African Journal of Biotechnology

Volume 16 Number 24, 14 June, 2017

ISSN 1684-5315

ABOUT AJB

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

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

Contact Us

Editorial Office: ajb@academicjournals.org

Help Desk: helpdesk@academicjournals.org

Website: http://www.academicjournals.org/journal/AJB

Submit manuscript online http://ms.academicjournals.me/

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. 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

African Journal of Biotechnology

Table of Content: Volume 16 Number 24 14 June, 2017

ARTICLES

Value addition of orange fruit wastes in the enzymatic production of xylooligosaccharides 1324
Praveen Kumar Gupta, Pushpa Agrawal and Prabha Hegde

Immunocapture and simple-direct-tube-reverse transcriptase polymerase chain reaction (RT-PCR) for detection of *Rice yellow mottle virus* 1331
Judith Hubert, Herman John Faraji Lyimo, Ashura Luzi-Kihupi and Ernest Rashid Mbega

Phytochemical screening and amino acids analysis of mushrooms from Burkina Faso 1338
Elise Sanon, Jean Claude W. Ouédraogo, Sylvain Ilboudo, Marie K. Laure Guissou, Pierre I. Guissou and Philippe Sankara

Establishment, multiplication, rooting and acclimatization of *Cabralea canjerana* (Vell.) Martius 1345
Eliseo Salvatierra Gimenes, Nathalia Pimentel, James Hancock and Dilson Antônio Bisognin

Optimised cetyltrimethylammonium bromide (CTAB) DNA extraction method of plant leaf with high polysaccharide and polyphenolic compounds for downstream reliable molecular analyses 1354
Turaki A. A.,, Ahmad B., Magaji U. F., Abdulrazak U. K., Yusuf B. A. and Hamza A. B.,

academicJournals

Vol. 16(24), pp. 1324-1330, 14 June, 2017
DOI: 10.5897/AJB2017.15927
Article Number: 4711BEE64715
ISSN 1684-5315
Copyright © 2017
Author(s) retain the copyright of this article
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Value addition of orange fruit wastes in the enzymatic production of xylooligosaccharides

Praveen Kumar Gupta*, Pushpa Agrawal and Prabha Hegde

Department of Biotechnology, R.V College of Engineering, Bangalore-560059, India.

Received 3 February, 2017; Accepted 30 May, 2017

Xylooligosaccharides (XOS) are sugar oligomers made up of xylose units with the chain length of 2 to 10 and are considered non-digestible food ingredients. It is mainly produced from xylan hydrolysis. XOS have a characteristic of prebiotic by promoting the growth of probiotic organisms. They have various physiological effects such as reducing cholesterol, maintaining the gastrointestinal health and improving the biological availability of calcium, animal feed, anti-cancerous. The production of XOS from agricultural residues offers great scope to the nutraceutical and pharmaceutical industries as the raw material is cheap and abundantly available. The driving force of this study was to produce XOS from the organic wastes such as orange fruit wastes. These wastes are rich in xylan which can be used as a renewable material for producing XOS. XOS can be obtained by chemical or enzymatic method, but due to the yield of toxic by-product, enzymatic production is preferred. In the enzymatic extraction method, acetic acid was used to prepare pellets from dried orange peels powder followed by xylanase enzyme degradation performed at 2, 4, 6 and 8 h. Samples containing XOS were chromatographed on HPLC system having a fluorescence detector (Ex320 nm, Em420 nm). The column used was Agilent C18 of length 250 mm and 4.6 mm internal diameter.

Key words: Xylooligosaccharide, xylobiose, xylotriose, prebiotic, xylan, enzymatic extraction, HPLC.

INTRODUCTION

The oligosaccharides with low degree of polymerization (DP 2-20 monomers) are considered as potential non-digestible sugars known for their benefits as dietary fibers. Recently, the significance of non-digestible oligosaccharides (NDOs) have been realized mainly due to their properties like sweeting ability water binding capacity, fat replacement value and notably their confrontation to digestion in higher region of gastrointestinal tract and maximum possibility of fermentation in the large bowel. The non-digestible oligosaccharides can be incorporated into processed food and could be promising functional ingredients in nutraceutical products (Gibson et al., 2004; Nyangale et al., 2012; Rossi et al., 2011). The utility is not the only parameters for these nutraceutical products; it is also economically and environmentally feasible as well as offers an opportunity for agriculture and food industries to produce value added products from agriculture or fruit

*Corresponding author. E-mail: praveenkgupta@rvce.edu.in. Tel: +91-9742620113.

Author(s) agree that this article remains permanently open access under the terms of the Creative Commons Attribution License 4.0 International License

wastes. As per the Stowell classification, the xylooligosaccharides (XOS) is categorized under emerging prebiotics whereas several other common prebiotics has different origin and chemical properties such as inulin, fructooligosacchrides (FOS), galactooloigosacchrides (GOS), lactulose and polydextrose, etc (Stowell, 2006).

According to a survey conducted on 1000 randomly selected consumers by the International Food Information Council (IFIC) in Washington, D.C. in 1998, it was observed that more than 95% of people believe that certain foods have various health assistances which completely depends on quality on rudimentary nutrition and can have a chance to reduce the risk of disease; whereas 92% people believe that they can control their own health by changing the life style, 78% of people reportedly name a particular food or component completely associated with various health benefits. Various food materials and green vegetables such as broccoli, carrots, fish and fish oil, oranges and orange juice, garlic, ginger, cucumber and milk were mentioned mostly by consumers, in that order (AsiaOne, 2015).

In this sense, orange have been shown to be a promising good food due to having variety of phytochemicals such as carotenoids which includes (beta-carotene, lutein and beta-cryptoxanthin), flavonoids in form of abundant volatile organic compounds producing orange aroma mainly including aldehydes, esters, terpenes, alcohols, and ketones (Elaine, 2011; Ensminger et al., 1986).

Orange fruit wastes mainly contain soluble sugars and pectin. According to Rivas et al. (2008), the orange fruit wastes constituted highest percentage of soluble sugars with 16.9% wt, followed by starch (3.75% wt), fiber which includes cellulose (9.21% wt), hemicelluloses (10.5% wt), lignin (0.84% wt) and pectines (42.5% wt). The high percent of ashes (3.50% wt) and proteins (6.50% wt). The least amount of fats, 1.95% wt is present in orange fruit wastes. The total cellulose content in orange fruit wastes ranged from 12.7 to 13.6% and hemicellulose from 5.3 to 6.1%. After all this consideration, the cost of materials becomes about 45% lower than that of conventional pulp of other fruits. Moreover, the dried orange fruit wastes showed lower fat concentration which will increase further the prebiotics components (Rivas et al., 2008; Beg et al., 2001). In addition, the recent studies showed that due to presence of hemicellulose and pectin the orange peels can be utilized to produce prebiotics materials such as xylooligosacchrides, fructooligosacchrides and pectin (Ma et al., 1993; Grohmann et al., 1995).

