afr. J. Food Agric. Nutr. Dev. (AJFAND), 4(1): 1 – 11.

Ginwal HS, Maurya SS (2010). Evaluation and optimization of DNA extraction method For Dalbergia sissoo leaf. Indian J Biotechnol. 9: 69 – 73.

Gupta S, Preet S (2012). Protocol optimization for genomic DNA extraction and RAPD – PCR in mosquito larvae (Diptera: Culicidae). Ann. Biol. Res. 3(3): 1553 – 1561.

Held PG (2001). Nucleic Acid Purity Assessment Using A_{260}/A_{280} Ratios. Classes.soeucsc.edu/bme2201/Spring11/Reading/PowerWave200_Nucleic_Acid_Purity_Assessmen. Accessed on 10 November, 2013.

ICS-Nigeria, USAID, IITA (2013). Growing Pepper in Nigeria – Commercial Crop Production Guide Series. www.cassavabiz.org/agroenterprise/ent%20images/pepper_01.pdf.

Idowu-Agida OO, Nwaguma EI, Adeoye IB (2010). Cost implications of wet and dry season pepper production in Ibadan, South Western Nigeria. Agric. Biol. J. N. Am. 1(4):495-500.

Jaliya MM, Sani BM (2006). Pepper production under irrigation. National Agricultural Extension Research Liaison Services, Ahmadu Bello University, Zaria, Extension Bulletin No. 206, Agricultural Engineering Series 7.

Khan S, Qureshi M, Kamaluddin AT, Abdin MZ (2007). Protocol for isolation of genomic DNA from dry and fresh roots of medicinal plants suitable for RAPD and restriction digestion. Afr. J. Biotechnol. 6(3): 175 – 178.

Lipp M, Shillito R, Giroux R, Spiegelhalter F, Charlton S, Pinero D, Song P (2005). Polymerase Chain Reaction Technology as Analytical Tool in Agricultural Biotechnology. J. AOAC Int. 88(1) : 136 – 155.

Madu EA, Uguru MI (2006). Inter-relations of growth and disease expression in pepper using principal component analysis (PCA). Afr. J. Biotechnol. 5(11): 1054 – 1057.

Mohammed AB, Ayanlere AF, Ekenta CM, Mohammed SA (2013). Cost and Return Analysis of Pepper Production in Ethiope West LGA of Delta State, Nigeria. Int. J. Appl. Res. Technol. 2(2): 3 – 7.

National Academy of Sciences (2006). Lost Crops of Africa, Vol. 11: Vegetables, National Academies Press, Washington, DC, 352pp.

Ogbonna PE (2013). Floral habits and seed production characteristics in “Egusi” melon (*Colocynthis citrullus* L.) J. Plant Breed. Crop Sci. 5(6): 137 – 140.

Onwubuya EA, Okporie EO, Nenna MG (2008). Nsukka yellow pepper processing and preservation techniques among women farmers in Enugu State. Afr. J. Agric. Res. 4(9): 859 – 863.

Owyoung P(2013).DNA Extraction Protocol. www.ehow.com/about_6192995_dna_extraction_protocol.html.Accessed on 27th November, 2013

Oxford Gene Technology (2011). Understanding and measuring variations in DNA Sample quality. http://www.ogt.co.uk/resources/literature/483_understanding_and_measuring_variations_in_dna_sample_quality. Accessed 14th November, 2013.

Pandey RN, Adams RP, Flournoy LE (1996). Inhibition of random amplified Polymorphic DNAs (RAPDs) by plant polysaccharides. Plant Mol. Biol. Rep.14: 17 – 22.

Porebski S, Balley LG, Baum BR (1997). Modification of a CTAB DNA extraction Protocol for plants containing high polysaccharides and polyphenols component. Plant Mol. Biol. Rep. 15: 8 – 15.

Promega (2013) How do I determine the concentration, yield and purity of a DNA sample? http://www.promega.co.uk/resources/articles/pubhub/enotes/how-do-i-determine-the-concentration-yield-and-purity-of-a-dna-sample/?. Accessed 14th November, 2013.

Sangwan NS, Sangwan RS, Kumar S (1998). Isolation of genomic DNA from the Antimalarial plant *Artemisia annua*. Plant Mol. Biol. Rep. 16(4): 1 – 9.

Sharma P, Purohit SD (2012). An improved method of DNA isolation from polysaccharide rich leaves of *Bosweltia serrata* Roxb. Indian J. Biotechnol. 11: 67 –71.

Suman PSK, Ajit KS, Darokar MP, Sushil K (1999). Rapid isolation of DNA from dry and fresh samples of plants producing large amounts of secondary metabolites and essential oils. Plant Mol. Biol. Rep. 17: 1 – 7.

Thermo Scientific (2013) Assessment of Nucleic Acid Purity. T042-Technical Bulletin Nano Drop Spectrophotometers, nano-drop.com/Library/TO42-NanoDrop-Spectrophotometer-Nucleic-Acid-Purity-Ratios.pdf. Accessed 20th November, 2013.

van der Vossen HAM, Denton OA, El Tahir IM (2004). Citrullus lanatus (Thunb). Matsum & Nakai [Internet] Record from PROTA4U. Grubben GJH, Denton OA (Editors). PROTA (Plant Resources of Tropical Africa/Ressources vegetale de l’Afrique tropicale), Wageningen, Netherlands. http://www.prota4u.org/search.asp. Accessed 2 November, 2013.

Vinod KK (2004). Total genomic DNA extraction, quality check and quantitation. In:Proceedings of the training programme on ‘Classical and Modern Plant Breeding Techniques – A hands on training’, Tamil Nadu Agricultural University,Coimbatore, India. http://kkvinod.webs.com. Accessed on 15th October, 2013, pp.109 – 121.

Wang J, Wu G, Li W, Zhao K, Zhu Z, Liu H, Li P, Bai L, Wei M, Pan A, Lu B, Tang X (2013). A DNA extraction method used for evaluation of diversity of the plant Rhizosphere microbial community. Afr. J. Microbiol. Res. 7(26): 3364 - 3372.

Yoon JY, Green SK, Tshanz AJ, Tsou SCS, Chenge LC (1989). Pepper Improvement for the tropics, Problems and Approach In: Tomato and Pepper Production in the Tropics, Asian Vegetable Research and Development Centre, AVRDC Shantine Taiwan. pp. 86 – 90.

academicJournals

Vol. 13(5), pp. 640-650, 29 January, 2014
DOI: 10.5897/AJB2013.13584
ISSN 1684-5315 ©2014 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Mechanism of biological control of *Rhizoctonia* damping-off of cucumber by a non-pathogenic isolate of binucleate *Rhizoctonia*

Mohsen Mohamed Elsharkawy[1], Naglaa Hassan[2,3], Remedios Villajuan-Abgona[2] and Mitsuro Hyakumachi[4]*

[1]Department of Agricultural Botany, Faculty of Agriculture, Kafr El-Sheikh University, Kafr El-Sheikh 33516, Egypt.
[2]United Graduate School of Agricultural Science, Gifu University, 1-1 Yanagido, Gifu 501-1193, Gifu, Japan.
[3]Department of Plant Pathology, Faculty of Agriculture, South Valley University, Qena 83523, Egypt.
[4]Laboratory of Plant Pathology, Faculty of Applied Biological Sciences, Gifu University, 1-1 Yanagido, Gifu City 501-1193, Japan.

Accepted 24 January, 2014

The interaction of binucleate *Rhizoctonia* (BNR) anastomosis group (AG)-A isolate W7, *Rhizoctonia solani* AG-4 and cucumber seedlings were investigated to elucidate the mechanism of biocontrol of *Rhizoctonia solani* by BNR. Hypocotyls of *Cucumis sativus* L. cv. Jibai were inoculated with a virulent isolate of *R. solani* AG-4 isolate C4 and examined with light microscopy and scanning and transmission electron microscopy. The hyphae of the virulent isolate colonized the outer surface of the hypocotyl and penetrated the epidermal and cortical cells to the pith of hypocotyls. The hyphae of a non-pathogenic species of BNR isolate W7 did not penetrate the cortical cells but instead colonized the outer epidermal cells 12 h after inoculation. Accumulation of mucilage occurred on the surface after dense colonization of the hypocotyls surface by the BNR. Observation of the hypocotyls surface pre-inoculated with BNR and challenged inoculated with *R. solani* showed constricted and deformed hyphae of *R. solani,* which were prevented to penetrate the epidermal cells. Pre-inoculating of BNR isolate caused profused formation of the mucilage which lysed BNR hyphae and restricted the growth of *R. solani*. The mucilage was identified as pectic substances. Analysis of pectin contents from dried hypocotyls tissues showed highly methylated pectin with low uronic acid contents in the seedlings inoculated with *R. solani* and unprotected by BNR while BNR- treated seedlings showed less methylated pectin and high amount of uronic acid. Furthermore, the BNR-inoculated seedlings showed significant (*P=0.05*) increase in calcium, indicating high amount of wall-bound cations in cell walls. Pectic substances accumulation and increased calcium in the cucumber tissues greatly contributed in the protection of cucumber seedlings against *Rhizoctonia* damping off by a non-pathogenic species of *Rhizoctonia.*

Key words: *Rhizoctonia solani*, binucleate *Rhizoctonia*, cucumber, biological control.

INTRODUCTION

The use of hypovirulent and non-pathogenic isolates of *Rhizoctonia* spp. has been potentially shown as effective biocontrol agents. Among *Rhizoctonia* spp., binucleate *Rhizoctonia* was effective in controlling diseases caused by *Rhizoctonia* spp. and *Pythium* spp. Although many studies have reported the role of binucleate *Rhizoctonia* in biological control of *Rhizoctonia* spp. and *Pythium* spp. in different plant species such as sugarbeet (Herr, 1988),

corn (Pascual et al., 2000), cotton (Jabaji-Hare and Neate, 2005), radish (Sneh et al., 2004), potato (Escande and Echandi, 1991), bean (Cardoso and Echandi, 1987a, 1987b), bedding plants (capsicum and celosia) and cucumber (Cubeta and Echandi, 1991; Villajuan-Abgona et al., 1996a), little is known about the mechanism of biological control.

The possible mechanisms of protection of creeping bentgrass, bean and cotton seedlings by a binucleate *Rhizoctonia* (BNR) and hypovirulent *Rhizoctonia solani* isolate have been studied but the results have led to different assumptions and conclusions. For instance, competition for nutrients and host induced resistance appeared to be the plausible mechanisms for protection of bean and creeping bentgrass (Burpee and Goulty, 1984; Cardoso and Echandi, 1987b). While in case of cotton seedlings, competition for infection sites or dense outer surface colonization was pointed out by Sneh et al. (1989).

Host-pathogen relationship between the virulent *Rhizoctonia* spp. and different hosts have been described in previous studies (Christou, 1962; Armentrout and Downer, 1987), but details on the histological study of host-fungus relationship which are essential for understanding the mode of action by hypovirulent or non-pathogenic isolates have not been thoroughly elucidated.

The present study was undertaken to describe in details, through histological study, the mode of colonization by the non-pathogenic BNR and the penetration of the hypocotyls surface and taproot of cucumber seedlings by a virulent *R. solani* isolate. To justify this initial finding, the result was compared with biochemical studies which revealed the importance of pectic substance and its influence in the suppression of the disease. Furthermore, the reasons of the protection provided by the non-pathogenic BNR to cucumber seedlings against *Rhizoctonia* damping-off were clarified.

MATERIALS AND METHODS

Fungal isolate

BNR isolate W7 (AG-A) obtained from tomato, non-pathogenic to several hosts and effectively control damping-off disease in cucumber (Villajuan-Abgona et al., 1996a) was used as the biocontrol agent. A virulent *R. solani* isolate C4 (AG-4), isolated from soil, causing severe damping-off disease to radish and cucumber was used as the challenge isolate.

Inoculation procedure

Cucumber (*Cucumis sativus* L. cv. Jibai) seeds were surface-disinfested in 70% ethyl alcohol (EtOH) for 1 min followed by 2% sodium hypochlorite with three drops of Tween 20 (polyoxyethylene sorbitan monolaurate) (Nacalai Tesque, Inc., Kyoto, Japan) for 30 min. The seeds were rinsed three times with sterile distilled water (SDW) and pre-germinated in between two layers of 90 mm diameter filter paper (Whatman No. 1, camlab, UK). Five seedlings were transferred to 2% water agar (WA) in Petri dish and allowed to grow for two days in growth chamber (16 h photoperiod with a daytime illuminance of 250 µE m^{-2s-1}) at 25°C. Mycelial disks (3-mm diameter) of the non-pathogenic BNR (from the advancing margin of three-day-old pure culture on potato dextrose agar (PDA)) were inoculated on the base of five hypocotyls of two day-old seedlings and incubated for 12 h and another set of treatment for 24 h. 1) After incubation of the non-pathogenic BNR for 12 and 24 h, 3 mm diameter mycelia disks of the virulent *R. solani* (grown on PDA) were inoculated on the same hypocotyls next to the previously inoculated mycelial disks of the non-pathogenic BNR and incubated for 12 and 24 h in the same growth chamber. Other treatments consist of 2) seedlings inoculated with non-pathogenic BNR alone, 3) seedlings inoculated with the virulent *R. solani* without the non-pathogenic BNR, 4) seedlings inoculated with 3 mm diameter disks of PDA only, 5) seedlings inoculated with 3 mm diameter mycelia disks of killed pathogen (autoclaved at 120°C for 30 min) and 6) un-inoculated seedlings.

All treatments were incubated for 12-24 h and each treatment was replicated three times. After incubation, the seedling hypocotyls and root portions were cut into segments for scanning electron microscopy (SEM) and paraffin sectioning for light microscopy. This study was repeated twice and observations were made on three replicates (with 10 segments per replicate) for each treatment of SEM and 100 paraffin sections were observed for each replicate in light microscopy.

Scanning electron microscopy

After incubation of the inoculated seedlings, the whole hypocotyl (including the areas inoculated with mycelia disks) and root portions of seedlings of all treatments were cut into segments (6 mm) and fixed for 2 h in 2% glutaraldehyde with 0.05M sodium cacodylate buffer (pH 7.4). The tissues were post-fixed in 1% OsO_4 with 0.05 M phosphate buffer (pH7.4) for 2 h and dehydrated through a graded ethyl alcohol (EtOH) series. The fixed specimens were then transferred to increasing concentrations of isoamyl acetate-EtOH mixture up to 100% isoamyl acetate concentration and critical dried point (JCPD-5, Japan Electron Optics Laboratory (Jeol), Ltd, Japan). Tissues were coated with gold on a JFC-1100 sputter coater (Jeol. Ltd, Japan) and examined with a field emission scanning microscope (JSM-820, Jeol, Ltd., Japan) at 10 kV and photographed. Replicate of the same samples were observed with a wavelength-dispersive X-ray microanalyzer (JSM-35CF/FCS, Jeol, Ltd, Japan) to study the distribution of elements on the hypocotyls surface of inoculated and un-inoculated cucumber seedlings.

Light microscopy

Hypocotyls and root segments (6 mm) were fixed for 24 h in FAA (37% formaldehyde-acetic acid- 50% EtOH) (6.5:2.5:91) (Johansen, 1940), washed with EtOH and dehydrated through increasing concentrations up to 100% butyl alcohol and embedded in paraffin (Wako, Osaka, Japan, 52-54°C mp). Transverse sections (16 µm

*Correponding author. E-mail: hyakumac@cc.gifu-u.ac.jp. Tel: +81-58-293-2847. Fax: +81-58-293-2847b.

Abbreviations: BNR, Binucleate *Rhizoctonia*; **SDW,** sterile distilled water; **WA,** water agar; **PDA,** potato dextrose agar; **EtOH,** ethyl alcohol; **FAA,** formaldehyde-acetic acid.

thick) of the cucumber hypocotyls and roots were cut using microtome (ERMA optical Works, Japan), mounted on glass slides with Haupt's adhesive (Johansen, 1940) and stained with phenyl-thionin (0.1% in 5% phenol solution) (Lauth's violet) (Nacalai Tesque, Inc., Kyoto, Japan) followed by staining in saturated solution of Orange G (Kishida chemicals Co., Ltd., Japan) in 100% EtOH (Stoughton, 1930). Thionin and Orange G were used to differentiate pectic substances and cellulose in cross-sections, respectively. Permanent mounts of the paraffin sections were examined with light microscopy (Olympus BH, Tokyo, Japan) and photographed in 135 ASA 100 film (Fujifilm, Tokyo, Japan) using camera AFM 35S (Nikon, Tokyo, Japan). Free-hand sections were taken from fresh samples, stained with aqueous ruthenium red (0.02%) (ammoniated ruthenium oxychloride) (Sigma Chemicals Co., St. Louis, MO) and used to identify pectin (Johansen, 1940; Sterling, 1970).

Inoculation procedure and assessment of disease severity

The same isolates were used and the above inoculation method was followed in this experiment consisting of four treatments: (1) seedlings inoculated with non-pathogenic BNR and incubated for two days and then challenge inoculated with virulent *R. solani* and again incubated for 6 days; 2) seedlings inoculated with the non-pathogenic BNR and incubated for 6 days; 3) seedlings inoculated with virulent *R. solani* without non-pathogenic BNR and incubated for three days; 4) and un-inoculated seedlings also incubated for six days). All treatments were kept in growth chamber (16 h photo-period with a daytime illuminance of 250 $\mu Em^{-2} s^{-1}$) after each inoculation. The treatments were prepared in five replicates and disease severity and sample preparations were done after six days. However, for seedlings inoculated with virulent *R. solani* without non-pathogenic BNR, disease severity and sample preparation were done three days after incubation because prolonged incubation can result in macerated or disintegrated hypocotyl tissues and make sample preparation difficult. Disease severity rating was determined using the following rating scheme (Villajuan-Abgona et al., 1996a), where 0 = healthy, no lesions on the hypocotyl; 1= one or two light brown lesions of <0.25 mm; 2 = light brown lesions of <0.5 mm and water soaked areas covering<10% of the hypocotyls; 3 = light to dark brown lesions>1.0 mm which coalesced with other lesions and water soaked areas covering >10% to <100% of the hypocotyl (leaves not yet wilted and hypocotyls still firm and upright); 4 = collapsed hypocotyls with wilted leaves of dead seedlings. Inoculation procedures for disease severity ratings and samples preparation for tissue analysis were done twice.

Analysis for pectic substances in hypocotyls tissues

The pectin contents of hypocotyls tissues were estimated following the procedure of Boos (1948). One hundred mg of dried and ground (40-mesh) samples of hypocotyls tissues were placed in 50 ml distillation flasks. 4 ml of 1 N NaOH were added to each flask and the mixture was allowed to stand for 30 min to saponify the pectin. This was followed by addition of 4.25 ml of 1 N HCl and distillation of the liberated methanol. The distillate was collected in 10 ml volumetric flask and diluted to 10 ml with water. Aliquot of 1 ml of the samples was transferred to a separate 10 ml volumetric flask and 3 µm diluted H_3PO_4 and 5 µl 5% $KMnO_4$ were added. The samples were held at room temperature for 10 min and occasionally swirled to ensure oxidation of CH_3OH to CH_2O. Then, 10 µl $NaHSO_3$ was added to reduce the excess permanganate. 4 ml concentrated H_2SO_4 was added slowly to each sample. The samples were placed in water bath at 60°C and held with occasional swirling for 15 min. The flasks were cooled in ice bath and water was added to make a 10 ml-volume. After the samples reached room temperature, the absorbance of the samples was determined spectrophotometrically (Hitachi U2000A, Hitachi Ltd., Tokyo, Japan) at 580 nm. The quantity of methanol was calculated from a standard curve and expressed in meq per gram dry weight of tissue and percentage of methanol was calculated.

Uronic acid contents of the hypocotyls tissues were determined following the procedures of McCready and McComb (1952). Dry tissues were ground to pass a 40-mesh screen and desugared by extraction with 70% EtOH. The extraction consisted of permitting the dry tissue to stand in 70% EtOH (100 ml/g tissue) for 18 h at room temperature followed by filtration on Whatman No. 1 filter paper and washing with one-fourth of the original volume of 70% EtOH. Desugared tissues were dried at 80°C and moistened with 95% EtOH. Two hundred ml of 0.5% Versene solution described by McCready and McComb (1952) were added to each sample to sequester multivalent cations. After adjusting the sample to pH 11.5, it was held at room temperature for 30 min to permit saponification of pectin. The pH was then adjusted to pH 5.0-5.5, and 0.1 g of pectinase (EC 3.2.1.15, from *Aspergillus niger*) (Sigma Chemical Co., St. Louis, MO) was added to each sample to solubilize the pectic substances. The samples were stirred for 1 h then diluted with water to 250 ml. The mixture was filtered through No. 1 Whatman filter paper (90 mm-diameter) discarding the first few millilitres. From the filtrate, 2 ml aliquot was diluted to 10 ml in a separate flask, mixed thoroughly and set aside to be used for analysis. In a separate tube, 12 ml of concentrated H_2SO_4 was cooled to 3°C and 2 ml from the mixture (2 ml aliquot + 10 ml water) was added and mixed thoroughly. The mixture was cooled to 3°C and then heated in boiling water for 10 min. After cooling the mixture to about 20°C, 1 ml of 0.15% carbazole reagent (Sigma Chemical Co., St. Louis, MO) was added to each tube, the contents were mixed thoroughly and allowed to stand at room temperature for 25±5 min. The absorbance of the samples was determined at 520 nm using Hitachi U2000A spectrophotometer (Hitachi Ltd., Tokyo, Japan). A standard curve was used to calculate the amount of anhydrouonic acid using galacturonic acid monohydrate as the standard. Results expressed as meq anhydrogalacturonic acid present per gram dry weight of tissue before desugaring.

Analysis for total elements in hypocotyls tissues

Inductively coupled plasma atomic emission spectrometry was used to determine the total elements present in hypocotyls of cucumber. Cucumber hypocotyls were separated from leaf and root portion of the seedlings, oven dried at 80°C and weighed. The oven dried tissues were ground to pass a 40-mesh screen and 0.5 g samples per treatment were digested to dryness in 10 ml HNO_3. The residue was redissolved in 10 ml 35%HCl and 1 ml Itolium standard solution (Kishida Chemicals Co., Ltd., Osaka, Japan) and diluted with 100 ml of cold purified distilled water. Analyses were done using inductively coupled plasma atomic emission spectrometer (Model JY48P) (Carrier gas- Argon) (Seiko Co., Osaka, Japan). The total concentrations of elements in samples were expressed in milli-equivalent per gram dry weight.

Data analysis

Experiments for biological study was laid out in 2 x 4 factorial in randomized complete block design with varied time of incubation period (12 and 24 h) while BNR and *R. solani* treatments as sub-plots. Results of histological study were qualitative observations with each assessment done based on the results of two experiments and on the total number observed per treatment. Each treatment was replicated three times. For analysis of pectin and total elements, the experiment was laid out in completely randomized design, repeated twice with five replicates. Data were

statistically analyzed using Microstat Analysis Progma 2.0 Release (Ecosoft Inc., Indianapolis, IN). Analysis of variance (ANOVA) was performed for the assessed data (each experiment considered as one replicate) in all parameters (disease severity, methanol, uronic acid, calcium content) to test the significance of each treatment. Treatment means were compared using Fisher's least significant difference (LSD, *P=0.05* and *P=0.01*) and Duncan's multiple range test (*P=0.05*).

RESULTS

Light and scanning electron microscopy

Morphological characteristics of the virulent *R. solani* isolate C4 showed slightly-melanized hyphae and irregularly-shaped and brownish sclerotia. Microscopic observation showed short-branched young hyphae produced by the virulent isolate while the non-pathogenic BNR produced long-branched young hyphae. The hyphal width for *R. solani* ranged from 5.0 to 8.0 μm while for the non-pathogenic BNR, ranged from 3.0 to 6.0 μm.

After inoculation on the hypocotyls surface, the hyphae of the virulent isolate of *R. solani* grew longitudinally, but did not grow along the anticlinal walls of the adjacent epidermal cells. It colonized and penetrated the outer surface of the hypocotyls, while the penetrating hyphae ramified through surface. Enzymatic substance was released by the fungus, which macerated the outer epidermal cells and disintegrated the host tissues (Figure 1A). The base portion of the hypocotyls until the older portion of the root was fully colonized and fully grown and young hyphae of *R. solani* were produced 24 h after inoculation (Figure 1B). Direct penetration was observed through hyphal tips which grew over the surface or between ridges of adjacent epidermal cells. The hyphae of *R. solani* penetrated the epidermal and cortical cells (Figure 2A). Penetration could be observed in the pith of the hypocotyls. Three days after inoculation, the virulent isolated occupied the parenchymatic cells and extensively macerated the tissues.

The non-pathogenic BNR densely colonized the outer surface of the lower hypocotyls, the whole taproot and a part of the lateral root adjacent to the taproot. The hyphae did not penetrate the cortical cells but colonized the outer surface of the epidermal cells 12 h after inoculation (Figure 1C). Upon inoculation of the BNR, accumulation of the mucilageinous material occurred on the surface of the hypocotyl until it became profused and lysed the hyphae of BNR (Figure 1D). Accumulation of mucilaginous material occurred not only on surfaces directly in contact with the hyphae but also on surface where no hyphae were found (Figure 1C). Stained section of the basal hypocotyls showed profused accumulation of mucilaginous material which lysed the hyphae of the non-pathogenic BNR on the epidermal and sub-epidermal layers as shown by the dark bluish- colored portion stained by phenyl-thionin (Figure 2B). The greater stain concentration was especially apparent outside the cell walls and the substance with affinity of phenyl-thionin appeared from the epidermal region. The cortical cells were still intact and no hypha of BNR was observed.

The formation of mucilaginous material which occurred on the hypocotyls surface upon inoculation of the non-pathogenic BNR restricted the growth of the virulent isolate. Pre-inoculation with the non-pathogenic BNR followed by challenge-inoculation with the virulent isolate showed constrict deformed hyphae of the virulent isolate which were prevented to penetrate the epidermal cells (Figure 1E). Hyphae of the virulent isolate of *R. solani*, which could not penetrate the host epidermal cells, were observed mainly above the lysed mycelia mat of the BNR. Later, the mycelia mat was lysed together with the hyphae of the non-pathogenic BNR and formed a gel layer (Figure1F). Hyocotyl sections pre-inoculated with non-pathogenic BNR followed by challenge-inoculated with virulent *R. solani* C4 after 24 h showed intact epidermal and cortical cells. Although the outer epidermal cells were sloughed-off (Figure 2D), radiating from this area for a distance of an additional three cells increased wall staining by phenyl-thionin which appeared blue. The affinity of cellulose in host cells to Orange G stain caused similar appearance of the stained sections of the hypocotyls of non-treated seedlings, seedlings inoculated with mycelia disks of killed pathogen and PDA only (Figure 2C).

Since phenyl-thioninn has an affinity to pectic materials such accretion of the stain on the cell walls indicate pectic substance accumulation. To verify this suggestion, ruthenium red was also used for free-hand cross-sections hypocotyls of seedlings which had the same treatment. A clear differentiation of red stained cell walls and outer epidermal cells was observed for seedlings treated with non-pathogenic BNR in contrast to deep pink stained cross section of healthy and unstained cross-section of seedlings inoculated with virulent *R. solani* (Table 1). The results reveal by ruthenium red staining indicated that the pectic substance present on cell walls and on hypocotyls surface is pectin.

Reduction in severity of *Rhizoctonia* damping-off disease

The cucumber seedlings grown on water agar and treated with mycelia disks of hypovirulent/ non-pathogenic BNR isolate W7 two days prior to inoculation of the virulent *R. solani* isolate C4 showed a low disease severity (DSI=1.4) which is highly significant compared with hypocotyls of seedling inoculated with virulent *R. solani* without non-pathogenic BNR (Figure 4).

The BNR isolate provided 63.2% protection to the seedling against the pathogen. The cucumber seedling inoculated with the non-pathogenic BNR alone exhibited low disease severity (0.2) which indicate faint lesion and slight browning of the root portion and can be considered as a non-pathogenic reaction.

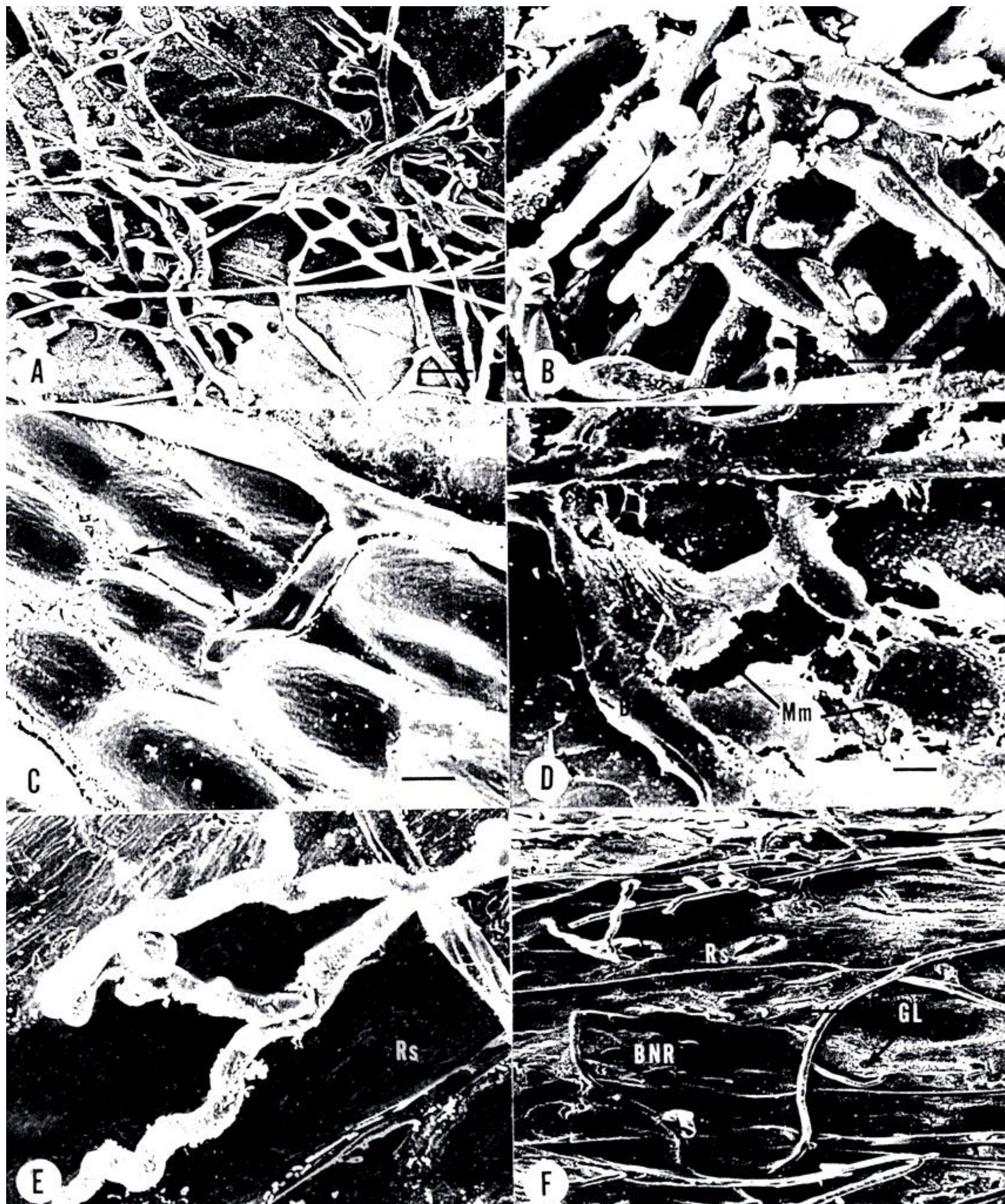


Figure 1. Scanning electron micrographs (SEM) of: **A)** cucumber hypocotyls surface inoculated with *R. solani* C4 only showing hyphae ramifying through the lesion with macerated epidermal cells and disintegrated host tissues 24 h after inoculation. Bar=50µm; **B)** hypocotyl surface inoculated with *R. solani* C4 only showing fully grown, young hyphae of *R. solani* 24 h after inoculation. Bar = 10 µm; **C)** colonization of the hypocotyls surface inoculated with BNR W7 only 12 h after inoculation and initial accumulation of mucilaginous material on the surface directly in contact with the hyphae and also in areas where hyphae was not found Bar = 10 µm; **D)** profused production of mucilaginous material which lysed the hyphae of BNR W7 on hypocotyls surface inoculated with BNR W7 only Bar = 10 µm; **E)** constricted and deformed hyphae of *R. solani* C4 on hypocotyls surface previously inoculated with BNR W7 and challenge inoculated with *R. solani* C4. Bar=10 µm; **F)** hypocotyl surface showing accumulation of mucilaginous material which occurred 12 h after inoculation with BNR W7, which subsequently lysed the BNR hyphae and turned into a gel layer while *R. solani* C4 which was challenge inoculated could not penetrate the epidermal cells and was observed mainly above the lysed mycelia mat of BNRW7. Bar= 50 µm. Mm, Mucilaginous material; GL, gel layer; BNR, binucleate *Rhizoctonia*; Rs, *R. solani*.

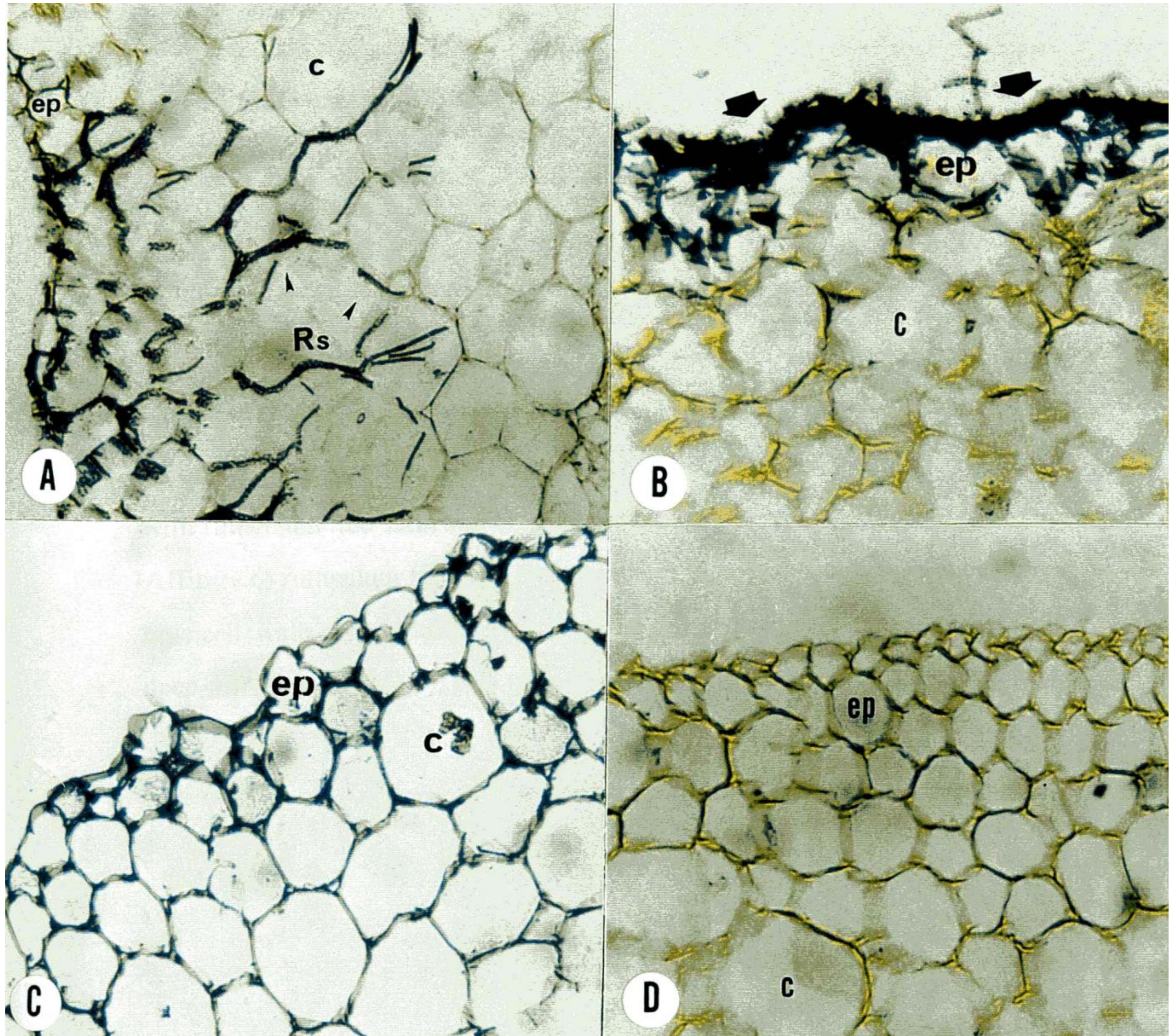


Figure 2. Light micrographs of cross-sections of cucumber hypocotyls stained with phenyl-thionin and counterstained with Orange G showing A, Hyphal penetration of *R solani* (Rs) into the epidermal and cortical cells of seedling hypocotyls inoculated with *R. solani* C4 only; B, cross-section inoculated with BNR W7 only showing accumulation of mucilaginous material (arrows) in the epidermal and sub-epidermal layers as shown by the dark-bluish colored portion stained by phenyl-thionin and non=penetration of BNR W7 into cortical cells; C, cross-section of uninoculated seedlings showing affinity to Orange G indicating undisturbed cells; D, cross-section previously inoculated with BNR and challenge inoculated with *R. solani* showing intact epidermal and cortical cells which are deeply-stained by phenyl-thionin indicating pectic substance accumulation on the cell walls. All micrographs were taken 40x. ep, Epidermal cells; C, cortical cells, Rs, *R. solani*; BNR, binucleate *Rhizoctonia*.

Earlier experiment had shown that un-inoculated seedlings did not show any symptoms of the disease and all treatments showed similar results. Thus, only the data for treatment of un-inoculated seedlings were considered for disease severity rating and tissue analysis.

Nature of pectic substance on hypocotyls tissues

Analysis of total pectin content of the dried hypocotyls tissues of cucumber seedlings indicated by percentage of methanol and amount of uronic acid. Results showed that percentage of methanol in hypocotyls tissues inoculated with virulent *R. solani* without non-pathogenic BNR were highly significant compared with other treatments (Table 2).

However, the methanol content of hypocotyls tissues inoculated with virulent *R. solani* and non-pathogenic BNR and hypocotyls tissues treated with non-pathogenic BNR alone showed similar results and were both significantly different than the control.

Table 1. Location of pectic substances using ruthenium red stain in hypocotyls tissues of cucumber seedlings inoculated with virulent *R. solani* with and without non-pathogenic BNR[1]

Treatment	Affinity to stain[2]
R. solani without BNR	-
R. solani with BNR	++
Nonpathogenic BNR	+++
Uninoculated	+

[1]Seedlings grown in 2% water agar were inoculated on the base of the hypocotyls with either non-pathogenic BNR or *R. solani*. Free-hand cross-sections (50 cross-sections) from each treatment were obtained on the site of inoculation and treated with ruthenium red stain and examined microscopically. [2]Affinity to ruthenium red stain indicate the presence of pectic substances in the host cell walls. Stain differentiation of the cell walls in each cross-section showed deep-red (+++), red (++), pale pink (+), and unstained.

Table 2. Pectin content in dried hypocotyls tissues of cucumber seedlings inoculated with virulent *R. solani* with and without the non-pathogenic binucleate *Rhizoctonia*.

Treatment	Methanol (%)	Uronic acid (meq/g)
R. solani without BNR	60.0[a]	7.0[c1]
R. solani with BNR	50.1[b]	7.7[b]
Nonpathogenic BNR alone	50.2[b]	8.3[a]
Uninoculated	29.3[c]	7.4[bc]

[1]Means values of two experiments. Values in columns followed by the same letter (s) are not significantly different (*P=0.05*) using Duncan's multiple range test.

Table 3. Analysis of total elements in the extracts of cucumber hypocotyls colonized by virulent *R. solani* with and without non-pathogenic binucleate *Rhizoctonia* using inductively coupled plasma atomic absorption spectrophotometer[1].

Treatment	Na	Mg	P	K	Ca	Mn	Fe	Cu	Zn
R. solani without BNR	18.4	12.9	41.9	13.4	8.8 b[2]	0	0.4	0	0
R. solani with BNR	17.2	15.6	38.6	14.2	10.5 a	0	0.5	0	0.3
Nonpathogenic BNR alone	24.2	20.5	45.2	15.7	15.4 a	0	0.9	0	0.2
Uninoculated	17.3	17.3	38.0	9.5	8.0 b	0	0.7	0	0.4

[1]Mean values of two experiments; [2]Values followed by the same letter are not significantly different (*P=0.05*) using Duncan's multiple range test.

The total uronic acid content of hypocotyls tissues inoculated with virulent *R. solani* without non-pathogenic BNR was significantly lower than the hypocotyls tissues inoculated with virulent *R. solani* and protected by non-pathogenic BNR. It was also significantly different when compared with hypocotyls inoculated with non-pathogenic BNR alone (Table 2). However, in comparison with control, hypocotyls tissues inoculated with virulent *R. solani* with and without non-pathogenic BNR showed similar results.

Total elements in extracts of hypocotyls tissues

Analysis for total elements revealed high amounts of Na, Mg, P, K, Ca and trace amount of Fe and Zn in hypocotyls tissues of BNR- treated seedlings when compared with hypocotyl tissues of seedlings inoculated with virulent *R. solani* and the control (Table 3). However, Zn was not obtained in *R. solani*-treated seedlings as well as Mn and Cu which were also absent in other treatments. Concentration of Ca increased in *R. solani*-treated seedlings and BNR treated seedlings compared with other treatments.

The amount of calcium obtained from the hypocotyls tissues inoculated with non- pathogenic BNR alone is double in proportion to the amount of uronic acid obtained in hypocotyls tissues having the same treatment. Additionally, Na content was increased in BNR

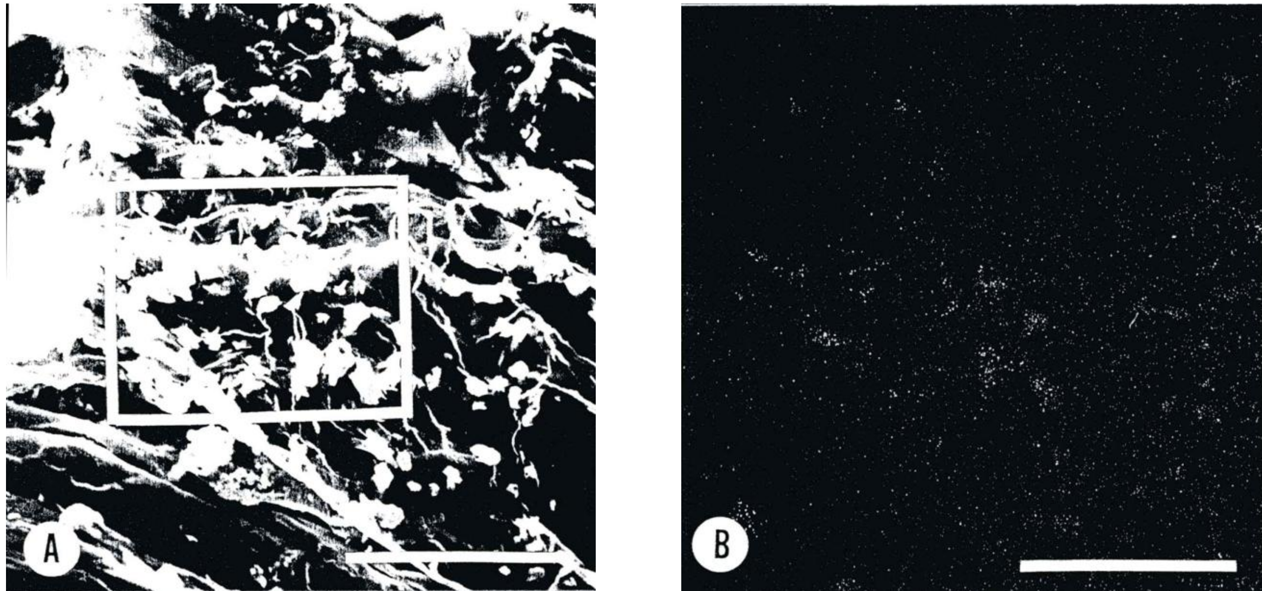


Figure 3. Scanning electron micrographs (SEM) of cucumber hypocotyls surface showing: A) distribution of crystal-like deposits on hypocotyls surface inoculated with BNR W7 only which appeared as calcium salts. The outline shows the area scanned with x-ray detector. Bar = 15 µm; **B)** x-ray dot map in which calcium intensity is shown by white spots. Bar =15 µm.

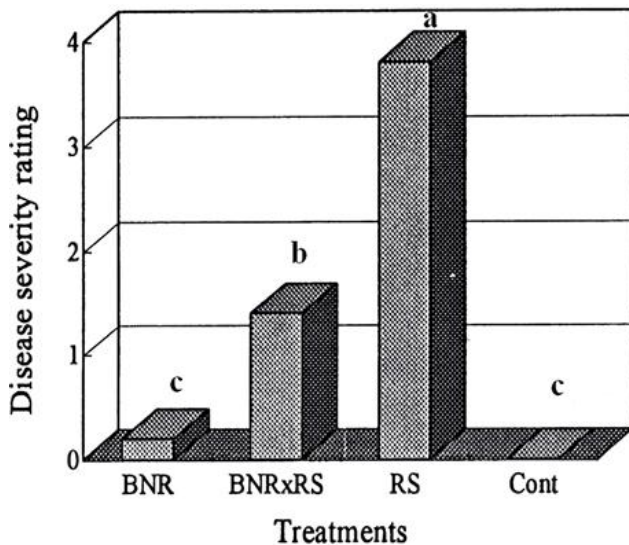


Figure 4. Reduction in severity of *Rhizoctonia* damping-off disease on cucumber seedlings grown in water agar caused by virulent *R. solani* C4 (AG4) as protected by non-pathogenic binucleate *Rhizoctonia* W7. LSD values for comparison of treatment means are 0.57 (*P=0.05*) and 1.70 (*P=0.1*).

alone-treated seedlings compared with other treatments.

Analysis with a wavelength-dispersive X-ray microanalyzer

Hypocotyls of seedlings inoculated with BNR isolates showed crystal-like deposits such as calcium salts (Figure 3A). These deposits were not observed on hypocotyls inoculated with *R. solani* without the BNR and un-inoculated seedlings. The area scanned with X-ray detector showed X-ray dot map shown as white spots which indicate high calcium intensity in areas where crystal-like deposits are present (Figure 3B).

DISCUSSION

The infection process by which the virulent *R. solani* Kuhn (*Thanatephorus cucumeris*, Donk.) penetrates the host surface was described in various ways (Floyd and Ohlorogge, 1970; Marshall and Rush, 1980; Staples and Macko, 1980; Armentrout and Downer, 1987; Villajuan-Abgona et al., 1993). In the present study, the hyphae of the virulent *R. solani* C4 (AG4) colonized the outer surface of the cucumber hypocotyls and directly penetrated the host tissues through hyphal tips which grew through the groves in between ridges of the outer epidermal cells.

Contrary to the findings observed on cucumber hypocotyls inoculated with virulent isolate, direct penetration by non-pathogenic BNR W7 was not observed and instead profused production of mucilaginous material was observed on the cuticular layer. The mere presence of the hyphae on the surface caused the preliminary synthetic of mucilage on the cuticular layer which lysed the BNR hyphae and later turned into a gel layer. This process was clearly revealed by the paraffin sections and hypocotyls surfaces examined through light and scanning electron microscopy, but this gel layer was entirely diffe-

rent from the reported mucilage (produced by the virulent *R. solani*) which is needed preliminarily for infection cushion formation (Armentrout et al., 1987; Sneh et al., 1989). In addition, the appearance of the reported mucilage was less dense than the gel layer which was produced on the hypocotyl surface upon inoculation with the non-pathogenic BNR. Since mucilaginous material was not observed on hypocotyls inoculated with virulent *R. solani* only, its presence on the hypocotyls surface was largely due to BNR colonization and not due to *R. solani*. The mucilage which was formed and lysed the hyphae of both the non-pathogenic BNR and *R. solani* prevented the virulent *R. solani* from penetrating the outer epidermis and completely covered the hypocotyls surface. The matrix of the mucilage which developed into a gel layer appeared homogenous and lacking any kind of structure.

Previous report discussed in plant nutrition through their effect on citation exchange and diffusion (Balandreau and Knowles, 1978). It occurred in several crops namely azalea (Leiser, 1968), onion (Scott et al., 1958), barley (Jenny and Grossenbacher, 1963), corn (Floyd and Ohlorogge, 1970; Wright and Northcote, 1974) and wheat (Northcote and Pickett-heaps, 1966) and were referred to as plant mucigels. However, the mucilage was not reported on cucumber and the mucilaginous material that was synthesized on the hypocotyls and root surface of cucumber seedlings upon inoculation of the non-pathogenic BNR was unknown.

The mucilage which turned into gel layer on the hypocotyls surface inoculated with non-pathogenic BNR was deeply stained by phenyl-thionin and strongly absorbed ruthenium red describing its nature as pectin (Leiser, 1968; Sterling, 1970). Pectic polysaccharides consist of galacturonan blocks which are heavily-branched with either large-methyl-esterified blocks and un-branched block which could aggregate through calcium binding to the junction zones which hold a gel together (Jarvis, 1984). Micro-scopic observation of the cucumber hypocotyls surface and its stained cross-sections showed that the gel-like substance exhibited affinity to phenyl-thionin and ruthenium red stains were indeed pectin. However, since pectic substances consist of acid and calcium gels in the junction zones (Rees and Wight, 1971), these sub-stances might have been detrimental to the hyphal growth of both the non-pathogenic BNR and the virulent *R. solani.* Pectic substances could cause lysis of the hyphae which later were incorporated into the gel layer. Lysis of the BNR hyphae could be primary due to the behaviour of the acidic galacturonic acid block which is an essential component of pectin. Pectin structure in host tissues of other crops was measured as *p*Ka (logarithmic acidic constant) = 3.52 under acidic condition (pH 3.6) (Jarvis, 1984). This acidic condition could not be tolerated by the non-pathogenic BNR hyphae and virulent *R. solani* which effectively grew and produced extracellular enzymes to catalyze cutin on cutinase selective medium only in conditions with pH ranges of 5.2 and 7.2, respectively (data not shown).

Results of the analysis of pectin through extracted anhydrouronic acid using the Versense-pectinase method (McCready and McComb, 1952) and liberated methanol by distillation method (Boos, 1948) from dried hypocotyls tissues further proved that the mucilage which was synthesized by the hypocotyls tissue was due to BNR treatment. Evidence has shown that pectic fragments released by fungal or host enzymes can elicit the cascade of defense reactions in plant cells which in favourable cases contain the growth of the pathogen (Hahn et al., 1981; Jin and West, 1984). In this experiment, it was evidently shown by significant differences in the proportion of pectin content obtained from hypocotyls inoculated with BNR when compared to the non-treated hypocotyls and hypocotyls treated with virulent *R. solani*. Galacturonic acid blocks consisting of methyl esters which are normally distributed are released as methoxyl groups after acid hydrolysis and expressed as percentage methanol (Boos, 1948). In this experiment, the methoxyl groups in the extracted pectin in the BNR-treated and non-treated hypocotyls were less methylated when compared to the highly methylated pectin of the *R. solani* -treated hypocotyls (Table 2). This indicates that methyl esters in pectic substances of the *R. solani*-treated hypocotyls were less-bound and were completely affected by the pectin esterase and polygalacturonase released by the pathogen. In addition, results of the analysis of calcium and potassium of *R. solani*- treated hypocotyls also indicate the direct loosening effect of the pectic fraction (Sentenac and Grignon, 1981; Jarvis, 1984). However, for BNR-treated seedling, high calcium content which is double in portion to the uronic acid content (Tables 2 and 3) resulted in more calcium cations which are wall-bound and pectin is considered a large aggregate unit (Jarvis, 1984). While in non-treated seedlings, calcium was of considerable amount in proportion to uronic acid content and can be considered wall-bound because it is a single primary unit. The same proportion of calcium and uronic was observed in *R. solani*-treated seedlings (Tables 2 and 3), but the cations were not wall-bound and eventually lose pectin due to pectic enzymes (Leiser, 1968). Similar study conducted by Bateman and Lumsden (1965) in older bean hypocotyls (resistance to damping-off) showed that more total calcium saturated its pectin in their cell walls and this implied that the legume stems contained pectin of large aggregates, if most of the calcium is in the cell wall (Jarvis, 1984). In another experiment (Tepfer and Taylor, 1981), Ca^{2+} showed a strong ability to form gels with purified, de-estrified pectin, when compared with Mg^{2+}, Mn^{2+}, Co^{2+} and Zn^{2+}. The same experiment also disproved the hypothesis that cation-induced inhibition of the growth response is caused by pectic gel formation in the cell wall but it rather played an important role in rendering the cell wall struc-

ture inaccessible for microorganisms.

Studies conducted regarding the presence of calcium cations in resistant host tissues showed its importance in disease control (Bateman and Lumsden, 1965; Stockwell and Hanchey, 1982; Akino et al., 1987; Yang et al., 1993; Biggs et al., 1994). Crystal deposits of calcium salts observed on the hypocotyls surface of BNR-treated seedlings (Figures 3A and B) proved its essential role in the suppression of damping-off in cucumber. This was supported by the results obtained by Akino et al. (1987) which revealed calcium salts on *R. solani* hyphae causing sugarbeet root rot. The authors correlated the appearance of calcium oxalate to the disease decline. In addition, a study revealed that calcium deprivation in cell walls of cucumber roots caused breakdown of pectic polysaccharide which stimulated polygalacturonase activity, thereby predisposing the cucumber seedlings to fungal attacks (Konno et al., 1984). On the other hand, previous study showed the positive effect of BNR isolate W7 on plant growth promotion in terms of significant increase in plant height ($P = 0.01$) and fresh weight ($P = 0.05$) (Villajuan-Abgona et al., 1996a).

Histochemical investigation of treated cucumber hypocotyls and biochemical analysis of extracts of host tissues evidently showed the importance of calcium and pectic substance accumulation as a result of BNR inoculation to the suppression of the disease caused by the virulent *R. solani* C4. However, the factor which elicited the physiological response of the cucumber tissues to produce profused mucilaginous pectic materials and increased calcium on hypocotyl surface is still obscure. Previous study discussed the secretion of mucigel on epidermal cell walls on wheat as a golgi-mediated response (Villajuan-Abogna et al., 1996b). The mucigel identified as pectic substance as shown by radioactive analysis, is a polysaccharide synthesized in the golgi apparatus of the root cap cells from where they move in through the cytoplasm and diffused to the plasmalemma (cell membrane) and ejected to the cell wall (pinocytosis). Another study supports the previous result which indicates the involvement of golgi apparatus in the secretion and targeting of cutinase by germinating spores of *Fusarium solani* f. sp. *pisi* on epidermal cell wall as a recognition response (Wyllie, 1962). Since colonization of the hypcotyl surface of the cucumber seedlings by BNR hyphae is necessary to synthesize pectic substances to effect the suppression of the disease, a fungal enzyme inherent in the non-pathogenic BNR might be responsible for this induced physiological response. Thus, a continuing study on the extraction and purification of fungal enzymes such as cutinase which could have elicited such physiological response is being conducted.

REFERENCES

Akino S, Hyakumachi M, Kobayashi K, Ogoshi A (1987). Crystal-like deposits on the hyphae of *Rhizoctonia solani*. Trans. Mycol. Soc. Japan 28:351-357.

Armentrout VN, Downer AJ (1987). Infections cushion development by *Rhizctonia solani* on cotton. Phytopathology 77:619-623.

Armentrout VN, Downer AJ, Grasmick DL, Weinhold AR (1987). Factor affecting infection cushion development by *Rhizctonia solani* on cotton. Phytopathology 77:623-630.

Balandreau J, Knowles R (1978). The rhizoshere. P 243-268 in: Interaction between non-pathogenic soil Microorganisms and plants. Y. R. Dommergues and S.V. Krupa, eds. Elsevier Scientific Publishing company, Amsterdam, The Netherlands.

Bateman DF, Lumsden RD (1965). Relation of calcium content and nature of pectic substances in bean hypocotyls of different ages to susceptibility to an isolate of *Rhizoctonia solani*. Phytopathology 55:734-738.

Biggs AR, El-kholi MM, El-Neshewy SM (1994). Effects of calcium salts on growth, pectic enzyme activity and colonization of peach twigs by *Leucostoma persoonii*. Plant Dis. 78:886-890.

Boos RN (1948). Quantitative colorimetric microdetermination of methanol with chromotropic acid. Anal. Chem. 20:964-965.

Burpee LL, Goulty LG (1984). Suppression of brown patch disease creeping bentgrass by isolates of non-pathogenic *Rhizoctonia* spp. Phytopathology 74:692-694.

Cardoso JE, Echandi E (1987a). Biological control of *Rhizoctonia* root rot of snap bean with binucleate *Rhizoctonia*-like fungi. Plant Dis. 71:167-170.

Cardoso JE, Echandi E (1987b). Nature of protection of bean seedling from *Rhizctonia* root rot by abinucleate *Rhizctonia*-like fungus .Phytopathology 77:1548-1551.

Christou T (1962). Penetration and host -parasite relationship of *Rhizctonia solani* in the bean plant. Phytopathology 52:381-389.

Cubeta MA, Echandi E (1991). Biological control of *Rhizctonia* and *Pythium* damping-off of cucumber: An integrated approach. Biol. Control 1:227-236.

Escande AR, Echandi E (1991). Protection of potato from *Rhizctonia* canker with binucleate *Rhizctonia* fungi. Plant Pathol. 40:197-202.

Floyd RA, Ohlorogge AJ (1970). Gel formation on nodal root surfaces of *Zea mays* l: Investigation of the gel's composition. Plant Soil 33:331-343.

Hahn MG, Darvill AG, Albersheim P (1981). Host-pathogen interactions. XIX. The endogenous elicitors, a fragment of a plant cell wall polysaccharide that elicits phytoalexin accumulation in soybeans. Plant Physiol. 68:1161-1169.

Herr LJ (1988). Biocontrol of *Rhizoctonia* crown and root rot of sugar beet by binucleate *Rhizoctonia* spp. and *Laetisaria arvalis*. Ann. Appl. Biol. 113:107-118.

Jabaji-Hare S, Neate SM (2005). Nonpathogenic binucleate *Rhizoctonia* spp. and benzothiadiazole protect cotton seedlings against *Rhizoctonia* damping-off and *Alternaria* leaf spot in cotton. Phytopathology 95:1030-1036.

Jarvis MC (1984). Structure and properties of pectin gels in plant cell walls. Plant Cell Environ. 7:153-164.

Jenny H, Grossenbacher K (1963). Root-soil boundary zone as seen in the electron microscope. Soil Sci. Soc. Amer. Proc. 27:273-277.

Jin DF, West CA (1984). Characterizatics of galacturonnic acid oligomers as elicitors of casbene synthetase activity in castor bean seedlings Plant Physiol. 74:989-992.

Johansen DA (1940). Plant Microtechnique. McGraw-Hill, New York. 523 pp.

Konno H, Tomoyuki Y, Matsumoto H (1984). Pectic polysaccharide breakdown of cell walls in cucumber roots grown with calcium starvation. Plant Physiol. 76:633-637.

Leiser AT (1968). A mucilaginous root sheath in Ericeae. Amer. J. Bot. 55(3):391-398.

Marshall DS, Rush MC (1980). Relation between infection by *Rhizctonia solani* and *Rhizctonia oryzae* and diseases severity in rice. Phytopathology 70:941-946.

McCready RM, McComb EA (1952). Extraction and determination of total pectic materials in fruits. Anal. Chem. 24 (12):1986-1988.

Northcote DH, Pickett-heaps JD (1966). A function of the golgi apparatus in polysaccharide synthesis and transport in the root cap cells of wheat. Biochem. J. 98(1):159-167.

Pascual CB, Raymundo AD, Hayakumachi M (2000). Efficacy of hypovi-

rulent binucleate *Rhizoctonia* sp. to control banded leaf and sheath blight in corn. J. Gen. Plant Pathol. 66:95-102
Rees DA, Wight AW (1971). Polysaccharide conformation VIII. Model building computations for alpha-1, 4- galacturonan and the kinking function of L-rhamnose residues in pectic substances. J. Chem. Soc. Section B. 1366-1372.
Scott FM, Hamner KC, Baker E, Bowler E (1958). Electron microscope studies of the epidermis of *Allium Cepa*. Amer. J. Bot. 45:449-460.
Sentenac H, Grignon C (1981). A model for predicting ionic equilibrium concentrations in cell walls. Plant Physiol. 68:415-419.
Sneh B, Ichielevich-Auster M, Scomer I (1989). Comparative anatomy of colonization of cotton hypocotyls and roots by virulent and hypovirulent isolates of *Rhizoctonia solani*. Can. J. Bot. 67:2142-2149.
Sneh B, Yamoah E, Stewart A (2004). Hypovirulent *Rhizctonia spp*. isolated from New Zealand soil protect radish seedling against damping-off by *R. solani*. New Zealand Plant Prot. 57:54-58.
Staples R, Macko V (1980). Formation of infection structures as a recognition response in fungi. Exp. Mycol. 4:2-16.
Sterling C (1970). Crystal structure of ruthenium red and stereochemistry of its pectin stain. Am. J. Bot. 57:172-175.
Stockwell V, Hanchey P (1982). Cyohistochemical techniques for calcium localization and the application to diseased plants. Plant Physiol. 70:244-451.
Stoughton RH (1930). Thionin and Orange G for thr differential staining of bacteria and fungi in plant tissues. Ann. Appl. Biol. 17:162-164.
Tepfer M, Taylor IEP (1981). The interaction of adjvalent cations with pectic substances and their influence on acid-induced cell wall loosening. Can. J. Bot. 59:1522-1525.
Villajuan-Abgona R, Kageyama K, Hyakumachi M (1993). Biological control of *Rhizctonia* damping-off of cucumber by binucleate *Rhizctonia:* Prevention of hyphal penetration. Ann. Phytopath. Soc. Japan 60(6):780.
Villajuan-Abgona R, Kageyama K, Hyakumachi M (1996a). Biological control of *Rhizctonia* damping -off of cucumber by non-pathogenic binucleate *Rhizctonia*. Eur. J. Plant Pathol. 102:227-235.
Villajuan-Abgona R, Katsuno N, Kageyama K, Hyakumachi M (1996b). Isolation and identification of hypovirulent *Rhizoctonia* spp. form soil. Plant Pathol. 45:896-904.
Wright K, Northcote DH (1974). The relationship of root-cap slimes to pectins. Biochem. 139:525-534.
Wyllie TD (1962). Effect of metabolic by -products of *Rhizctonia solani* on the roots of Chippewa soybean seedlings. Phytopathology 52:202-206.
Yang J, Tewari JP, Verma PR (1993). Calcium oxalate crystal formation in *Rhizoctonia solani* AG2-1 culture and infected crucifer tissue: relationship between host calcium and resistance. Mycol. Res. 97(12):1516-1522.

academicJournals

Vol. 13(5), pp. 651-656, 29 January, 2014
DOI: 10.5897/AJB2013.13200
ISSN 1684-5315 ©2014 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Assessment of growth and yield of some high-and low-cyanide cassava genotypes in acid ultisols of south eastern Nigeria

Okpara[1], D. A., Mbah[2], E. U. and Chukwu[1], E. I.

[1]Department of Agronomy, Michael Okpara University of Agriculture, Umudike, Abia State, Nigeria.
[2]Department of Crop Production Technology, Federal College of Agriculture, P. M. B. 7008, Ishiagu, Ebonyi State, Nigeria.

Accepted 20 January, 2014

Thirty-eight high and low cyanide cassava genotypes were evaluated for growth and yield. The experiment was fitted in a randomized complete block design with three replications. Analysis of variance (ANOVA) revealed significant difference ($P \leq 0.05$) in growth and yield parameters, indicating strong genetic variations among the genotypes. Significant ($P \leq 0.05$) highest average storage root weight of 1.5 and 1.8 kg were obtained from TMS 94/0035 (high cyanide cassava genotype) and TMS 98/0505 (low cyanide cassava genotype), respectively. Correlation analyses indicated significant ($P \leq 0.05$) and positive associations between number of storage roots per plant, average storage root weight and root yield in the two types of cassava genotypes tested, indicating that the parameters are good indices that can be improved upon during breeding and selection to enhance root yield. Four high cyanide cassava genotypes (TMS 99/2123, TMS 96/1642, TMS 98/0068 and TMS 94/3200) were outstanding in fresh root yield performance. To improve fresh root yield in cassava, the inter-relationships among the various agronomic character of the cassava genotypes demands appropriate attention. The study gives more information on the improvement of fresh storage root yield of cassava.

Key words: High cyanide cassava genotypes, low cyanide cassava genotypes, correlations, growth, yield.

INTRODUCTION

In the humid and sub-humid topics, cassava, which is one of the most important food crops play a dominant role in the rural economy and food security of the people living there. The root crop is characterized by the presence of cyanoglycosides (linamarin and lotoaustralin), which make both the roots and leaves potentially toxic to man and animals (Padmaja, 1995). Two distinct cassava genotypes exists namely, the bitter (high cyanide) and sweet (low cyanide) genotypes and their performance in terms of growth, photosynthetic efficiency and yield vary greatly. Santana et al. (2002) in their physiological studies on cassava reported that the content and quantity of cyanoglycosides in cassava roots depend on the genotypes and the growth conditions the plants are exposed to such as drought and soil composition. Eke-Okoro (2000) assessed the photosynthetic efficiency and productivity of low and high cyanide cassava genotypes and reported that high cyanide cassava genotypes had better photosynthetic efficiency and higher storage root yield than low cyanide cassava genotypes. Also, cassava genotypes with profuse branching characters have the tendency to produce higher fresh root yield than the less branching types (Eke-Okoro et al., 2001).

The breeding and release of cassava varieties by the

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

Table 1. High- and low-cyanide cassava genotypes used in the study.

Cassava genotype	Level of cyanide in storage root
TMS 91/02324	High
TMS 92/0067	High
TMS 94/0026	High
TMS 94/0561	High
TMS 94/0039	High
TMS 95/0379	High
TMS 95/0166	High
TMS 96/0523	High
TMS 96/1632	High
TMS 96/1569	High
TMS 96/1642	High
TMS 96/1317	High
TMS 96/0603	High
TMS 97/4763	High
TMS 97/0211	High
TMS 97/3200	High
TMS 97/4779	High
TMS 98/0581	High
TMS 98/2226	High
TMS 98/0068	High
TMS 98/2101	High
TMS 98/0002	High
TMS 99/3073	High
TMS 99/2123	High
TMS 30572	High
TMS 82/0058	Low
TMS 92/0326	Low
TMS 92/0057	Low
TMS 92^B/0068	Low
TMS 97/4769	Low
TMS 97/0325	Low
TMS 97/6012	Low
TMS 98/0510	Low
TMS ^{m}98/0028	Low
TMS 98/0040	Low
TMS 98/0505	Low
TMS 4(2)1425	Low
TME 419	Low

National Root Crops Research Institute, Umudike and International Institute of Tropical Agriculture, Ibadan, Nigeria has necessitated the evaluation of the new cultivars for the purpose of advising farmers on their yield potentials. A number of researchers have stressed that tuber bulking and yield in cassava are determined by the characteristics of the genotype, assimilation supply of the plant and environmental factors (Cock, 1986; Akoroda, 2005; and IITA, 1990). Furthermore, Githunguri et al. (2004) reported that cassava cultivated under humid conditions with high rainfalls have higher tuberous root bulking ability and lower cyanogenic potential than those cultivated in drier conditions. In recent years, studies have been directed at improving fresh storage root yield of cassava, one of which involves the analysis of inter-relationship among important agronomic characters in high- and low-cyanide cassava genotypes and some of them include the works of Asante and Dixon (2002), Akinwale et al. (2011) and Mulualem and Ayenew (2012).

The purpose of the study therefore was to assess the effect of genotypic difference of high and low cyanide cassava on root yield and other related characters, assess the performance of the genotypes as basis for ascertaining their acceptability and recommendation to farmers in the humid agro-ecozone of south-eastern Nigeria. Also, to clarify the inter-relationship between storage root yield and other agronomic characters of the genotypes as to elicit information that can be useful in boosting root yield of cassava during breeding and selection.

MATERIALS AND METHODS

Thirty-eight high- and low-cyanide cassava genotypes were obtained from the germplasm unit of National Root Crops Research Institute, Umudike, Nigeria and used for the study (Table 1). The experiment was carried out at Michael Okpara University of Agriculture, Umudike, Research Farm (latitude 05° 29' N, longitude 07° 33' E, altitude 122 m) in the low-land humid tropics of south eastern Nigeria in 2004 and 2005 cropping seasons. The treatments were laid out in a randomized complete block design (RCBD) with three replications. The total annual rainfall was 1,911.4 mm (2004) and 2,064.8 mm (2005). The soil, which is a sandy loam classified as ultisol (Paleulstalt), had, at 0 – 20 cm depth a pH of 4.86 (1:2.5; Soil:Water), 1.62% organic matter; 0.56% total N; 26.0 mg/kg available P, which was high; 0.164 cmol/kg exchangeable K; and 0.34, 2.40 and 0.80 cmol/kg of Na, Ca and Mg, respectively.

The soil samples from the experimental plots were bulked, thoroughly mixed and then one composite sample obtained from which, a sub-sample was collected, air-dried and subjected to analyses in the laboratory. Soil pH was measured potentiametrically in a glass electrode in deionized water (pH water) at soil:water solution ratio of 1:2.5. Percentage organic matter was analysed using wet oxidation method by Walkley and Black (1934), while total nitrogen (N) was obtained by Microkjeldahl method of wet oxidation (Bremner, 1996). Available phosphorus was determined by calorimetric method (Olson and Sommers, 1982). Exchangeable potassium (K) and sodium (Na) were determined by Flamephoto-metory (Udo and Ogunwale, 1978), while calcium (Ca) and magnesium (Mg) were determined by ethylenediaminetetraacetate (EDTA) titration method (Olson and Sommers, 1982).

Twenty-five (25) cm long planting setts obtained from 12-month old matured cassava stems were planted at a spacing of 1 m apart on the crest of ridges spaced 1 m apart, which gave a plant population of 10,000 plants/ha. Each genotype occupied two rows of ridges measuring 20 m in length. A mixture of grammaxon (2 L/ha) and primextra (3 litres/ha) was applied to the field immediately after planting and two manual weeding regimes were carried out at 4 and 8 months after planting (MAP) to control weeds. N:P:K:Mg 12:12:17:2 fertilizer was applied at 1 MAP at the rate of 400 kg/ha.

Growth data were taken on plant height, number of stems per plant, number of internodes per plant and number of leaves per plant at 6 MAP. Yield data collected were on number of storage

Table 2. Growth and yield characters of twenty five high-cyanide cassava genotypes in 2004 and 2005 cropping seasons.

Cassava genotypes	Plant height (m)	Number of stems/ plant	Number of inter-nodes/ plant	Number of leaves/ plant	Number of storage roots/ plant	Average storage root weight (kg)	Fresh storage root yield (t/ha)
TMS 91/02324	2.1	2.3	280.0	71.7	4.2	0.5	45.8
TMS 92/0067	1.4	1.7	265.0	71.0	4.5	0.6	28.5
TMS 94/0026	1.7	1.3	290.3	85.7	45	0.5	23.7
TMS 94/0561	1.6	1.0	366.3	231.0	3.5	0.8	27.3
TMS 94/0039	1.6	1.6	195.0	56.0	3.6	1.5	49.0
TMS 95/0379	1.3	2.0	298.3	101.0	3.8	0.6	24.0
TMS 95/0166	1.2	2.3	316.3	93.7	4.0	1.0	38.0
TMS 96/0523	1.8	1.7	295.7	124.0	5.5	0.9	42.5
TMS 96/1632	1.8	1.1	270.0	137.7	5.0	0.9	42.7
TMS 96/1569	1.8	1.3	444.0	294.7	4.8	1.0	47.7
TMS 96/1642	2.1	1.0	373.7	168.7	6.5	0.8	54.4
TMS 96/1317	2.1	2.6	298.3	69.0	3.5	0.7	25.7
TMS 96/0603	2.0	2.3	347.7	153.7	4.5	0.5	25.1
TMS 97/4763	1.7	1.3	319.7	279.7	4.2	0.9	26.6
TMS 97/0211	1.5	1.0	405.0	232.3	3.7	1.1	39.3
TMS 97/3200	2.1	2.0	438.7	288.7	4.3	1.2	50.6
TMS 97/4779	2.3	2.0	349.3	176.7	6.0	0.7	40.6
TMS 98/0581	2.3	2.0	221.7	52.0	4.2	0.8	34.1
TMS 98/2226	1.7	1.0	286.3	134.7	3.5	0.9	29.1
TMS 98/0068	2.0	2.0	359.0	251.7	6.4	0.8	52.0
TMS 98/2101	1.5	2.0	360.0	205.7	4.2	0.9	37.4
TMS 98/0002	1.3	2.0	361.0	166.0	4.0	0.8	32.7
TMS 99/3073	1.3	2.0	202.0	152.0	4.5	0.7	31.3
TMS 99/2123	2.3	1.3	402.0	212.0	5.5	1.1	63.4
TMS30572	1.4	1.7	347.3	283.7	4.2	0.6	25.6
$LSD_{0.05}$	0.3	0.7	87.0	95.3	1.9	0.4	17.9

roots per plant, average storage root weight (kg) and fresh storage root yield (t/ha) at 12 MAP. Analysis of variance (ANOVA) was performed on growth and yield related traits following the procedure outlined for randomized complete block design (Steel et al., 1997). Data were also subjected to simple and partial correlation and regression analyses using SPSS statistical package for windows version 17.0 (2010). Mean separation was done using least significant difference (LSD) at 5% probability level according to Obi and Obi (2002).

RESULTS AND DISCUSSION

The ANOVA revealed that there was significant difference ($P \leq 0.05$) among the cassava genotypes in all the plant characters assessed such as plant height, number of stems per plant, number of internodes per plant, number of leaves per plant, number of storage roots per plant, average storage root weight and fresh storage root yield (t/ha) (Table 2), indicating the presence of genetic differences. Plant height, number of stems per plant, number of internodes per plant and number of leaves per plant ranged between 1.20 and 2.30, 1.0 and 2.6, 195.0 and 444.0 as well as 52.0 and 288.7, respectively.

TMS 99/2123, TMS 98/0581, and TMS 97/4779 cassava genotypes were significantly ($P \leq 0.05$) taller compared with the other genotypes except TMS 91/02324, TMS 96/1642, TMS 97/3200, TMS 96/1317, TMS 98/0068 and TMS 96/0603 while TMS 98/0581 significantly ($P > 0.05$) had the lowest number of leaves per plant (52.0) relative to the other cassava genotypes. TMS 96/1569 had significantly highest number of internodes per plant (444.0) followed by TMS 97/3200 (438.7), which also had significantly highest number of leaves per plant, while TMS 96/1317 gave the highest number of stems per plant (2.6) relative to the other high cyanide cassava varieties. The results obtained corroborated studies by Okonkwo (2002) on evaluation of cassava genotypes for yield and biotic stress in which he reported significant differences in plant height and other growth parameters between cassava genotypes.

In high cyanide cassava genotypes, number of storages roots per plant, average storage root weight (kg) and fresh storage root yield (t/ha) ranged from 3.5 – 6.5, 0.5–1.5 kg and 23.7 – 63.4 t/ha, respectively. TMS 96/1642 gave the highest ($P \leq 0.05$) number of storage roots per

Table 3. Growth and yield characters of thirteen low-cyanide cassava genotypes in 2004 and 2005 cropping seasons.

Cassava genotype	Plant height (m)	Number of stems/plant	Number of inter-nodes/plant	Number of leaves/plant	Number of storage roots/plant	Average storage root weight (kg)	Fresh storage root yield (t/ha)
TMS 82/0058	2.2	2.0	300.3	111.0	3.0	0.7	22.3
TMS 92/0326	2.2	2.3	392.0	213.7	4.3	1.1	44.0
TMS 92/0057	1.6	1.0	448.0	267.7	4.5	1.2	56.0
TMS 92^B/0068	2.3	2.0	238.3	102.6	5.2	1.7	37.1
TMS 97/4769	1.7	1.3	304.3	231.0	3.8	0.7	25.0
TMS 97/0325	1.1	1.0	188.0	84.3	4.0	1.2	36.7
TMS 97/6012	3.0	1.3	240.7	45.7	3.3	1.0	32.1
TMS 98/0510	1.7	1.7	234.3	87.0	2.8	0.9	26.0
TMS ^{m}98/0028	1.6	1.3	493.0	377.0	5.0	1.1	54.0
TMS 98/0040	2.3	1.7	281.0	148.0	4.0	1.0	46.7
TMS 98/0505	2.1	2.0	548.0	301.3	3.5	1.8	60.7
TMS 4(2)1425	1.4	2.3	427.0	168.3	3.7	1.1	29.3
TME 419	2.7	2.0	217.3	48.0	4.0	1.3	43.7
$LSD_{0.05}$	0.4	0.7	61.6	64.8	ns	0.5	ns

plant (6.5) but had very low average storage root weight (0.8 kg) compared to the other genotypes assessed, while TMS 98/2226, TMS 94/0561 and TMS 96/1317 with the lowest (3.5) number of storage roots per plant also had very low average storage root weight. Data on fresh storage root yield indicated significant difference ($P \leq 0.05$) in the yield of TMS 99/2123 (63.4 t/ha) relative to the other cassava genotypes except TMS 96/1642 (54.4 t/ha), TMS 98/0068 (52.0 t/ha) and TMS 97/3200 (50.6 t/ha). The yield results obtained were higher compared with yields obtained from similar works by Eke-Okoro (2000) on evaluation of photosynthetic efficiency and productivity of sweet and bitter cassava varieties in the humid tropics as well as Okonkwo (2002) on some cassava genotypes in the cool highlands of Jos Plateau, Nigeria.