Though the orange fruit wastes contain maximum amount of pectic oligosaccharides, it has low prebiotic potential as compared to XOS and even FOS. Also, the cost of producing prebiotic materials such as XOS becomes 45% lower than that of other conventional fruit pulp industries as shown in the earlier research (Olano-Martin et al., 2002). Hence, the current research prefers XOS production from orange fruit wastes.

The pre-treatment of hemicellulose is one of the most important steps in the process to recover xylan from dried orange fruit wastes powder. The most common NaOH alkaline pre-treatment methods is used to separate hemicellulose content from other cellulosic waste materials such as lignin, pectines, fat substances and protein materials (Samanta et al., 2012a, b).

In order to produce the maximum xylan content, high alkaline concentration is required. Hence in the current research work the alkaline concentration was taken in varying concentration with water such as 2, 4, 6, 8, 10 and 12%, respectively. The main goal of pre-treatment alkaline or enzymatic hydrolysis is to modify or eliminate the structural and compositional inhibitions of waste materials present in hemicellulose other than desired materials to increase the yields of intended products such as XOS (Hendriks and Zeeman, 2009).

MATERIALS AND METHODS

Xylan extraction and production of XOS from extracted xylan were two major important things in the current research work.

Phase 1: xylan extraction

Xylan extraction involves the treatment of powdered orange fruit wastes with alkaline solution based on the use of NaOH (Sigma, India) followed by steam extraction. Based upon earlier investigation as well as trial and error analysis, 5 g of sample was chosen initially to extract xylan. To evaluate and optimize the condition, minimum of three trials were performed at each parameter using different concentrations (2, 4, 8 and 12%) of NaOH followed by steam treatment (120°C, 15 lbs pressure for 45 min). The solid to liquid ratio was 1:10. The alkali-solubilized xylan was centrifuged at 5,000 rpm for 20 min and filtered first by zero filter paper followed by Whatman filter paper 40. By using glacial acetic acid at pH 5.0, the acidified condition was maintained to supernatant. On addition of 3 vol. of ice-cold, 70% ethanol xylan was extracted. Later, the solution centrifuged at 8,000 rpm for 10 min at room temperature and xylan was precipitated and dried in hot air oven at 60°C till it reached persistent weight. LastLY, the dried pellets were stoked in powder form at room temperature for further analyses (Samanta et al., 2012a, b; 2013; Hsiao, 2006). The extracted xylan was characterized using Fourier Transform – Infrared Spectra (FTIR) and the true yield of xylan was calculated using the following formula:

$$\text{True yield (\%)} = \frac{\text{Dry weight of extracted xylan (g)} \times 100}{\text{Weight of the sample (g)}}$$

Production of XOS from extracted xylan

The production of XOS was performed using enzymatic hydrolysis on extracted xylan pellets obtained from dried orange fruit wastes powder. The enzymatic hydrolysis steps were carried out using endoxylanase enzyme extracted from *Trichoderma viride* procured from Sigma, India. In order to carry out enzymatic hydrolysis process, first enzyme buffer solution was prepared. Therefore,

Table 1. Variables for condition optimization.

Variables	XOS concentration	Ideal condition to maximize the response
pH		
4.5	9.48071 E-2±.0004	
5.5	6.303752 E-1 ± .0009	pH: 5.5
6.5	9.20759 E-2± .0003	
P value	2.99E-06	
Temperature		
30	1101479±.0007	
40	630.3752 ± .0009	Temperature: 40
50	0493784±.0004	
P value	2.58E-05	
Enzyme dose		
2.65	3.7040E-4±.0002	
5.30	1.51320 E-2±.0003	
7.95	8.47607 E-2 ±.004	Enzyme concentration 10.60
10.60	630.3752 ± .0009	
13.25	1.03005 E-1 ± .004	
P value	4.68E-05	
Alkaline concentration		
2% (5 g in 250 ml)	6.10963 E-2±.0007	
4% (10 g in 250 ml)	5.73150 E-2±.0004	
8% (20 g in 250 ml	2.13.2163 E-1±.0004	Alkaline concentration: 12%
12% (30 g in 250 ml)	630.3752 ± .0009	
15% (37.5 g in 250 ml)	7.30759 E-2 ± .0006	
P value	0.0007	
Incubation period (h)		
2	0.1700659 ± .008	
4	0.0817579 ± .005	
6	630.3752 ± .0009	Incubation period: 6 h
8	0.04159974 ± .006	
10	0.0055916± .001	
12	0.01565706± .002	

initially, 10 ml of sodium citrate buffer was taken in round neck conical flask. The initial substrate concentration (2%) was chosen as per the previous research (Akpinar et al., 2009). The pH (4.5, 5.5, 6.5), temperature (30–50°C) and enzyme dose (2U, 4U, 6U 8U and 10U when 1U= 2.65) were varied to optimise the conditions, in order to produce maximal conversion of xylan to XOS. The flasks, containing the reaction mixtures, were put in the incubator having shaking speed of 150 rpm. Aliquots were taken at fixed time intervals (2, 4, 6, 8, 10 and 12 h, respectively) and assayed. The enzymatic reaction was stopped by boiling it for 10 min before being subjected to the various analyses. Duplicate samples for enzyme hydrolysis were set up for each of the experiments. To quantify the final product, first reducing sugars, followed by HPLC analysis were considered. For HPLC analysis, the refractive index detector having ZORBAX carbohydrate column (Agilent, USA) were used (Samanta et al., 2012a, b; 2013; Hsiao, 2006).

Statistical methods

The statistical approach was required to enhance the production of XOS under different variables used in the current investigation. The main purpose of the statistical analysis was to increase the XOS production (X1-X5). The variables were identified based upon earlier investigation and optimized mainly based upon trial and error analysis. The statistical analysis was performed using MS Excel 2010 statistical add on tools in order to justify the maximum significant variation in XOS production. Table 1 shows the variables identified for condition optimization.