Table 3 shows that there was significant difference ($P \leq 0.05$) among the low cyanide cassava genotypes for plant height, number of stems per plant, number of inter-nodes per plant, number of leaves per plant and average storage root weight. TMS 99/6012 significantly ($P \leq 0.05$) exhibited the highest value for plant height (3.0 m) but had lowest number of leaves per plant (45.7) compared to the other genotypes. The differences in number of stems per plant and number of internodes per plant were significant ($P \leq 0.05$). TMS 98/0505 produced the highest number of internodes per plant (548.0), which was higher by 65.7% relative to TMS 97/0325, which had the lowest number of internodes per plant (188.0), while TMS 4(2)1425 and TMS 92/0326 exhibited the highest values of 2.3 stems per plant. TMS ^{TM}98/0028 had the highest number of leaves per plant while TMS 98/0505, TMS 92/0057 TMS 97/4769, and TMS 92/0326 were intermediate. The other genotypes exhibited the lowest values. These findings were in consonance with studies by Naskar et al. (1989) in which they surmised that differences in growth parameters could be basically due to high genetic variability among cassava genotypes, though environmental factors may also be considered. The number of storage roots per plant and fresh storage yield among low cyanide genotypes did not differ statistically ($P>0.05$). However, average storage root weight was significantly ($P \leq 0.05$) highest in TMS 98/0505 (1.8 kg), followed by TMS 92^B/0068 (1.7 kg), while lowest values were obtained in TMS 97/4769 and TMS 82/0058 (0.7 kg).

The correlation between fresh storage root yield and the plant characters studied showed significant ($P \leq 0.05$) and positive relationship between number of storage roots per plant and fresh storage root yield with correlation coefficients (r) of (0.59) as well as between average storage root weight and storage root yield with (r = 0.58) in high cyanide cassava genotypes, an indication that these yield parameters are good indices that can be improved upon to boost cassava storage crop yield during breeding and selection (Table 4). The other parameters (plant height, number of stems per plant, number of internodes per plant and number of leaves per plant) did not show any correlated response with storage root yield of cassava. In low cyanide cassava genotypes, number of leaves per plant, number of internodes per plant and number of tubers per plant were significantly ($P \leq 0.05$) correlated with fresh storage root yield while average storage root weight had highly significant ($P \leq 0.01$) correlation with fresh storage root yield of cassava with (r = 0.74) (Table 5). These parameters demand close attention in breeding for improved storage root yield. Similar studies by Amadi et al. (2008) on potato genotypes showed that tuber number and average tuber weight are major individual contributors to crop yield.

Table 6 shows that analysing the plant characters using the method of partial correlation coefficients further highlighted the importance of average storage root weight and number of storage roots per plant with correlation

Table 4. Simple correlation matrix for the relationship between high-cyanide cassava genotype characters.

Plant character	Plant height (cm)	Number of stems/plant	Number of inter-nodes/plant	Number of leaves/plant	Number of storage roots/plant	Average storage root weight (kg)
Number of stems/plant	0.06 ns					
Number of inter-nodes/plant	0.19 ns	-0.20				
Number of leaves/plant	0.22ns	-0.33	0.77**			
Number of storage roots/plant	0.49 ns	0.18ns	0.25 ns	0.22 ns		
Average storage root weight (kg)	0.01 ns	-0.30	0.15 ns	0.22 ns	-0.09	
Fresh storage root yield(t/ha)	0.48 ns	-0.16	0.34 ns	0.22 ns	0.59*	0.58*

*, Significant at 5 % level of probability; **, significant at 1 % level of probability; ns, non-significant.

Table 5. Simple correlation matrix for the relationship between low-cyanide cassava genotype characters.

Plant character	Plant height (cm)	Number of stems/ plant	Number of inter-nodes/ plant	Number of leaves/ plant	Number of storage roots/ plant	Average storage root weight (kg)
Number of stems/plant	0.32ns					
Number of inter-nodes/plant	-0.25	-0.14				
Number of leaves/plant	-0.40	-0.13	-0.21			
Number of storage roots/plant	-0.12	-0.13	0.21 ns	0.40 ns		
Average storage root weight (kg)	-0.04	0.05 ns	0.53*	0.34 ns	0.01 ns	
Fresh storage root yield (t/ha)	0.02 ns	-0.12	0.62*	0.61*	0.52*	0.74**

*, Significant at 5 % level of probability; **, significant at 1 % level of probability; ns, non-significant.

Table 6. Partial correlation coefficients between fresh storage root yield of cassava and six agronomic characters of high- and low-cyanide cassava genotypes.

Plant attributes	Yield (t/ha)	Partial Correlation coefficients (r)	
		HC	LC
Plant height (cm)	Fresh storage root	0.28ns	0.61*
Number of stems/plant	Fresh storage root	0.13ns	-0.33ns
Number of internodes/plant	Fresh storage root	0.33 ns	-0.04ns
Number of leaves/plant	Fresh storage root	0.29ns	-0.32 ns
Number of storage roots/plant	Fresh storage root	0.73**	0.76 **
Average storage root weight (Kg/plant)	Fresh storage root	0.81**	0.87**

*, Significant at 5 % level of probability; **, significant at 1 % level of probability; ns, non-significant; HC, high cyanide cassava genotypes; LC, low cyanide cassava genotypes.

coefficients (partial r) of 0.81 and 0.73, respectively, for high cyanide cassava genotypes, as well as 0.87 and 0.76, respectively for low-cyanide cassava genotypes as vital characters that contributed greatly to storage root yield of cassava. Similar studies by Ntawuruhunga et al. (2001) on twenty broad-based cassava genotypes in the forest savanna, northern and southern guinea savanna as well as Sudan savanna agro-ecozones of Nigeria indicated increased storage root yield of cassava, which was mainly due to increase in number of storage roots per plant as well as individual storage root weight of the tested genotypes. This implies that for breeding and selection, premium should be placed on increasing the number of storage roots per plant and weight of average storage root in other to obtain higher root yield in both high- and low-cyanide cassava genotypes.

Conclusion

The results showed that among the thirty-eight high and low cyanide cassava genotype tested, four high cyanide cassava genotypes (TMS 99/2123, TMS 96/1642, TMS ^{m}98/0068 and TMS 97/3200) were outstanding in fresh storage root yield performance, hence can be considered for recommendation to farmers in the humid agro-eco-zone of southeastern Nigeria. Furthermore, the correlation studies specifically reviewed the inter-relationships among the various agronomic character of the cassava genotypes, which indicated that cassava selection programmes based on number of storage roots per plant and average weight of storage root, demands appropriate attention, especially when the challenge is aimed at improving fresh storage root yield in cassava.

REFERENCES

Akinwale MG, Akinyele BO, Odiyi AC, Dixion AGO (2011). Genotype X environment interaction and yield performance of 43 improved cassaa (*Manihot esculenta* Crantz) genotypes at three agro-climatic zones in Nigeria. Br. Biotechnol. J. 1 (3): 68 – 84.

Akoroda MO (2005). Root Crops. Institute of Agricultural Research, Ngaounudere, Cameroon. p. 537.

Amadi CO, Ene-Obong EE, Okonkwo JC, Lenka DM (2008). Preliminary studies on yield and some yield attributes of potato grown under high and low ambient temperatures. Niger. Agric. J. 39 (1): 65 -75.

Asante K, Dixon AGO (2002). Heritability studies of some cassava genotypes. West Afr. J. Appl. Ecol. 3: 49 – 53.

Bremner JM (1996). Nitrogen-Total. In: Spark, DL (ed). Methods of soil analysis. Part 3, chemical method. 2nd ed. SSSA Book Series. No. 5. SSSA Madison, W. I. pp. 1085 – 1121.

Cock JH (1986). Cassava: Its calories can overcome malnutrition. International Center for Tropical Agriculture (CIAT), Cali Colombia, Topic. 147: 30-33.

Eke-Okoro ON (2000). Evaluation of photosynthetic efficiency and productivity of sweet and bitter cassava cultivars. J. Sustain. Agric. Environ. 3 (1): 165 – 170.

Eke-Okoro ON, Okereke OU, Okeke JE (2001). Effect of stem sizes on some growth indices and yield of three cassava cultivars. J. Agric. Sci. Camb. 37: 419 – 426.

Githunguri CM, Kanayake IJ, Waithaka K (2004). Effect of the growth environment and bulking rate on cyanogenic potential of cassava tuberous roots. Agric. Res. Inst. Nairobi (Kenya). Proc. 8th KARI Biennial Sci. Conf. held 11th – 15th November. pp. 151 - 157.

IITA (International Institute of Tropical Agriculture) (1990). Cassava in Topical Africa, IITA. Cassava Reference Manual, Chayce Publication Services, UK. p. 196.

Mulualem T, Ayenew B (2012). Cassava (Mannihot esculenta Crantz) varieties and Harvesting Stages Influenced Yield and Yield Related Components. J. Nat. Sci. Res. 2(10): 122 - 128.

Naskar SK, Singh DP, Srinivasan G (1989). Performance of cassava varieties in the laterite soils of Bhubaneswar and their yield stability. J. Root Crops 15: 29 - 31.

Ntawuruhunga P, Rubaihayo PR, Whye JBA, Dizon AGO, Osiru DSO (2001). Inter-relationship, among traits and path analysis for yield components of cassava. A search for storage root yield indicators. Afr. Crop Sci. J. 9 (4): 599 - 606.

Obi IU, Obi E (2002). Statistical Methods of Detecting Difference Between Treatment Means and Research Methodology. Issues in Laboratory and Field Experiments, (ed. 2nd) p. 117.

Okonkwo JC (2002). Evaluation of cassava genotypes for yield and response to biotic stress in Jos Plateau. J. Sustain. Agric. Environ. 4 (1): 29 – 35.

Olson SR, Sommers LE (1982). Phosphorus. In: Page AL (ed). Method of Soil Analysis. Part 2, Argon. Monogr. No. 9. Madison, W. I., pp. 403 – 431.

Padmaja G (1995). Cyanide detoxification in cassava for food and feed uses. Crit. Rev. Food Sci. Nutr. 35: 299 – 339.

Santana MA, Vasquez V, Matehu J, Aldao RR (2002). Linamarase expression in cassava cultivars with roots of low- and high- cyanide content. Plant Physiol. 129 (4): 1686 – 1694.

SPSS Statistical Package for Windows (2010). SPSS for windows. Release 17.0 Standard Version.

Steel RDG, Torrie JH, Dickey DA (1997). Principles and Procedures of Statistics: A Biometrical Approach. 3rd ed.McGraw-Hill. p. 666.

Udo EJ, Ogunwale JA (1978). Laboratory manual for analysis of soil and water samples. Department of Agronomy, University of Ibadan, pp. 87 – 99.

Walkley A, Black AI (1934). In: Examination of the Degtaga-reff. Methods of determining soil organic matter and a proposed modification of the chronic acid titration method. Soil Sci. 37: 29 – 38.

academicJournals

Vol. 13(5), pp. 657-663, 29 January, 2014
DOI: 10.5897/AJB2013.13334
ISSN 1684-5315 ©2014 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Identification of putative candidate gene markers for grain zinc content using recombinant inbred lines (RIL) population of IRRI38 X Jeerigesanna

Naveen Kumar Gande[1,2], Pavan J Kundur[1], Rakhi Soman[1,2], Rajeswari Ambati[1], Ashwathanarayana R[1], Berhanu Dagnaw Bekele[3] and Shashidhar H.E[1]

[1]Department of Plant Biotechnology, University of Agricultural Sciences, GKVK, Bangalore-560065 Karnataka, India.
[2]Department of Biotechnology, Karpagam University, Coimbatore - 641021, Tamilnadu, India.
[3]Department of Biotechnology, University of Gondar, P. O. Box: 196, Gondar, Ethiopia.

Accepted 16 January, 2014

Nutrients in food crops can be enriched by adopting biofortification. Identifying the target quantitative trait loci (QTL) genes will help achieve biofortification with greater precision and accuracy. The objective of this experiment is to estimate grain zinc content, evaluation of candidate gene markers in recombinant inbred lines (RIL) derived from IRRI38 X Jeerigesanna and validation of putative candidate gene markers with rice accessions. Grain zinc content ranged from 16.1 to 35.5 ppm with an average of 23.7 ppm. Among twenty four candidate gene markers, eight showed polymorphism and out of three simple sequence repeats (SSR) markers, three showed polymorphism. Single marker analysis revealed that four (OsNAC, OsZIP8a, OsZIP8c and OsZIP4b) candidate gene markers showed significant variation among RIL population with a phenotypic variation of 4.5, 19.0, 5.1 and 10.2% respectively. Validation with 96 rice genotypes showed three markers (OsZIP8a, OsNAC and OsZIP4b) with phenotypic variation of 11.0, 5.8 and 4.8%, respectively.

Key words: Zinc, biofortification, single-marker analysis (SMS) and marker assisted selection (MAS).

INTRODUCTION

Rice (*Oryza sativa* L.) is a staple food for most of the people across the world. About 50% world's populations depends on rice as their main source of nutrition (White, 1994). However, rice is a poor source of micronutrients (Bouis and Welch, 2010). Micronutrients deficiency is a global problem contributing to world's malnutrition. This in turn leads to high rate of mortality in women and children (WHO, 1996). Zinc is one of the essential nutrients for increasing the immunity and it works as a cofactor for over 300 enzymes. It also plays a critical role in synthesis of protein and DNA (Rosal et al., 2010). Fortification of zinc and food supplementation approaches are also can be selected but these inventions are not easy to implement in developing countries due to its cost effective (Bouis, 2003; Stein et al., 2007; Pfeiffer and McClafferty, 2007).

Biofortification of the zinc content using conventional breeding and biotechnological methods can enhance the nutrient content in grains of rice (Bouis, 2003; Pfeiffer and McClafferty, 2007; Howarth et al., 2010). Realizing the

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

Abbreviations: QTL, Quantitative trait loci; **RIL,** recombinant inbred lines; XRF, X-ray florescence; **CTAB,** cethyltrimethyl ammoniac bromide; **PCR,** polymerase chain reaction; **SSR,** simple sequence repeats; **SMA,** single marker analysis.

importance of biofortification, several studies were undertaken for the evaluation of germplasm and advanced breeding lines for grain Zn content (Gregorio et al., 1999; Lu et al., 2008). Graham et al. (1999) reported the zinc concentration among 939 genotypes studied ranged from 13.5 to 58.4 ppm±1. Literature shows that 70% microntrients are lost during polishing (Sellappan et al., 2009). Selection of varieties with trait for higher Zn content using marker assisted selection in rice grain is an effective strategy to address widespread dietary deficiency in human populations. The genetic basis of accumulation of micronutrients in the grains and mapping of the quantitative trait loci (QTL) will provide the basis for preparing the strategies and improving grain micronutrient content through marker assisted selection. DNA markers which are closely linked with desired traits allow the selection of plants possessing those traits prior to trait expression. Earlier reports have cited that grain zinc content in rice is governed by a number of QTL located on different regions of the chromosome with different phenotypic effects (Biradar et al., 2007; Lu et al., 2008; Garcia-Olivera et al., 2009; Zhang et al., 2011). Chandel et al. (2011) reported three QTL (qZN-5, qZN-7 and qZN-11) for grain zinc content on chromosome 5, 7 and 11, respectively. To use this approach, preliminary steps are required to characterize molecular markers linked to QTL for grain Zn content and study their phenotypic variation. In rice, around 43 candidate genes were identified belonging to different gene families OsZIPs, OsNRAMPs, OsYSLs, OsFROs and Ferritin (Gross et al., 2003).

Markers identified through marker-trait association studies using one single mapping population has to be validated in different genetic backgrounds to determine its consistency (Miklas, 2007). Markers showing greater association and tighter linkage with the trait of interest can be used for marker assisted selection. The objective of this experiment was to evaluate candidate gene markers in recombinant inbred lines (RIL) population derived from IRRI38 X Jeerigesanna, grown under aerobic condition and validation of putative candidate gene markers with germplasms accessions.

MATERIALS AND METHODS

Plant material

Experiment was carried out during fall 2011 and 2012 using augmented experimental design as described by Federer (1960). One hundred sixty RILs derived from IRRI38 X Jeerigesanna were sown in 16 blocks using IRRI38 and Jeerigesanna as checks under aerobic condition.

Estimation of zinc

Seeds were harvested from RILs and a hand threshing was done to avoid contamination. Dehusking was done manually and seeds were washed immediately with 0.1 N HCl and with double distilled water to avoid surface contamination. Washed seeds were dried in an oven at 70°C for 72 h. Grain zinc content was estimated using X-ray florescence (XRF) (OXFORD Instruments X-Supreme 8000, Nicholas et al., 2012). Five gram (5 g) of dehusked rice grains of each sample was used for analysis. Measurement conditions were followed as recommended by the manufacture for analysis of Zn and Fe in a cellulose matrix.

Analysis time for each sample was 186 s, which included 60 s acquisition time for the separate Zn and Fe conditions as well as 66 s 'dead time' during which the XRF establishes each measurement condition. Scans was conducted in sample cups assembled from 21 mm diameter all cups combined with polypropylene inner cups sealed at one end with 4 µm Poly-4 XRF sample film. Calibration of instrument was done using known ICP-OES values of high, low zinc and iron containing genotypes.

Designing of candidate gene markers

For the designing of candidate gene primers, the gene sequence information was downloaded from National Centre for Biotechnology Information (NCBI) and primers were designed using primer-3 tool. Genes used for primer synthesis are shown in Table 1. Oligonucleotide synthesis was done by Eurofins genomics.

Molecular analysis of RILs using candidate gene and SSR markers

DNA isolation was done by cethyltrimethyl ammoniac bromide (CTAB) method from 21 day old leaves (Doyle and Doyle, 1990). Twenty four designed candidate gene primers and three SSR markers (Berhanu et al., 2013) (Table 2) were used for finding the association of zinc accumulation in the grains of rice. The polymerase chain reaction (PCR) mixture contained 50 ng of template DNA, 1 X PCR buffer (10 mM Tris, pH8.4, 50 mM KCl, 1.5 mM $MgCl_2$ and 0.01 mg mL^{-1} gelatin), 2.5 mM of $Mgcl_2$, five picomoles of forward and reverse primer, 0.05 mM of dNTPs and 1 U of Taq polymerase in a 20 µl of reaction volume. Template DNA was initially denatured at 95°C for five minutes followed by 38 cycles of PCR (Applied Biosystems 2720) amplification with the following parameters. A 30 s of denaturation at 95°C, 1 min of annealing at 60°C and 1 min of elongation at 72°C. A final elongation was done at 72°C for 10 min. The amplified product was resolved electrophoretically on a 3% agarose gel for 2 – 3 h, visualized under UV trans-illuminator and documented (Alpha Innotech FluorochemFC2).

Single marker analysis (SMA) and validation

SMA was done with t-test and regression analysis using SPSS 16.0 (SPSS Inc.) to find the association between molecular markers and grain zinc content. Polymorphic candidate markers which showed significant association with grain zinc content were used for validation with different germplasm accessions.

RESULTS AND DISCUSSION

Estimation of zinc

RIL lines were developed by hybridization and advanced by continuous selfing up to F_7 generation without any trait selection by single seed descent method. During the developmental process RIL lines underwent continuous recombinations for stabilization of trait. The grain zinc content in brown rice of IRRI38 and Jeerigesanna was

Table 1. Genes used for designing the primer.

Name	Chromosome	Location	Reference
OsYSL2	2	High grain zn content	Chandel, 2011
OsNAAT1	2	High grain zn content	Chandel, 2011
OsNAC	3	High grain zn content	Chandel, 2011
OsZIP1	3	High grain zn content	Chandel, 2011
OsZIP3	4	Leaf blade, root, stem, anther, ovary and embryo.	Bashir 2012
OsZIP7	5	High grain zn content	Chandel, 2011
OsNRAMP4	5	Mid grain filling stage	Chandel 2011
OsNRAMP5	7	Mid grain filling stage	Chandel 2011
OsZIP8	7	Leaf blade, root, stem, anther, ovary and embryo.	Bashir 2012
OsZIP4	8	Leaf blade, root, stem, anther, ovary and embryo.	Bashir 2012
OsVIT1	NM	High grain zn content	Chandel, 2011
OsNRAMP7	11	High grain zn content	Chandel, 2011

NM, Not mapped.

Table 2. Candidate gene primers were designed using NCBI and Primer-3 tool.

Genes	Chr. No	Source	Forward primer	Reverse primer	Annealing temp	Exp Product size
OsYSL2a	2	NCBI	CCGCTCCCGAGATAGAGA	AAGGCCATCCCCCATGAT	NA	1010
OsYSL2b	2	NCBI	TCACTGCTAAGAGCCTGCAT	CTAGCTTCCGGGAGTGAACTT	60.0	980
OsVIT1	NA	Chandel et al 2011	AAGAGCGAGGCAGACCATTA	GGAATGGACGGTTTCCAGTA	63.0	980
OsNAAT1	2	Chandel et al 2011	CATCTTCCTAACCGCTGGAG	CCTTTGGCAGAAGGATTTGA	58.0	700
OsNAC	3	Chandel et al 2011	AGCGAGAAGCAAGCAAGAAG	ATGCCCTGGATATCGTCGTA	58.0	600
OsZIP1a	3	NCBI	GCTCTTGCTCGCTGCAATTC	CCAACCAGTACCCGTTCTCC	59.0	883
OsZIP1b	3	NCBI	GAACTGTTTCGGCCACGATT	TGAGATGAATTGCAGCGGAGC	59.5	561
OsZIP3a	4	NCBI	ACCCATCATTGCCTCCATCT	AGAACCTGCATGGCCAAA	59.0	1131
OSZIP3bII	4	NCBI	GGGAATCTTGGTGCATTCAGT	GATCACCTGAGATAAGCTTTGG	59.0	1104
OsZIP3b	4	NCBI	CCTGCTGAGGCTGAGTTGAA	CGAGAACAAAGTAACAGGCTGC	61.5	370
OsZIP7c	5	NCBI	GCATCGAATCCAATCCAATC	GCATTAATGAACTCAGCCTCCA	NA	940
OsZIP7d	5	NCBI	GTTTCTTGCAGATACTTGAGATGG	CTGGGAATATCAAAGTCCGATT	57.0	1032
OsZIP7e	5	NCBI	AGACTGCTATGCTTCTCATAACG	GGGAGTATACATCACATGATCACA	49.5	940
OsZIP8a	7	NCBI	ATGAGGACGAACACCACCAC	CGGAGGGAGGGAGTAGTAATG	67.0	880
OsZIP8b	7	NCBI	GGGAGTAGTACGTACGTTTCAAATA	GCTCCCTTTTCCCTCTTTACAT	52.0	1064
OsZIP8c	7	NCBI	TGTAAAGAGGGAAAAGGGAGCTA	GGCGAGTACATTCACTTCCATT	52.0	927
OsZIP4a	8	NCBI	GTGTTCTTCGCCGTCAAGG	GAAATGGATGGTGGCAAAGT	60.0	641
OsZIP4b	8	NCBI	TGAGCTCATCATCACCAACCGTC	CACCTCCACCATCAAGGACG	61.5	673
OsZIP4d	8	NCBI	TTGATGGGAGAAAGGCATGT	CCATTTCTGAAAACGCGGTA	60.0	963
OsZIP4c	8	NCBI	ACTGTATCCACTATCCAGTGCTGA	GCTGGGGATTATTTGATTCTTCAC	56.0	1000
OsNAC5a	11	NCBI	CGAAAGCTTCCATTAGCGACT	CCAATTTGGCACACCTTTCA	53.0	916
OsNAC5b	11	NCBI	TGGCTGTAGCCGCTAGGTAT	GATCGATCGAGCACGGTTA	55.0	885
OsNAC5c	11	NCBI	CTCCACCGGCAGATCAAAAT	CATGTCGCAATCACCCTTAC	53.0	600
OsNRAMP 7	12	Chandel et al 2011	CGGGGCAGACTAGTACCATAACG	CAGCAAGAGATAGCCATTGATCG	60.0	2000
RM263	2	Gramene	CCCAGGCTAGCTCATGAACC	GCTACGTTTGAGCTACCACG	55.0	199
RM152	8	Gramene	GAAACCACCACACCTCACCG	CCGTAGACCTTCTTGAAGTAG	56.0	151
RM21	11	Gramene	ACAGTATTCCGTAGGCACGG	GCTCCATGAGGGTGGTAGAG	55.0	157

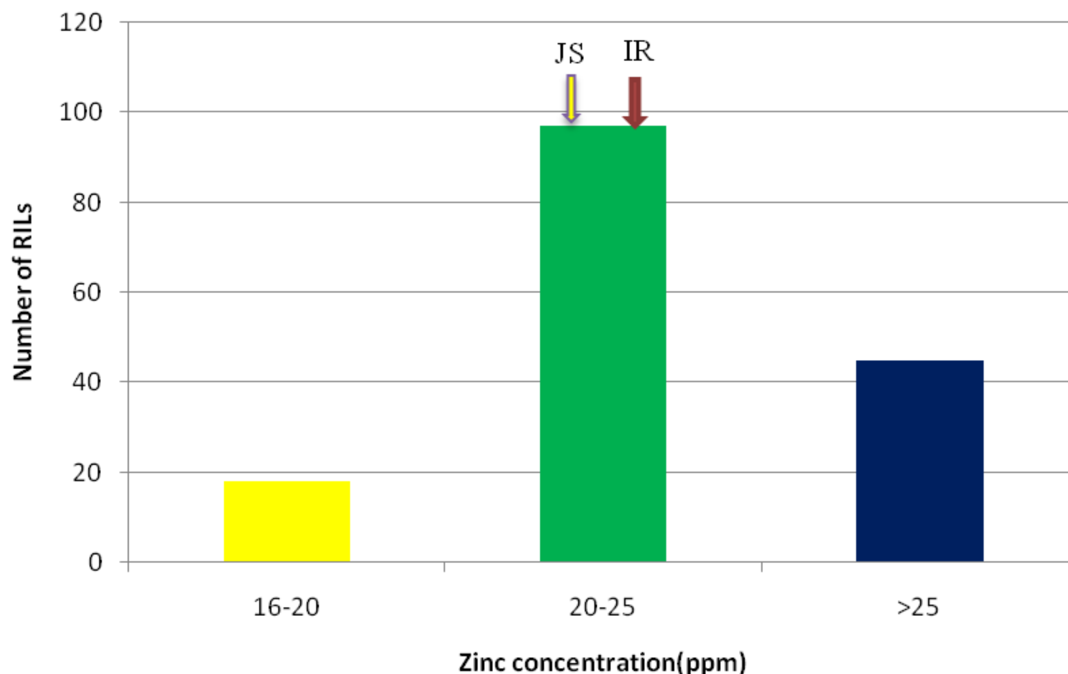


Figure 1. Distribution of grain zinc concentration (ppm) among RILs, arrow marks indicates for parents IR: IRRI38, JS: Jeerigesanna.

23.49 and 21.59 ppm, respectively. Among the RILs grain, zinc content in brown rice ranged from 16.1 to 35.5 ppm with an average zinc content of 23.7 ppm (Figure 1). Zinc estimation showed wide range of variability among RILs. This estimation revealed that the RIL lines had more zinc content than parents. 45 RILs had more zinc content (>25 ppm) than both the parents. Similar results were reported by Tiwari et al. (2009) on grain zinc content in wheat mapping population, ranging from 19.9 to 64.2 ppm. Grain zinc content ranged from 0.4 to 104 ppm in rice germplasm accessions (Anuradha et al., 2012) and Berhanu et al. (2013) reported 16.1 to 88.6 ppm for the rice RIL population. Depending on soil properties grain zinc content varies, pH, organic matter also showed effect on grain zinc content (Chandel et al., 2010; Pandian et al., 2011). Variation in zinc values in different samples of the same accession was observed due to absence or presence of embryo, time of harvest, sampling procedure such as seeds from only main panicle or all panicles or from the entire plant and different digestion or analytical methods (Chandel et al., 2010). Grain zinc content of brown rice was significantly influenced by native soil properties (Banerjee et al., 2010; Chandel et al., 2010; Suwarto, 2011).

Designing of candidate gene markers

Candidate gene markers for grain zinc content of different gene families are designed and synthesized as shown in Table 2.

Molecular analysis of RILs using candidate gene and SSR markers

Marker assisted technology hastens the breeding process and helps in more accurate QTL detection. Among 24 candidate gene markers used in the study eight markers (33.33%), (OsNAC, OsZIP3b, OsZIP3bII, OsZIP7e, OsZIP8a, OsZIP8c, OsVIT1 and OsZIP4b) showed polymorphism (Figure 2) among RIL population on the chromosome numbers 3, 4, 4, 5, 7, 7, not mapped and 8, respectively. Sixteen candidate gene markers showed monomorphism (Figure 3) among the RIL population, out of three SSR markers {RM263 (Figure 2), (RM152 and RM21)} three (100%), showed polymorphism among the RIL population on the chromosome numbers 2, 8 and 11 respectively. These polymorphic markers were used for single marker analysis to find the association.

Single marker analysis and validation

Single marker analysis revealed that out of 11 polymorphic markers, four (OsNAC, OsZIP8a, OsZIP8c and OsZIP4) showed association with a phenotypic variation of 4.5, 19.0, 5.1 and 10.2%, respectively (Table 3) among the RIL population. SSR markers did not show any significance difference among the RIL population. Similar results were reported for candidate markers by Sarala Neelamraju et al. (2012). They reported six QTL for grain zinc content showing >30% phenotypic variance. Anuradha et al. (2012) reported phenotypic variance in grain iron

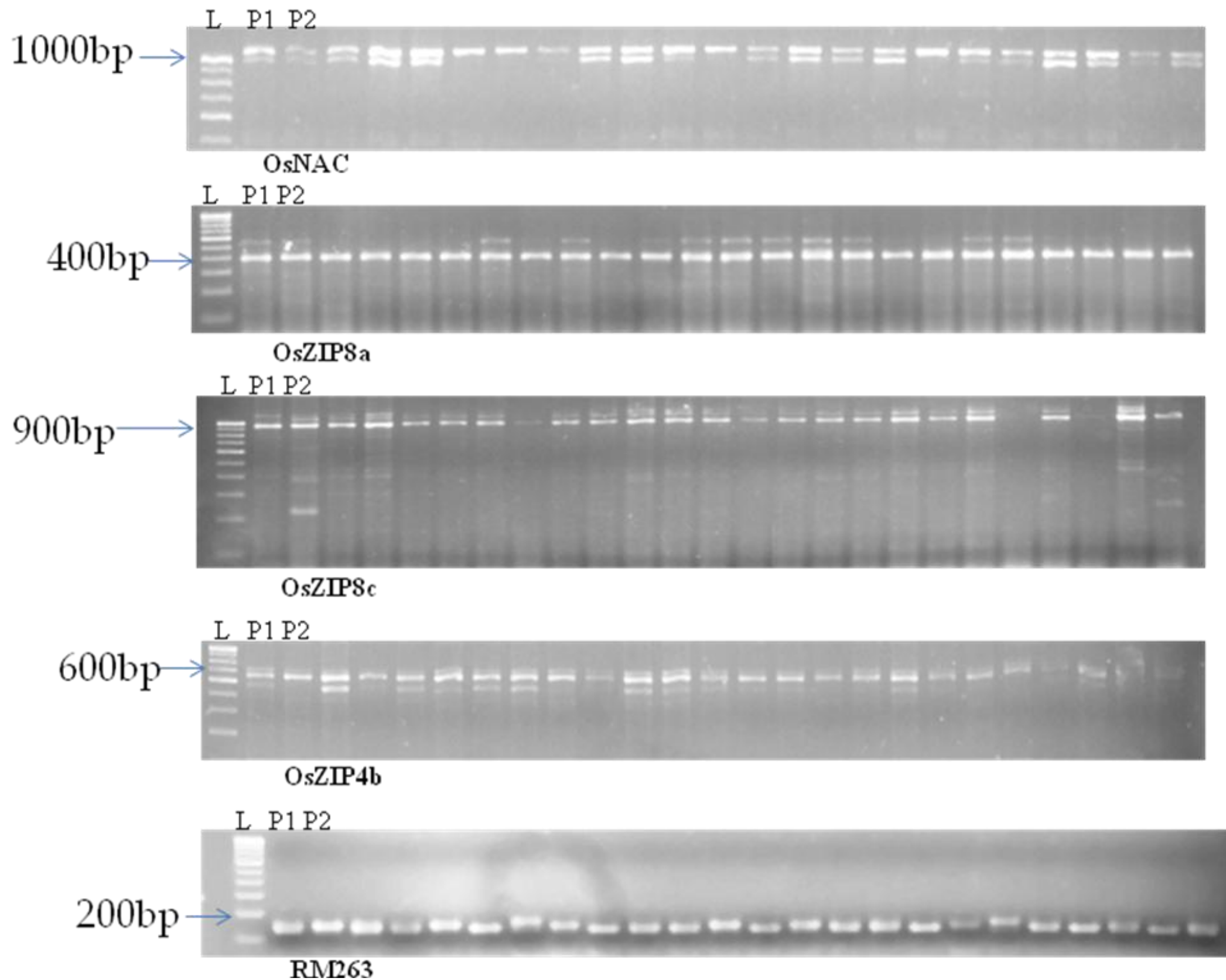


Figure 2. Polymorphic candidate gene and SSR markers.

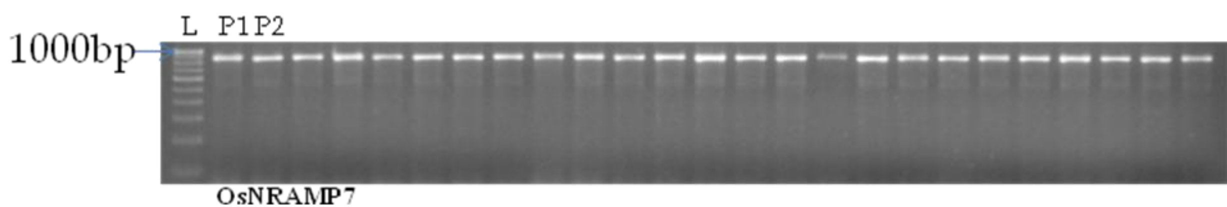


Figure 3. Monomorphic candidate gene markers. L, 100bp ladder; P1, IRRI38; P2, Jeerigesanna.

content with 69 to 71% variability (OsYSL1 and OsMTP1) and with zinc content of 29 to 35% variability (OsARD2, OsIRT1, OsNAS1 and OsNAS2). Grain zinc content for SSR markers (RM152, RM263 and RM21) with 6.1 to 11.7% phenotypic variability was reported by Berhanu et al. (2013). Nagesh et al. (2013) reported similar results from F_2 population of grain iron and zinc content (OsZIP1) with 13.09 and 19.51% variability, respectively.

Validation of putative markers is used to confirm the reproducibility of usefulness in marker aided breeding program. Validation of four candidate gene markers with 96 germplasm accessions showed significant association for three markers (OSZIP8a, OsNAC and OsZIP4b) with a phenotypic variation of 11.0, 5.8 and 4.8% respectively (Table 4). These markers can be further used in maker aided selection for zinc biofortification programs.

The present study revealed that RILs having high grain zinc content with high genetic variability. Single marker analysis showed four candidates gene markers with a significant phenotypic variation among the RIL population. Three putative candidate gene markers (OsZIP8a, OsNAC and OsZIP4b) with a phenotypic variation of 11.0,

Table 3. Single marker analysis (SMA) showing P and R^2 values of candidate gene and SSR markers in RILs of IRRI38 X Jeerigesanna for grain zinc content.

S/N	Marker	P	R^2 (%)	Mean Difference	Estimated effect
1	OsZIP3b	0.34	2.1	1.2	4.2
2	RM263	0.59	0.7	1.8	1.4
3	RM21	0.28	1.6	0.6	3.2
4	RM152	0.98	0	0.4	0.0
5	OsNAC	0.03*	4.5	1.7	9.0
6	OsZIP3bII	0.41	0.4	0.3	0.8
7	OsZIP8a	0.00**	19	3.9	38.0
8	OsZIP8c	0.02*	5.1	1.6	10.2
9	OsVIT1	0.73	0.4	0.2	0.8
10	OsZIP4b	0.00**	10.2	2.5	20.4
11	OsZIP7e	0.71	0.4	1.8	0.8
Mean	23.7ppm				
SD	3.37				

P, Significance; R^2**,** percentage of phenotype variability.

Table 4. Single marker analysis showing P and R^2 values of significant candidate gene markers with genotypes for grain zinc content.

S/N	Marker	P	R^2 (%)	Mean Difference	Estimated effect
1	OsNAC	0.02	5.8	4.2	22
2	OsZIP8a	0.01	11	2.6	2.8
3	OsZIP8c	0.51	1.4	1.8	11.6
4	OsZIP4b	0.03	4.8	3.2	9.6
Mean	29.35				
SD	6.26				

P, Significance; R^2**,** percentage of phenotype variability.

5.8 and 4.8% were found. These putative markers can be used in biofortification programs by breeders and bio-technologists.

ACKNOWLEDGEMENTS

Authors are thankful to Department of Biotechnology, New Delhi, India for grant support (Ab/Ac: 8270), University of Agricultural Science for providing facilities to execute work, M. S. Swaminathan Research Foundation for their support in estimation of zinc content using XRF instrument and Karpagam University for their constant support during research period.

REFERENCES

Anuradha K, Surekha Agarwal, Venkateswara Rao Y, Rao KV,
Viraktamath BC, Sarla N (2012). Mapping QTLs and candidate genes for iron and zinc concentrations in unpolished rice of Madhukar×Swarna RILs. Gene 508: 233 – 240.

Bashir K, Ishimaru Y, Nishizawa, NK (2012). Molecular mechanisms of zinc uptake and translocation in rice. Plant soil 361:189-201

Banerjee S, Sharma DJ, Verulkar, SB, Chandel G (2010). Use of in silico and semiquantitative RT-PCR approaches to develop nutrient rich rice (*Oryza sativa* L.). Indian. J. Biotechnol. 9: 203–212.

Berhanu DB, Naveen GK, Rakhi S, Shashidhar HE (2013). Genetic Evaluation of Recombinant Lines of Rice (*Oryza sativa* L.) for Grain Zinc Concentrations, Yield Related Traits and Identification of Associated SSR markers. Pakistan J. Biol. Sci. 16: 1714 - 1721.

Biradar H, Bhargavi MV, Sasalwad R, Parama R, Hittalmani S (2007). Identification of QTL associated with silicon and zinc content in rice *(Oryza sativa* L.) and their role in blast disease resistance. Indian J. Genet. 67: 105 - 109.

Bouis HE (2003). Genetically modified food crops and their contribution to human nutrition and food quality. Trends Food Sci. Technol. 14: 191 - 209.