RESULTS AND DISCUSSION

FTIR analysis

In the current investigation, the FTIR spectroscopy analysis was applied to the 12% alkaline concentration xylan pellets obtained after the treatment of dried orange peels powder with 12% alkaline solution prepared from NaOH followed by steam treatment (120°C, 15 lbs

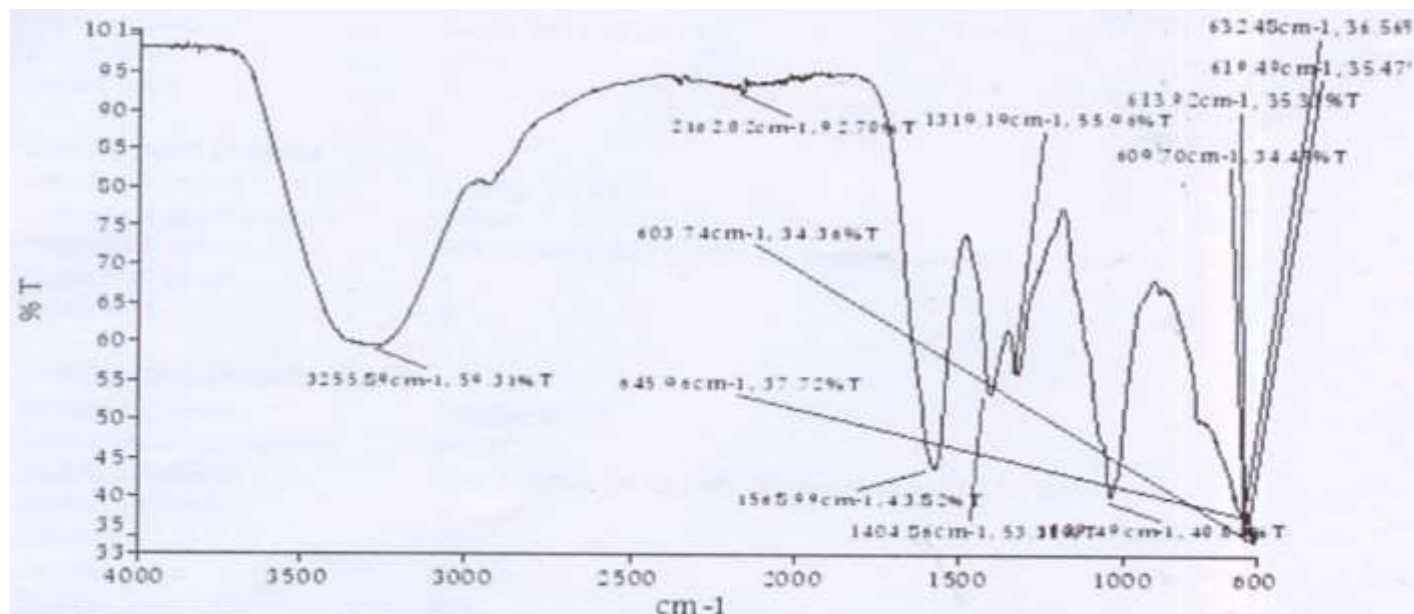


Figure 1. FT-IR spectra of the xylan obtained from dried orange peels powder treated 12% NaOH solution followed by steam application for 45 min.

pressure for 45 min). The solid to liquid ratio was 1:10. The alkali-solubilized xylan was centrifuged at 5,000 rpm for 20 min and filtered first by zero filter paper followed by Whatman filter paper 40. By using glacial acetic acid at pH 5.0 the acidified condition was maintained to supernatant. On addition of 3 volume of ice-cold 70% ethanol, xylan was extracted (Griffiths and De Haseth, 2007).

Figure 1 shows the observed absorption bands at 3000-3300 = 3255 cm^{-1} which recognizably extended the H-bonded OH groups existing in extracted xylan. Hence, it is confirmed that there was removal of acetyl, uronic acid, or ester groups in the alkaline treatment because no band at 1,736 cm^{-1} was observed. Although it is difficult, it is possible to identify removal of all lignocellulosic-based impurities in the extracted xylan using FTIR technique. However, in one study it has been reported that the xylan has got strong affinity towards water molecules which was not shown in band but may be due to xylan being prepared from completely dried orange peels powder (Samanta et al., 2013). The current FTIR was operated at 4000–650 cm^{-1} spectral range having a resolution of 0.9 cm^{-1} using a MIR source and KBr beam splitter and LiTa03 Detector (7800 to 350 cm^{-1}) (Software version CPU32 main 00.09.9951).

From the FTIR spectra, it can be said that the presence of the C–H and C–O or OH are confirmed because the intense bands at 1505 cm^{-1} were observed. The C–H and C–O or OH comprises bending vibrations in xylan. Similarly, the C–O, C–C stretching, or C–OH bending was confirmed in the xylan due to observation found at 1,027 cm^{-1} in form of vibrations. Two additional absorption bands were also seen in the FTIR spectra of current investigation at the range of 645 and 600 cm^{-1}. It may be due to possible stretching or bending of C–C–H or C– O–C (Samanta et al., 2012a, b; 2013; Hsiao, 2006).

Dinitrosalicylic (DNS) acid method

The current research work was conducted at fixed condition on precisely quantifying the enzyme activities of endoxylanase on extracted xylan pellets in order to measure the reducing sugar amount. The investigation was based upon different concentration of standards taken in increasing volumes of concentrations of 1 mg/100 ml along with samples of increasing volumes of concentrations of 2, 4, 8, and 12%. Incubation period was varied in 2, 4, 6, 8, 10 and 12 h, respectively.

The varying sample determination was chosen based upon a method explained by Nelson-Somogyi (also known as arsenomolybdate assay) (Somogyi, 1952). Each experiment was done in triplicate to avoid any ambiguity in OD values. The xylose concentration was calculated using the equation below:

Concentration of reducing sugar = Extrapolation on the X axis x Dilution factor

The graph was plotted using MS Excel 2007 and the R value was measured (Somogyi, 1952; Akpinar et al., 2007). For each different concentration of standards and samples, ANOVA was analyzed separately using MS

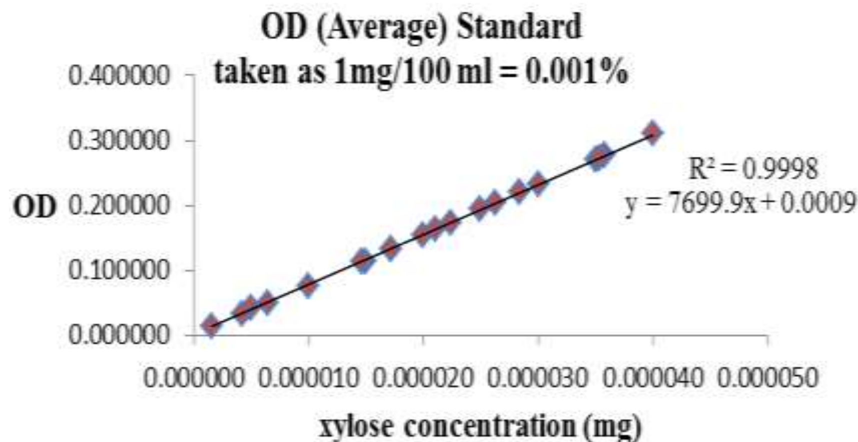


Figure 2. Determination of xylose by using DNS assay method. Standard taken as 1 mg/100 ml = 0.001%.