Bouis HE, Welch RM (2010). Biofortification-a sustainable agricultural strategy for reducing micronutrient malnutrition in the global south. Crop Sci. 50: 20 – 32.

Chandel G, Banerjee S, See S, Meena R, Sharma DJ and Verulkar SB (2010). Effects of different nitrogen fertilizer levels and native soil properties on rice grain Fe, Zn and protein contents. Rice Sci. 17: 213–227.

Doyle JJ and Doyle JL (1990). Isolation of plant DNA from fresh tissue. Focus 12: 13 - 15.

Federer MT (1960). Augmented designs with one-way elimination of heterogeneity. Cornell University, New York, U.S.A.

Garcia-oliveira AL, Lubin T, Yongcai F, Chuanqing S (2009). Genetic Identification of quantitative trait loci for contents of mineral nutrients in rice grain. Journal of Integrative. Plant Biol. 51: 84 - 92.

Chandel GP, Samuel M, Dubey R, Meena (2011). *In silico* expression analysis of QTL specific candidate genes for grain micronutrient (Fe/Zn) content using ESTs and MPSS signature analysis in rice (*Oryza sativa* L.). Journal of Plant Genetics and Transgenics 2: 11 - 22.

Graham RD, Senadhira D, Beebe S, Iglesias C, Monasterio I (1999). Breeding for micronutrient density in edible portions of staple food crops: conventional approaches. Field Crops Research 60: 57 – 80.

Gregorio GB, Senadhira D, Graham RD, Htut T (1999). Improving iron and zinc value of rice for human nutrition. Agric. Dev. 23: 68 – 81.

Gross J, Stein RJ, Fett-Neto AG, Fett JP (2003). Iron homeostasis related genes in rice. Genet. Mol. Biol. 26: 477 – 497.

Howarth EB, Ross MW (2010). Biofortification-A Sustainable Agricultural Strategy for Reducing Micronutrient Malnutrition in the Global South. Crop Sci. 50: 20 - 32.

Lu K, Li L, Zheng X, Zhang Z, Mou T, Hu Z (2008). Quantitative trait loci controlling Cu, Ca, Zn, Mn and Fe content in rice grains. J. Genet. 87: 305–310.

Miklas PN (2007). Marker assisted backcrossing QTL for partial resistance to *Sclerotinia* white mold in dry bean. Crop Sci. 47: 935 - 942.

Nagesh P, Usharani G, Neeraja N, Ravindra BV, Dayakar RT (2013). Molecular Mapping of High Iron and Zinc Rich Regions in Rice (*Oryza sativa* L.) Grains Using Microsatellite Markers. Helix 1: 231 - 237.

Neelamraju S, Mallikarjuna Swamy BP, Kaladhar K, Anuradha K, Venkateshwar Rao Y, Batchu AK, Agarwal S, Babu AP, Sudhakar T, Sreenu K, Longvah T, Surekha K, Rao KV, Ashoka Reddy G, Roja TV, Kiranmayi SL, Radhika K, Manorama K, Cheralu C, Viraktamath BC (2012). Increasing iron and zinc in rice grains using deep water rices and wild species – identifying genomic segments and candidate genes. Q. Assur. Safety Crops Foods 4: 138.

Nicholas GP, Lachlan JP, Paul JM, Georgia EG, James CRS (2012). Energy - dispersive X-ray fluorescence analysis of zinc and iron concentration in rice and pearl millet grain. Plant soil 361: 251 - 260..

Pandian SS (2011). Influence of intrinsic soil factors on genotype-by-environment interactions governing micronutrient content of milled rice grains. Austr. J. Crop Sci. 5: 1737–1744.

Pfeiffer WH, McClafferty B (2007). Harvest Plus: breeding crops for better nutrition. International plant breeding symposium. Crop Sci. 47: 88 - 105.

Rosa SR, Ribeiro ND, Jost E, Reiniger LRS, Rosa DP, Cerutti T, Possobom M (2010). Potential for increasing the zinc content in common bean using genetic improvement. Euphytica 175: 207 – 210.

Sellappan K, Datta K, Parkhi V, Datta SK (2009). Rice caryopsis structure in relation to distribution of micronutrients (iron, zinc, b-carotene) of rice cultivars including transgenic indica rice. Plant Sci. 177: 557 – 562.

Stein AJ, Nestel P, Meenakshi JV, Qaim M, Sachdev HPS, Bhutta ZA (2007). Plant breeding to control zinc deficiency in India: how cost-effective is biofortification. Publ. Health Nutr. 10: 492 – 501.

Suwarto N (2011). Genotype×environment interaction for iron concentration of rice in central Java of Indonesia. Rice Science 18: 75–78.

White PT (1994). Rice: The essential harvest. Nat. Geo. 185: 48 - 79.

World health organization (1996). Trace elements in human nutrition and health, WHO report, Geneva, Switzerland.

Zhang X, Zhang G, Guo L, Wang H, Zeng D, Dong G, Qian Q, Xue D (2011). Identification of quantitative trait loci for Cd and Zn concentrations of brown rice grown in Cd-polluted soils. Euphytica 180: 173 – 179.

academicJournals

Vol. 13(5), pp. 664-672, 29 January, 2014
DOI: 10.5897/AJB2013.13023
ISSN 1684-5315 ©2014 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Comparsion of bioleaching of copper sulphides by *Acidithiobacillus ferrooxidans*

Kaibin Fu[1,2*], Hai Lin[2], Deqiang Luo[1], Wufei Jiang[1] and Ping Zeng[1]

[1]Key Laboratory of Solid Waste Treatment and Resource Recycle, Ministry of Education, Southwest University of Science and Technology, Mianyang 621010, China.
[2]School of Civil and Environmental Engineering, University of Science and Technology Beijing, Beijing 100083, China.

Accepted 5 September, 2013

Bioleaching of copper sulphides were investigated. The results show that the copper extraction from djurleite, bornite, covellite and chalcopyrite under the optimal conditions were 95.12, 84.5, 54.1, and 18.33%, respectively. A ranking for the bioleaching of copper sulfides was obtained: djurleite > bornite > covellite > chalcopyrite. A determination of the extent of adsorption of *A. ferrooxidans* on the surface of the minerals showed that the cell density of *Acidithiobacillus ferrooxidans* on djurleite was the highest. The order of adsorption on the copper sulphides was: djurleite > bornite > covellite > chalcopyrite. The results suggest a difference in affinity of bacteria for different copper sulphides. The higher the leaching rate of copper sulphides, the greater the density of bacteria absorbed on the surface of minerals.

Key words: Bioleaching, copper sulphides, *Acidithiobacillus ferrooxidans*, cell density, copper extraction.

INTRODUCTION

Bacterial heap leaching of low-grade copper sulphides is a developing technology. Bioleaching has been applied successfully to the extraction of copper from secondary sulphide minerals, such as chalcocite, at a number of operations worldwide (Watling, 2006). However, heap bioleaching of the refractory primary copper sulphide, chalcopyrite, is still a major challenge due to slow kinetics and poor extraction (Zhou et al., 2009). A study at Billiton Process Research showed bioleaching of secondary sulphide minerals with adapted mesophilic bacterial cultures is technically feasible. The preferential order in which sulphide minerals are leached by such mesophilic cultures is: Chalcocite> bornite> cubanite> covellite> pyrite> enargite> carrolite>> chalcopyrite (Dew et al., 1999). Regrettably, the reason for difference of bioleaching of sulphide minerals was nott analyzed.

Rodríguez et al. (2003) confirmed that a relationship exists between attachment and mineral dissolution rates. The bioleaching process can be divided into three stages. An initial stage with extensive bacterial attachment to the minerals is of major importance in order to obtain high dissolution rates. In a second stage, the bacterial attachment diminishes due to the saturation of the surface by the attached cells. Finally, in a third stage, a balance between free and attached cells is reached, giving rise to a cooperative mechanism (Sand et al., 1995; Rojas-Chapana and Tributsch, 2004). Attached cells play important roles in the whole bioleaching process.

The present work is a comparative study of the bioleaching of several copper sulfide minerals, djurleite ($Cu_{31}S_{16}$), bornite (Cu_5FeS_4), covellite (CuS), chalcopyrite ($CuFeS_2$). Experiments were conducted to characterize the adsorption

*Corresponding author. E-mail: fukaibin@126.com. Tel: +86-15884633446.

Abbreviations: XRD, X-ray diffraction; **AAS,** atomic absorption spectrometry; **ORP,** oxidation reduction potential; **SEM,** scanning electron microscopy.

Table 1. Chemical analysis of copper sulphides.

Mineral	Cu (%)	Fe (%)	S (%)	Purity (%)
Djurleite	71.94	1.22	20.53	90.65
Bornite	50.17	10.67	24.38	79.22
Covellite	60.26	3.20	33.52	90.48
chalcopyrite	27.88	28.29	32.36	80.62

of *Acidithiobacillus ferrooxidans* on copper sulphides as functions of time and cell concentration. A relationship between attachment and mineral dissolution rates will be discussed.

MATERIALS AND METHODS

Minerals

Four copper sulphides were used in the experiments. These minerals included djurleite, bornite, covellite and chalcopyrite. They were supplied by the Bofang copper mine in Hunan province, the Dongxiang copper mine in Jiangxi province, the Zijinshan copper mine in Fujian province, and the Dexing copper mine in Jiangxi province, respectively. The chemical analysis of the copper sulphides is shown in Table 1.

X-ray diffraction (XRD) analysis showed that djurleite ($Cu_{31}S_{16}$) contained small amounts of chacocite, bornite and quartz; bornite (Cu_5FeS_4) contained small amounts of chalcopyrite, pyrite (FeS_2), helvite, and quartz; covellite (CuS) contained small amounts pyrite and enargite; chalcopyrite ($CuFeS_2$) contained small amounts of pyrite and quartz. The handpicked samples were ground in a porcelain ball mill to a particle size of less than 74 μm, then stored under nitrogen in a sealed container. The specific surface area of djurleite, bornite, covellite and chalcopyrite were 302.67, 818.93, 1319.34 and 1362.47 cm^2/g, respectively.

Bacteria

An *A. ferrooxidans* (*Atf.6*) strain used in this study was isolated from a copper mine in Daye (China), and then screened through different stages of adaptation. Bacteria were grown in optimized 4.5 K medium (2.0 g/L $(NH_4)_2SO_4$, 0.25 g/L K_2HPO_4, 0.25 g/L $MgSO_4 \cdot 7H_2O$, 0.1 g/L KCl, and 22.0 g/L $FeSO_4 \cdot 7H_2O$) (Fu et al., 2011) and harvested from the late logarithmic phase of growth. The optimal cultivation conditions of *Atf.6* were temperature of 30°C, pH of 2.0, and a rotation speed of 160 rpm.

Bioleaching experiments

Bioleaching experiments were performed in 250 mL flasks, at pulp density of 20 g/L. When the pH was adjusted to 2, nutrients and inoculum were added to the erlenmeyer flask. The flasks were kept at 30°C and shaken at 160 rpm. The number of viable bacteria, pH, redox potential, and copper ion concentration in the leaching solution were determined at certain intervals. During the leaching process, distilled water was supplied to compensate for evaporation, and to maintain a solution volume of 100 mL in the flasks. Finally, the mineral residues were washed with Milli-Q water, and vacuum freeze-dried.

Adsorption studies

The bacterial cultures were fully grown in a sterile modified medium (for 60 h) and filtered through a Whatman No. 1 filter paper to remove all of the insoluble compounds. The filtrate was centrifuged at 10,000 rpm for 10 min, washed several times with distilled water, and then dispersed in distilled water at pH of 2.0 (Jia et al., 2008). Attachment experiments were carried out in 50 mL KCl solution with an ionic strength of 0.001 mol/L, containing 2.0×10^{10} cells and 0.5 g mineral (50 μm), on a rotary shaker (120 rpm) at 30°C.

The number of attached cells was calculated by subtraction of the remaining planktonic cells from the total numbers of cells inoculated.

Analytical methods

The concentration of dissolved copper ions in the leaching solution was analyzed by atomic absorption spectrometry (AAS). The ferrous iron was determined by titration with potassium dichromate (K_2CrO_7). The pH value and redox potential were measured by S20 Seven easy pH/Eh process controller. The bacterial number was determined by hemocytometer under ZBM-300E biological microscope.

SEM observations

SEM studies were carried out to observe bacterial cells attachment to mineral surfaces. After the adsorption equilibrium of *A. ferrooxidans* on copper sulfides, the mineral samples were gently washed twice with distilled water to remove loosely attached bacteria.

The samples were then vacuum freeze-dried and coated with carbon under vacuum. Images were obtained using a LEO-1450 model scanning electron microscope.

RESULTS AND DISCUSSION

Bioleaching of copper sulphides

Inoculum

The media contained 0 g/L of Fe^{2+}. The effect of cell concentration in the inoculum on the copper extraction from the copper sulphides is shown in Figure 1. When the pH was adjusted to 2, the copper extraction from djurleite, bornite, chalcocite, and chalcopyrite reached 36.45, 18.38, 9.92, and 0.34%, respectively. Figure 1 shows the effect of cell concentration on copper extraction of copper sulphides. In the initial stages of leaching, copper extraction increased with the increase of inocula. Generally, within the range of a cell concentration, as bacterial inocula increases, more bacterial cells were adsorbed on the mineral surface. Rapid dissolution rate depends on adsorption of large numbers of bacteria on the surface of metal sulphide mineral, which results in the release of Fe^{2+} through “contact” bioleaching (Pich otero et al., 1995;

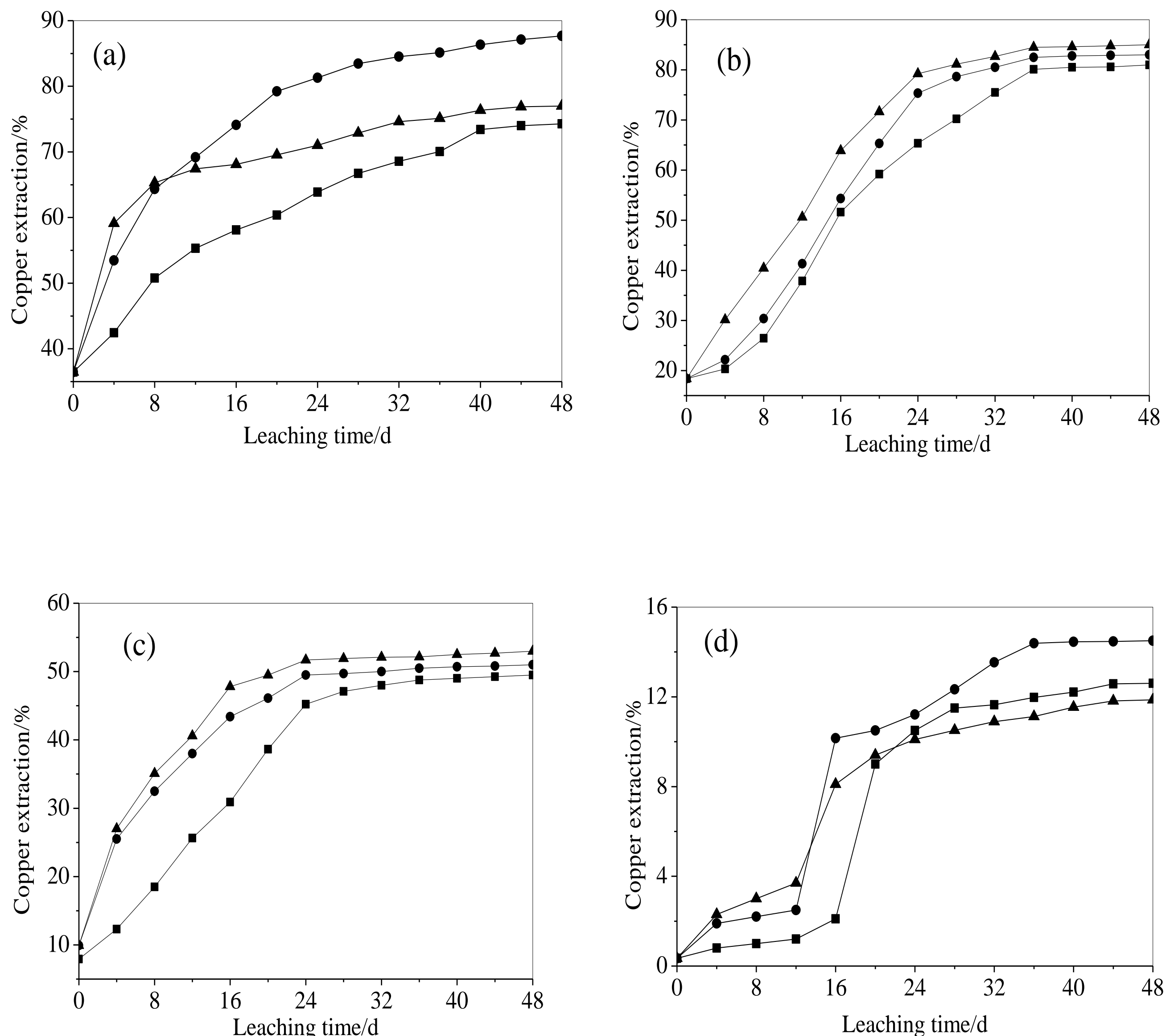


Figure 1. Effect of cell concentration in inocula (■, 0.5×10^8 cells; ●, 1.0×10^8 cells; ▲, 1.5×10^8 cells) on copper mobilization from djurleite (a), bornite (b), covellite (c), and chalcopyrite (d).

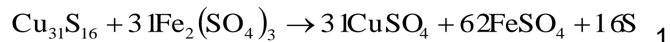
Rodríguez et al., 2003). As the duration of leaching lengthened, the differences in leaching rate at different inoculum sizes became smaller. Copper extraction from djurleite with inoculum of 1.0×10^8 cells was greater than that with an inoculum of 1.5×10^8 cells from day 10 to day 48. More copper was extracted from chalcopyrite with an inoculum of 0.5×10^8 cells than that with 1.5×10^8 cells. The optimum inoculation quantity is beneficial to bioleaching of copper sulphides. The maximal copper recovery of djurleite and chalcopyrite were achieved when the inoculation amount is 1.0×10^8 cells. After 48 day of bioleaching, the copper dissolution of djurleite and chalcopyrite were 87.67 and 14.5%, respectively. The maximum copper extraction of bornite and covellite were 85 and 53%, respectively, when the inoculation amount is 1.5×10^8 cells. The results present in Figure 1 indicated the preferential order of mineral bioleaching: djurleite> bornite> covellite> chalcopyrite.

Initial ferrous ion concentration

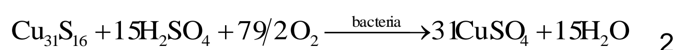
Other than the bacterial inoculum, the initial ferrous iron concentration is an important parameter in the bioleaching of copper sulphides (Van et al., 2010; Kinnunen et al., 2006). The effect of the initial ferrous ion concentration on the bioleaching of copper sulphides is shown in Figure 2. Substantial work with regards to the mechanism of bioleaching has been done with *A. ferrooxidans*. Since the discovery of this bacterium in acid mine drainage, two bio-oxidation mechanism have been discussed: the direct

Figure 2. Effect of $[Fe^{2+}]_{initial}$ (■, 0g/L ; ●, 1.5g/L ; ▲, 2.5g/L ; ▼, 4.5g/L) on bioleaching of djurleite (a), bornite (b), covellite (c), and chalcopyrite (d).

one and the indirect one (Schippers and Sand, 1999; Colmer and Hinkle, 1947). The direct mechanism assumes the action of a metal sulfide-attached cell oxidizing the mineral by an enzyme system with oxygen to sulfate and metal cations. In contrast, the indirect mechanism basically involving the oxidation of the metal sulphide by Fe^{3+} produced by the bacteria in the oxidation of Fe^{2+}. In the course of metal sulphide oxidation by Fe^{3+}, Fe^{2+} and elemental sulfur (S_8) are generated. These products are then biologically oxidized to Fe^{3+} and sulfate (Sand et al., 2001; Tributsch, 2001). Accordingly, the bioleaching of copper sulphides are summarized by the following equations (Fu et al., 2013; Ahmadi et al., 2012):



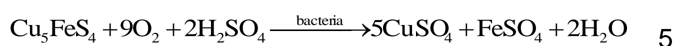
$$Cu_{31}S_{16} + 31Fe_2(SO_4)_3 \rightarrow 31CuSO_4 + 62FeSO_4 + 16S \quad 1$$



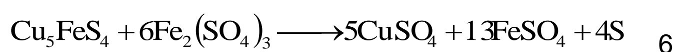
$$Cu_{31}S_{16} + 15H_2SO_4 + 79/2O_2 \xrightarrow{bacteria} 31CuSO_4 + 15H_2O \quad 2$$

$$CuS + 2O_2 \xrightarrow{bacteria} CuSO_4 \quad 3$$

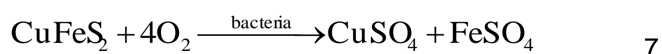
$$CuS + Fe_2(SO_4)_3 \longrightarrow CuSO_4 + 2FeSO_4 + S \quad 4$$



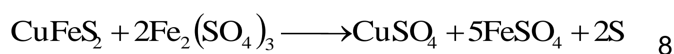
$$Cu_5FeS_4 + 9O_2 + 2H_2SO_4 \xrightarrow{bacteria} 5CuSO_4 + FeSO_4 + 2H_2O \quad 5$$



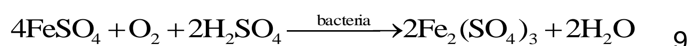
$$Cu_5FeS_4 + 6Fe_2(SO_4)_3 \longrightarrow 5CuSO_4 + 13FeSO_4 + 4S \quad 6$$



$$CuFeS_2 + 4O_2 \xrightarrow{bacteria} CuSO_4 + FeSO_4 \quad 7$$

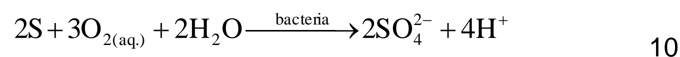


$$CuFeS_2 + 2Fe_2(SO_4)_3 \longrightarrow CuSO_4 + 5FeSO_4 + 2S \quad 8$$



$$4FeSO_4 + O_2 + 2H_2SO_4 \xrightarrow{bacteria} 2Fe_2(SO_4)_3 + 2H_2O \quad 9$$

Figure 2. Effect of $[Fe^{2+}]_{initial}$ (■, 0g/L ; ●, 1.5g/L ; ▲, 2.5g/L ; ▼, 4.5g/L) on bioleaching of djurleite (a), bornite (b), covellite (c), and chalcopyrite (d).



$$2S + 3O_{2(aq.)} + 2H_2O \xrightarrow{\text{bacteria}} 2SO_4^{2-} + 4H^+ \quad (10)$$

Figure 2 shows copper dissolution was initially fast, but slowed down rapidly after approximately 24 day. Copper extraction inceased with an increase in initial ferrous iron concentration. The results indicate the oxidizing action of Fe^{3+} played a leading role in bioleaching of copper sulfides. In the lag phase, copper sulfides were oxidized by Fe^{3+} (Equations 1, 4, 6 and 8). After a short lag phase, the bacteria adapted to copper ores environment, and multiplied rapidly. *A. ferrooxidans*, together with Fe^{3+}, facilitates dissolution of copper sulfides. With the dissolution of minerals, the excess of iron ions has a negative effect on the copper dissolution rate. Jarosite formed gradually, and coated on the surface of the remaining unreacted ore. This jarosite precipitation restricted the leaching of the sulphide mineral. The ferrous ion, as energy source of *A. ferrooxidans* plays a very important role in extracting copper and will affect the recovery of copper. The apropriate initial ferrous ion concentration is in favor of dissolution of copper sulfides. After 48 day of bioleaching, when the initial ferrous ion concentration was 1.5 g/L, the maximal copper dissolution of djurleite and chalcopyrite achieved were 95.12 and 18.33%, respectively. The ferrous ion would hinder the dissolution of bornite, the bornite dissolution was 84.5% at 0 g/L of the initial ferrous ion concentration. The covellite dissolution was 54.1% at 4.5 g/L of $[Fe^{2+}]_{initial}$. The preferential order of bioleaching of copper sulfides still is: djurleite> bornite> covellite> chalcopyrite.

Bioleaching under optimal conditions

Based on the results determined in the previous tests, the optimum conditions of bioleaching of djurleite and chalco-

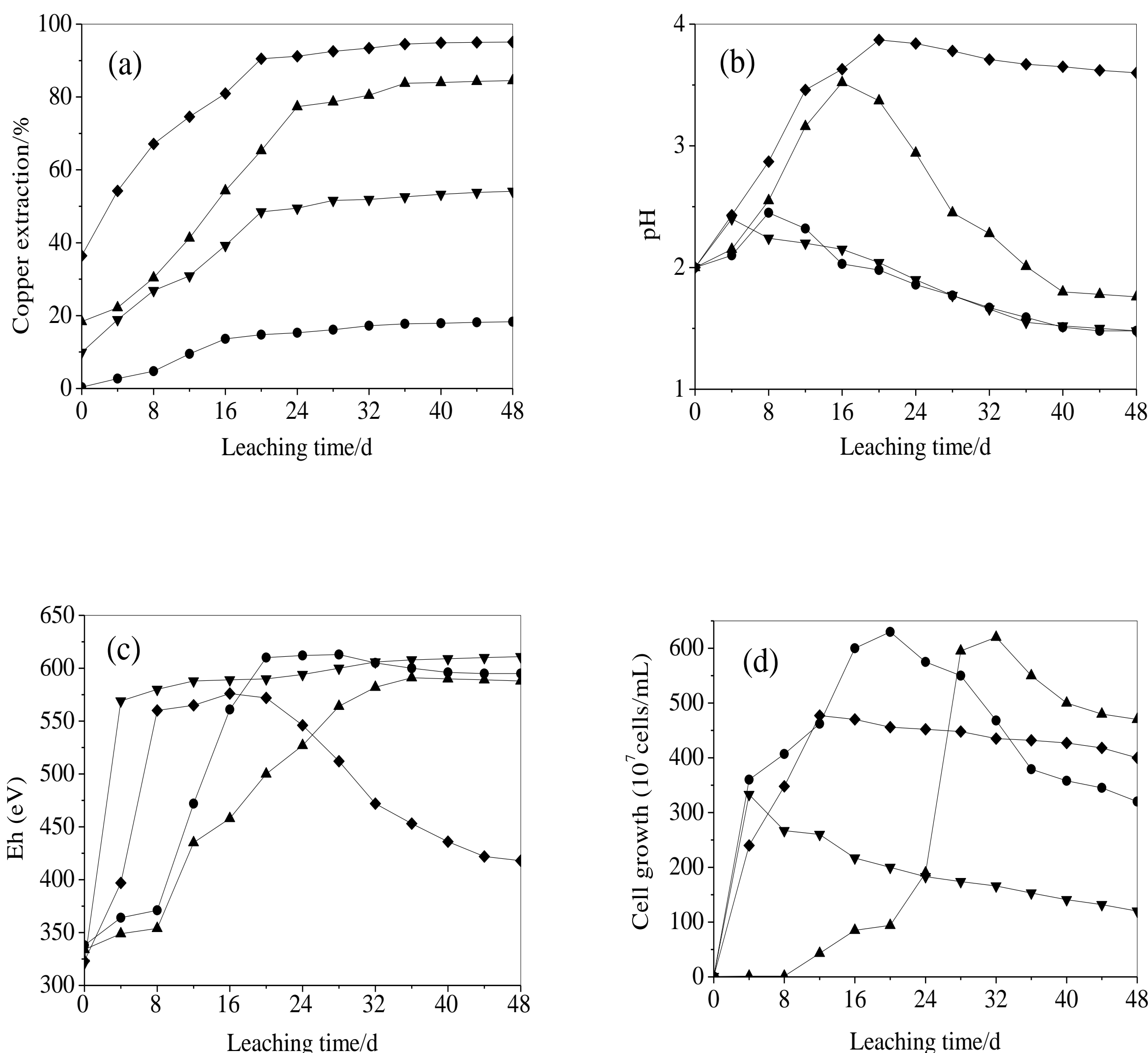


Figure 3. Bioleaching of djurleite (◆), bornite (▲), covellite (▼), and chalcopyrite (●) under the optimal conditions. Variations of copper extraction (a), pH (b), Eh (c) and cell growth (d).

pyrite were determined as follows: An inoculum of 0.5×10^8 cells, and the initial ferrous ion concentration of 1.5 g/L. The optimal inoculum of bornite and covellite were 1.5×10^8 cells. The appropriate initial ferrous ion concentration of bornite and covellite were 0 and 4.5 g/L, respectively. The bioleaching of copper sulfides under the optimal conditions is shwon in Figure 3. Figure 3a shows the copper extraction from djurleite, bornite, covellite and chalcopyrite were 95.12, 84.5, 54.1, and 18.33%, respectively. The ranking for the bioleaching of copper sulfides is obtained: djurleite> bornite> covellite> chalcopyrite. The results are in good agreement with those obtained by Dew et al. (1999).

Figure 3b shows the change of pH during the bioleaching of copper sulfides. The pH in the bioleaching of covellite and chalcopyrite was lower than 2.5, as the leaching reaction progressed, the pH decreased, low pH resulted in high ratio of [Fe^{3+}] to [Fe^{2+}] (Figure 3c). The oxidation reduction potential (ORP) in the solution of covellite and chalcopyrite was higher than 550 eV. Sandstrom et al. (2005) reported chalcopyrite leached rapidly at the lower potential, dropping at the higher redox value. The bioleaching experiments of Third et al. (2002) showed that the dissolution of chalcopyrite was inhibited by redox potentials> 420 mV. High [Fe^{3+}]/[Fe^{2+}] might hinder the dissolution of covellite and chalcopyrite. The pH in bioleaching of djurleite and bornite were higher than that in bioleaching of covellite and chalcopyrite after 8 day of bioleaching. The pH of djurleite bioleaching was higher than 3.0 after 9 day (Figure 3b). The ORP in the solution of djurleite gradually decreased after 20 day of bioleaching (Figure 3c), and was 418 eV at the end of bioleaching. *A. ferrooxidans* obtains energy from the simultaneous oxidation of Fe^{2+} and elemental sulfur, their growth rate is given by the following equation (Vilcaez and Inoue 2009):

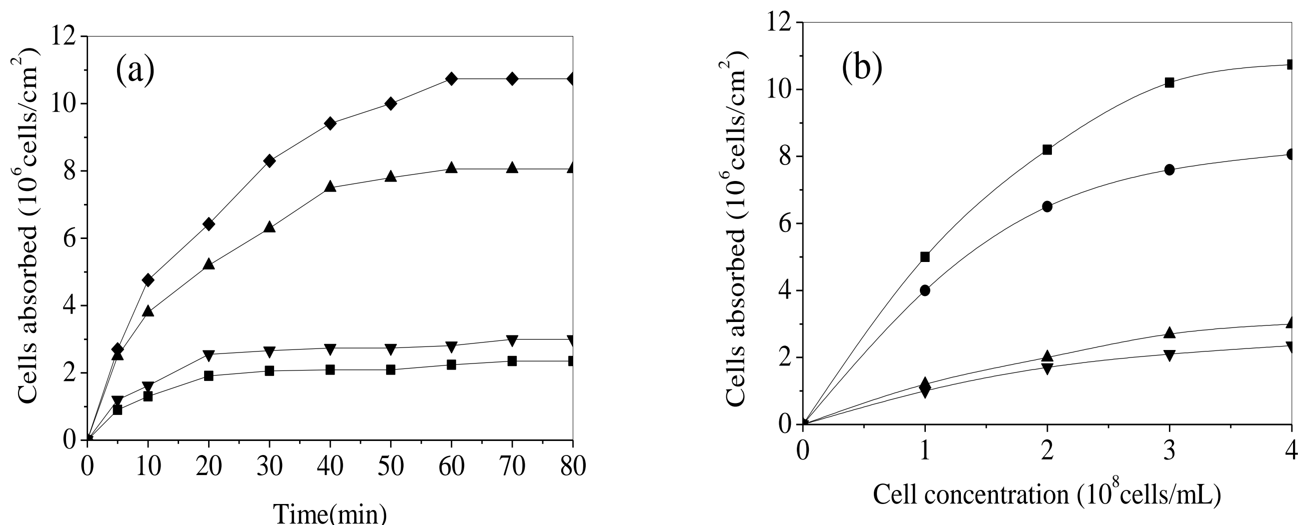


Figure 4. Adsorption of *A. ferrooxidans* on djurleite (◆), bornite (▲), covellite (▼), and chalcopyrite (●). A, Time; B, cell concentration.

$$\frac{d[N]}{dt} = \mu_{Fe}\frac{[Fe^{2+}]}{K_{Fe}+[Fe^{2+}]}[N] + \mu_S\frac{[S^0]}{K_S+[S^0]}[N] \qquad 11$$

Where, N is the biomass concentration, μ_{Fe} is the specific growth coefficient of mesophiles in the oxidation of iron, K_{Fe} is the half-saturation coefficient of the oxidation of iron, μ_S is the specific rate coefficient in the oxidation of elemental sulfur by mesophiles, K_S is the saturation coefficient in the oxidation of elemental sulfur.

When the initial ferrous ion concentration was 0 g/L, *A. ferrooxidans* obtained energy from the oxidation of copper sulphide, the lag phase of bacteria in solution of bornite lengthened (Figure 3d). The initial ferrous ion concentration in the solution of djurleite, chalcopyrite and covellite were 1.5, 1.5 and 4.5 g/L, respectively. *A. ferrooxidans* obtained easily energy from the oxidation of Fe^{2+} to Fe^{3+}. The lag phase of *A. ferrooxidans* in the solution of djurleite, chalcopyrite and covellite shortened. The appropriate initial ferrous ion is beneficial to the bacterial growth. The cell concentration in the solution of covellite decreased after 4 days of bioleaching. This might be atttibuted to the low pH value. Deveci et al. (2004) reported that the increase in the acidity to pH 1.2-1.4 led to a decrease in the oxidising activity of bacteria, indicating the inhibitory effect of increased acidity on bacteria.

Adsorption experiment

The adsorption of *A. ferrooxidans* on copper sulphides at pH=2.0 is shown in Figure 4. Figure 4a is the adsorption curve of *A. ferrooxidans* on copper sulphides. Figure 4b is the adsorption isotherm of *A. ferrooxidans* on copper sulphides. Figure 4a shows the adsorption quantity increased with the extension of time. The adsorption equilibrium of *A. ferrooxidans* on covellite and chalcopyrite were attained after 20 min. However, the adsorption equilibrium of *A. ferrooxidans* on djurleite and bornite were attained after 60 min. As for as the four copper sulphides, the cell density of *A. ferrooxidans* on djurleite was the highest. The order of adsorption of cells on copper sulphides is: djurleite> bornite> covellite> chalcopyrite. The order is in accordance with their copper dissolution rate.

The shape of adsorption isotherms enables characterization of adsorption process. Adsorption isotherms have been classified by Giles et al. (1960) into four main groups: L, S, H, and C. According to the classification, the isotherm of adsorption of *A. ferrooxidans* on copper sulphides displayed an L curve pattern (Figure 4b). The L shape of the adsorption isotherms means that there is no strong competition between solvent and the bacteria to occupy the copper sulphides.

As can be seen from Figure 4b, the values of experimental maximum adsorption of *A. ferrooxidans* on djurleite, bornite, covellite, and chalcopyrite were about 10.74×10^6, 8.06×10^6, 3.0×10^6, and 2.35×10^6 cells/cm^2, respectively. Several mathematical models can be used to describe experimental data of an adsorption isotherm. The equilibrium data in Figure 4b were modeled with the Langmuir and Freundlich models, respectively. The linearized equations of adsorption isotherm of *A. ferrooxidans* on copper sulphides and the corresponding correlation coefficient are shown in Table 2.

Based on Table 1, the Langmuir isotherm was more applicable to the experimental data than the Freundlich adsorption isotherm because of the higher values of correlation coefficient (R^2). The coefficients of correlation of the Langmuir adsorption isotherm are high (≥0.9944), which shows a good linearity. The result shows the ad-sorption of *A. ferrooxidans* on copper sulphides is mono-layer adsorption. This was verified by the SEM image of adsorption of *A. ferrooxidans* on copper sulphides (Figure 5).

Table 1. Chemical analysis of copper sulphides.

Mineral	Cu (%)	Fe (%)	S (%)	Purity (%)
Djurleite	71.94	1.22	20.53	90.65
Bornite	50.17	10.67	24.38	79.22
Covellite	60.26	3.20	33.52	90.48
chalcopyrite	27.88	28.29	32.36	80.62

Table 2. Adsorption isotherm of *A. ferrooxidans* on copper sulphides.

Mineral	Langmuir adsorption isotherm		Freundlich adsorption isotherm	
	Linear form	R^2	Linear form	R^2
Djurleite	$1/q_e=0.040/C_e+0.018$	0.9947	$lgq_e=0.53lgC_e+4.95$	0.963
Bornite	$1/q_e=0.013/C_e+0.011$	0.9944	$lgq_e=0.44lgC_e+6.08$	0.9646
Covellite	$1/q_e=0.042/C_e+0.013$	0.9983	$lgq_e=0.65lgC_e+4.07$	0.9895
Chalcopyrite	$1/q_e=0.048/C_e+0.017$	0.9976	$lgq_e=0.59lgC_e+4.41$	0.9803

q_e, Equilibrium adsorption quantity, cells/cm^2; C_e, equilibrium concentration of bacterial cells, cells/mL; R, correlation coefficient.