Excel 2007 version.

Figure 2 shows the xylose as standard (1 mg/100 ml = 0.001% carried out) and the concentration of reducing sugar was found to be 3.58E-05 and 3.54E-05 in 2 and 4 ml respectively for 12% unknown sample solution. The result was analyzed using ANOVA and interpreted mainly based on the F value and lowest P value. With this criteria, the P value was observed to be P = 1.06E-09*** which is lower than P =0.005. Also, F value is higher than P and Fcrit which was also found in earlier investigations (Samanta et al., 2012b, 2013; Hsiao, 2006; Akpinar et al., 2009) (*< = 0.5, ** <= 0.01 to 0.05 and *** <= 0.001-0.005).

High performance liquid chromatography analysis

The present investigation carried out HPLC analysis in order to perform qualitative and quantitative analysis of XOS based on their variable DP ranges. The HPLC system (Agilent, USA) was equipped with refractive index detector and the mobile phase used for XOS elution comprising acetonitrile and water in the proportion of 63:37. The flow rate was maintained at 0.5 ml/min. During the investigation the ZORBAX carbohydrate column (Agilent, USA) was used and the column temperature was fixed at 25°C (Samanta et al., 2012b). The sample was filtered using 0.2 µm membrane (Minigen USA- MG-25020PVDF) and around 20 ul samples was injected in the column using manual injector. The total HPLC investigation for one sample was achieved in around 30 min. The quantification of sample XOS was based upon comparison of average peak areas compared with the standards XOS (X1–X5= (xylose, xylobiose, xylotriose, xylotetrose and xylopentose). The quantification was expressed in ng/ml and converted to mg/ml for further analysis (Samanta et al., 2012b; Akpinar et al., 2007). All standards were provided and analysis was performed at National Institute of Animal Nutrition and Physiology Bangalore.

The Figure 3 displayed the quantification of HPLC result at 12% alkaline concentration and varying incubation period which showed that the 6 h incubation period has produced maximum amount of XOS (X1-X5) 630.38 ng/ul. Hence 1 g of xylan will produce 0.63 mg of XOS in given condition which is maximum in all other incubation periods. Therefore, the 6 h incubation period was chosen to best carry out further analysis.

Orange fruit wastes are becoming one of the largest amount of organic waste which are dumbed with other garbage and litters and since these waste are unprocessed, therefore the chance for development of air borne disease, bad odor and air pollutions are very high (Wu et al., 2004; Peng et al., 2009; Sukri et al., 2014; Marín et al., 2007). In this context few study research showed that around 50-60% of the total orange fruits are converted into peel waste (Wu et al., 2004; Peng et al., 2009) which gives a consequence that the continuous increasing in peel waste day to day especially near to fruits processing industries. Due to unawareness towards disposal method and the need to recycle these wastes, these organic wastes are becoming a serious environmental issues and health associated disorders considerations (Sukri et al., 2014; Marín et al., 2007). Among the different agro industrial residues, the usages of orange peels for XOS production have given new organic waste sources which were not mentioned in the earlier investigations. The present study demonstrated the application of alkali and steam treatment for extraction and recovery of xylan from orange peels waste. Extraction with sodium hydroxide in combination with steam application was used for achieving higher recovery of xylan. The enzymatic hydrolysis of xylan using the commercial endoxylanase enzyme from *T.*

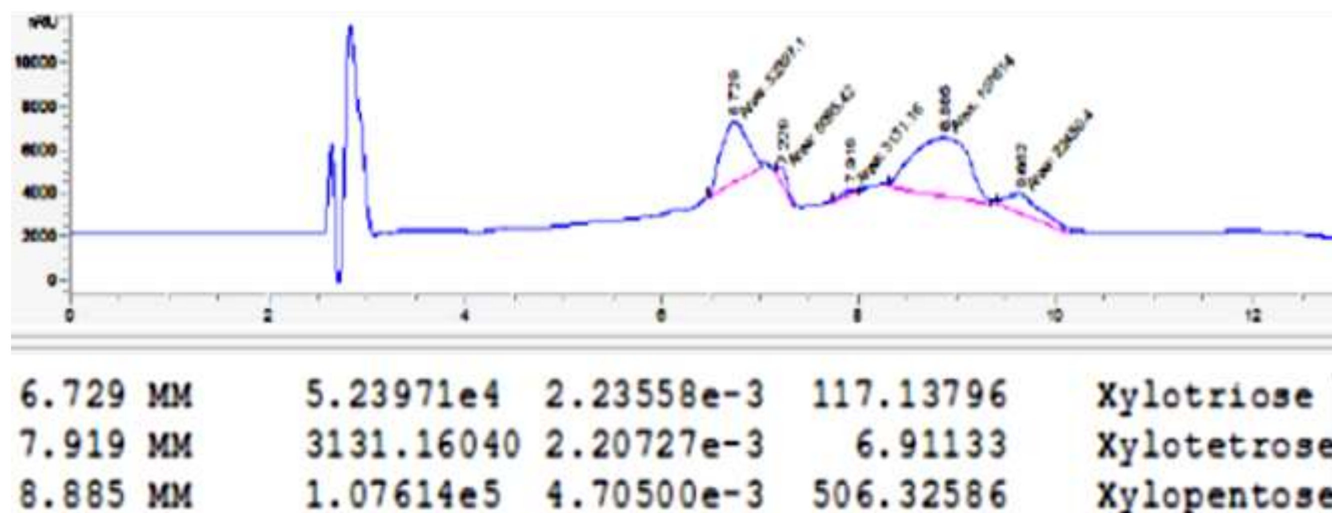


Figure 3. HPLC Chromatogram -Condition fixed at 12% alkaline concentration 6 h incubation period.

viride enabled production of XOS in the DP range of 1-5, that is, xylose and xylopentose.

Though the XOS production techniques uses both chemical and enzymatic classically, in the current investigation, the enzymatic method is preferred to avoid any toxic material production and to improve edibility performance which is significantly higher in terms of toxicity and lower edibility. Moreover, the pH is much more balanced in enzymatic method of XOS production. The statistical methods employed defined the point wherein maximum production of XOS could be achieved which was further proved experimentally.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors are grateful to the Principal and all other staff members of Department of Biotechnology, RVCE Bangalore for granting the permission to carry out this research work along with extending the financial support to undertake the proposed research.

REFERENCES

Akpinar O, Ak O, Kavas A, Bakir U, Yilmaz L (2007). Enzymatic production of xylooligosaccharides from cotton stalks. J. Agric. Food Chem. 55:5544-5551.

Akpinar O, Erdogan K, Bostanci S (2009). Enzymatic production of Xylooligosaccharide from selected agricultural wastes. Food Bioproducts Process. 87:145-151.

AsiaOne (2015). 4 Food that act as natural medicine, Feb 13. Retrieved from http://www.asiaone.com/print/News/Latest%2BNews/Health/Story/A1Story20121002-374998.html.

Beg QK, Kapoor M, Mahajan L, Hoondal GS(2001). Microbial Xylanases and their industrial applications: A review. Appl. Microbiol Biotechnol. 56:326-338.

Elaine W (2011). Survey shows sharp rise in awareness of pre- and probiotics (2011 Aug 08). Retrieved on (2014 July) from http://www.foodnavigator-usa.com/Markets/Survey-shows-sharp-rise-in-awareness-of-pre-and-probiotics.

Ensminger AH, Ensminger ME, Konlande JE, Robson JRK (1986). Food for Health: A Nutrition Encyclopedia. Clovis, California: Pegus Press. 1:178.

Gibson GR, Probert HM, Van Loo JAE, Roberfroid MB (2004). Dietary modulation of the human colonic microbiota: Updating the concept of prebiotics. Nutr. Res. Rev. 17:257-259.

Griffiths PR, De Haseth JA (2007). Fourier Transform Infrared Spectrometry. John Wiley & Sons, 2nd Edition, ISBN 9780470106297.

Grohmann K, Cameron RG, Buslig BS (1995). Fractionation and pretreatment of orange peel by dilute acid hydrolysis. Bioresour. Technol. 54:129-141.

Hendriks ATWM, Zeeman G (2009). Pretreatments to enhance the digestibility of lignocellulosic biomass. Bioresour. Technol. 100(1):10-18.

Hsiao SY (2006). Study in production of xylooligosaccharides from rice straw and sugarcane bagasse. Ph.D. dissertation. Retrieved Dec. 2014 at http://www.aspergillus.org.uk/content/study-production-xylooligosaccharides-rice-straw-and-sugarcane-bagasse.

Ma E, Cervera Q, Mejía Sánchez GM (1993). Integrated utilization of orange peel. Bioresour. Technol. 44:61-63.

Marín FR, Soler-Rivas C, Benavente-García O, Castillo J, Pérez-Alvarez JA (2007). By-products from different citrus processes as a source of customized functional fibres. Food Chem. 100(2):736-741.

Nyangale EP, Mottram DS, Gibson GR (2012). Gut microbial activity, implications for health and disease: the potential role of metabolite analysis. J. Proteome Res. 11:5573-5585.

Olano-Martin E, Gibson GR, Rastall RA (2002). Comparison of the *in vitro* bifidogenic properties of pectins and pectic-oligosaccharides. J. Appl. Microbiol. 93:505-511.

Peng F, Ren JL, Xu F, Bian J, Peng P, Sun RC (2009). Comparative study of hemicelluloses obtained by graded ethanol precipitation from sugarcane bagasse. J. Agric. Food Chem. 57:6305-6317.

Rivas B, Torrado A, Torre P, Converti A, Domínguez JM (2008). Submerged citric acid fermentation on orange peel autohydrolysate. J. Agric. Food Chem. 56:2380-2387.

Rossi M, Amaretti A, Raimondi S (2011). Folate production by probiotic bacteria. Nutrients 3:118-134.

Samanta AK, Jayapal N, Kolte AP, Senani S, Sridhar M, Mishra S, Prasad CS, Suresh KP (2013). Application of Pigeon Pea (Cajanus cajan) Stalks as Raw Material for Xylooligosaccharides Production. Appl. Biochem. Biotechnol. 169:2392-2404

Samanta AK, Jayapal N, Kolte AP, Senani S, Sridhar M, Suresh KP, Sampath KT (2012a). Enzymatic production of xylooligosaccharides from alkali solubilized xylan of natural grass (Sehima nervosum). Bioresour. Technol. 112:199-205.

Samanta AK, Senani S, Kolte AP, Sridhar M, Sampath KT, Jayapal N, Devi A (2012b). Production and *in vitro* evaluation of xylooligosaccharides generated from corn cobs. Food Bioproducts Process. 90:466-474.

Somogyi M (1952). Notes on sugar determination. J. Biol. Chem. 195:19-23.

Stowell J (2006). Calorie control and weight management. In. Mitchell H (ed) Sweeteners and sugar alternatives in food technology, Blackwell Publishing Ltd.

Sukri SS, Rahman RA, Illias RM, Yaakob H (2014). Optimisation of Alkaline Pretreatment Conditions of Oil Palm Fronds in Improving the Lignocelluloses Contents for Reducing Sugar Production. Rom. Biotechnol. Lett. 19(1):9006-9018.

Wu N, Dempsey J, Yehl PM, Dovletoglu A, Ellison A, Wyvratt J (2004). Practical aspects of fast HPLC separations for pharmaceutical process development using monolithic columns. J. Anal. Chim. Acta 523(2):149-156.

academicJournals

Vol. 16(24), pp. 1331-1337, 14 June, 2017
DOI: 10.5897/AJB2017.16036
Article Number: AA612A664717
ISSN 1684-5315
Copyright © 2017
Author(s) retain the copyright of this article
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Immunocapture and simple-direct-tube-reverse transcriptase polymerase chain reaction (RT-PCR) for detection of *Rice yellow mottle virus*

Judith Hubert[1,2]*, Herman John Faraji Lyimo[1], Ashura Luzi-Kihupi[1] and Ernest Rashid Mbega[3]

[1]Department of Crop Science and Horticulture, Sokoine University of Agriculture, P. O. Box 3005, Morogoro, Tanzania.
[2]Africa Rice Center (AfricaRice), P. O. Box 33581, Dar es Salaam, Tanzania.
[3]School of Life Science and Bio-Engineering, Nelson Mandela African Institution of Science and Technology, P. O. Box 447, Tengeru, Arusha, Tanzania.