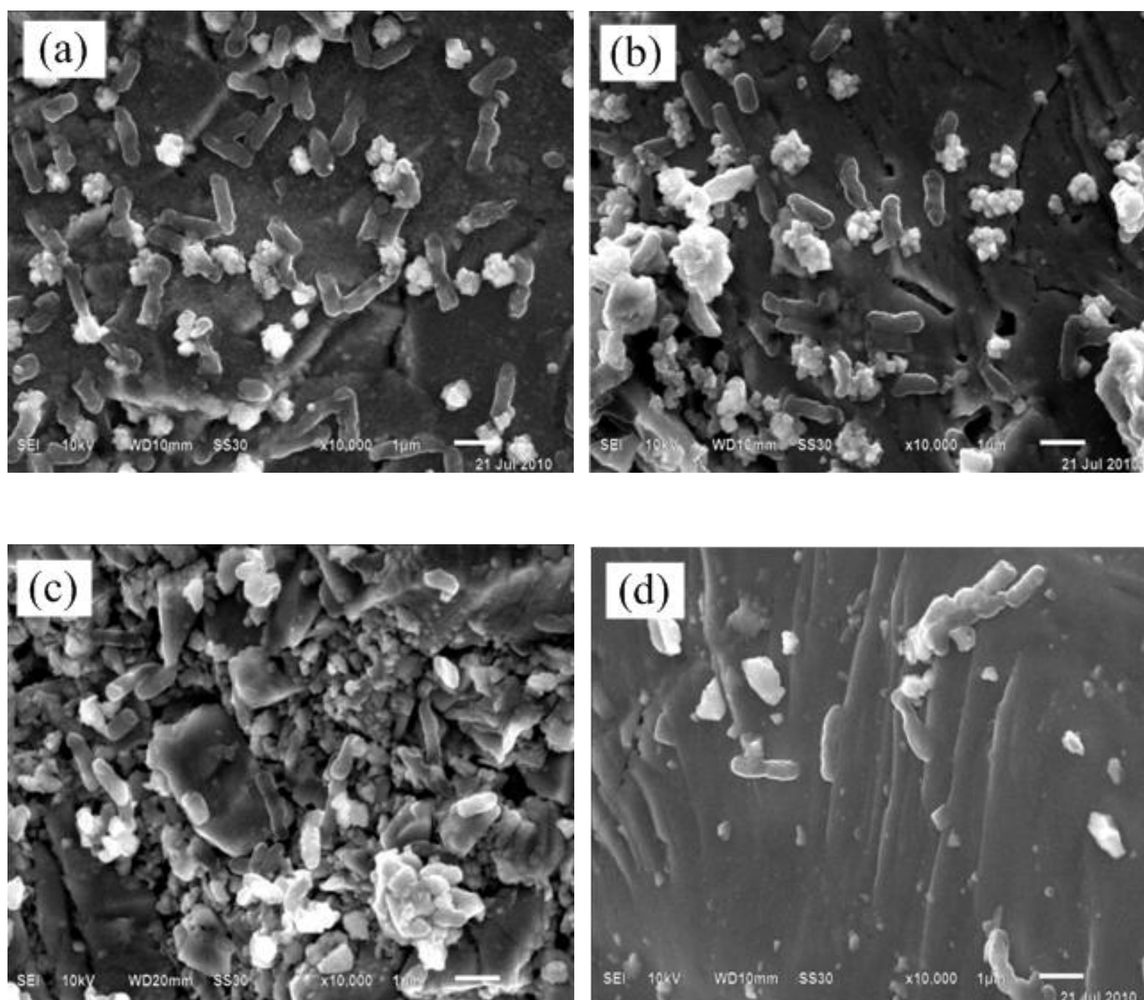


Figure 5. SEM image of adsorption of *A. ferrooxidans* on djurleite (a), bornite (b), covellite (c), and chalcopyrite (d).

Scanning electron microscopy (SEM) photomicrographs in Figure 5 shows patterns of adherence of *A. ferrooxidans* on djurleite, bornite, covellite, and chalcopyrite, respectively. Figure 5 shows an obvious difference in the adsorp-

tion density of bacteria on copper sulphides. This may be associated with the distribution and the atom number proportion of Cu, Fe, and S on the surface of copper sulphides (Sanhueza et al., 1999). The adsorbed bacteria on the surface of djurleite, bornite, and covellite were much more evenly distributed, and were mainly present as isolated bacteria. Attached bacteria on the surface of chalcopyrite were unevenly distributed, and present as short-string-like chains with two or three bacteria in few zones.

The values of surface density of attached bacteria were calculated from the population of bacteria observed in the SEM micrographs (Figure 5), and their order is: djurleite> bornite> covellite> chalcopyrite. The results suggest that the high density of *A. ferrooxidans* on the surface of a mineral and the evenly distributed the bacteria would be beneficial to each of the mineral surface. A direct enzymatic attack on the mineral surface could initiate the leaching reaction (Vilcáez and Inoue, 2008).

Conclusions

The bioleaching of copper sulphides showed that copper extraction from djurleite, bornite, covellite and chalcopyrite under the optimal conditions were 95.12, 84.5, 54.1, and 18.33%, respectively. The ranking for the bioleaching of copper sulfides is obtained: djurleite> bornite> covellite> chalcopyrite.

The adsorption of *A. ferrooxidans* on copper sulphides is monolayer adsorption. After the saturation of the surface by the attached cells, the values of adsorption density of *A. ferrooxidans* on djurleite, bornite, covellite, and chalcopyrite were about 10.74×10^6, 8.06×10^6, 3.0×10^6, and 2.35×10^6 cells/cm^2, respectively. The cell density of *A. ferrooxidans* on djurleite was the highest. The order of adsorption on copper sulphides is: djurleite> bornite> covellite> chalcopyrite. It seems that cells adsorbed by the minerals are beneficial to bioleaching ofcopper sulphides. The results suggest that the high density of *A. ferrooxidans* on the surface of a mineral and the evenly distributed the bacteria would be beneficial to each of the mineral surface. On the other hand, the higher the leaching rate of copper sulphides, the greater the density of bacteria absorbed on the surface of minerals.

ACKNOWLEDGEMENTS

The authors would like to thank Doctoral Foundation of Southwest University of Science and Technology (No. 12zx7113), College Student Innovation Fund Project of Southwest University of Science and Technology (No. CX13-022) and the National Natural Science Foundation of China (No. 51304162) for support of this research.

REFERENCES

Ahmadi A, Ranjbar M, Schaffie M, Petersend J (2012). Kinetic modeling of bioleaching of copper sulfide concentrates in conventional and electrochemically controlled systems. Hydrometallurgy 127-128:6-23.

Colmer AR, Hinkle ME (1947). The role of microorganisms in acid mine drainage: A preliminary report. 106(2751): 253-256.

Deveci H, Akcil A, Alp I (2004). Bioleaching of complex zinc sulphides using mesophilic and thermophilic bacteria: comparative importance of pH and iron. Hydrometallurgy 73(3-4): 293-303.

Dew DW, Van BC, Mcewan K, Bowker C (1999). Bioleaching of base metal sulphide concentrates: A comparison of mesophile and thermopile bacterial cultures//Amils, R., Ballester, A. Biohydrometallurgy and environment toward the mining of 21st century, parts A. Amsterdam, Netherlands, Elsevier, 229-238.

Fu KB, Lin H, Cheng H, Mo XL, Dong YB (2013). Bioleaching of djurleite using *Acidithiobacillus ferrooxidans*. Minerals Eng. 40: 38-41.

Fu KB, Lin H, Mo XL, Dong YB, Zhou L (2011). Study on bioleaching of different types of chalcopyrites. J. Univ. Sci. Technol. Beijing 33(7): 806-811.

Giles CH, MacEwan TH, Nakhwa SN (1960). Studies in adsorption. Part XI. A system of classification of solution adsorption isotherms, and its use in diagnosis of adsorption mechanisms and in measurements of specific surface areas of solids. J. Chem. Society 10: 3973-3993.

Jia CY, Wei DZ, Liu WG, Han C, Gao SL, Wang YJ (2008). Selective adsorption of bacteria on sulfide minerals surface, Trans. Nonferrous Met. Soc. China 18(5): 1247-1252.

Kinnunen PHM, Heimala S, Riekkola-Vanhanen ML, Puhakkaa JA (2006). Chalcopyrite concentrate leaching with biologically produced ferric sulphate. Bioresour. Technol. 97(44): 1727-1734.

Pich Otero A, Curutchet G, Donati E, Tedesco P (1995). Action of *Thiobacillus thiooxidans* on sulphur in the presence of a surfactant agent and its application in the indirect dissolution of phosphorus. Proc. Biochem. 30(8): 747-750.

Rodríguez Y, Ballester A, Blázquez ML, González F, Muñoz JA (2003). Study of bacterial attachment during the bioleaching of pyrite, chalcopyrite, and sphalerite. Geomicrobiol. J. 20(2): 131-141.

Rojas-Chapana JA, Tributsch H (2004). Interfacial activity and leaching patterns of *Leptospirillum ferrooxidans* on pyrite. FEMS Microbiol. Ecol. 47(1): 19-29.

Sanhueza A, Ferrer IJ, Vargas T, Amils R, Sánchez C (1999). Attachment of *Thiobacillus ferrooxidans* on synthetic pyrite of varying structural and electronic properties. Hydrometallurgy 51(1): 115-129.

Sandstrom A, Shchukarev A, Paul J (2005). XPS characterisation of chalcopyrite chemically and bioleached at high and low redox potential. Minerals Eng. 18(5): 505-515.

Sand W, Gehrke T, Hallmann R (1995). Sulfur chemistry, biofilm, and the (in) direct attack mechanism a critical evaluation of bacterial leaching. Appl. Microbiol. Biotechnol. 43(6): 961-966.

Sand W, Gehrke T, Jozsa PG, Schippers A (2001). (Bio) chemistry of bacterial leaching-direct vs. indirect. Hydrometallurgy 59(2-3):159-175.

Schippers A, Sand W (1999). Bacterial leaching of metal sulfide proceeds by two indirect mechanisms via thiosulfate or via polysulfides and sulfur. Appl. Environ. Microbiol. 65(1): 319-321.

Third KA, Cord-Ruwisch R, Watling HR (2002). Control of the redox potential by oxygen limitation improves bacterial leaching of chalcopyrite. Biotechnology and Bioengineering 78(4): 433-441.

Tributsch H (2001). Direct versus indirect bioleaching. Hydrometallurgy 59(1-2): 177-185.

Van HRP, Van ZAW, Spurr NRL, Harrison STL (2010). Investigating heap bioleaching: Effect of feed iron concentration on bioleaching performance. Minerals Eng. 23(6): 518-525.

Vilcáez J, Inoue C (2008). Bioleaching of chalcopyrite with thermophiles: Temperature–pH–ORP dependence. Int. J. Mineral Proc. 88(1-2): 37-44.

Vilcaez J, Inoue C (2009). Mathematical modeling of thermophilic bioleaching of chalcopyrite. Minerals Eng. 22(11): 951-960.

Watling HR (2006). The bioleaching of sulphide minerals with emphasis n copper sulphides — A review. Hydrometallurgy 84(1-2): 81-108.

Zhou HB, Zeng WM, Yang ZF, Xie YJ, Qiu GZ (2009). Bioleaching of chalcopyrite concentrate by a moderately thermophilic culture in a stirred tank reactor. Bioresour. Technol. 100(2): 515-520.

academicJournals

Vol. 13(5), pp. 673-677, 29 January, 2014
DOI: 10.5897/AJB2013.12187
ISSN 1684-5315 ©2014 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Characterization of moulds associated with processed garri stored for 40 days at ambient temperature in Makurdi, Nigeria

Aguoru, Celestine U.*, Onda, Mercy Atoye, Omoni, Victor Taghoghor and Ogbonna, Innocent Okonkwo

Department of Biological Sciences, Faculty of Science, University of Agriculture, P. M. B 2373, Makurdi, Nigeria.

Accepted 12 August, 2013

Characterization of moulds associated with processed white and yellow garri stored at ambient temperature for 40 days was investigated. The moulds isolated from white garri (%) were: *Aspergillus* spp 35.3, *Penicillium* spp 23.53, *Fusarium* spp 2.94, *Mucor* spp 17.65, *Alternaria* spp 5.88, *Cladosporium* sp 2.94 and *Rhizopus* 11.76%. For yellow garri: *Aspergillus* spp 37.04, *Penicillium* spp 23.53, *Fusarium* spp 7.41, *Mucor* spp 18.52, *Rhizopus* spp 14.81, *Alternaria* spp 0% *Cladosporium* sp. and *Aspergillus* spp had the highest frequency of occurrence in both white and yellow garri. Higher moulds species were isolated from white garri (34) compared to yellow (27) samples. The mean total fungal counts from the three hostels were 6.22×10^3, 7.22×10^4 and 9.67×10^5 $CFUg^{-1}$ in white garri, and 3.56×10^3, 4.22×10^4 and 5.78×10^5 $CFUg^{-1}$ in yellow garri. There were significant differences in total mean fungal counts in the various dilutions of white and yellow garri at $p< 0.05$. Results also revealed that the longer the storage time, the higher the pH and moisture content. Proper storage is recommended owing to the public health concern due to mycotoxins, food safety, shelf life and biostability of this product.

Key words: Garri, moulds, storage, pH, moisture content.

INTRODUCTION

Garri is made from peeled, washed, grated, fermented and toasted fresh cassava tuber (*Manihot esculenta* Crantz). It is the most popular fermented cassava products in Africa (Oluwole et al., 2004; Ernesto et al., 2000). It is consumed by several millions of people in West Africa where it forms a major part of their diet (Edem et al., 2001; Kostinek et al., 2005; Ogiehor et al., 2007). In Nigeria, its acceptability cuts across the various ethnic and socio-economic classes, making it the commonest food item (Jekayinfa and Olajide, 2007; Ogiehor et al., 2007). Garri is stored and marketed in a ready-to-eat form and prepared into a stiff paste or dough-like called 'Eba' by adding the granules into hot water and stirring to make a paste of varied consistency. Eba can be consumed with local soups or stews of various types by chewing or swallowing in morsels (Asegbeloyin and Onyimonyi, 2007; Ogiehor et al., 2007). Garri can also be deliciously consumed directly (without cooking) with groundnut, smoked fish, coconut, cowpeas, moimoi, or taken as a fast food when soaked in cold water (Ogugbue and Obi, 2011). Sometimes, it is taken with beverages mixed with cold water or warm water with salt depending on the choice of the individual. Microbial growth, deterioration and spoilage of garri are major cause of food borne illnesses and threat to public health. However, some unhygienic practices involved in production, processing of cassava to garri and post processing handling such as spreading on the floor and mats after frying, displaying in

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

open bowl or buckets in the markets during sales; the use of various packaging materials to transfer finished products from rural to urban areas and the use of bare hands during handling and sales may lead to microbial contamination due to deposition of bioaerosols on exposed products and transfer of infectious agent during handling (Ogiehor et al., 2007; Ogugbue and Obi, 2011; Ogugbue et al., 2011).

The main biological agents that contaminate and spoil garri are moulds, insects and mites (Igbeka, 1987; Ogiehor et al., 2005). Garri is rich in carbohydrate and therefore, suitable for fungal growth. Moulds such as *Aspergillus*, *Penicillium*, *Fusarium*, *Rhizopus*, *Cladosporium* and *Mucor* have been associated with garri during storage and distribution (Ekundayo, 1984; Ogugbue et al., 2011). Several reports have revealed high occurrence of microorganisms in market samples of garri (Ijabadeniyi, 2007; Amadi and Adebola, 2008; Ogiehor et al., 2007). The growth of moulds in garri results in changes in the organoleptic, microbiological and nutritive quality which lead to spoilage of the food product (Efiuvwevwere and Isaiah, 1998). Some moulds such as *Aspergillus flavus*, *Aspergillus parasiticus* and *Penicillium* spp. can also produce aflatoxins (SubbaRao, 2000; Frazier and Westhoff, 2000; Ogiehor et al., 2007), which can have serious effects depending on the dosage consumed. Garri is a major food consumed among students in Africa, especially in Nigeria; hence, the present study was based on the need to assess the microbiological safety of garri consumed by these students in the University environment with respect to their storage conditions and packages used in storage.

MATERIALS AND METHODS

Sample collection

Two hundred (200) samples of garri (100 yellow and 100 white) were collected from three hostels within the University environment and transferred into clean factory sterilized polythene bags, labelled appropriately and transported to Microbiology Laboratory of the University for microbiological Examination. The samples were immediately analysed within 5 h of collection to avoid contamination from external source.

Moisture content and pH determination

Each sample was determined by a modification of method described by AOAC (1990). Briefly, the moisture content was determined by weighing 5.0 g each of all the samples and drying in an oven maintained at 105°C for 7 to 8 h for a constant weight to be obtained. Thereafter, they were stored in desiccators to cool and then re-weighed. The difference in weight was used to obtain the moisture content. Moisture content determination was done at every 5- day interval within 40 days. The pH of each garri samples were determined using a reference electrode pH meter by homogenizing 10 g of each garri sample in 10 ml of sterile distilled water and the pH of the sample determined (Ogiehor and Ikenebomeh, 2005).

Microbiological examination of garri samples

Sample preparation

The spread plate count method was used by weighing 10 g of each processed sample aseptically into 90 ml of 0.1% (w/v) sterile peptone water in a sterile 500 ml beaker, and allowed to stand for 5 min with occasional stirring using a magnetic stirrer as described by the method of Ogiehor and Ikenebomeh (2005). Furthermore, serial dilution method described by Jonathan and Olowolafe (2001) was carried out using 1 ml of initial suspension in the beaker in 9 ml of sterile distilled water in a 1:10 dilution. Further, serial dilutions were carried out using sterile pipettes to obtain up to 10^{-5} dilutions.

Isolation and identification of moulds

A 0.1 ml aliquot of each dilution of the suspension prepared earlier was pipetted and spread-plated in sterile Petri dish containing potato dextrose agar (PDA) supplemented with 0.1% concentrated lactic acid to inhibit bacterial growth. Inoculated plates were thereafter incubated at 28°C for 5 to 7 days. Total viable fungal counts were carried out and expressed as colony forming units per gram ($CFUg^{-1}$) of sample after incubation. Identification of the isolated moulds based on their morphological and cultural characteristics was carried out using the descriptions of Alexopoulus et al. (1996).

Statistical analyses

The various data obtained from this study were subjected to statistical analyses: ANOVA and student's t test to compare significant differences of means ($p < 0.05$) in the white and yellow garri using statistical software, SPSS version 17.0.

RESULTS

Changes in moisture content of both white and yellow garri samples are presented in Figure 1. The results indicate that there was slight increase in the moisture contents of both white and yellow garri sample at every 5-day interval within 40 days. The ranges of the moisture contents for the white and yellow garri were 7.6 to 12.5 and 6.3 to 11.7%, respectively. In the samples, the higher the storage, the higher the pH. The pH changes of both white and yellow garri samples are presented in Table 1. A lower pH range of 2.9 to 3.6 was recorded for yellow garri compared to 3.5 to 4.3 of white garri. However, both samples recorded a slight increase in pH value as the storage progressed. Generally, higher moulds species were isolated from white garri samples compared to yellow garri. The mean of total fungal counts from the three hostels were 6.22×10^3, 7.22×10^4 and 9.67×10^5 $CFUg^{-1}$ in white garri, and 3.56×10^3, 4.22×10^4 and 5.78×10^5 $CFUg^{-1}$ in yellow garri (Table 2). There were significant differences in total mean fungal counts in the various dilutions of white and yellow garri at $p < 0.05$.

A total of seven mould species (*Aspergillus* spp., *Penicillium* spp., *Fusarium* spp., *Mucor* spp., *Alternaria* sp., *Cladosporium* sp and *Rhizopus* sp) were isolated from both white and yellow garri samples (Table 2). The prevalence of moulds in white garri were *Aspergillus* spp (35.3%), *Penicillium* spp (23.53%), *Fusarium* spp (2.94%), *Mucor* spp (17.65%), *Alternaria* sp (5.88%),

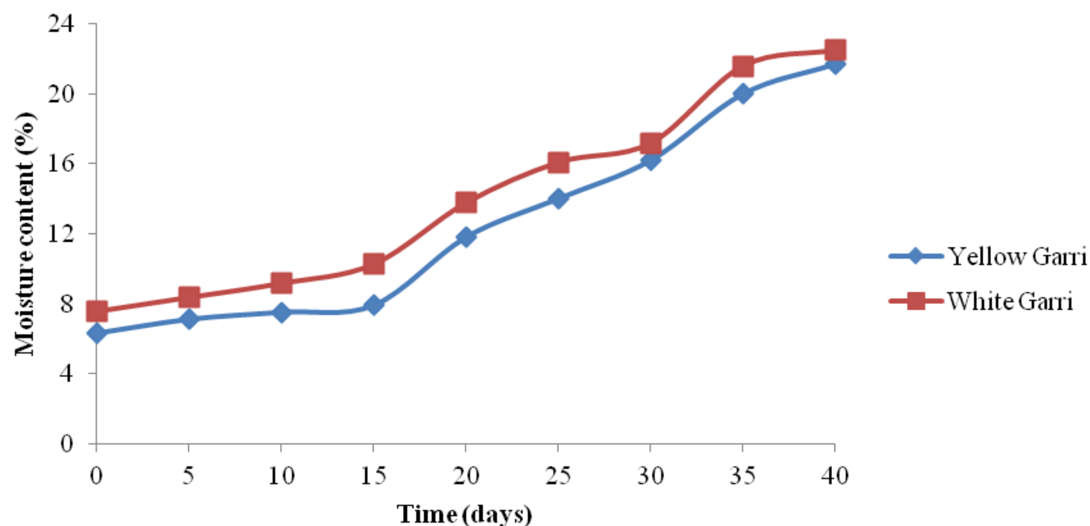


Figure 1. Changes in moisture content of the two types of garri stored at ambient temperature.

Table 1. Changes in pH of white and yellow garri stored at ambient temperature.

Time (day)	White garri	Yellow garri
0	4.3	3.6
5	4.3	3.6
10	4.3	3.3
15	4.3	3.1
20	3.9	3.1
25	3.9	3.1
30	3.5	3.1
35	3.5	2.9
40	3.5	2.9

Table 2. Changes in total viable fungal counts of white and yellow garri obtained from the university hostels during storage at ambient temperature.

Time (day)	Total fungal counts (TFC)					
	Hostel A (CFUg^{-1} × 10^{3})		Hostel B (CFUg^{-1} × 10^{4})		Hostel C (CFUg^{-1} × 10^{5})	
	White garri	Yellow garri	White garri	Yellow garri	White garri	Yellow garri
0	1	1	3	ND	7	3
5	3	1	7	1	9	3
10	6	2	3	4	8	4
15	5	4	8	2	11	5
20	7	4	9	9	9	9
25	7	4	6	2	11	6
30	5	6	7	6	9	4
35	11	4	13	6	10	9
40	11	6	9	8	13	9
Average TFC	6.22	3.56	7.22	4.22	9.67	5.78

ND, Not detected.

Cladosporium sp (2.94%) and *Rhizopus* (11.76%) while in yellow garri, the percentage occurrence were *Aspergillus* spp (37.04%), *Penicillium* spp (23.53%), *Fusarium* spp (7.41%), *Mucor* spp (18.52%) and *Rhizopus* (14.81%). How-

Table 3. Frequency of occurrence of mould species obtained from garri samples during the period of study.

Mould	Percentage (%) occurrence	
	Yellow garri	White garri
Aspergillus spp	10 (37.04)	12 (35.3)
Penicillium spp	6 (22.22)	8 (23.53)
Fusarium spp	2 (7.41)	1 (2.94)
Mucor spp	5 (18.52)	6 (17.65)
Alternaria sp	-	2 (5.88)
Cladosporium sp	-	1 (2.94)
Rhizopus sp	4 (14.81)	4 (11.76)
Total	27 (100)	34 (100)

However, *Alternaria* sp and *Cladosporium* sp were absent in yellow garri but present in white garri.

Aspergillus spp had the highest frequency of occurrence in both white (35.3%) and yellow (37.04%) garri from the overall isolates from the samples collected.

DISCUSSION

The percentage moisture contents of yellow garri (12.50%) and white garri (11.70%) supported the values of 12.7 to 13.6% previously reported (Halliday et al., 1967; Adeniyi, 1976). Ogugbue and Obi (2011) also reported higher moisture content in garri stored in different packages above the safe limit. In this study, it was observed that white garri had higher moisture content than yellow garri from day 0 to 40th day. The results of this study also reveal that the moisture content increased as the number of days increased and could eventually cause deterioration of the stored products. Halliday et al. (1967) reported that the major important factor that could encourage mould contamination and proliferation of garri is the high initial moisture content or increase in moisture content during storage.

The pH range of white garri (3.5 to 4.3) had higher values than yellow garri (2.9 to 3.6) at the end of the study. The pH of the yellow garri were within the pH range of 3.4 to 4.0 reported by Achinewhu et al. (1998) and white garri within the range of 4.19 to 4.58 reported by Owuamanam et al. (2011). The slight increase in pH obtained in both white and yellow garri may be due to production of acidic metabolites by microorganisms during their growth and proliferation (Ogugbue et al., 2011). The mould isolated from both yellow and white garri sample are *Aspergillus* spp., *Penicillium* spp., *Fusarium* spp., *Mucor* spp., *Alternaria* sp., *Cladosporium* sp and *Rhizopus* sp.

Obadina et al. (2009) also reported the isolation of similar moulds from their study on garri during assessment of some fermented cassava products. *Aspergillus* spp were the most predominant isolates in the present study. Several authors have isolated and identified number of mould species in garri under various storage conditions (Obadina et al., 2009; Ogugbue et al., 2011; Jonathan et al., 2011; Thomas et al., 2012). The mould contamination could have been introduced during local method of processing, exposure to bioaerosols during sale in the market and post processing handling of such product (Aboaba and Amisike, 1991; Okigbo, 2003; Ogeihor and Ikenebomeh, 2005; Amadi and Adebola, 2008; Ogubue et al., 2011). Samson et al. (2000) reported that almost all the common *Aspergillus* spp have been recovered at some time from agricultural products. The result of this study revealed that the longer the storage time, the higher the bio-load, pH and moisture content. Therefore, proper storage methods such as dry environment (with very low relative humidity and moisture) is required to prevent the growth and survival of moulds associated with garri and the public health concerns (such as food intoxication and illnesses) that may result from mycotoxins harbours by some of these fungi when they are consumed by the students.

REFERENCES

Aboaba OO, Amisike J (1991). Storage of melon seeds. Niger. J. Bot. 4: 213-219

Achinewhu SC, Barber LI, Ijeoma IO (1998). Physicochemical properties and *garri*fication (*garri* yield) of selected cassava cultivars in Rivers State, Nigeria. Plant Foods Hum. Nutr. 52(2): 133-140.

Adeniyi MO (1976). Fungi Associated with the Deterioration of Garri. Niger. J. Plant Prot. 2: 74-77.

Alexopoulus CJ, Mims CW, Blackwell M (1996). Introductory Mycology, (4th Edition), John Wiley, New York. pp. 1-50.

Amadi JE, Adebola MO (2008). Effect of Moisture Content and Storage Conditions on the Stability of Garri. Afr. J. Pure Appl. Sci. 7(24): 4591-4594.

AOAC (1990). Official Methods of Analysis, 15th ed. Association of Official Analytical Chemists, Arlington, VA.

Asegbeloyin JN, Onyimonyi AE (2007). The effect of different processing methods on the residual cyanide of 'Gari'. Pak. J. Nutr. 6(2): 163-166

Edem DO, Ayatse JOI, Itam EH (2001). Effect of soy protein supplementation on the nutritive value of 'gari' (farina) from *Manihot esculenta*. Food Chem. 75: 57-62

Efiuvwevwere BJO, Isaiah AU (1998). Effects of hygienic handling in combination with potassium sorbate treatment and smoking on the microbial quality and shelf-stability of croaker (*Micropogonias furnieri*). Z lebensm unters forsch. 207: 13-17.

Ekundayo CA (1984). Microbial spoilage of packaged garri in storage. Microbiol. Lett. 23: 271-278.
Ernesto M, Cardoso AP, Cliff J, Bradbury JH (2000). Cyanogens in cassava flour and roots and urinary thiocyanate concentration in Mozambique. J. Food Compos. Anal. 13: 1-12.
Frazier WC, Westhoff DC (2000). Food Microbiology. McGraw-Hill Inc., New York. pp. 17-48, 441-443
Halliday DJ, Quareshi AH, Broadbent JA (1967). Investigations on the storage of garri. Rep. Niger. Stored Prod. Res. Inst. Tech. Rep. 16: 131-141.
Igbeka JC (1987). Stimulation of moisture profile in stored garri. J. Food Agric. 1: 5-9.
Ijabadeniyi AO (2007). Microbiological safety of garri, lafun and ogiri in Akure metropolis, Nigeria. Afr. J. Biotechnol. 6(22): 2633-2635.
Jekayinfa SO, Olajide JO (2007). Analysis of energy usage in the production of three selected cassava-based foods in Nigeria. J. Food Eng. 82: 217-226.
Jonathan G, Ajayi I, Omitade Y (2011). Nutritional compositions, fungi and aflatoxins detection in stored ‘gbodo’ (fermented *Dioscorea rotundata*) and ‘elubo ogede’ (fermented *Musa parasidiaca*) from South western Nigeria. Afr. J. Food Sci. 5(2): 105-110
Jonathan SG, Olowolafe TB (2001). Studies on nutrient contents and microorganisms associated with dodo Ikire a plantain snack from Western Nigeria. NISEB J. 1(1): 27 – 30
Kostinek M, Specht I, Edward VA, Schillinger U, Hertel C, Holzapfel, WH, Franz CMAP (2005). Diversity and technological properties of predominant lactic acid bacteria from fermented cassava used for the preparation of Gari, a traditional African food. Syst. Appl. Microbiol. 28(6): 527-540.
Obadina AO, Oyewole OB, Odusami AO (2009). Microbiological safety and quality assessment of some fermented cassava products (*lafun*, *fufu*, *gari*). Sci. Res. Essay 4(5): 432-435
Ogiehor IS, Ikenebomeh MJ (2005). Extension of Shelf Life of Garri by Hygienic Handling and Sodium Benzoate Treatment. Afr. J. Biotechnol. 4(7): 744-748.
Ogiehor IS, Ikenebomeh MJ, Ekundayo AO (2007). The bioload and aflatoxin content of market Garri from some selected states in southern Nigeria. Afr. Health Sci. 7(4): 223-227.
Ogiehor, IS, Ekundayo, AO, Okwu GI (2005). Shelf stability of agidi produced from maize (*Zea mays*) and the effects of sodium benzoate treatment in combination with low temperature storage. Afr. J. Biotechnol. 4 (7): 738-743
Ogugbue CJ, Mbakwem-Aniebo C, Akubuenyi F (2011). Assessment of microbial air contamination of post processed garri on sale in markets. Afr. J. Food Sci. 5(8):503-512
Ogugbue CJ, Obi G (2011). Bioburden of Garri Stored in Different Packaging Materials under Tropical Market Conditions. Middle-East J. Sci. Res. 7(5): 741-745
Okigbo RN (2003). Fungi Associated with Peels of Post Harvest Yams in Storage. Glob. J. Pure Appl. Sci. 9(1): 19-23.
Oluwole OB, Olatunji OO, Odunfa SF (2004). A process technology for conversion of dried cassava chips into gari. Niger. Food J. 22: 65-73.
Owuamanam CI, Ogueke CC, Achinewhu SC, Barimala IS (2011). Quality characteristics of *garri* as affected by preferment liquor, temperature and duration of fermentation. Am. J. Food Tech. 6(5): 374-384
Samson RA, Hoekstral ES, Frisvad JC, Filtenborg O (2000). Fungi and foods. The Netherland. pp. 1-32.
SubbaRao NS (2000). Soil Microbiology (4th Edn). Oxford and IBH publishing Co. PUT Ltd New Dehli. pp. 283-286.
Thomas BT, Effedua HI, Agu G, Musa OS, Adeyemi MT, Odunsi OD, Adesoga KO, Ogundero O, Oluwadun A (2012). Fungi Associated with the Deterioration of Garri (a traditional fermented cassava product) in Ogun State, Nigeria. Researcher 4(2):1-5.

academicJournals

Vol. 13(5), pp. 678-682, 29 January, 2014
DOI: 10.5897/AJB2013.12803
ISSN 1684-5315 ©2014 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Acute toxicity, lipid peroxidation and ameliorative properties of *Alstonia boonei* ethanol leaf extract on the kidney markers of alloxan induced diabetic rats

Enechi, O.C.*, Oluka, Ikenna H. and Ugwu, Okechukwu P.C.

Department of Biochemistry, University of Nigeria, Nsukka, Enugu State, Nigeria.

Accepted 20 January, 2014

The lethal toxicity and lipid peroxidation studies of *Alstonia boonei* on alloxan induced diabetic rats were analysed. The effect of ethanol leaf extract of *A. boonei* on the kidney markers of diabetic rat was also determined. The acute toxicity of the ethanol extract of *A. boonei* was found to be more than 5000 mg/kg body weight of the extract after 24 h of constant observation. The malondialdehyde concentration of the rats treated with 200 and 400 mg/kg body weights of the extract significantly decreased (p<0.05) when compared with the untreated diabetic rats. There was no significant difference (p>0.05) when malondialdehyde concentrations of rat treated with 200 and 400 mg/kg body weights of the extracts were compared with the standard control. But the creatinine concentration decreased significantly (p<0.05) when 200 and 400 mg/kg body weights of the extract of the treated diabetic animals were compared with the diabetic control.

Key words: *Alstonia boonei,* diabetic rats, malondialdehyde, creatinine and urea.

INTRODUCTION

Diabetes mellitus represents a group of metabolic disorders in which there is an impaired glucose utilization inducing hyperglycemia which is an increase in the blood group level beyond normal values (Hazuda, 1991; Adonu et al., 2013). Diabetes is a chronic disease characterized by elevated blood glucose level and disturbances in carbohydrate, fat and protein metabolism (Sky, 2000; Rother, 2007; Andrew et al., 2013). In the local markets in West and Central Africa, *Alstonia boonei* is often among the most common sold plant as crude drugs. Parts of the plant are employed for the treatment of a variety of ailments in Africa and the stem bark has been listed in the Africa Pharmacopoeia as an antimalarial drug (Bello et al ., 2009).

A. boonei is a popular plant (Figure 1). It has many medicinal properties such as antifungal, antibacterial, anti-inflammatory, antiplasmodial, antimalarial, antipyretic, analgesic and hypotensive effects (Olajide et al., 2000). In this study, acute toxicity of *A. boonei* ethanol leaf extract and the effects of *A. boonei* ethanol leaf extract on the kidney markers and lipid peroxidation of diabetic rats were evaluated.

MATERIALS AND METHODS

Materials

Plant materials

Matured leaves of *A. boonei* were collected in Nsukka environs, Enugu State of Nigeria. The plants were identified and authentic-cated by Mr. A. Ozioko of Bioresource Development and Conser-vation Programme (BDCP) Research Centre, Nsukka, where the voucher specimens were deposited in the *herbarium* of the Depart-ment of Botany, University of Nigeria, Nsukka.

*Corresponding author. E-mail: oky9992000@yahoo.com. Tel: +2348050356279 and +23430799187.

Figure 1. Pictorial view of *Alstonia boonei* leaves (Sidiyasa, 1998).

Animals

Adult Wistar albino rats of between 10 and 16 weeks with average weight of 160 ± 13 g were obtained from the Animal House of the Faculty of Biological Sciences, University of Nigeria, Nsukka. The animals were acclimatised for 7 days under standard environmental conditions, with a 12 h light/dark cycle maintained on a regular feed (vital feed) and water *ad libitum*.

Chemicals/reagents/samples

All chemicals used in this study were of analytical grade and products of May and Baker, England; BDH, England and Merck, Darmstadt, Germany. Reagents used for all the assays were commercial kits and products of Randox, USA; Teco (TC), USA; Biosystem Reagents and Instruments, Spain; Glucose Analyzer, Accu Chek Active of Germany; alloxanmonohydrate, Sigma St. Louis, MO, USA.

Instruments/equipment

Water bath (Gallenkamp, England), Chemical Balance (Gallenkamp, England), Conical Flasks (Pyrex, England), Hotbox (Gallenkamp, England), Centrifuge (3,500 rpm, PIC, England), Digital Photo Calorimeter (El 312 Model, Japan), Adjustable Micropipette (Perfect, U.S.A.), Refrigerator (Kelvinator, Germany), pH Meter (Pye, Unicam 293, England), multi-well microtiter plate reader (Tecan, Austria), Glucose Analyzer, Accu Chek Active of Germany.

Methods

Experimental protocol/design

A total of twenty eight (28) albino Wistar rats were divided into seven (7) groups of four (4) rats each. The animals were grouped as follows: Group 1: (Reference/normal control): Rats were fed with normal diet and water *ad libitum*; Group 2: diabetic untreated rats (diabetic control); Group 3: rats were administered with 2 mg/kg b.w. of glibenclamide (standard drug/control); Group 4, diabetic rats were administered with low dose (200 mg/kg b. w.) of the ethanol leaf extract of *A. boonei*; Group 5: diabetic rats were administered with high dose (400 mg/kg b. w.) of the ethanol leaf extract of *A. boonei*; Group 6: normal rats were administered with low dose (200 mg/kg b. w.) of the ethanol leaf extract of *A. boonei*; Group 7: normal rats were administered with high dose (400 mg/kg b. w.) of the ethanol leaf extract of *A. boonei*.

Collection and preparation of extracts

Matured leaves of *A. boonei* were obtained from the environs of Nsukka Local Government Area, of Enugu State of Nigeria. Specimens of these leaves were authenticated in the Department of Bioresource Development and Conservation Programme (BDCP) Research Centre, Nsukka, and voucher specimens deposited in the *herbarium.* Leaves of *A. boonei* were separately dried under shade for 7 days, and then pulverized into powder. A weight of 208.1 g of the sample (powder) was agitated in 70% ethanol organic solvent for 48 h. The macerated extract was filtered with a cheese cloth and the filtrates concentrated *in vacuo* to 10% of the original volume at 40°C. Concentrates were dried at 37°C to obtain percentage extract yield (35%) of the crude extract from the leaves of *A. boonei*. The extracts were reconstituted prior to administration.

Induction of experimental diabetes

Diabetes was induced by intraperitoneal injection of 120 mg/kg body weight of alloxanmonohydrate (Sigma St. Louis, MO, USA) in overnight-fasted animals after acclimatization. Diabetes was confirmed five (5) to eight (8) days later in the alloxan-treated animals showing random blood glucose (RBG) level ≥200mg/dl (11.1 mmol/L). Diabetes status determination was monitored on blood obtained from tail vein puncture using an automated glucose sensor machine GlucometerAnalyser (Accu Chek Active).

Determination of extract yield

The percentage yield of the ethanol extract of leaves of *A. boonei* was determined by weighing the pulverized leaf powder before extraction and the concentrated extract was obtained after extraction and then calculated using the formula:

$$\text{Percentage yield} = \frac{\text{Weight of leaf powder}}{\text{Weight of extract}} \times 100$$

Toxicological studies

Acute toxicity and lethality (LD_{50}) test

Acute toxicity (LD_{50}) of the ethanol extract of *A. boonei* leaves were determined by Lorke's method (1983). A total of eighteen (18) adult albino mice were used for this investigation. The studies were conducted in two stages. In the first stage, three groups of three mice each were administered (p.o.) 10, 100, 1000 mg/kg body weight of the extract, respectively. The mice were observed for 24 h for a number of death and behavioural changes. In the second stage, based on the percentage survival rates, further increased doses of 1600, 2900 and 5000 mg/kg body weight were administered (p.o.) to three additional mice for each group, respectively, and the fourth mice received only solvent (5% Tween80) which served as the control. The mice were observed for 24 h and the number of deaths was recorded. The LD_{50} was calculated as the geo

Table 1. The percentage yield of ethanol extract of *Alstonia boonei* leaves.

Initial weight of ground leaf powder (g)	Final weight of extract (g)	Percentage (%)
208.1	26.31	12.64

Table 2. Results of the acute toxicity (LD_{50}) of the ethanol extract of *Alstonia boonei* leaves.

Dose (mg/kg body weight)	No. of animals before oral administration	No. of deaths after administration
10	3	-
100	3	-
1000	3	-
1900	3	-
2600	3	-
5000	3	-

geometric mean of the highest non-lethal and the lowest lethal doses.