Received 17 April, 2017; Accepted 30 May, 2017

This study aimed at optimizing the immunocapture (IC) and simple-direct-tube (SDT) -reverse transcriptase polymerase chain reaction (RT-PCR) techniques for detection of *Rice yellow mottle virus* (RYMV) in order to avoid the extraction of high quality RNA required for molecular methods and avoid costs involved. *Rice yellow mottle virus* strains and phylotypes were obtained from infected rice leaf samples collected from Morogoro, Arusha and Kilimanjaro regions. The efficacy and sensitivity of IC and SDT methods was demonstrated using the aliquots from infected plant sap obtained by grinding rice leaves and binding onto PCR tube using coating buffer and in phosphate buffer saline with 0.5% Tween-20 (PBST 1X), respectively, and assayed by RT-PCR with RYMVIIIF/RYMVIIR primers. Analysis of the PCR product was performed by electrophoresis on 1% agarose gel, pre-stained with 2.5 µl of ethidium bromide (10 µg of ethidium bromide per ml of 0.5x Tris-acetate-ethylenediaminetetraacetic acid (TAE) buffer) at 100 V cm^{-1} for 30 min and visualized under UV light. The results indicate that SDT-RT-PCR and IC-RT-PCR detected RYMV in all tested infected leaf samples at the expected band size of 720 bp and had the same sensitivity as virus extraction RNA-RT PCR technique, implying that the methods can be useful for detection of wide range of RYMV strains. The negative control did not yield any amplicons. The results also show that these techniques are rapid methods for characterization of RYMV strains and may be recommended for use soon after periodical surveys to quickly identify new strains for breeding purposes at low cost. However, SDT protocol was easier and faster than IC and it was also cost-effective in terms of reagents for the detection of RYMV.

Key words: *Rice yellow mottle virus*, detection, immunocapture-RT-PCR, simple-direct-tube-RT-PCR.

INTRODUCTION

Rice yellow mottle virus (RYMV) is a variable and very damaging rice disease in Africa. A number of biotic stresses including RYMV have been reported to contribute to low yields of rice in Africa (Lamo et al.,

*Corresponding author. E-mail: hubertjudith@yahoo.com Tel: (+255 688 410 067).

Author(s) agree that this article remains permanently open access under the terms of the Creative Commons Attribution License 4.0 International License

2015). Yield estimates of 2.5 t ha^{-1} have been reported in Africa compared to 4.4 t ha^{-1} reported in Asia (Wilson and Lewis, 2015). Rice is an important staple food crop and is affected by RYMV disease with suspected high yield losses ranging from 25 to 100% (Kouassi et al., 2005; Luzi-Kihupi et al., 2009). The virus disease was first recorded in 1966 at Otonglo near Lake Victoria, Kenya (Bakker, 1970) and until now the RYMV is present in most of the rice-growing countries in Africa and Madagascar (Abo et al., 1998; Kouassi et al., 2005; Séré et al., 2008; Traoré et al., 2009). The virus belongs to the genus *Sobemovirus* (Tamm and Truve, 2000; Fauquet et al., 2005) and is characterized by icosahedral particles of about 30 nm in diameter (Fauquet et al., 2005) and one single strand positive sense genomic ribonucleic acid (RNA). The virus is associated with viroid-like satellite RNA ranging from 220 to 390 nucleotides (Tamm and Truve, 2000).

The RYMV is highly diverse in nature due to high rates of its mutation and recombination (Hébrard et al., 2006). The subgroup diversity of RYMV strains was determined serologically (N'Guessan et al., 2000) and molecularly by RNA sequencing using primers that target sequences encoding coat protein (Pinel et al., 2000). The studies indicated that RYMV encompasses six strains, each having a specific and restricted geographical range. Strains S1, S2 and S3 have been reported to exist in West Africa with latitudes and longitudes between 4°N and 28°N and 15°E and 16°W, respectively. Strains S4, S5 and S6 are reported to dominate in East Africa which lies between latitudes 23°N and 12°S and longitudes 22°E and 51°E (Fargette et al., 2002; Abubakar et al., 2003; Banwo et al., 2004; Kanyeka et al., 2007).

Tanzania has been reported as one of the biodiversity hotspot of RYMV. The emergence of new virulent phylotypes of RYMV in rice crop in different geographical areas is increasing and becoming a common occurrence (N'guessan et al., 2001; Kanyeka et al., 2007; Pinel-Galzi et al., 2007; Ochola et al., 2015), possibly as a consequence of the constantly growing susceptible varieties (Hubert et al., 2016). Therefore, such situations constitute a gap for the demand for reliable methods of sensitive but also rapid detection of these new RYMV phylotypes for control of RYMV in order to improve rice productivity.

Variations of enzyme-linked immunosorbent assay (ELISA) are currently used for large-scale routine testing for viruses. Recently, new ELISA methods for detection of *Rice dwarf virus* (RDV) in rice such as plate-trapped antigen enzyme-linked immunosorbent assay (PTA-ELISA) and dot enzyme-linked immunosorbent assay (dot-ELISA) have been developed (Wu et al., 2014). However, established antigen-trapping ELISA and virus amplification techniques for virus diagnosis require the presence of intact live virus, which depends on sample quality. This situation raised necessity of complementary PCR-based methods such as immuno-capture- and simple-direct-tube RT-PCR (Silva et al., 2011). One area where PCR has shown great value is the detection of viral pathogens, particularly those for which culturing is difficult or where serologically based detection systems are inadequate (Chandler et al., 1998). Polymerase chain reaction has been used successfully to detect a range of viruses including both DNA and RNA viruses (Chandler et al., 1998). However, detection of viral RNA by PCR requires reverse transcription (RT) of viral RNA prior to the reaction (Lopez et al., 2003). Rice viruses, except *Rice tungro bacilliform virus* (RTBV), are RNA viruses, and synthesis of cDNA of the viral genome by reverse transcription (RT) is necessary before the target RNA sequence is amplified (Uehara-Ichiki et al., 2013).

Several variations of RT-PCR have been developed, including nested- (Kiliç and Yardimci, 2012), one step- (Uga and Tsuda, 2005), multiplex- (Grieco and Gallitelli, 1999), real-time (Osman et al., 2007), immunocupture- (Wetzel et al., 1992) and simple-direct-tube RT-PCR (Suehiro et al., 2005). Immuno-capture-RT PCR is designed for highly specific detection of templates present in very low amounts (Wetzel et al., 1992). This method combines capture of virus particles by antibodies through the practical conditions of ELISA with amplification by RT-PCR. In this assay the virus particles are trapped onto a micro centrifuge tube or ELISA plate using virus-specific antibodies. The adsorbed virus particles are disrupted and the released viral nucleic acid is amplified by RT-PCR. Immunocapture (IC)-RT-PCR has been used successfully to detect several plant viruses such as *banana streak virus*, *beet necrotic yellow vein virus*, *cassava common mosaic virus*, *cucumber mosaic virus*, *lily symptomless virus, lily mottle virus*, *pepino mosaic virus*, *plum pox virus* and *sugarcane streak mosaic virus* (Wetzel et al., 1992; Harper et al., 1999; Hema et al., 2003; Mansilla et al., 2003; Ling et al., 2007; Zein et al., 2008; Silva et al., 2011; Ha et al., 2012; Kiliç, and Yardimci; 2012). Generally, immunocapture-RT-PCR protocol has been established for the virus sensitivity improvement in detection (Silva et al., 2011; Yang et al., 2012).

Preparation of plant extracts is a critical aspect of RT-PCR (Rowhani et al., 2004). Nucleic acid extraction from plant tissues is the most laborious, costly and time-consuming step in the detection of a virus (Rowhani et al., 2004). As the RT-PCR is effective in detecting several plant viruses, attempts have been made in this study to optimize simple and rapid techniques such as simple-direct-tube (SDT) method for detection of RYMV. Since 1 h was required to extract viral RNAs from infected tissues, an easy and rapid procedure designated SDT method was optimized for preparing viral RNA for cDNA synthesis.

The extraction of viral RNAs using SDT method may be completed in approximately 15 min and does not require the use of antiserum, filtering and centrifugation (Suehiro et al., 2005). This protocol involves grinding of plant

tissues in phosphate buffer saline with 0.5% Tween-20 (PBST 1X) and placing the extract in a micro-centrifuge tube for two minutes and allowing adsorption of virus particles to the tube wall. Simple direct tube method was successfully used for detection of *Turnip mosaic virus*, *Cucumber mosaic virus* and *Cucumber green mottle mosaic virus* in infected plants (Kobori et al., 2005; Suehiro et al., 2005). The purpose of this work was to optimize and evaluate the efficiency of IC- and SDT- RT-PCR methods for RYMV detection in infected plant materials.

MATERIALS AND METHODS

Rice yellow mottle virus strains used in this study

Infected leaves of RYMV isolates obtained from different strains used for IC and SDT-RT-PCR techniques were collected from farmers' rice fields in the cropping season, April to May 2014 in selected rice growing areas in Tanzania (Hubert et al., 2017). Isolation and characterization of RYMV isolates was done at Institut de Recherche pour le Developpment/French National Research Institute of Sustainable Development (IRD), France. For their characterization, total RNA was extracted with the RNeasy Plant Mini kit (Qiagen, Germany) (Pinel et al., 2000). The coat protein (CP) and the viral protein genome-linked (VPg) genes of the RYMV were amplified by RT-PCR (Fargette et al., 2002; Hébrard et al., 2006). The phylogenetic analysis for identification of RYMV strains and phylotypes was done to compare CP sequences using the maximum likelihood method with default parameters in SeaView software (Gouy et al., 2010). These include RYMV strains and phylotypes S4lm (Tz526), S5 (Tz429, Tz450) , S6c (Tz486) and S6w (Tz539) collected from Kilombero and Ulanga districts (latitudes 7° S and 9° S and longitudes 35°E and 37°E and at an elevation of about 300 m above sea level), Morogoro region. *Rice yellow mottle virus* phylotypes S4lv (Tz516) and S4ug (Tz601) collected from Arusha and Kilimanjaro regions, respectively, were also included. The viral extraction RNA of serological isolate of RYMV phylotype S4lm (Tz554) collected from Ulanga district, Morogoro region was used as control.

Immunocapture (IC) technique

Sterile PCR tubes were coated with 100 µl polyclonal antibodies in coating buffer (pH 9.6, 1:500 IgGs dilution) and incubated at 37°C for 4 h (Silva et al., 2011). The tubes were washed three and two times with phosphate buffer saline with 0.5 % Tween-20 (PBST 1X) and sterile distilled water (SDW), respectively. Two hundred milligrams of each infected leaf sample were ground separately in 400 µl extraction buffer (79.5g NaCL, 1.9 g KH_2P0_4, 11.36 g Na_2HP0_4.anhydrous, 1.93 g KCL, 2% polyvinyl pyrrolidine-PVP, 0.05% Tween 20 in 1000 ml SDW) and centrifuged at 7000 rpm for 7 minutes. Then, 100 µl of each sample supernatant was added accordingly to each PCR tube and incubated at 4°C for 12 h. The tubes were then washed three times with washing buffer and finally with SDW. The tubes were left to air dry for 2 min before proceeding to the RT-PCR step.

Simple-direct-tube (SDT) technique

Half a gram of infected leaves of RYMV isolates were ground using a mortar and pestle in a PBST 1X buffer at a ratio of 1:1 w/v (Suehiro et al., 2005). The crude sap (100 µl) was carefully placed into a sterile PCR tube using micropipette with a truncated tip and incubated at room temperature (25-30°C) for 15 min. The sap was removed from the tube and the tube was then washed twice with 100 µl PBST to remove any residual tissue. Then, 30 µl diethylpyrocarbonate-treated water containing 15 units of RNase inhibitor (DEPC, Sigma, Germany) was added to the tube and immediately denatured at 94°C for 1 min. The resulting solution in the tube was allowed to cool on ice for 1 min ready for RT-PCR.

Immunocapture (IC) RT reaction

The synthesis of complementary deoxyribonucleic acid (cDNA) was prepared in a final volume of 15 µl. A mixture of 1 µl antisense primer RYMV II at 100 µM and 9 µl sterile distilled water (SDW) per one sample was added into the tube containing the virus particles (Pinel et al., 2000; Silva et al., 2011). The mixture was denatured at 70°C for 5 min. The reverse transcriptase (RT)-cDNA synthesis 15 µl mixture of 9 µl SDW, 2 µl dNTPs (5 mM), 2.5 µl buffer RT x10 (Sigma, Munich, Germany) 1X final, 1 µl Moloney-Murine leukemia virus reverse transcriptase (M-MLV-RT) (Sigma, Munich, Germany) 200 U/µl and 0.5 µl RNase inhibitor 40U/µl (Sigma, Missouri, USA) were added to each denatured sample and incubated at 42°C for 1 h.