Determination of serum urea concentration

The concentration of serum urea was determined using the method of Tietz (1994) as outlined in Randox kits, UK.

Determination of serum creatinine concentration

The concentration of serum creatinine was determined using the method of Tietz (1994) as outlined in Randox kits, UK.

Determination of lipid peroxidation (malondialdehyde, MDA)

The concentration of malondialdehyde was determined using the method of Draper and Hadley (1990).

Statistical analysis

Data were reported as means ± SEM, where appropriate. Both one- and two-way analyses of variance (ANOVA) were used to analyse the experimental data and Duncan multiple test range was used to compare the group means obtained after each treatment with control measurements. Differences were considered significant when $p \leq 0.05$.

RESULTS

Percentage yield of the extract

From the result in Table 1, the (%) yield of the ethanol extract of *A. boonei* leaves was found to be 12.64%.

Acute toxicity studies of *Alstonia boonei* leaves

The acute toxicity studies (LD_{50}) of the ethanol extract of *A. boonei* leaves showed that no animal died in any group after receiving increasing doses (between 10 and 5000 mg/kg b.w.) of the ethanol extract of *A. boonei* leaves (Table 2).

Effects of the ethanol extract of *Alstonia boonei* leaves on urea concentration in normal and diabetic rats

Results in Figure 2 showed neither significant increase nor decrease ($p>0.05$) in the urea concentration of the diabetic animals administered with 200 mg/kg b.w. of the extract compared with the normal control. Significantly elevated ($p<0.05$) concentration of urea was observed in the 400 mg/kg b.w of extract treated diabetic rats compared with the normal control. The 200 and 400 mg/kg b.w. of extract treated diabetic animals showed no significant increase or decrease ($p>0.05$) compared to the diabetic control (group 2) as shown in Figure 2. The diabetic animals treated with low and high doses of the extract (200 and 400 mg/kg b.w.) showed significant decrease ($p<0.05$) and increase ($p<0.05$), respectively, compared with the diabetic animals treated with the standard drug. The normal animals administered low and high doses (200 and 400 mg/kg b.w.) of the extract showed no significant difference compared with the normal and diabetic controls (Groups 1 and 2) as shown in Figure 2. Interestingly, the 200 mg/kg b.w. treated normal animals showed a significant depreciation ($P<0.05$) in urea concentration compared with the glibenclamide-treated diabetic animals (standard control).

Effects of ethanol extract of *Alstonia Boonei* leaves on creatinine concentration in normal and diabetic animals

Creatinine concentration decreased significantly ($P<0.05$) in the animals treated with low dose (200 mg/kg b.w.) of the extract compared with the standard control as shown in Figure 3. There was no significant increase or decrease ($P>0.05$) in the creatinine concentration of both the 200 and 400 mg/kg b.w. treated diabetic animals as compared with the diabetic control. There was, however, a significant increase ($p<0.05$) in the creatinine concentra-

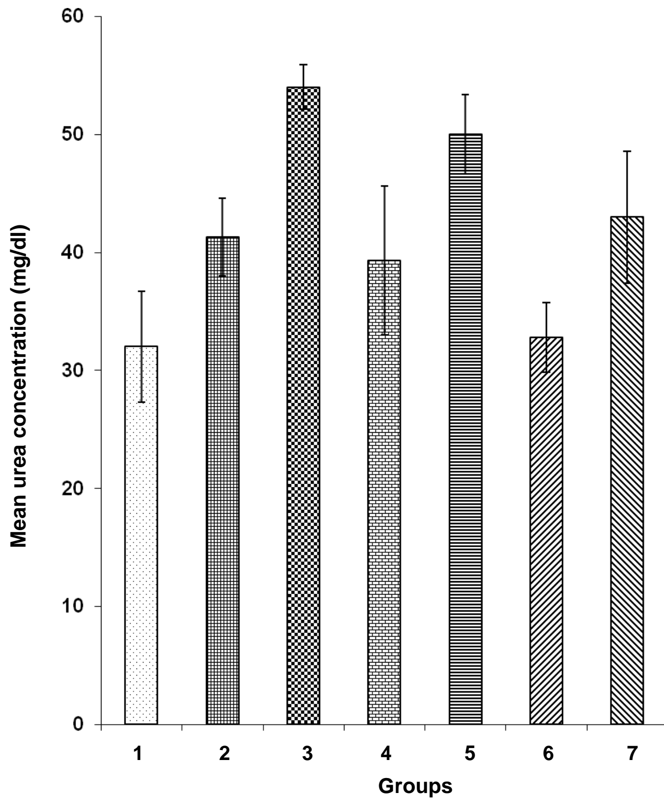


Figure 2. Effect of ethanol leaf extract of *Alstonia boonei* on the urea concentration of normal and diabetic rats. Group 1: Normal control; Group 2: diabetic control (Untreated); Group 3: diabetic + glibenclamide; Group 4: diabetic + 200 mg/kg of extract; Group 5: diabetic + 400 mg/kg of extract; Group 6: 200 mg/kg of extract; Group 7: 400 mg/kg of extract.

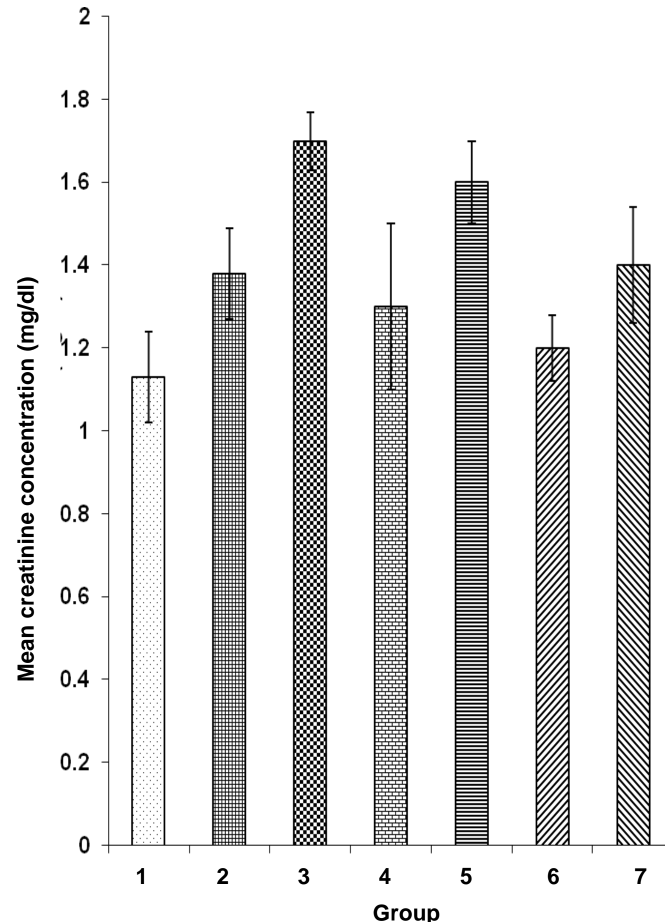


Figure 3. Effect of ethanol leaf extract of *Alstonia boonei* on the creatinine concentration of normal and diabetic rats. Group 1: Normal control; Group 2: diabetic control (untreated); Group 3: diabetic + glibenclamide; Group 4: diabetic + 200 mg/kg of extract; Group 5: diabetic + 400 mg/kg of extract; Group 6: 200 mg/kg of extract; Group 7: 400 mg/kg of extract.

tion of the diabetic animals administered high dose (400 mg/kg b.w.) of the extract compared with the normal control as shown in Figure 3.

Effects of the ethanol extract of *Alstonia Boonei* leaves on malondialdehyde (MDA) concentration in normal and diabetic rats

Observation carried out in Figure 4 reveals significant decrease ($p<0.05$) in the MDA concentration of the diabetic rats treated with low and high (200 and 400 mg/kg b.w.) doses of the extract compared with the diabetic and control (Group 2). There was however no significant difference ($p>0.05$) in the MDA concentration of these diabetic test animals (Groups 4 and 5) compared with the normal and standard controls. There was a significantly elevated ($p<0.05$) concentration of MDA in the untreated diabetic rats (Group 2) compared with the rats administered with the standard drug. It is also pertinent to mention that the MDA concentration of the normal rats administered 400 mg/kg b.w. of the extract increased significantly ($p<0.05$) compared with the normal and standard controls. The normal rats administered 400 mg/kg b.w. of the extract also showed a significantly elevated ($p<0.05$) MDA concentration compared with the diabetic rats treated with low and high doses of the extract, and the normal rats administered low dose (200mg/kg) of the extract as shown in Figure 4.

DISCUSSION

Ayurvedic system of medicine relies on the administration of crude extract or concentration of crude extract of the plant material for the treatment of diabetes mellitus (Salahuddin and Jalalpure, 2010). Literature survey indicates that there is no scientific evidence to support the antidiabetic effect of *A. boonei*. Therefore, the present study is undertaken to investigate acute toxicity of *A. boonei* ethanol leaf extract and the effects of *A. boonei* ethanol leaf extract on the kidney markers and lipid peroxidation of diabetic rats. In severe diabetic conditions, the kidneys lose their ability to remove wastes

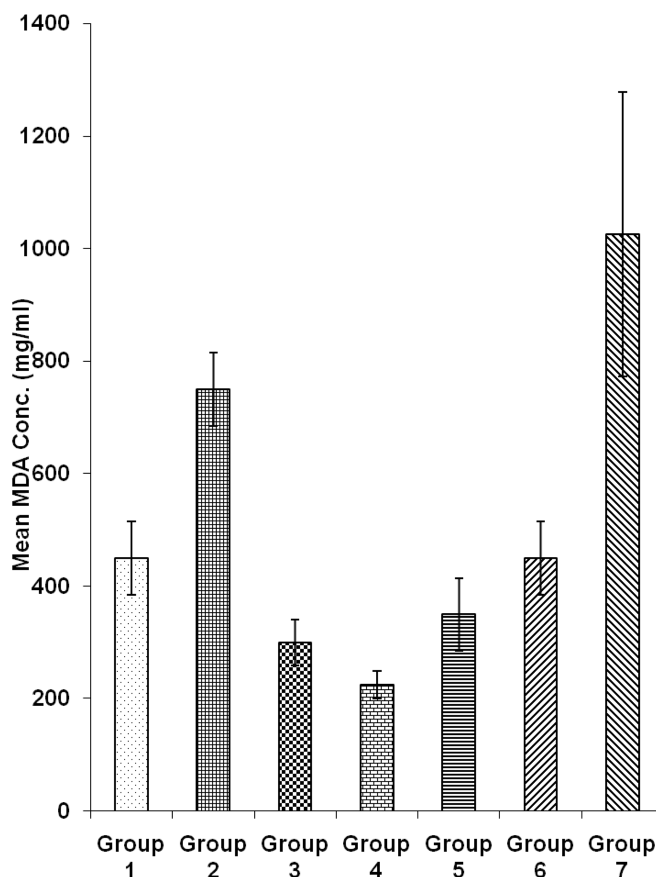


Figure 4. Effect of Ethanol leaf extract of *Alstonia boonei* on the malondialdehyde concentration on normal and diabetic rats.

products, such as creatinine and urea, from the blood.

From the result of the acute toxicity test of the ethanol extract of *A. boonei* leaves in mice, none of the mice died in any of the groups administered 10, 100, 1000, 1900, 2600 and 5000 mg/kg b.w. of the extract after 24 h. Consequently, it could be suggested that the ethanol extract of *A. boonei* leaves could be generally regarded as safe (GRAS) (Lorke, 1984). However, further studies are needed to ascertain the effects of prolonged feeding of high doses of the extract on the organs and tissues of experimental animals.

It was also observed that the extract had no reparatory effect on urea and creatinine excretion by the kidney in the diabetic animals since there was no significant difference (p>0.05) in the urea concentration of the treated diabetic animals compared with the diabetic control. Interestingly, the MDA concentration of the 200 and 400 mg/kg b.w. treated diabetic rats showed a significant decrease (p<0.05) compared with the untreated diabetic rats. There was no observed significant difference (p>0.05) in MDA concentration of the treated diabetic rats compared with the standard control. This shows that the extract was able to prevent lipid peroxidation by reducing the action of the alloxan-induced free radicals. Consequently, the ethanol concentration of *A. boonei* leaves has a potential antioxidant activity which can be a possible mechanism of its antidiabetic action. The flavonoids (antioxidants and free radical scavengers) in this plant may contribute to the effect of the plant extract in reducing MDA concentration and concomitant lipid peroxidetion (Afolabi et al., 2007). The capacity of flavonoids to act as antioxidants depends on their molecular structure. The position of hydroxyl groups and other features in the chemical structure of flavonoids are important for their antioxidant and free radical scavenging activities. Quercetin, the most abundant dietary flavonol, is a potent antioxidant because it has all the right structural features for free radical scavenging activity (Frei, 2006).

Conclusion

In conclusion, the ethanol leaf extract of *A. boonei* has been shown to prevent lipid peroxidation and at the same time had no reparatory effects on the kidney markers. This prevention of lipid peroxidation could be the reason ethanol extracts of *A. boonei* leaf have been used in numerous ethnomedicinal practices in combating ailments.

REFERENCES

Adonu CC, Ugwu OPC, Bawa A, Ossai EC, Nwaka AC (2013). Intrinsic Blood Coagulation Studies in Patients Suffering From Both Diabetes and Hypertension. Int. J. Pharma Med. Biol. Sci. 2 (2): 36-47.

Afolabi C, Akinmoladun EO, Ibuku Emmanuel Afor, Akinrinlola BL, Onibon TR, Akinboboye AO, Obuotor EM, Farombi EO (2007). Chemical constituents and antioxidant activity of *Alstonia boonei*. Afr. J. Biotechnol. 6 (10):1197-1201

Andrew NC, Peter UN, Onyechi O, Ugwu OPC, Ossai EC, Chigor VN (2013). Effect of Soyabean Diet on the Serum Immunoglobulin Profile and Cd4+ Count of Male Diabetic Rats Challenged With K.Pneumoniae. Int. J. Pharm. Biol. Sci. 3 (1): 563-573.

Bello IS, Oduola T, Adeosun OG, Omisore NOA, Raheem GO, Ademosun AA (2009). Evaluation of antimalarial activity of various fractions of *Morinda lucinda* leaf extract and *Alstonia boonei* stem bark. Glob. J. Pharmocol. 3(3): 163-165.

Draper HH, Hadley M (1990). A review of recent studies on the metabolism of exogenous and endogenous malondialdehyde. Xenobiotika 20(9): 901-907.

Frei B (2006). Consumption of flavonoid-rich food and increased plasma antioxidant capacity in humans. Free Radic. Biol. Med. 35(6): 1237-1238.

Hazuda HP, Knap JA, Stern MP (1999). Genetic and Environmental Determinants of Type II Diabetes in Mexian Americans. Diabetic Care 14(7):649-54.

Lorke D (1984). A new approach to practical acute toxicity testing. Arch. Toxicol. 53: 275-287.

Olajide OA, Awe SO, Makinde JM, Ekhelar AI, Olusola A, Morebise O, Okpako DT (2000). Studies on the anti-inflammatory, antipyretic and analgesic properties of *Alstonia boonei* stem bark. J. Ethnopharmacol. 71(1–2): 179–186.

Rother KI (2007). Diabetes Treatment Bridging the Divide. N. Engl. J. Med. 356 (150): 1499-1501.

Sidiyasa K (1998). Taxonomy, phylogeny, and wood anatomy of *Alstonia* (Apocynaceae). International Association of Plant Taxonomy 24(2): 16-23.

Sky GM (2000). Kinetics of insulin secretion underlying notabolism events in diabetes mellitus. Diabetes mellitus: A fundamental and clinical text. Lippicott willians and Wilkins, Plucadelphia. pp. 2-11.

Tietz NW (1994). Textbook of Clinical Chemistry. 2nd Edn. Burtis CA, Ashwood ER, W.B. Saunders Company, Philadelphia. p. 751.

academicJournals

Vol. 13(5), pp. 683-692, 29 January, 2014
DOI: 10.5897/AJB2013.13205
ISSN 1684-5315 ©2014 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Antioxidant properties of volatile oils obtained from *Artemisia taurica* Willd. and *Salvia kronenburgii* Rech. Fil. plants and their effects on xanthine oxidase

Mahire BAYRAMOĞLU[1]* and Ferda CANDAN[2]

[1]Department of Chemistry, Division of Biochemistry, Yuzuncu Yil University, Van, Turkey.
[2]Department of Chemistry, Division of Biochemistry, Cumhuriyet University, Sivas, Turkey

Accepted 20 January, 2014

In this study, the antioxidant properties of volatile oils obtained from the earth parts of the *Artemisia taurica* Willd. and *Salvia kronenburgii* Rech. Fil. plants and their effects on xanthine oxidase enzyme were studied. The chemical contents of each volatile oil were determined by applying gas chromatograpghy-mass spectrometry (GC-MS) analysis. The total phenol and flavonoid amounts of each volatile oil and the total antioxidant capacities were determined and it was observed that there was a positive correlation among these values. It was found out that the volatile oils were effective on inhibiting the reactive oxygen kinds and that they scavenged the superoxide radical made up with xanthine-xanthine oxidase system and the hydroxyl radical made up with Fe^{+3} ascorbate EDTA-H_2O_2 system. Moreover, it was observed that both volatile oil samples reduced the 2,2-diphenyl-1-picrylhydrazyl (DPPH), a determined independent. It was also found out that *A. taurica* and *S. kronenburgii* volatile oils inhibited the xanthine oxidase enzyme and that *Artemisia* volatile oil was more effective on inhibiting this enzyme than *Salvia* volatile oil. When the inhibition kinetics were studied, it was observed that the inhibition kinds was successively competitive inhibition for *A. taurica* and uncompetitive inhibition for *S. kronenburgii.*

Key words: *Artemisia taurica* Willd, *Salvia kronenburgii* Rech.Fil, antioxidant activity, free radical, xanthine oxidase.

INTRODUCTION

Changing living conditions, environmental pollution, industrial wastes, solar rays, exhaust gases, heavy metals, cigarette, alcohol, ozone and miscellaneous chemicals are inescapable elements for today's humanity. These unfavourable conditions lie behind the nascence of free radicals (Dorman et al., 2000; Candan et al., 2003). Free radicals may damnify the basic cellular constituents such as lipid, protein, DNA, carbonhydrate and enzyme (Çakatay and Kayalı, 2006; Prakash et al., 2011). It is thought that the cellular injury associated with free radicals contributes to the complication of several chronic diseases. It is thought that the cellular injury resulting from free radicals conduces to the complications of cardiovascular diseases, and many chronic and neuro-degenerative diseases like diabet mellitus, cancer, and aging (Fu et al., 2011; Prasad et al., 2012).

*Corresponding author. E-mail: m_bayramoglu65@hotmail.com

Abbreviations: XO, Xanthine oxidase; **DPPH,** 2,2-diphenyl-1-picrylhydrazyl; **TBA,** thiobarbituric acid; **NBT,** nitrobluetetrazolium; **EDTA,** ethylenediaminetetraacetate; **TCA,** trichloroacetic acid; BHT, butylated hydroxytoluene; **GC-MS,** Gas chromatography-mass spectrometry.

The enzyme xanthine oxidase (XO) (EC. 1.1.3.2) is a Mo-containing flavoprotein from the oxidoreductase group (Pei and Li, 2000; Çete et al., 2005). Xanthine oxidase is one of the vital sources of superoxide anion which is a crucial free oxygen radical. This enzyme is a metalo flavoprotein existing in all nucleate cells. It is known as a member of the enzyme group functioning in hydroxylation of molybdenum, iron and flavine in catalyzing the hydroxylation of purines. It functions as a rate limiting enzyme in purine nucleotide metabolism. Xanthine oxidoreductase is found in two forms convertible to each other. These are xanthine dehydro-genase and xanthine oxidase (Cai and Harrison, 2000). While xanthine dehydrogenase reduces both NAD^+ and oxygen, XO reduces only O_2 and, as a consequence, it forms O_2^-• and H_2O_2. Both enzymes catalyze the con-version of hypoxan-thine to xanthine and the conversion of xanthine to uric acid. Uric acid is the last product of purine destruction in humans (Champe and Harvey, 1997). In the meantime, molecular oxygen is converted to super-oxide anion by way of reduction (Li and Jackson, 2002).

Today, many plants found in their natural flora are both used as a folkremedy because of their contentand studied academically in medical researches. Especially essential (volatile) oils have been used in medication since antiquity. After it was discovered that they have lots of functional characteristics as well as antimicrobial, antioxidant and anticancer activities, studies on the bioactive principles of essential oils extracted from various plants and spices have gained popularity because adverse effects that are not or hardly seen in natural drugs are found with significantly higher quantities in synthetic drugs (Candan et al., 2003; Vardar et al., 2003).

Labiatae are a large family with nearly 200 genera and 3000 species, including some plants like sideritis, sage, mint and thyme. This family is important in terms of medicinal and aromatical plants (Satıl et al., 2007). Among the Labiatae family, Salvia L. is one of the most common genera with shrubs and herbaceous forms spreading naturally from the temperate zones to the hot zones across the world. It contains approximately 900 species all over the world. In Turkey, this genus is identified with 89 species and 94 taxons, and 50% of these is reported to be endemic (Başer, 2002). *Salvia kronenburgii* in the genus Salvia L. is an endemic grown in 1500-2500 metre-high regions peculiar to the Lake Van basin. The localities where *S. kronenburgii* is found are of brown and chestnut soil.

The plants were collected in steep, sloping, shallow stony and highly erosive soil. The genus has a cluster-like, shrubbier, and quite deep taproot. Its stem is puberulent, quadrangular, upright and ascendant. *Salvia* species are highly rich in essential oil. Since the ancient times, many plants peculiar to this species have been used for therapeutic purposes and as odorizer and flavour as well as ornamental plants in food industry as they have aromatic effects (Demirci et al., 2003). Diterpenoids obtained from the *Salvia* species are reported to have antioxidant, antibacterial or cytotoxic features (Perry et al., 2003).

Asteraceae is known to be the richest family of angiospermae with approximately 1000 genera and 20000 species (Bağcı and Kocak, 2008). Most of the plants are two or more years old, nonarboreal, in the form of shrub or tree. In this family, essential oil, inuline, and latex are the most common compounds. Owing to these compounds, most of the plants are used in pharmaceutics, food and other industrial fields (Seçmen et al., 1998; Tanker et al., 2004).

Artemisia taurica Willd. from the genus *Artemisia* is generally 900-1900 m high, and it spreads in prairies and steppes. It is a perennial species of Artemisia with an upright and ramified stem, ascendant up to 60 cm, with lignified leaves at the bottom, upper parts with dense white hairs, fragmental leaves with white hairs on both sides (Davis, 1975). Artemisia species have also been used for medicinal purposes since the time of ancient Egyptians. Nearly 20 Artemisia species are grown in Anatolia and some of these are used for medicinal purposes under different names (Baytop, 1999). Artemisia species are generally used in pharma-ceutics, perfumery, and flavouring industry (Scora and Kumamoto, 1984). They are used as appetizer, roborant and stimulant by the people. They have an antifebrile effect as well (Baytop, 1991).

Given these characteristics, this study aims to conduct a chemical analysis regarding the essential oils of *A. taurica* Willd. and *S. kronenburgii* Rech.Fil., and to examine whether they show antioxidant effects by scavenging free radicals (superoxide, hydroxyl and 2,2-diphenyl-1-picrylhydrazyl (DPPH)), and their effect on xanthine oxidase enzyme, thus to ensure the conscious consumption, and to light the way for the next pharmacological studies on those plants.

MATERIALS AND METHODS

Used chemicals

In this study, we procured gallic acid, quercetin, allopurinol, thiobarbituric acid (TBA), nitrobluetetrazolium (NBT), xanthine, xanthine oxidase from Sigma (USA) Company; ethylenediamine-tetraacetate (EDTA), trichloroacetic acid (TCA), $FeCl_3$, K_2HPO_4, KH_2PO_4, NaH_2PO_4, $NaHPO_4$, $AlCl_3$, butylated hydroxytoluene (BHT), hydrogen peroxide, folin reagent, ascorbic acid, methanol, hexane, HCl, H_2SO_4 chemicals from Merck (Germany) Company, and deoxyribose from Aldrich-Chem.

Used plant materials

Supraterra parts of *S. kronenburgii* Rech.Fil. were collected from the dip slopes in the site of Kurubaş Geçidi (Kurubaş Strait) in northwest of Gürpınar/Van in May 2008; those of *A. taurica* Willd. were collected from the northern slopes of Zernek Dam in Gürpınar/Van in August 2007.

Table 1. Plants' essential oil yields.

Örnekler	Percentage (v/w)
Artemisia Taurica Willd.	0.590 ± 0.065
Salvia Kronenburgii Rech.Fil.	0.716 ± 0.035

Obtaining essential oil from plant material

One hundred gram (100 g) herbal samples that were exsiccated in the shade and powderized were subjected to steam distillation with 500 mL water via Clevenger apparatus for 3 h. Obtained essential oils were preserved at + 4°C.

Gas chromatography-mass spectrometry (GC-MS) analysis of essential oils

Qualitative and quantitative analyses of the essential oils were conducted in Adana Çukurova University via Thermo Finnigan-Trace GC-MS using an autosampler. By using split method, a sample size of 1 µL was injected at the split rate of 50. Chromatographic separation was done with the split mode injection at the split rate of 50 via TR-MS capillary column (5%-phenyl 95%-dimethylpolysiloxane, length= 60 m, inner diameter=0.25 mm and film thickness 25 µm). The analysis was performed at a flow rate of 1 mL min^{-1}. by using helium carrier gas. The column temperature was programmed with 3°C min^{-1}. increases between 50-250°C. Temperatures of injection and ion source were 250 and 200°C, respectively. An ionization voltage of 70 eV and a mass range of 41-400 a.m.u. were applied. Separated components were determined by comparing the data of NIST and Wiley mass spectral library.

Determining the total phenolic content

The Folin-Ciocelteu reagent was used in determining the total phenolic content of the essential oil (Gamez-Meza et al., 1999). Phenolic content of the essential oils was given as gallic acid equivalent to mg g^{-1} oil.

Determining the total flavanoid content

In determining the flavonoid content of the essential oils, a methanolic $AlCl_3$ solution of 1 mL 2% was added to 1 mL essential oil dissolved in methanol. Absorbance of the samples was read against the check sample at the wavelength of 394 nm (Lamasion et al., 1990). Total flavanoid quantity of the essential oils was given as quercetin equivalent to mg g^{-1} oil.

Determining the total antioxidant capacity

This method is based on the formation of green phosphate/Mo(V) complex in acidic pH by reducing acidic Mo (VI) to Mo (V) (Prieto et al., 1999). Total antioxidant capacity of the essential oils was given as mM α-tocopherol acetate g^{-1} oil.

DPPH radical scavenging activity

In order to determine the obtained essential oils' feature of scavenging the DPPH radical, a DPPH solution of 5 mL (% 0.004) was added to the solutions in different concentrations of the essential oils which were diluted with methanol, and it was incubated for 30 min. Absorbance of the samples was read against the check sample at the wavelength of 517 nm (Cuendet et al., 1997; Al-Reza et al., 2010).

Hydroxyl radical scavenging activity

The obtained oils' feature of scavenging the hydroxyl radical was determined by measuring the thiobarbituric acid-reactive substances revealed with the deformation of deoxyribose by the hydroxyl radicals formed via the system of Fe^{+3}/ascorbate/EDTA/H_2O_2 (Kunchandy and Rao, 1990).

Superoxide radical scavenging activity

Scavenging of the superoxide radical by the essential oils of *Salvia* and *Artemisia* was determined with the reduction of NBT by the superoxide radical formed via xanthine/xanthine oxidase system (Robak and Gryglewski., 1988; Lee et al., 2002 Al-Reza et al., 2010).

Xanthine oxidase studies

Activity of xanthine oxidase (XO) (EC. 1.1.3.2.) was assessed according to the method of Prajda and Weber with spectrophotometric measurement of the absorbance increase at 293 nm during the formation of uric acid from xanthine (Prajda and Weber, 1975).

RESULTS

Essential oil products of plants

Plants' essential oil yield were given as dry plant of mL/100 g in Table 1.

GC-MS analysis of essential oil of *Salvia kronenburgii* Rech. Fill.

The chromatogram according to the GC-MS analysis of *Salvia* essential oil is presented in Figure 1, and the components, retention time and relative availibility precentages of the essential oil are given in Table 2.

GC-MS analysis of essential oil in *Artemisia taurica* Willd.

The chromatogram according to the GC-MS analysis of *Artemisia* essential oil is presented in Figure 2, and the components, retention time and relative availibility precentages of the essential oil are given in Table 3.

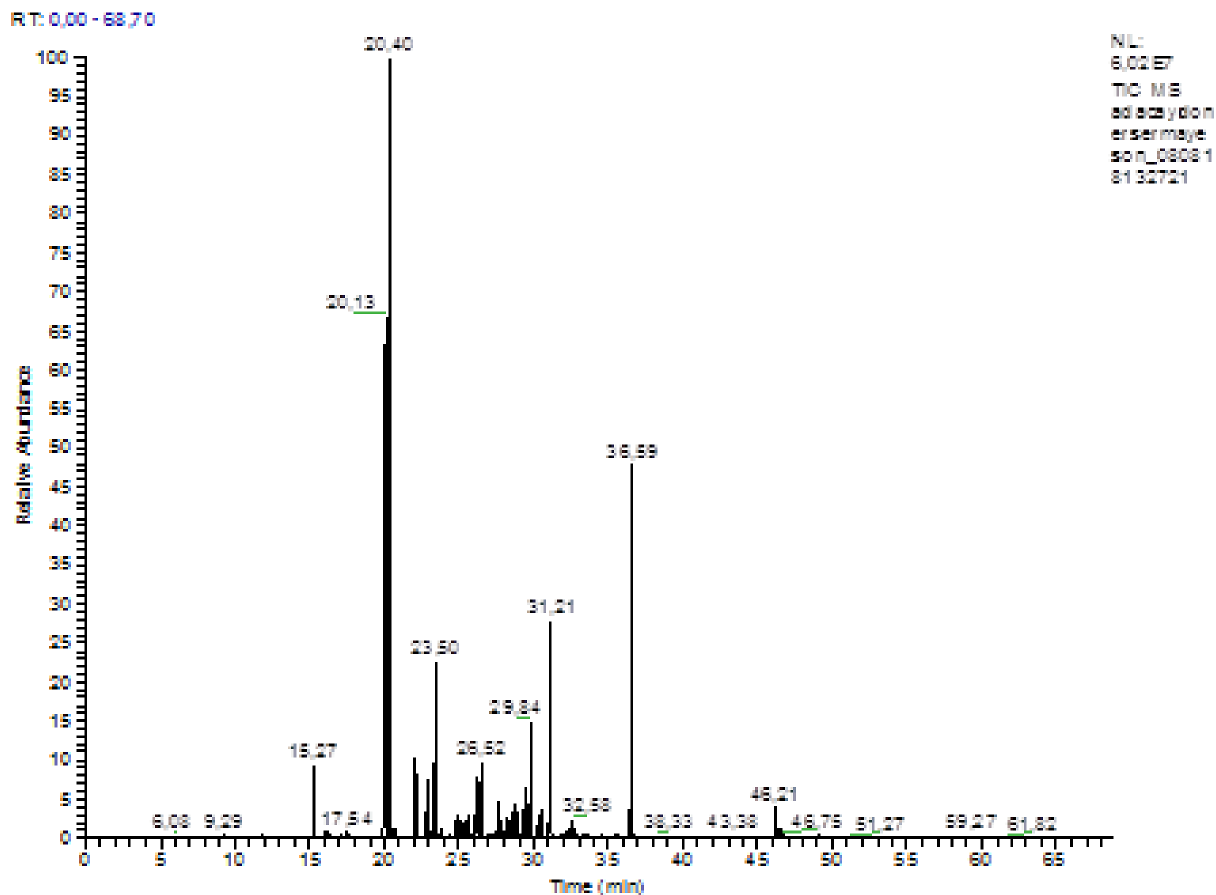


Figure 1. Essential oil chromatogram of *Salvia kronenburgii.*

Table 2. Chemical composition of the essential oil from *Salvia kronenburgii.*

Number	Component	RT	Area	Composition
1	α-Pinene	15.27	15909137	1.58
2	Camphane	16.17	1296833	0.13
3	β-Pinene	17.54	1327824	0.13
4	o-Cymene	19.86	1430258	0.14
5	DL-Limonene	20.13	207236972	20.62
6	Eucalyptol	20.4	248721241	24.75
7	(E)-β-ocimene	20.62	2181889	0.22
8	Linalool oxide trans	22.12	21507022	2.14
9	Linalool oxide cis	22.93	16966827	1.69
10	Linalool	23.5	58134949	5.78
11	6-Methyl-hepta-3,5-dien-2-one	23.9	2284962	0.23
12	α-Camphenolic aldehyde	25.17	3880727	0.39
13	2,6-dimethyl-1,3,5,7-octatetraene	25.63	6846047	0.68
14	trans- Pinocarveol	26.03	6963486	0.69
15	(E)-3-Caren-2-ol	26.21	21725354	2.16
16	β-Terpineol	26.29	2586624	0.26
17	L(-)-Camphor	26.52	22545951	2.24
18	Dihydrocarveol	27.21	2073294	0.21
19	α-Terpinenol	27.4	2106112	0.21
20	Borneol	27.64	8431329	0.84

Table 2. Contd.

21	cis-p-mentha-2,8-dien-1-ol	27.77	4510259	0.45
22	4-carvomenthenol	27.84	2084334	0.21
23	p-mentha-6,8-dien-2-ol	28.22	6065094	0.60
24	o-Acetyltoluene	28.36	4193256	0.42
25	α-Terpinenol	28.61	7963446	0.79
26	1,2-dimethyl-3-vinyl-1,4-cyclohexadiene	28.83	9375753	0.93
27	Terpinyl asetat	29.2	1793994	0.18
28	1-Verbonene	29.44	12813970	1.28
29	L-Carveol	29.84	48806273	4.86
30	Isobornyl formate	30.2	2739070	0.27
31	cis-Carveol	30.46	6631774	0.66
32	Geraniol formate	30.57	8710832	0.87
33	Cis-Geraniol	30.88	3210580	0.32
34	Carvone	31.21	83426017	8.30
35	p-Mentha-1,8-dien-3-on	32.34	1671112	0.17
36	cis-Carvon oxsit	32.58	4545397	0.45
37	p-mentha-1,8-dien-7-al	32.69	2775185	0.28
38	Geranyl Acetate	36.59	127360711	12.67
39	Dehydroaromadendrene	46.21	6966524	0.69
40	Ledene oxide	46.46	2327805	0.23
Total				99.37

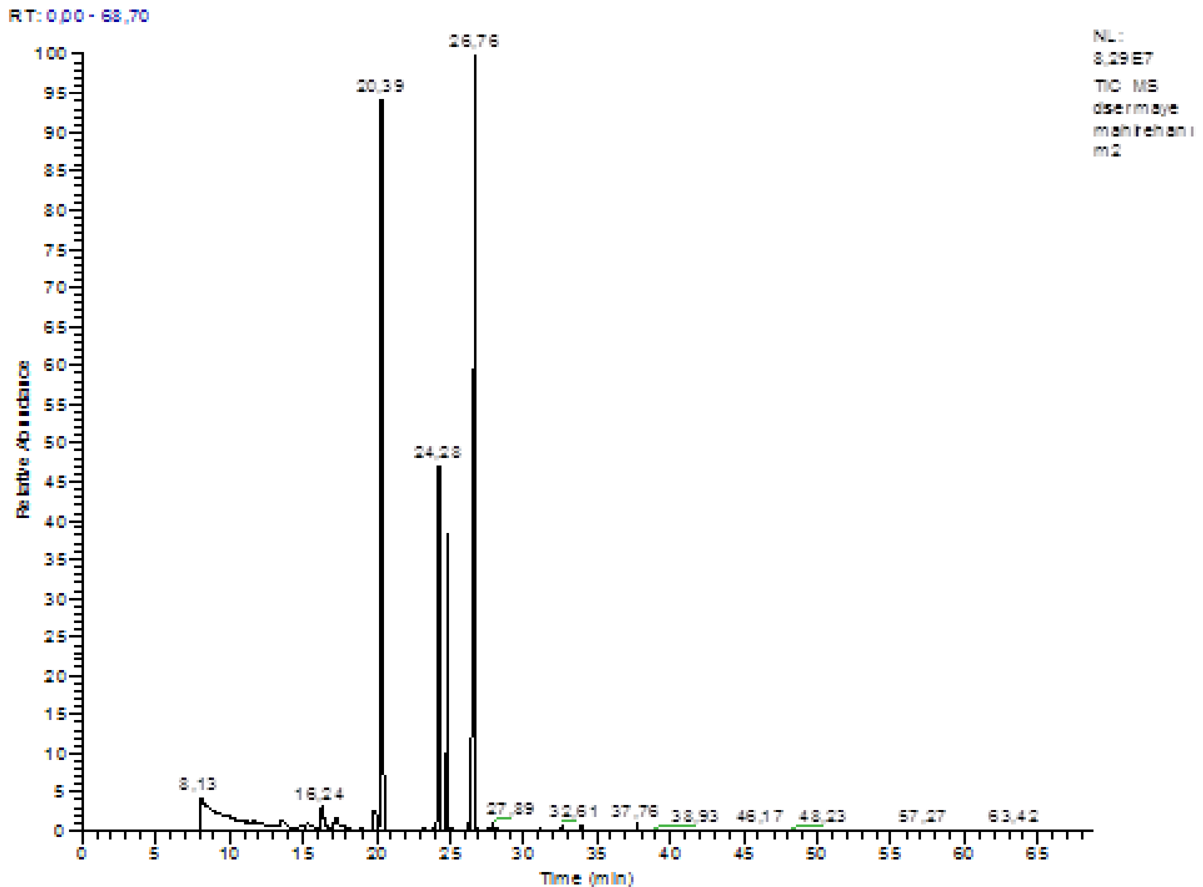


Figure 2. Essential oil chromatogram of *Artemisia taurica*.

Table 3. Chemical composition of essential oil from *Artemisia taurica*.

Number	Component	RT	Area	Composition
1	Santolina triene	13.58	13138504	0.58
2	α- Pinene	15.34	8123008	0.36
3	Camphene	16.24	39954195	1.75
4	Sabinene	17.23	11457823	0.50
5	o-Cymene	19.88	27318607	1.20
6	Eucalyptol	20.39	810254681	35.57
7	Trans-Caryophyllene	24.28	317842287	13.95
8	Thujone	24.84	178052243	7.82
9	Comphor	26.76	858268028	37.68
	Total			99.42

Table 4. Total phenol and total flavonoid value, total antioxidant capacity of essential oils *Artemisia taurica* and *Salvia kronenburgii*.

Sample	Total phenol (mg gallic acid g^{-1} oil)	Total Flavonoid (mg quercetin g^{-1})	Total antioxidant capacitiy (mM α-Tocopherol acetate g^{-1})
Artemisia essential oil	5.137 ± 0.050	7.984 ± 0.233	438.4 ± 4.198
Salvia essential oil	10.848 ± 0.077	12.600 ± 0.224	818.4 ± 7.563

Total phenol-flavonoid quantity and total antioxidant capacity of essential oils

Total phenol quantity, total flavonoid quantity and total antioxidant capacity of the obtained essential oils are shown in Table 4.

DPPH radical scavenging activity

As a result of our studies and calculations, the highest percentage in inhibiting the DPPH radical by herbal essential oils and positive controls, and their values of concentration values (IC_{50}) that inhibit the DPPH radical by 50% are shown in Table 5.

Hydroxyl radical scavenging activity

Table 5 demonstrates the highest inhibition percentage shown by essential oils and positive controls in inhibiting the formation of hydroxyl radical and their concentration that inhibits the formation of hydroxyl radical by 50%.

Superoxide radical scavenging activity

The highest inhibition percentage shown by essential oils and positive controls in inhibiting the superoxide formation and their concentration that inhibits the formation of superoxide radical by 50% are given in Table 5.

Xanthine oxidase studies

The highest percentage shown by essential oils in xanthine oxidase inhibition and IC_{50} values are demonstrated in Table 5. Maximum velocity (V_{max}) and substrate concentration at half of the maximum velocity (K_m) calculated via the Lineweaver-Burk plot which was drawn to determine the inhibition type are shown in Table 6.

DISCUSSION

Plants are the main sources of natural antioxidant compounds. Antioxidant compounds existing in fruits, vegetables, spices, vegetable oils and seeds have been discussed in lots of researches, and it has been shown that their antioxidant effects stem from phenolic compounds, particularly from the flavonoid structure (Dapkevicius et al., 1998; Merken et al., 2001). Antioxidant effect of phenolic compounds originates from their characteristics such as taking electron or hydrogen atom from free radicals, scavenging free radicals by ending the chain reactions, solvating with transition metals, and preventing or reducing the singlet oxygen atom (Cotelle, 2001).

Generally phenolic compounds are represented by flavonoids, tannins and phenolic acids (Giampieri et al., 2012). Flavonoids are one of the functional nutrients and important components having antioxidant features (Cotelle, 2001). Flavonoids show their antioxidative activity by way of inhibiting such enzymes as xanthine

Table 5. The highest percentage in inhibiting the DPPH, hydroxyl, superoxide anion radical and xanthine oxidase by *Artemisia* and *Salvia* essential oils and positive controls, and their concentration values (IC_{50}) which inhibit the DPPH, hydroxyl, superoxide anion radical and xanthine oxidase by 50%.

Parameter	Inhibition (%)	IC_{50} (μL mL^{-1})
DPPH		
Artemisia essential oil	82.175 ± 0.366	14.238 ± 0.129
Salvia essential oil	83.712 ± 0.408	7.438 ± 0.100
BHT	52.420 ± 0.990	24.210 ± 0.926
Ascorbic acid	60.000 ± 1.699	16.752 ± 0.804
Hydroxyl radical		
Artemisia essential oil	76.206 ± 0.344	0.404 ± 0.016
Salvia essential oil	78.275 ± 1.503	0.192 ± 0.001
BHT	58.212 ± 0.276	29.984 ± 1.149
Superoxide radical		
Artemisia essential oil	60.000 ± 4.810	0.205 ± 0.047
Salvia essential oil	57.142 ± 3.092	0.335 ± 0.045
BHT	52.891 ± 0.858	55.242 ± 1.196
Ascorbic acid	50.217 ± 0.716	82.512 ± 3.854
Xanthine Oxidase		
Artemisia essential oil	75.29 ± 0.1	0.321 ± 0.3
Salvia essential oil	41.03 ± 0.7	-

Table 6. The Vmax and Km values obtained from the interaction of Xanthine Oxidase with the different concentrations of *Artemisia taurica* and *Salvia kronenburgii* essential oils.

Essential oil	Concentration (μL mL^{-1})	Vmax (μmol dk^{-1})	K_m ($μM^{-1}$)
Artemisia taurica	Control	0.0309	90.91
	0.64	0.0309	161.29
	0.32	0.0309	133.33
	0.156	0.0308	107.52
Salvia kronenburgii	Control	0.0329	90.91
	0.3	0.0284	80.00
	0.8	0.0213	60.61
	1.0	0.0149	47.85

oxidase, lipoxygenase, cyclooxygenase, forming chelation with metal ions, interacting with other antioxidants, grabbing such free radicals as superoxide anions, lipid peroxyl and hydroxyl. Flavonoids have antiviral, anti-allergic and antitumor features as well.

We examined the total antioxidant capacity, total phenol and flavonoid content of the both essential oils we used in our study. It is seen that there is a direct proportion between the total antioxidant capacity and the total phenol-flavonoid quantities of those essential oils.

GC-MS analysis was carried out in order to determine the chemical composition of the essential oils obtained from the plants. As a result of the analysis, camphor, 1.8-cineole and trans caryophyllene were detected as the first three main compounds in the essential oil of *A. taurica*. As a result of the GC-MS analysis of the essential oil of *S.*

kronenburgii which is endemic to the vicinity of Van, 1.8-cineole, limonene and geranyl acetate were detected as the first three main compounds. Given the first three main compounds of the two essential oils in consequence of their GC-MS analyses, we see that 1.8-cineole is the shared compound for the essential oils of *Artemisia* and *Salvia*. 1.8-cineole has antibacterial, sedative, antilaryngiticand hypotensive features. Furthermore, it is a monoterpene used in treating such diseases as asthma and bronchitis due to its antiinflammatory effect (Juergens et al., 2003; Faleiro et al., 2003).

Being the second main compound in the essential oil of *Salvia*, L-limonen is a monoterpene commonly found in citrus and several other plant species. Due to its fragrance, it is used in cosmetic industry and cleaning products. Studies proved that limonen has antiinflammatory, anticancer and antimicrobial effects; according to some experiments on animals, it has also an antitumor effect. In addition to these, it is effective in cardio-atherosclerosis (Mazzanti et al., 1998; Sever and Özbek, 2005; Özbek et al., 2007). The third main compound of the *Salvia* essential oil, geranyl acetate is described to have sedative, antibacterial, antivirus and antidepressant effects (Lima et al., 1996; Duarte et al., 2006).

Camphor, the first main compound of the *Artemisia* essential oil, is a bicyclic monoterpene found in many plantal structures in nature. It is antibacterial and has some features to invigorate blood circulation, to clear up cardiac dysfunction and pneumonopathy (Baricevic et al., 2001; Mirva et al., 2001; Capek et al., 2003). Caryophyllene, the third main compound constituing 19.95 percent of the *Artemisia* essential oil, is reported to show an antiinflammatory feature (Kamatou et al., 2005; Duarte et al., 2006).

Since antiquity, it is known that plants, plant extracts and essential oils have antimicrobial, antioxidant, antifungal among others effects to different extents. Extracts of medicinal plants are used as a preserver in foods and as raw materials in a great deal of sectors such as medicine, pharmaceutics, perfumery and cosmetics. Therefore, they have been scrutinized in several studies and there are significant results related to their antioxidant effects (Nielson and Rios, 2000; Karanika et al., 2001). Given the DPPH radical-inhibition percentages of the essential oils, the highest DPPH radical-scavenging activity was observed in the essential oil of *Salvia*. When we looked at the essential oil concentrations which scavenged 50 percent of the DPPH radical, we observed a high radical-scavenging effect in Salvia parallel to its percental inhibition value.

Hydroxyl is the most harmful free radical for molecules in living cells such as DNA base, lipids, aminoacids and carbonhydrates. It was found that the destruction of deoxyribose by the hydroxyl radical generated via the system of Fe^{+3}/ascorbate/EDTA/H_2O_2 is inhibited by the studied oil samples.

In our study, it was seen that the essential oils of *Artemisia* and *Salvia* are more effective than the positive controls in scavenging the hydroxyl radical. As it is known, superoxide radical is a reactive oxygen species which is generated primarily and most easily in enzymatic and non-enzymatic reactions in both environmental agents and organisms. Due to its long half-life, it can be moved to further regions from its birthplace. So it is a significant radical in biological systems. When we compared the superoxide radical-inhibition percentages of the essential oils, we found that the essential oil of *Artemisia* is more effective than that of *Salvia*.

In the last stage of our experiment, we examined the effect of essential oils on the enzyme xanthine oxidase. As it is known, xanthine oxidase is one of the crucial sources of superoxide anion. It catalyzes the reactions which convert hypoxanthine to xanthine and xanthine to uric acid. Mean while, molecular oxygen is reduced and then converted to superoxide anion. There are a great deal of studies about plant extracts' inhibition of xanthine oxidase and their inhibition kinetics (Sweeney et al., 2001; Candan, 2003; Filha et al., 2006; Lin et al., 2008). It has been found out that some flavonoids like chrysene, luteolin, flavone and quercetin cause xantine oxidase inhibition as well (Nagao et al., 1999). When inhibitions of essential oils were assessed according to IC_{50}values, it was seen that *Artemisia* is the most effective essential oil in the inhibition of xanthine oxidase with a 75 percent inhibition value (IC_{50}= 0.321 ± 0.3 µL mL^{-1}). It was seen that *Salvia* essential oil has a 41 percent inhibition value; its IC_{50} value was not observed because it did not show an activity to inhibit 50 percent of xanthine oxidase.

As it was seen that the essential oils contain inhibitory for the enzyme xanthine oxidase, their inhibition kinetics was examined to determine the inhibition type of the reaction. For this purpose, a Lineweaver-Burk plot was drawn and values of V_{max} and K_m were determined for the reactions. While V_{max} did not change with the increasing concentrations of the *Artemisia* essential oil, K_m increased, so it was put forth that the inhibition type is competitive. On the other hand, despite the increasing concentrations of the *S. kronenburgii* essential oil, both V_{max} and K_m decreased, so it was put forth that the inhibition type is uncompetitive.

In consequence of the all in-vitro studies, it was observed that the essential oils of *A. taurica* and *S. kronenburgii* are effective in inhibiting reactive oxygen species and xanthine oxidase. It was seen that the *Artemisia* essential oil is more effective than *Salvia*'s in inhibiting the superoxide radical and xanthine oxidase; on the other hand, the Salvia essential oil is effective in scavenging the hydroxyl radical and DPPH. In accordance with all these results, it is observed that these plants are antioxidant and can be used in treating numerous diseases caused by reactive oxygen species. It may even be used in treating such diseases as gout just as allopurinol that is an xanthine oxidase inhibitor. However, these prospective cogitations need to be sup-

ported by *in vivo* studies.

REFERENCES

Al-Reza MS, Rahman A, Sattar AM, Rahman OM, Fida MH (2010). Essential oil composition and antioxidant activites of Curcuma aromatica Salibs. Food Chem. Toxicol. 48:1757-1760.

Bağcı E, Kocak A (2008). Composition of Essential oils of Salvia palaestina Bentham and S. Tomentosa Miller species, a chemataxonomical approach. Sci. Eng. J. Fırat Univ. 20(1):35-41.

Baricevic D, Sosa S, Loggıa RD, Tubaro A, Simorovska B, Kransa A, Zupannic A (2001). Tropical antiinflamatory activity of Salvia officinallis L. J. Ethnopharmacol. 75:125-132.

Başer HKC (2002). Aromatic biodiversity among the flowering plant taxa of Turkey. Pure Appl. Chem. 4 (74):527-545.

Baytop A (1991). Textbook of Pharmaceutical Botany. Istanbul University, Faculty of Pharmacy Publications. pp. 255-256.

Baytop T (1999). Treatment with Medicinal Plants in Turkey. Istanbul University, Faculty of Pharmacy Publications. Second Edition. pp. 275-350.

Cai H, Harrıson DG (2000). Endothelial Dysfunction in Cardiovasculer Diseases : The Role of Oxidant Stres. American Heart Assocation, Inc. Circ Res. 87:840-844.

Candan F (2003). Effect of Rhus coriaria L. (Anarcidiaceae) on Superoxide Radical Scavenging and Xanthine Oxidase Activity. J. Enzyme Inhib. Med. Chem. 18(1):59-62.

Candan F, Unlu M, Tepe B, Daferera D, Polissiou M, Somken A, Akpulat H (2003). Antioxidant and antimicrobial activity of the essential oil and methanol extracts of Achillea millefolium subsp. millefolium Afan. (Asteraceae). J. Ethnopharmacol. 87(2-3):215-20.

Capek P, Hribalova V, Suondova E, Ebringerova A, Sasınkova V, Massorova C (2003). Charecterization of ımmunomodulatory polysaccharides from Salvia officinallis L. Int. J. Biol. Macromol. 33:113-119.

Champe PC, Harvey RA (1997). Degradation of Purine Nucleotides. (Editors:Tokullugil, A., Dirican, E., Ulukaya, E.) Lippincott's Illustrated Reviews, Biochemistry. Second Edition, Nobel Tip Kitabevi, İstanbul. pp. 348-351.

Cotelle N (2001). Role of Flavanoids In Oxidative Stress. Curr. Top. Med. Chem. 1:569-590.

Cuendet M, Hostettmann K, Potterat O (1997). Iridoid glucosides with free radical scavenging properties from Fagraea blumei. Helv. Chim. Acta. 80:1144-1152.

Çakatay U, Kayalı R (2006). The evulation of free radical biochemstry in historical perspective. Cerrahpaşa J. Med. 37:162-167.

Çete S, Arslan F, Yaşar A (2005). Investigation of antimicrobial effects aganist some mıcroorganısms of Aloe Vera and Nerıum Oleander Also examination of the effects on the xantine oxidase activity ın liver tıssue treated wıth cyclosporin. G.U. J. Sci. 18(3):375-380.

Dapkevicius A, Venskotonis R, Beek TA, Linssen JPH (1998). Antioxidant Activity, of Extracts Obtained by Different Isolation Procedures from Some Aromatic Herbs Grown in Lithuania. J. Sci. Food Agric. 77:140-146.

Davis PH (1975). Flora of Turkey and East Aegan Islands. Edınburg at the university press. Vol:5. 323.

Demirci B, Baser KH, Yıldız B, Bahcecioglu Z (2003). Composition of the essential oils of six endemic Salvia spp. From Turkey. Flavour Fragr. J. 18:116-121.

Dorman HJD, Surai P, Deans SG (2000). Aromatic & Med Plant Grp, Auchincruive KA6 5HW, Scotland. *In vitro* antioxidant activity of a number of plant essential oils and phytoconstituents. J . Essent. Oil Res. 12(2):241-248.

Duarte MC, Leme EE, Delarmelina C, Soares AA, Fıgueıra GM, Sartora A (2006). Activity of essential oils from Brazilian medicinol plants on Escherichia coli. J. Ethnopharmacol. 111(2):197-201.

Faleiro ML, Migvel MG, Ladeiro F, Venancio F, Tavares R, Brito JC, Figueiredo AC, Barroso JG, Pedro LG (2003). Antimicrobial activity of essential oils isolated from Portuguese Endemic Species of Thymus. Lett. Appl. Microbiol. 36(1):35-40.

Filha-Feraz Z, Vitolo IF, Fietto LG, Lombardi AJ, Guimaraes D (2006). Xanthine oxidase inhibitory activity of Lychnophora species from Brazil (Arnica). J. Ethnopharmacol. 107:79-82.

Fu L, Xu TB, Xu XR, Gan RY, Zhang Y, Xia EQ, Li HB (2011). Antioxidant capacites and total phenolic content of 62 fruits. Food Chem. 129:345-350.

Gamez-Meza N, Noriega-Rodriguez JA, Medina-Juarez LA, Ortega-Garcia J, Cazarez-Casanova R, Argulo-Guerreo O (1999). Antioxidant Activity in Soybean Oil of Extracts from Thompson Grape Bagasse. Jaocs. 76:1445-1447.

Giampieri F, Tulipani S, Alvarez-Suarez JM, Quiles Jl, Mezzetti B, Battino M (2012). The Strawberry: Composition nutritional quality and impact on human health. Nutrition 28:9-19.

Juergens UR, Dethlefsen U, Steinkamp G, Gillissen A, Repges R, Vetter H (2003). Antiinflamatory activity of 1,8- cineol in broncihial asthma:a double blind-placebo controlled trial. Respir. Med. 97(3):250-256.

Kamatou GP, Viljoen A, Gano-Blowyo AB, Vuuren SF, Lourens AC, Başer H, Demirci B, Lindsey KL, Van Staden J, Steen Kamp P (2005). The *in vivo* pharmacolocial activites and chemical investigation of three South African Salvia species. J. Ethnopharmacol. 102(3):382-390.

Karanika MS, Komaıtıs M, Aggelis G (2001). Effect of Aqueous Extract of Some Plants of Lamiaceae Family on the Growth of Yarrowia Lipolytia. Int. J. Food Microbiol. 64:175-181.

Kunchandy E, Rao MNA (1990). Oxygen radical scavenging activity of curcumin. Int. J. Pharmacog. 58:237-240.

Lamasion JL, Carnat A, Petitjean C (1990). Tannin content and inhibiting activity of elastase in rosaceae. Ann. Pharm. 48(6):335-340.

Lee JC, Kim H, Kim J, Jang YS (2002). Antioxidant activity of etanol extract of the stem of Opuntia ficus-indica var. saboten. J. Agric. Food Chem. 50:6490-6496.

Li C, Jackson RM (2002). Reactive species mechanism of cellular hypoxia-reoxygenation injury. Am. J. Physiol. Cell Physiol. 282(2):227-241.

Lima MA, Silverio ER, Margues MS, Santos RH, Gombordela MT (1996). Biological activite flavanoids and terpenoids from Egletcs viscosa. Phytochemstry 41(1):217-23.

Lin SY, Wang CC, Lu YL, Wu WC, Hou WC (2008). Antioxidant, anti-semicarbazine sensitive amine oxidase and anti hypertensie activites of geraniin isolated from Phyllanthus urinaria. Food Chem. Toxicol. 46:2484-2492.

Mazzanti G, Barttınelli L, Salvatore G (1998). Antimicrobial Properties of the Linanol Rch Essential Oil of Hyssopos officinalis L. Var. (Lamiaceae). Flavour Fragr. J. 13:289-294

Merken HM, Merken CD, Beecher, GR (2001). Kinetics Method for the Quantitation of Anthocyanins, Flavonols, and Flavones in Food. J. Agric. Food Chem. 49:2727-2732.

Mirva K, Kikuzaki H, Nakatani N (2001). Apianane terpenois from Salvia officinallis. Phytochemistry 58:1171-1175.

Nagao A, Seki M, Kobayashı H (1999). Inhibition of Xanthine Oxidase by Flavonoids. Biosci. Biotechnol. Biochem. 63(10):1787-1999.

Nielson PV, Rios R (2000). Inhibition of Fungal Growth on Bread by Volatile Components from Spices and Herb and the Possible Aplication in Active Packaging with Special Emphas on Mustard Essential Oil. Int. J. Food Microbiol. 60:219-229.

Özbek H, Bahadır Ö, Kaplanoğlu V, Öntür H (2007). Studying the anti-inflammatory effect of the essential oil of basil (Ocimum basilicum L.). J. Gen. Med. 17(14):201-204.

Pei J, Li X (2000). Xanthine and hypoxantine sensors based on xanthine oxidase immobilized on a $CuPtCl_6$ chemically modified electrode and liquid chromatography electrochemical detection. Anal. Chim. Acta. 414:205-229.

Perry N, Bollen C, Perry EK, Ballard C (2003). Salvia for dementia therapy: review of pharmacological activity and pilot tolerability clinical trial. Pharmacol. Biochem. Behav. 75:651-659.

Prajda N, Weber G (1975). Malign transformation-linked imbalance: decreased XO activity in hepatomas. FEBS Lett. 59:245-249.

Prakash B, Shukla R, Priyanka S, Mishra PK, Dubey NK, Kharwar RN (2011). Efficacy of chemically characterized Ocimum gratisssium L. essential oils as an antioxidant and a plant based antimicrobial aganist fungal and aflatoxin B1 contamination of spices. Food Res. Int. 44:385-390.

Prasad S, Sung B, Aggarwal BB (2012). Age-associated chronic

diseases require age old medicine: Role of chronic inflammation. Prev. Med. 54:29-30.

Prieto PM, Pineda M, Aguilar M (1999). Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E. Anal Biochem. 269(2):337-341.

Robak J, Gryglewski, RJ (1988). Flavonoids are scavengers of superoxide anions. Biochem. Pharmacol. 37:837-841.

Satil F, Ünal M, Hopa E (2007). Comparative Morphological and Anatomical Studies of Hymenocrater bituminosus Fisch. C.A.Mey. (Lamiaceae) in Turkey. Turk J. Bot. 31:269-275.

Scora RW, Kumamoto J (1984). Ontogenetic Variation and Dıurnal Study in the Composition of Essential Oil of Artemisia Douglasıana. J. Nat. Prod. 47(2):279-284.

Seçmen Ö, Gemici Y, Leblebici E, Bekat L (1998). Systematic of Seed Plants. Ege University Faculty of Science publications. Fifth Edition.

Sever B, Özbek H (2005). Studying the total dose levels and anti-inflammatory activities of (R)- (+) Limonene on mice and rats. Turkish Pharmacology Association, 18. National Pharmacology Congress, 28 September-1 October.

Sweeney AP, Wyllie SG, Shalliker JL (2001). Xanthine oxidase inhibitory activity of selected Australian native plants. J. Ethnopharmacol. 75:273-277.

Tanker N, Koyuncu M, Coşkun M (2004). Pharmaceutical Botany. Ankara University, Faculty of Pharmacy Publications.

Vardar GU, Candan F, Somken A, Daferera D, Polissiou M, Dönmez E, Tepe B (2003). Antimicrobial and Antioxidant Activity of the Essential Oil and Methanol Extracts of Thymus pectinatus Fisch. et Mey. Var. pectinatus (Lamiaceae). J. Agrıc. Food Chem. 51(1):63-67.

academicJournals

Vol. 13(5), pp. 693-698, 29 January, 2014
DOI: 10.5897/AJB2013.13225
ISSN 1684-5315 ©2014 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Fractionation and determination of total antioxidant capacity, total phenolic and total flavonoids contents of aqueous, ethanol and n-hexane extracts of *Vitex doniana* leaves

Yakubu, O.E.[1,2], Nwodo, O.F.C.[1], Joshua, P.E.[1], Ugwu, M.N.[2], Odu, A.D.[2] and Okwo F.[2]

[1]Department of Biochemistry, Faculty of Biological Sciences, University of Nigeria, Nsukka, Nigeria.
[2]Department of Medical Biochemistry, Cross River University of Technology, Calabar, Nigeria.

Accepted 20 January, 2014

As a result of normal metabolic processes, the human body produces reactive oxygen species capable of oxidizing biomolecules that can damage DNA, cells and also contribute to the development of chronic diseases. The process can be attenuated or perhaps reversed by herbs and diets containing components that can scavenge reactive oxygen species. In this study, the total antioxidant capacity (TAC), total polyphenolic content (TPC) and total flavonoids content (TFC) of aqueous, ethanol, n-Hexane extract as well as ethanol extract fractions of *Vitex doniana* leaves were determined. Ethanol extract showed the highest 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity (69.01±1.13) followed by aqueous extract (66.14±1.12) and n-hexane extract (50.05±2.11). The total flavonoids content is in the order; aqueous (304±4.14) > ethanol (276 ±4.69) > n-Hexane (88±3.45). Hence, the total phenolic content is in a similar order as that of total antioxidant capacity. Chloroform : ethyl acetate fraction has the highest antioxidant capacity (165mg/ml). methanol : H_2O fraction (76mg/ml) and 100% methanol (76mg/ml). Similarly, the total flavonoids content is in the order of fractions; 1>6>4>13>12>2 and others. Total phenolics were in the order of fractions; 1>5>4>12>7>2. There was a strong relationship (R^2 = 0.77) between total antioxidant activity and total flavonoid contents and (R^2 = 0.6517) for total phenolic content of the fractions. The present study demonstrated that *V. doniana* leaves extracts contain high amounts of flavonoids and phenolic compounds so that these compounds are efficient free radical scavengers.

Key words: 1,1-Diphenyl-2-picrylhydrazyl (DPPH), polyphenols, flavonoids, *Vitex doniana*.

INTRODUCTION

Natural products are important sources for biologically active drugs and wild herbs have been investigated for their antioxidant properties (Gazzaneo et al., 2005). Medicinal plants containing active chemical constituents with high antioxidant property play an important role in the prevention of various degenerative diseases and have potential benefit to the society (Lukmanul et al., 2008). Natural antioxidants from plant sources are potent and safe

*Corresponding author. E-mail: oj4real_2007@yahoo.co.uk.

Abbreviations: DPPH, 1,1-Diphenyl-2-picrylhydrazyl; **TAC,** total antioxidant capacity; **TE,** trolox equivalent; **GAE,** gallic acid equivalent; **QE,** quercetin equivalent; **TFC,** total flavonoid content.

due to their harmless nature. A free radical in each molecule is determined as an unpaired electron that occupies an atomic or molecular orbital on its own. This reactive molecule is to another electron to pair, this in step an uncontrolled chain reaction that can damage the natural function of the living cell, resulting in different diseases (Zhishen et al., 1999). Many fruits and vegetables, herbs, cereals, seeds that contain natural antioxidants can abstract the lone electron from free-radical molecules and help humans to keep control on these harmful species. Most of these antioxidants in plants are highly coloured anthocyanines, proanthocyaninidins, flavans, flavonoids, and their glycosides, carotenoids, like β-carotene and lycopene (Matkowski et al., 2009). Isolation of antioxidants from plants depends on the polarity of these compounds. First distribution of antioxidants between a polar (aqueous, hydro ethanol) and a semi-polar solvent (n-butanol, ethyl acetate) can be used to determine the distribution factor of the compounds between phases (Matkowski et al., 2009).

Vitex doniana sweet, (family *Verbanaceae*) is a perennial shrub widely distributed in tropical West Africa, and some East African countries including Uganda, Kenya and Tanzania and high rainfall areas. It is found in the middle belt of Nigeria particularly Kogi, Benue, and parts of the savannah regions of Kaduna, Sokoto and Kano states (Etta, 1984). It is variously called *vitex* (English), *dinya* (Hausa), *dinchi* (Gbagyi), *uchakoro* (Igbo), *oriri* (Yoruba) *ejiji* (Igala) and *olih* (Etsako) (Burkill, 2000). *V. doniana* is employed in the treatment of a variety of diseases. Hot aqueous extracts of the leaves are used in the treatment of stomach and rheumatic pains, inflammatory disorders, diarrhoea dysentery and diabetes (Irvine, 1961; Etta, 1984). Yakubu et al. (2012; 2013) reported the antidiabetic properties of the leaves. The roots and leaves are used for nausea, colic and epilepsy (Bouquet et al., 1971; Iwu, 1993). In North-Central and eastern parts of Nigeria, the young leaves are used as vegetables or sauces and porridge for meals, especially for diabetic patients.

MATERIALS AND METHODS

Collection and preparation of plant materials

Fresh leaves of *V. doniana* were collected from its natural habitat in Ankpa, Kogi State. It was identified and authenticated by the Ethnobotanist in the Department of Medicinal Plant Research and Traditional Medicine of the National Institute for Pharmaceutical Research and Development (NIPRD) Abuja, Nigeria. A voucher specimen number NIPRD/H/6415 was deposited at the herbarium of the department. The plant material was dried in the laboratory at room temperature and pulverized using laboratory mortar and pestle.

Aqueous extraction

About 400 g of the pulverized sample was soaked in 2 l of distilled water (1:5 W/V) and was allowed to stand for 24 h at room temperature according to the study of Iwueke and Nwodo (2008).
The extract was filtered and the filtrate was concentrated using rotary evaporator under reduced pressure. It was allowed to dry at room temperature and stored in refrigerator prior to usage.

Ethanol / n-hexane extraction

About 400 g of the pulverized sample was soaked in 2 l (1:5 w/v) of ethanol/n-Hexane (2:1 v/v) for 24 h. The extract was filtered under reduced pressure using filter paper, membrane filter and vacuum pump. Ethanol extract was separated from the n-hexane extract using separatory funnel and the filtrates were concentrated using rotary evaporator under reduced pressure, respectively.

Fractionation

The ethanol extract was subjected to column chromatograph to separate the extract into its component fractions. Silica gel was used in packing the column while varying solvent combinations of increasing polarity were used as the mobile phase.

Packing of column

In the packing of the column, the lower part of the glass column was stocked with glass wool with the aid of glass rod. 75 g of silica gel ($G_{60\text{-}200}$ mesh size) was dissolved in 180 ml of absolute chloroform to make the slurry. The chromatographic column (30mm diameter by 40 mm height) was packed with silica gel and was allowed free flow of the solvent into a conical flask below. The set up was seen to be in order when the solvent drained freely without carrying either the silica gel or glass wool into the tap. At the end of the packing process, the tap was locked and the column was allowed 24 h to stabilize after which, the clear solvent at the top of the silica gel was allowed to drain down the silica gel meniscus

Elution

The ethanol extract (2 g) was dissolved in 2 ml absolute methanol and the solution was applied unto a chromatographic column (30 mm diameter by 400 mm height). Elution of the extract was done with solvent system of gradually increasing polarity, beginning from chloroform, ethyl acetate, methanol and finally water. The following ratios of solvent combinations were sequentially used in the elution process: Chloroform : ethyl acetate 100:0, 80:20, 60:40, 40:60, 20:80, 0:100; ethyl acetate : methanol 80:20, 60:40, 40:60, 20:80, 0;100; methanol : water 50:50 and 0:100. A measured volume (400 ml) of each solvent combination was poured into the column each time using separator funnel. The eluted fractions were collected in aliquots of 10 ml in test tubes.

Total antioxidant capacity

The scavenging action of the plant extracts and the resulting fractions from ethanol extract on 1,1-diphenyl-2-picrylhydrazyl (DPPH) was determined colorimetrically at 517 nm using Trolox as standard according to the method described by Singleton et al. (2002). About 1.0 ml tris HCl buffer was added to test tube containing 1.0 ml absolute ethanol, 2.0 ml DPPH (0.1 mM) solution was added and the solution was thoroughly mixed. The absorbance was measured within 30 s after addition of sample at 517 nm. The absorbance was measured in triplicate for each extract/fraction. Total antioxidant capacity (TAC) was calculated as mg/ml of trolox equivalent (TE) using the regression equation from calibration curve.

Total polyphenol content (TPC)

Total polyphenol component was estimated colorimetrically at 765 nm as described by Lachman et al. (2000), using Follin-Ciocalteu reagent and expressed as gallic acid equivalent (GAE). Exactly 0.25 ml sample was added to test tube containing 2.50 ml Follin reagent. Sodium carbonate solution (2.0 ml) was added and was allowed to stand for 15-20 min at room temperature. The reactions were conducted in triplicates and absorbance of the sample was measured against the reagent blank. The results were expressed as GAE.

Estimation of total flavonoids content (TFC)

Flavonoids were determined using the aluminum chloride colorimetric method of Chang et al. (2002). Quercetin was used for derivation of the calibration curve. Exactly 0.5 ml of the diluted sample was added into test tube containing 1.5 ml methanol. 0.1 ml of 10% aluminum chloride ($AlCl_3$) solution and 0.1 ml potassium acetate (CH_3COOK) were added. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm and the concentration of flavonoids in the sample was estimated from the calibration curve. The amount of 10% aluminum chloride was substituted by the same amount of distilled water in blank. Total flavonoids were expressed as mg/ml quercetin equivalent (QE).

RESULTS AND DISCUSSION

Total antioxidant capacity, total flavonoids and total polyphenolic contents of aqueous, ethanol and n-Hexane extracts of *Vitex doniana* leaves

Ethanol extract showed the highest antioxidant activity (69.01±1.13) followed by aqueous extract (66.14±1.12) and n-hexane extract (50.05±2.11). The total flavonoids content is in the order; aqueous (304±4.14) > ethanol (276±4.69) > n-hexane (88±3.45). Hence, the total phenolic content is in a similar order as that of total antioxidant capacity.

Total antioxidant capacity, total flavonoids and total polyphenolic contents of different fractions obtained from ethanol extract of *Vitex doniana* leaves

Table 2 unveils the total antioxidant capacity, total flavonoids and total phenolic contents of different fractions obtained from the ethanol extract of *V. doniana* leaves. Chloroform: ethyl acetate fraction has the highest antioxidant capacity (165 mg/ml). Methanol: H_2O fraction (76 mg/ml) and 100% methanol (76 mg/ml). Similarly, the total flavonoids content is in the order of fractions; 1>6>4>13>12>2 and others. Total phenolics is in the order of fractions; 1>5>4>12>7>2.

Correlation between total antioxidant capacity and total flavonoids content of different fractions obtained from ethanol extract of *V. doniana* leaves

Strong positive correlation (R^2 = 0.77) between total antioxidant capacity and total flavonoids content of ethanol extract fractions of *V. doniana* leaves was observed (Figure 1).

Correlation between total antioxidant capacity and total polyphenolic content of different fractions obtained from ethanol extract of *V. doniana* leaves

Linear correlation between total antioxidant capacity and total polyphenolic content of fractions obtained from ethanol extract of *V. doniana* leaves showed positive correlation (R^2 = 0.6517) (Figure 2).

Correlation between total polyphenolic and total flavonoids contents of different fractions obtained from ethanol extract of *V. doniana* leaves

There was a strong positive correlation (R^2 = 0.8825) between total flavonoids and total polyphenolic contents of fractions obtained from *V. doniana* ethanol extract of leaves (Figure 3).

DISCUSSION

Flavonoids as antioxidant compounds in our study reportted in range of 88-304 mg/ml QE (Table 1) and 100-390 mg/ml QE dry weight of fractions (Table 2). The TFC for ethanol extract was 304±4.14 mg/ml QE. After partial purification, TFC of the fractions was found within the range 100-390 mg/ml QE which is higher than the TFC of the whole extract. This implies that the extract contains a lot of phytochemicals other than flavonoids. Furthermore, total phenolic content of the aqueous extract was 460±2.24 and 380±1.97 mg/mlGAE for ethanol extract while TFC for aqueous extract was 276.69±mg//mQE and 304±4.14mg/mlQE for ethanol extract. It means aqueous extract contained higher concentration of phenolics than ethanol extract, but lower concentration of flavonoids than ethanol extract. This is an indication that flavonoids take 80% of total phenolic content of ethanol extract and 60% of aqueous extract.

The TAC of the extracts are given as follow; ethanol > aqueous > n-Hexane extract (Table 1). This difference may be attributed to differences in extraction or hydrolysis time (Ismail et al., 2004; Andarwulan et al., 2010). In our study, flavonoids content were correlated with antioxidant activity in the DPPH. It is known that flavonoids (Wojdyło et al., 2007) have the strongest radical-scavenging power among all natural phenolic compounds. Moreover, it is a potent antioxidant against lipid peroxidation in mitochondrion and microsome (Wang et al., 2010); therefore, absolute ethanol, as a polar solvent, was the better extraction solvent for antioxidant capacity and TFC in this study.

Moore and Adler (2001) reported that apolar solvents are among the most employed solvents for removing polyphenols from water (Moure et al., 2001; Anjaneyulu and Chopra 2004). Several studies have reported on the relationships between phenolic content and antioxidant

Table 1. Total antioxidant capacity, total flavonoids and total polyphenolic contents of aqueous, ethanol and n-hexane extracts of *Vitex doniana* leaves.

Extracts	TAC (mg/ml TE)	TFC (mg/ml QE)	TPC (mg/ml GAE)
Aqueous	66±1.12	276±4.69	460±2.24
Ethanol	69±1.23	304±4.14	380±1.97
Hexane	50±2.11	83. ±3.45	202±1.11

Data are mean SD of triplicate determinations.

Table 2. Total antioxidant capacity, total flavonoids and total polyphenolic contents of different fractions obtained from ethanol extract of *Vitex doniana* leaves.

Fraction	Solvent combination	Ratio	TAC (mg/ml TE)	TFC (mg/ml QE)	TPC (mg/ml GAE)
1	Chloroform : ethyl acetate	10:00	165	390	680
2	Chloroform : ethyl acetate	08:02	64	200	300
3	Chloroform : ethyl acetate	06:04	53	87	75
4	Chloroform : ethyl acetate	04:06	66	265	430
5	Chloroform : ethyl acetate	02:08	71	140	180
6	Chloroform : ethyl acetate	00:10	72	287	475
7	Ethyl acetate : methanol	08:02	71	105	320
8	Ethyl acetate : methanol	06:04	71	100	100
9	Ethyl acetate : methanol	04:06	74	140	180
10	Ethyl acetate : methanol	02:08	73	107	115
11	Ethyl acetate : methanol	00:10	76	107	115
12	Methanol : H_{2O}	05:05	76	212	325
13	Methanol : H_2O	00:10	76	240	380

Data are mean of triplicate determinations.

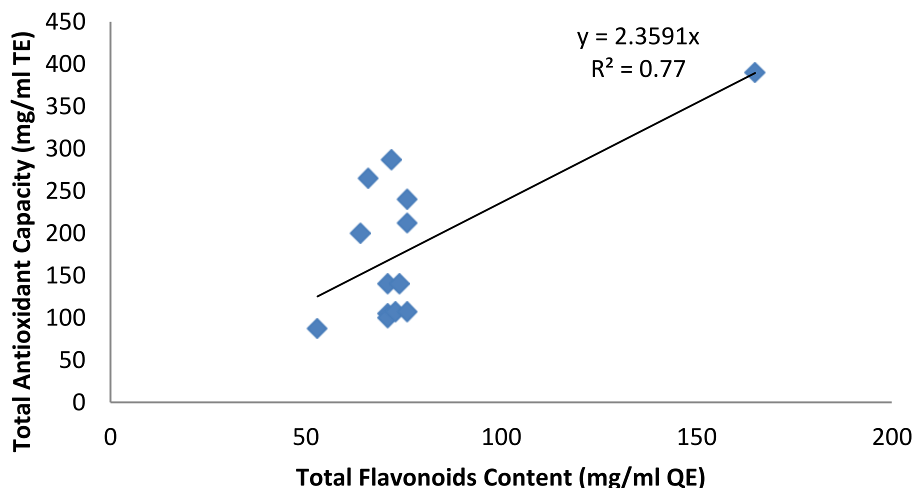


Figure 1. Linear correlation between total antioxidant capacity and total flavonoids content of fractions obtained from ethanol extract of *Vitex doniana* leaves.

activity (Ismail et al., 2004). Velioglu et al. (1998) reported a strong relationship between total phenolic content and antioxidant activity in selected fruits and vegetables.

In our study, there was strong relationship (R^2 = 0.77) between antioxidant activity and total flavonoid contents and (R^2 = 0.6517) for total phenolic content of the fractions. It could be deduced however that the antioxidant capacity of the fractions is majorly dependent on its flavonoids content although there is a wide grade of variation between different phenolic compounds in their effec-

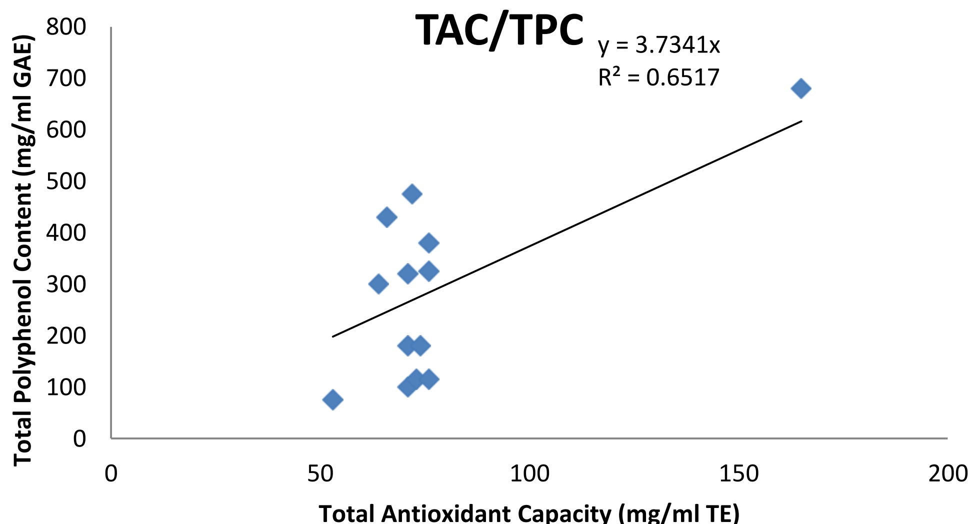


Figure 2. Linear correlation between total antioxidant capacity and total polyphenolic content of fractions obtained from ethanol extract of *Vitex doniana* leaves.

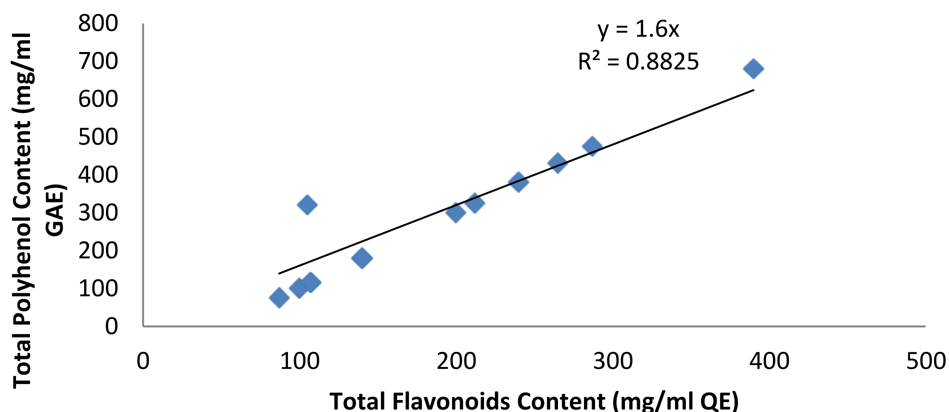


Figure 3. Linear correlation between total flavonoids content and total polyphenolic content of fractions obtained from ethanol extract of *Vitex doniana* leaves.

tiveness as antioxidant (Robards et al., 1999; Bjelakovic et al., 2007). Hence, concentration and pH can also play role in the antioxidant activity of phenolics (Bouayed et al., 2011). In addition, the chemical structure of phenolics play a role in the free radical scavenging activity, mainly depending on the number and position of hydrogen donating hydroxyl groups on the aromatic rings of the phenolic molecules (Bouayed et al., 2011).

The temperature during drying and extraction, affects the compound stability due to chemical and enzymatic degradation, casualties by volatilization or thermal analysis, these latter have been suggested to be the main mechanism causing the reduction in polyphenol content (Moure et al., 2001). Also, for synthetic antioxidants, evaporation and analysis were the main mechanisms for the loss of activity. Of course, the temperature during extraction can affect the extractable compounds differently: boiling and static increased the total phenol content on the other hand, proanthocyanidin content decreased. The antioxidant activity depends on the extract concentration. The results of the present study showed that *V. doniana* leaves are rich in flavonoids and phenolic constituents and demonstrated good antioxidant activity. This plant, rich in flavonoids and phenolic acids could be a good source of natural antioxidant.

REFERENCES

Andarwulan N, Batari R, Sandrasari DA, Bolling B, Wijaya H (2010). Flavonoid content and antioxidant activity of vegetables from Indonesia. J. Cell Biochem. 102:61-72.

Anjaneyulu M, Chopra K (2004). Quercetin, an anti-oxidant bioflavonoid, attenuates diabetic nephropathy in rats. Clin. Exp. Pharmacol. Physiol. 31:244-8.

Bjelakovic G, Nikolova D, Gluud LL, Simonetti RG, Gluud C (2007). Mortality in randomized trials of antioxidant supplements for primary and secondary prevention: systematic review and meta-analysis. JAMA. 297:842–57.

Bouayed J, Hoffmann L, Bohn T (2011). Total phenolics, flavonoids, anthocyanins and antioxidant activity following simulated gastro-intestinal contents. JAMA 291:84–87.

Burkill HM (2000). Useful plants of West Tropical Africa. Royal Botanic Garden Kew 2:272-275.

Chang CC, Yang MH, Wen HM, Chm JC (2002). Estimation of total flavonoids in propolis by two complementary colorimetric methods. J. Food Drug Anal. 10:178-182.

Ismail A, Marjan Z M, Foong CW (2004). Total antioxidant activity and phenolic content in selected vegetables. Food Chem. 87:581-86.

Iwueke AV, Nwodo OFC (2008). Anthihyperglycaemic effect of aqueous extract of Daniella oliveri and Sarcocephalus latifolius roots on key carbohydrate metabolic enzymes and glycogen in experimental diabetes. Biokemistri 10(2):63-70.

Lachman J, Hamouz K, Orsak M, Pivec V (2000). Potato tubers as a significant source of antioxidants on human nutrition. Rostl. Vyr. 46:231-236.

Matkowski A, Hajones M, Skalicka-Wozniak K, Slusarczy K (2009). Antioxidant activity of polyphenols from Lycopus lucidus Turcz. Food Chem. 120(4):88–89,

Moore C, Adler R (2001). Herbal vitamins: Lead toxicity and develop-ment delay. Pediatrics 160: 600-602.

Moure A, Cruz JM, Franco D, Domoanguez JM, Sineiro J, DomõÂnguez (2001). Natural antioxidants from residual sources. Food Chem. 72:145-71.

Robards K, Prenzler PD, Tucker G, Swatsitang P, Glover W (1999). Phenolic compounds and their role in oxidative processes in fruits. Food Chem. 66:401-436.

Singleton VL, Orthofer R, Lamuela-Raventos RM (2002). Analysis of total phenol and other oxidative substrates and antioxidants by means of Folin-ciocalteu reagent. Methods Enzymol. 299:152-178.

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

Wojdyło A, Oszmian´ski J, Czemerys R (2007). Antioxidant activity and phenolic compounds in 32 selected herbs. Food Chem.105: 940-49.

Yakubu OE, Nwodo, OFC, Nwaneri-Chidozie VO, Ojogbane E (2012). Amelioration of lipid peroxidation and oxidative stress in hepatocytes of streptozotocin-induced diabetic rats treated with aqueous extract of *Vitex doniana* leaves. Int. J. Basic Appl. Chem. Sci. 2 (4). 89-98.

Yakubu OE, Ojogbane E, Nwodo OFC, Nwaneri-Chidozie VO, Dasofunjo K (2013). Effect of *Vitex doniana* ethanol leaf extract on renal function in diabetic rats. Euro. J. Exp. Biol. 3(3):630-634.

academicJournals

Vol. 13(5), pp. 699-704, 29 January, 2014
DOI: 10.5897/AJB2013.12273
ISSN 1684-5315 ©2014 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Synergistic effects of ethanolic plant extract mixtures against food-borne pathogen bacteria

Salvador Eduardo Vásquez Rivera[1], Mayra Alejandra Escobar-Saucedo[1], Diana Morales[2], Cristóbal Noé Aguilar[1] and Raúl Rodríguez-Herrera[1]*

[1]Food Research Department, School of Chemistry, Universidad Autónoma de Coahuila. Blvd. V. Carranza and Ing. José Cárdenas V. s/n. Col. República Ote. Saltillo 25280, Coahuila, México.
[2]Fitokimica Industrial SA de CV, Ramos Arizpe, Coahuila, México.

Accepted 5 January, 2014

Plant extracts are an important part in agroecology, as they benefit environment in combating pathogenic organisms, without resorting to synthetic chemicals. The objective of this study was to evaluate the antibacterial activity of mixtures of ethanol extracts from semi-desert plants [creosote bush (*Larrea tridentata*), tarbush (*Flourensia cernua*) and paddle cactus (*Opuntia ficus-indica*)] against *Enterobacter aerogenes*, *Escherichia coli*, *Salmonella typhi* and *Staphylococcus aureus*. The maximum antimicrobial activity was achieved with the creosote bush–tarbush-paddle cactus mix (1:1:1) v/v, followed by paddle cactus-tarbush (1:1) v/v. *E. coli* was the bacterial strain that showed the highest growth inhibition as consequence of the concentration of plant extracts (4000 and 5000 ppm of tarbush). While with the creosote bush plant extracts, the highest inhibition halos were observed. Synergistic effects were observed when mixtures of ethanolic plant extract against food-borne pathogen bacteria were used, so this may be a better way to design alternative pathogen control methodologies for food-borne pathogen bacteria.

Key words: *Larrea tridentate*, *Flourensia cernua*, *Opuntia ficus-indica*, ethanolic plant extract mixtures, food-borne pathogen.

INTRODUCTION

Food-borne pathogen bacteria are one of the major public concerns worldwide (Tayel and El-Tras, 2010). A variety of microorganisms also lead to food spoilage that is encountered as one of the most important matter concerning the food industry. So far, many pathogens microorganisms have been reported as causal agents of food-borne diseases and/ food spoilage (Natta et al., 2008). Prevention of pathogenic and spoilage microorganisms in food is usually achieved by using synthetic chemical preservatives but some of them are responsible for many carcinogenic and teratogenic attributes as well as residual toxicity, and with growing concern of microbial resistance toward conventional synthetic preservatives (Pundir et al., 2010). In addition, the continuous spreads of multi-drug resistant pathogens have become a serious threat to public health and a major concern for infection control practitioners (Iwalokun et al., 2004). All of the above mentioned are concerns that have put pressure on the food industry for progressive removal of synthetic chemical preservatives and adoption of natural alternatives to obtain its goals concerning safe food with long shelf lives (Agatemor, 2009). In addition, at the present time, there

*Corresponding author. E-mail: raul.rodriguez@uadec.edu.mx. Tel: (+52) 01844-4169213.

Abbreviation: CT, Condensed Tannins; **HT,** hydrolysable tannins; **C,** creosote bush; **T,** tarbush; **P,** paddle cactus.

is a tendency to create environmentally friendly products (Lira-Saldivar et al., 2003).

Many plant derived products such as spices, fruit preparations, vegetal preparations or plant extracts have been used for centuries for preservation and extension of shelf life of foods (Pundir et al., 2010). Today, scientific research reveals that not only chemicals from plants have an effect against a particular disease. But also the antioxidant property from plant extracts gives a beneficial effect to human health (Puangpronpitag and Sittiwet, 2009). Different extracts of plants used for treatment of some gastrointestinal disorders, may be successfully applied to elimination of food borne bacteria (Tayel and El-Tras, 2010). On the other hand, the perception that there is a lower incidence of adverse reactions to plant preparations compared to synthetic pharmaceuticals and the reduced cost of plant preparations (Cock, 2008), suggests the idea that some plant extracts may be used as natural antimicrobial additives to reclaim the shelf –life of foods (Pundir et al., 2010). Considerable research on antimicrobial plant extracts have been reported (Sharma and Hashinaga, 2004; Dupont et al., 2006; Sutherland et al., 2009). But rarely the combined effect of two or three plant extracts on growth of food-borne pathogen bacteria has been reported. Mexican flora is one of the most diverse worldwide due to number of species, biological variability levels and climatic diversity. In this country, more than 200 plant species with antifungal and antibacterial activities, mainly against plant pathogens have been reported (Montes et al., 2000).

The state of Coahuila is a region with a high biodiversity (endemic flora and fauna); some representative plants of the flora region are creosote bush (*Larrea tridentata)*, tarbush (*Flourensia cernua*) and paddle cactus (*Opuntia ficus-indica*). These plants are characterized by a large number and variety of polyphenols. Among the phenolic compounds present are: gallotannins, ellagitannins and condensed tannins, which have protective functions against microorganisms (Osorio et al., 2010). Plant extract consist of a combination of active products of plant metabolism. The large number of chemical compounds that extracts have may interact synergistically, so the resulting effects are higher than those using each component separately (Viveros and Castaño, 2006). Currently, agriculture and food industry uses various synthetic chemicals, but, some of them may promote environmental and health risks, so, there is a tendency to create natural and environmentally friendly products. The use of different solvents like hexane, chloroform and methanol is not allowed in the context of organic production systems (Lira-Saldivar et al., 2003). For this reason, it is importance to study the use of plant extracts obtained organically and identify their applications on inhibition of food-borne pathogens.

The aim of this study was to evaluate the effect of ethanol extract mixtures from *L. tridentata*, *F. cernua* and *O. ficus-indica*, on inhibition of food-borne pathogen bacteria.

MATERIALS AND METHODS

Vegetal material

Leaves and stems of creosote bush (*L. tridentata*), tarbush (*F. cernua)* and paddle cactus (*O. ficus-indica*) were collected from areas nearby to Saltillo, Coahuila Mexico (samples were collected by Diana Morales, and were identified with the numbers CB-03-2010, TB-24-2010 and PC-09-2010 for creosote bush, tarbush and paddle cactus, respectively). Each vegetal tissue was dehydrated at 60°C for two days. The dried samples were grinded in a miller and the powder was sieved at 1 mm. The fine powder obtained was stored in amber bottles or dark plastic bags at room temperature until phytochemical compounds extraction was performed.

Extraction of phytochemical compounds

Each fine powder sample (100 g) was mixed in an Erlenmeyer flask in a 1:4 (w/v) ratio with 70% ethanol. The flask was covered with aluminium foil to avoid light exposition. The mixture was refluxed at 60°C for 7 h. After this, extracts were filtered using Whatman filter paper No. 4. The solvent was removed using a rotary evaporator (Yamato RE540) using a temperature below 60°C, the sample was stored at 5°C in containers covered with aluminum foil until phytochemical analysis were performed.

Analysis of phytochemical compounds

Assay for quantification of tannins of the ethanol extracts was performed using the method reported by Waterman and Mole (1994).

Tannins concentration

Concentration of Condensed Tannins (CT) was spectrophotometrically determined using the method proposed by Swain and Hillis (1959). For condensed tannins determination, an aliquot of 0.5 ml of plant extract was placed in a tube, with 3 ml of HCl/butanol (1:9) and 0.1 ml of ferric reagent.

Analytical standard

On the other hand, it was added to a tube assay serie, [Catechin standard, (+)-Catechin 43412 Fluka from Sigma-Aldrich] in distilled water at different concentrations (0, 200, 400, 600, 800 and 1000 ppm) to obtain a reference curve. The tubes were plugged tightly and heated for 1 h in a water bath at 100°C. After that, they were left to cool and absorbances were read at 460 nm. The concentration of hydrolysable tannins (HT) was determined by the traditional method of Folin-Ciocalteu according to the protocol reported by Makkar (1999), a reference curve was done placing gallic acid to different concentrations (0, 200, 400, 600 and 800 ppm) in assay tubes. The solution of stock gallic acid was to a concentration of 500 ppm and prepared using distilled water. Each one of plant extracts was diluted in a test tube then immediately a 400 µL of commercial Folin-Ciocalteu reagent was added to each tube and the samples were vortexed and left for 5 min. Then, 400 µL of Na_2CO_3 (0.01 M) and 2.5 ml of distilled water was added. Finally, absorbances were read at 725 nm in an UV/visible spectrophotometer.

Antibacterial activity evaluation

For this study, we used four food-borne pathogenic bacteria, *Enterobacter aerogenes*, *Escherichia coli*, *Salmonella typhi* and *Staphylococcus aureus* which were supplied by the Coahuila Public

Table 1. Effect of plant extracts and concentrations on growth inhibition of four different food-borne pathogen bacteria.

Source of variation	Degree of freedom	Mean squares	Pr>F
Replication	2	0.04	0.2603
Concentration (c)	4	10.62	<0.0001
Extracts (e)	12	18.75	<0.0001
Bacteria (b)	3	130.47	<0.0001
c*e	48	0.29	<0.0001
c*b	12	3.53	<0.0001
e*b	36	9.58	<0.0001
c*e*b	144	0.31	<0.0001
Error	518	0.03	<0.0001
Total	779		

Table 2. Area (cm^2) of inhibition halo of four food-borne pathogen bacteria, as an effect of different plant extract mixtures and concentrations.

Bacteria	Inhibition halo (cm^2)
Enterobacter aerogenes	2.71[a]
Salmonella typhi	1.94[b]
Escherichia coli	1.01[c]
Staphylococcus aureus	1.01[c]

Means with the same letter in the same column are not significantly different (p ≤ 0.05) according to Tukey's multiple range test.

Health State Laboratory (Saltillo, Coahuila Mexico). Each bacterial strain was grown in brain-heart-infusion broth (BHIB). Antibacterial activity of plant extracts against four food-borne pathogens was evaluated in test tubes (16 × 150 cm) with screw cap. Each test tube was filled with 5 ml of sterile BHIB medium, and bacteria were added with a sterile inoculating loop [approximately 1.5 × 10(8) bacterial cells/ml]. Inoculated test tubes were incubated at 37°C for 24 h.

Extracts preparation

Pure extracts of creosote bush (C), tarbush (T) and paddle cactus (P) were obtained at five different concentrations (1000, 2000, 3000, 4000 and 5000 ppm). Also, the extract mixtures (C-P, C-T, P-T) in 1:1, 1:3 and 3:1 relations and the mixture (C-T-P) in a relation 1:1:1 were also included. All these mixtures were used in the same five concentrations too. As control we used a tablet of trimethoprim with sulfamethoxazole with a concentration of 4 and 0.8 mg, respectively. Three Petri plates were used for each treatment, in addition two control treatments (sterile water and BHIB medium without extract) were also included in this study. In each Petri plate we placed disks of paper filters (0.7 cm in diameter) soaked in the extract solution corresponding to a concentration.

Experimental design and data analysis

The experiment to determine the effect of different extract mixtures and concentrations on growth inhibition of four food-borne pathogen bacteria was established under a randomized complete block design with three replications under a factorial arrangement of treatments; where the considered factors were: treatments with thirteen levels (all extract combinations), concentrations with five levels (1000, 2000, 3000, 4000 and 5000 ppm) and bacterial species with four levels (*E. aerogenes*, *E. coli*, *S. typhi* and *S. aureus*). The response variable was the area (cm^2) of the bacterial growth inhibition halo. The data were analyzed using ANOVA. When it was needed, Tukey's multiple range procedure was used for treatments mean separation. In this case, statistical analyses were performed using InfoStat software. In order to determine if the extract combinations had an effect on bacterial growth inhibition, an analysis of mixture experiments design under a simplex centroide design was performed using Statgraphics software.

RESULTS

ANOVA results for the effect of three different plant extracts at different concentration on growth inhibition of four food-borne pathogens are showed in Table 1. There was no observed significant differences among replications, but significant differences (P<0.001) were observed for extract concentrations, extracts, bacteria and all interactions among these sources of variation. In addition, it was observed that as extract concentration increased so does bacteria growth inhibition. *E. aerogenes* and *S. typhi* were the food-borne pathogens where the growth was most inhibited as consequence of the plant extract mixtures and their concentrations. While S. *aureus* was the bacteria less inhibited (Table 2). Figure 1 shows that combination of three extracts in a 1:1:1 proportion was the best treatment for inhibition of bacterial growth, suggesting synergical effects among the extracts, with an inhibition halo of 3.13 cm. The pure extract of tarbush and its combinations with creosote bush or paddle cactus have the same inhibition halo that the pure extract of creosote bush (non-significant differences). *E. coli* and *S. aureus* were the lowest inhibited by the extracts and their combinations. Different combinations of extracts were tested, the lowest 1000 ppm; however, *E. coli* was only inhibited with the highest concentrations of tarbush extracts (4000 or 5000 ppm). On the other hand, *S. aureus* growth was inhibited only with the highest concentrations (5000 ppm) of the C: T (25-75) and C: P (25-75) extract

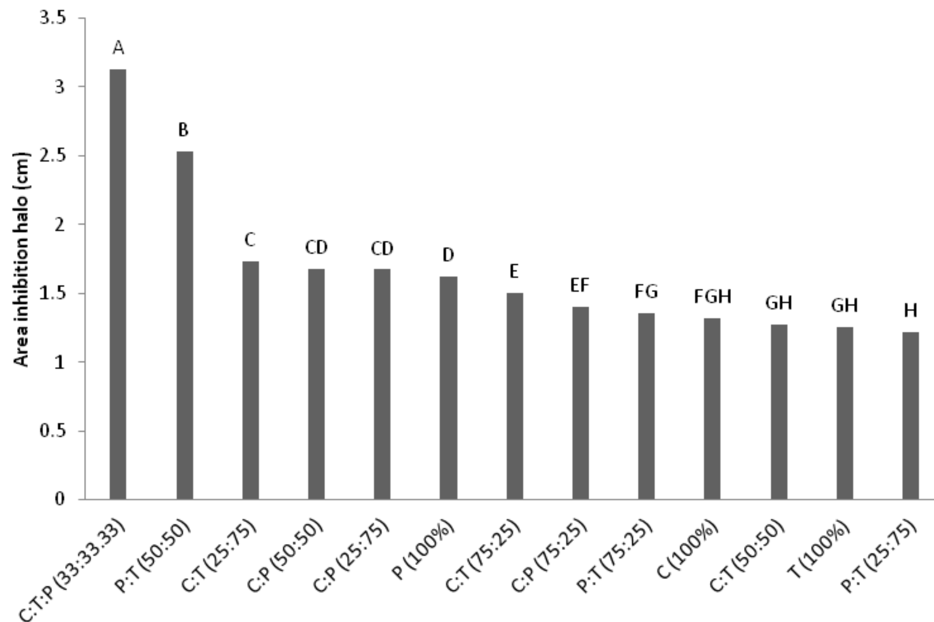


Figure 1. Evaluation of plant extracts as inhibitors, creosote bush (C), paddle cactus (P) and tarbush (T).

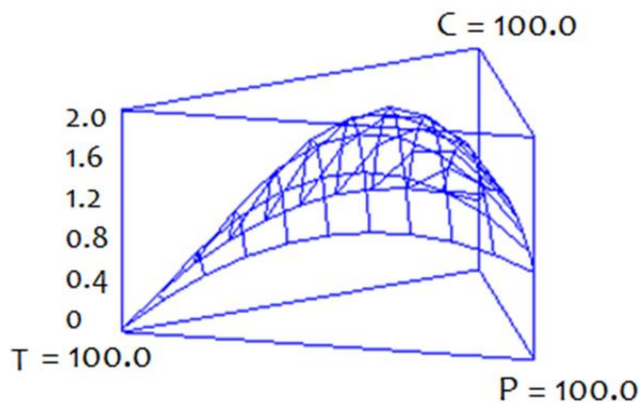


Figure 2. Growth inhibition of *Enterobacter aerogenes* by different plant extracts combinations.

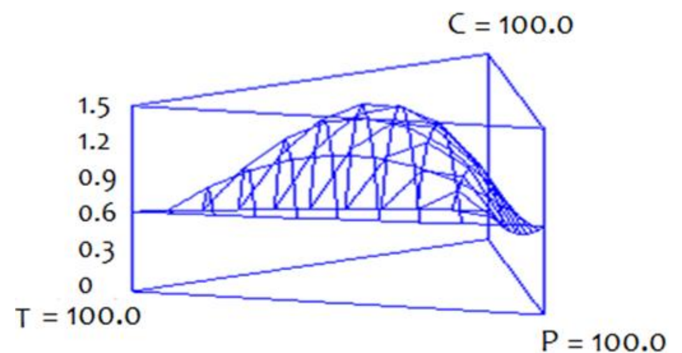


Figure 3. Growth inhibition of *Salmonella typhi* by different extract combinations.

combinations. Most of extract combinations had an inhibitor effect on *E. aerogenes* and *S. typhi*; therefore a mixture analysis was performed under a cubic model, and this analysis was performed using Statgraphics software.

The graphic of response surface showed that the combination of the three extracts (1:1:1) provoked the highest level of growth inhibition of *E. aerogenes* and *S. typhi* (Figures 2 and 3).

DISCUSSION

The significant differences observed among bacteria suggest that extract mixtures affect in a different way each of the tested food-borne pathogen bacteria; while differences among extract mixtures suggest that at least one extract mixture affect in a different way the bacteria growth. In addition, extract concentration has an effect on bacteria growth. A significant difference for the extract concentration-bacteria interaction indicates that a specific extract concentration promotes the highest growth inhibition of a specific bacterium. In same way, the extract-bacteria interaction showed significant differences, showing that a specific extract promotes the highest growth inhibition of a specific bacterium. In addition, the significant differences for the concentration-extract interactions suggest that a specific concentration in each extract is the best for growth inhibition of food-borne pathogen bacteria. Food-borne pathogenic bacteria were more inhibited by the highest extract concentration (Figure 4). It has been reported that extract concentration is very important for microbial control. Alvarez (1999) studied the effect of

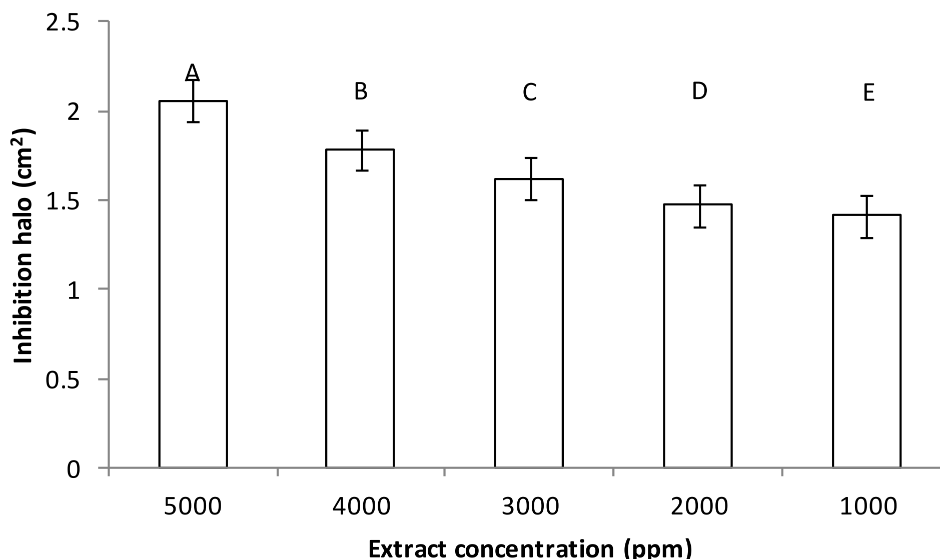


Figure 4. Influence of the extract concentration on bacterial inhibition halo area. Means with the same latter are not different, according to the Tukey multiple range test p<0.05. cm^2.

oregano powder and essential oil on growth inhibition of *S. aureus*, *E. coli* and *S. thyphimurium* finding better inhibitory effects with 150 to 200 ppm of essential oil and 1500 ppm of oregano powder for these three bacteria. On the other hand, Guerrero-Rodríguez et al. (2007) evaluated the effect of fresh leaves extract from *F. cernua* on mycelium inhibition and sporulation of three plant pathogens (*Alternaria alternata*, *Collectotrichum gloesporioides* and *Penicillium digitatum*). These extracts were obtained with a mixture of solvents [methanol:chloroform (1:1v/v)], and by sequential extractions with hexane, diethyl ether and ethanol. The highest *A. alternate* mycelial inhibition occurred when the extract was obtained using hexane and methanol:chloroform at 4000 ppm. *E. aerogenes* and *S. typhi* growth was most inhibited by the plant extracts (Table 2). Castillo-Godina (2008) reported that creosote bush, tarbush, pecan nut shells, damiana and leatherstem extracts promoted *E. aerogenes* growth inhibition as high as 60%. These results are similar to those obtained in this study. The different percentages of bacterial growth inhibition can be attributed mainly to the different chemical composition of these plant extracts. That may contain compounds such as diterpenes and flavones that may cause breakdown of bacterial cell membranes by action of terpenes and the bacterial specie membrane composition, which may be a barrier against the chemical extract (Urzua et al., 2006; Cowan, 1999). Figure 1 shows that combination of three extracts in a 1:1:1 proportion was the best treatment for inhibition of bacterial growth, suggesting synergical effects among the extracts, with an inhibition halo of 3.13 cm.

It has been reported that alcoholic extract of creosote bush has antifungal activity against species of *Aspergillus*, *Penicillium* and *Fusarium* (Tequida et al., 2002). López-Guerra et al. (2007) reported that creosote bush is a good source of condensed and hydrolysable tannins which represent 61.12% of the total phenolic content of this plant species. The amount of phytochemicals present in different extracts may be the explanation for the differences of antimicrobial activity among different plant extracts. Scalbert and Williamson (2000) mentioned that the antimicrobial properties of tannins may be because: tannins can form complexes with microbial enzymes and some proteins inhibiting their functions, tannins also inhibit electron transport through membranes and can alter ions like iron and copper thus inhibiting activity of some enzymes which may be essential for microbial life. *E. coli* was only inhibited with the highest concentrations of tarbush extracts; these extracts were those that had the highest polyphenols content and promoted the highest bacterial growth inhibition. Susceptibility of *E. coli* to plant extract has been reported previously; Mounchid et al. (2005) determined that *E. coli* is susceptible to essential oils of *Rosmarinus officinalis* L. and *Eucalyptus globules*. *S. aureus* growth was inhibited only with the highest concentrations (5000 ppm) of the C: T (25-75) and C: P (25-75) extract combinations. High concentration of plant extract for *S. aureus* inhibition had been reported before. Castaño et al. (2010) evaluated the bacterial activity of ethanolic extract and essential oil from rosemary *R. officinalis* L. leaves on microorganisms of interest in food industry: *E. coli*, *S. aureus*, *S. typhimurium*, *Shigella sonnei*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Bacillus cereus* and *Lactobacillus plantarum*. The growth inhibition of *E. coli* was observed with a high concentration (4092 ppm) of rosemary essential oils.

On the other hand, Burt (2004) determined the minimum inhibitory concentration (MIC) of the rosemary essential oil against *S. typhimurium*, *B. cereus* and *S. aureus*, obtaining values of 20000, 2000 and 8000 ppm, respec-

tively. Synergic effect among creosote bush, paddle cactus and tarbush extracts at a specific concentration (1:1:1) is a good alternative for growth inhibition of *E. aerogenes* and *S. typhi* while that of *S. aureus* and *E. coli* showed the lowest growth inhibition. The highest bacterial growth inhibition was observed with the creosote bush and tarbush extracts and their combinations; this may be attributed to higher concentration of polyphenolic compounds in these plants.

ACKNOWLEDGMENTS

This investigation was supported by Fitokimica Industrial SA de CV and Universidad Autonoma de Coahuila

REFERENCES

Agatemor C (2009). Antimicrobial activity of aqueous and ethanol extracts of nine Nigerian spices against four food borne bacteria. EJEAFChe 8: 195–200.

Alvarez CA (1999). Determination and quantification of antimicrobial and antioxidant capacities of polar fraction of oregano (*Origanum vulgare L.*). MSc. Thesis. School of Chemistry. Universidad Autónoma de Chihuahua. Mexico

Burt S (2004). Essential oils: their antibacterial properties and potential applications in foods-a review. Int. J. Food Microbiol. 2: 223-253.

Castaño HI, Ciro G, Zapata JE, Silva L (2010). Antibacterial activity of an ethanolic extract and essential oil from *Rosmarinus officinalis* L. leaves on some bacteria with food interest. Revista de la Facultad de Química Farmacéutica. 17(2): 149-154. (in Spanish)

Castillo-Godina R (2008). Antibacterial and antifungal activity of polyphenolic compounds from typical semi-desert plants. [BSc thesis]. Universidad Autónoma de Coahuila unidad Saltillo, México, p. 60 (In Spanish).

Cock IE (2008). Antibacterial activity of selected Australian native plant extracts. Int. J. Microbiol. 4: 2.

Cowan MM (1999). Plant products as antimicrobial agents. Clin. Microbiol. Rev. 12: 564–582.

Dupont S, Caffin N, Bhandari B, Dykes GA (2006). *In vitro* antibacterial activity of Australian native herb extracts against food related bacteria. Food Control 17: 929–932.

Guerrero-Rodríguez E, Solís-Gaona S, Hernández-Castillo FD, Flores-Olivas A, Sandoval-López V, Jasso-Cantú D (2007). Biological activity *in vitro* of *Flourensia cernua* D. C. extracts against post-harvest pathogens: *Alternaria alternata* (Fr.:Fr.) Keissl. *Colletotrichum gloeosporioides ((*Penz.) Pens. y Sacc). y *Penicillium digitatum* (Pers.:Fr.) Sacc. Revista Mexicana de Fitopatología 25(1): 48-53 (in Spanish).

Iwalokun BA, Ogunledun A, Ogbolu DO, Bamiro SB, Jimi-Omojola J (2004). *In vitro* antimicrobial properties of aqueous garlic extract against multidrug-resistant bacteria and C*andida* species from Nigeria. J. Med. Food 7: 327-333.

Lira-Saldivar RH, Balvantín GF, Hernández-Castillo FD, Gamboa A, Jasso de Rodriguez D, Jiménez DF (2003). Evaluation of resin content and antifungal effect of *Larrea tridentata* (Sesse and Moc. Ex D. C. Coville) extracts against *Pythium* sp. Pringsh. Rev. Mex. Fitopatol. 21: 97–101 (in Spanish).

López-Guerra NL, Belmares-Cerda R, Contreras-Esquivel C, Rodríguez-Herrera R, Aguilar CN (2007). Biotechnological potential of Creosote bush (*Larrea tridentata* cov.) Revista CienciAcierta 11: 33-35 (in Spanish).

Makkar PS (1999). Quantification of Tannins in Tree Foliage: A Laboratory Manual for FAO/IAEA. Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, Vienna, Austria. pp. 5–7.

Montes BR, Cruz CV, Martinez MG, Sandoval GG, García LR, Zilch DS, Bravo LL, Bermudez TL, Flores HE (2000). Antifungal properties in superior plants. Retrospective analysis of researches. Rev. Mex. Fitopatol. 18: 125–131 (In Spanish).

Mounchid K, Bourjilat F, Dersi N, Aboussaouira T, Rachidai A, Tantaoui-Elaraki A, Alaoui-Ismaili M (2005). The susceptibility of *Escherichia coli* strains to essential oils of *Rormarinus officinalis* and *Eucalyptus globulus*. Afr. J. Biotechnol. 4(10): 1175-1176.

Natta L, Orapin K, Krittika N, Pantip B (2008). Essential oil from five *Zingiberaceae* for anti food-borne bacteria. Int. Food Res. J. 15: 337–346.

Osorio E, Flores M, Hernández D, Ventura J, Rodríguez R, Aguilar C (2010). Biological efficiency of polyphenolic extracts from pecan nuts shell (*Carya illinoensis*), pomegranate husk (*Punica granatum*) and creosote bush leaves (*Larrea tridentata* Cov.) against plant pathogenic fungi. Ind. Crop. Prod. 31(1): 153-157.

Puangpronpitag D, Sittiwet C (2009). Antimicrobial properties of *Cinnamomum verum* aqueous extracts. Asian J. Biol. Sci. 2: 49–53.

Pundir RK, Jain P, Sharma C (2010). Antimicrobial activity of ethanolic extracts of *Syzygium aromaticum* and *Allium sativum* against food associated bacteria and fungi. Ethnobotanical Leaflets 14: 344–360.

Scalbert A, Williamson G (2000). Dietary intake and bioavailability of polyphenols. J. Nutr. 130: 2073–2085

Sharma KD, Hashinga F (2004). Alpinia leaf extract: A prospective natural food preservative. J. Sci. Ind. Res. 63: 689-693.

Sutherland J, Miles M, Hedderley D, Li J, Devoy S, Sutton K, Lauren D (2009). *In vitro* effects of food extracts on selective probiotic and pathogenic bacteria. Int. J. Food Sci. Nutr. 60:717-727.

Swain T, Hillis WE (1959). The phenolic constituents of *Prunus domestica* L. the quantitative analysis of phenolic constituents. J. Sci. Food Agric. 10: 63–68.

Tayel AA, El-Tras WF (2010). Anticandidal activity of pomegranate peel extract aerosol as an applicable sanitizing method. Mycoses 53: 117–122.

Tequida M, Cortez R, Rosas B, López S, Corrales M (2002). Effect of alcoholic extracts of wild plants on the inhibition of growth of *Aspergillus flavus*, *Aspergillus niger*, *Penicillium chrysogenum*, *Penicillium, expansum*, *Fusarium moniliforme* and *Fusarium poae* moulds. Rev. Iberoam. Micol. 19: 84–88 (in Spanish).

Urzua A, Jara F, Tojo E, Wilkens M, Mendoza L, Rezende MC (2006). A new antibacterial clerodane diterpenoid from the resinous exudate of *Haplopappus uncinatus*. J. Ethnopharmacol. 103: 297–301.

Viveros-Folleco J, Castaño-Zapata J (2006). Evaluation *in vitro* of vegetal extracts against *Mycosphaerella fijiensis* Morelet. Agron. 14(1): 37-50 (in Spanish).

Waterman PG, Mole S (1994). Methods in ecology. Analysis of phenolic plant metabolites. Blackwell Scientific publications. p. 237.

UPCOMING CONFERENCES

3rd Biotechnology World Congress, Dubai, UAE, 10 Feb 2014

3rd Biotechnology World Congress

February 10th - 12th, 2014, Dubai, UAE

7th Annual World Congress of Industrial Biotechnology (IBIO-2014), Dalian, China, 25 Apr 2014

ibio-2014

BIT's 7th Annual Congress of

Industrial Biotechnology

Theme: Greener Manufacturing in Synbio Era

Date: April 25-28, 2014 Place: Dalian, China

Conferences and Advert

February 2014

3rd Biotechnology World Congress, Dubai, UAE, 10 Feb 2014

International Biotechnology, Chemical Engineering and Life Science Conference (IBCELC), Honolulu, USA, 14 Feb 2014

April 2014

XI International Symposium on Plant Biotechnology, Jardines del Rey archipelago, Cuba, 9 Apr 2014

5th International Conference on Biotechnology and Food Science (ICBFS 2014), Erzurum, Turkey, 24 Apr 2014

7th Annual World Congress of Industrial Biotechnology (IBIO-2014), Dalian, China, 25 Apr 2014

African Journal of Biotechnology

Related Journals Published by Academic Journals

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

academicJournals