Simple-direct-tube (SDT) RT reaction

Reverse transcription was performed using cDNA synthesis from RYMV infected leaves (Temaja et al. 2012). Two scenarios were tested to determine the amount of template needed for optimal amplification results. These were categorized into: one plant sample in which high amount (7 µl) of final solution containing RNA was used, the other was a plant sample which has low amount (4 µl) of final solution containing RNA. This was done to determine a range of template that can be added to obtain optimal amplification. The reaction mixture consisted of total volume of 15 µl: 7 µl final solution containing viral RNA, 1 µl antisense primer 5′CTCCCCCACCCATCCCGAGAATT3′ at 100 µM, 1 µl of 0.2 M Dithiotreitol (DTT), 1 µl M-MLV- RT (sigma) 200 U/µl, 2.5 µl Buffer RT x10 (sigma) 1X final, 2 µl dNTPs 5 mM chacuum 2.0 mM final, 0.5 µl Rnase inhibitor 40 U/µl was incubated at 42°C for 1 h.

Extraction of RYMV RNA

Total RNA of RYMV was extracted from frozen infected rice leaves using the Rneasy Plant Mini Kit (Qiagen, Germany) (Pinel et al., 2000). Viral suspension were collected in 2 ml Eppendorf tube with sterile steel beads, frozen in liquid nitrogen and ground with high speed TissueLyser II mechanical shaker for 1 min at 30 rpm. The RTL lysis buffer was added, mixed by vortexing then incubated in water bath at 56°C for 2 min and centrifuged at 7000 rpm for 7 min. Tissues were separated by 225 µl of 100% ethanol followed by spinning at 10,000 rpm for 1 min, and then the supernatants were transferred into 2 ml Eppendorf tubes. Proteins of RYMV were removed by adding 700 µl RW1 and 500 µl RPE buffer, respectively and separately, centrifuged as above then the supernatant liquid was discarded and transferred into sterile 2 ml tubes. Ribonucleic acid was washed in 500 µl RPE buffer by spinning at 13,000 rpm for two minutes. Nucleic acids were eluted by 30 µl RNase free water directly to the spin column membrane and placed into clean sterile 1.5 ml tubes then centrifuged at 10,000 rpm for 1 min at 25°C. The obtained RNAs were stored in the freezer at -20°C for RT-PCR amplification.

PCR amplification of viral coat protein gene

A reaction of PCR to amplify the coat protein gene consisted of a

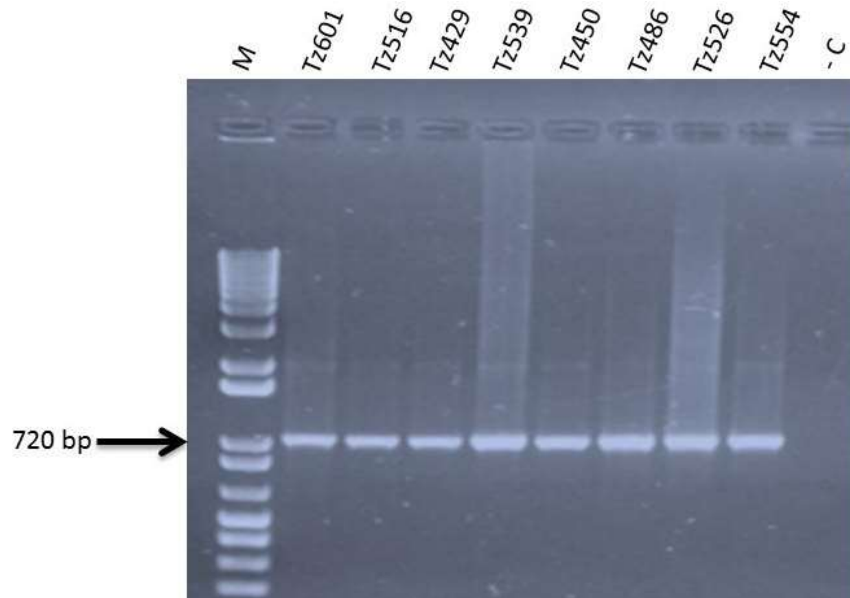


Figure 1. Immunocapture-RT-PCR amplification products obtained from *Rice yellow mottle virus* isolates of different strains S4ug (Tz601), S4lv (Tz516), S4lm (Tz526), S5 (Tz429, Tz450), S6c (Tz486), S6w (Tz539), virus extraction RNA S4lm (Tz554) and healthy plant (- C). M = DNA molecular marker (100-bp ladder). The PCR products were analyzed in 1% agarose gel.

total volume of 50 µl of a mixture (Pinel et al., 2000). The mixture included 10 µl of 5X PCR buffer, 1X final, 2 µl dNTP 5 mM chacum 2.0 mM final, 1 µl antisense primer RYMV II at 100 µM, 3 µl sense primer RYMV III at 100 µM, and 1 µl dynazyme, 30.5 µl SDW and 2.5 µl RT reaction per sample. The primer set of 5′CTCCCCCACCCATCCCGAGAATT3′ (reverse primer) and 5′CAAAGATGGCCAGGAA3′ (forward primer) were used as internal control of PCR tests to amplify the 720 nucleotide CP gene of RYMV (Fargette et al., 2002). The amplification involved three processes which were denaturation, annealing and elongation. The mixture was first heated at 94°C for 5 min to denature the sides of the double-stranded DNA. This was followed by 30 cycles whereas the mixture was heated again at 94°C for 1 min to separate the sides of the double-stranded DNA. In the same cycles, the mixture was then cooled at 55°C for 30 s to allow primers to find and bind to their complementary sequences on separated strands and elongated at 72°C for 1 min for polymerase to extend the primers into new complementary strands. The repeated heating and cooling cycles multiplied the target DNA exponentially because each new double strand separated to become two templates for further synthesis. Finally, the mixture was extended and stopped at 72°C for 10 min. The amplified PCR products were confirmed using 1% agarose gel (in 0.5X TAE buffer) electrophoresis, pre-stained with 2.5 µl ethidium bromide (10 mg/ml), and visualized under UV light.

RESULTS

The IC-RT-PCR results indicate that, all tested infected leaves of RYMV strains and phylotypes S4lv (Tz516), S4lm (Tz526), S4ug (Tz601), S5 (Tz429, Tz450), S6c (Tz486) and S6w (Tz539) yielded amplicon of the expected size at 720 bp (Figure 1). The healthy plant control (-C) gave no amplification indicating that the sample did not contain virus and that the primers did not amplify part of the plant's genome. The leaf samples infected with RYMV strain S4lm (Tz554) that RNA was extracted using RNase kit yielded amplicon at expected size of 720 bp (Figures 1 and 2).

The primers used were 3' RYMV II at 100 µM and 5' RYMV III at 100 µM in order to compare the results with the immunocapture results. The results show that the plant samples containing high amount of final solution (7 µl) yielded amplicon at expected size (720 bp) in electrophoresis analysis (Figure 2). Amplification was not obtained when low amount of template (4 µl sap) was used. The results presented in Figure 2 show that the infected leaves of different RYMV strains tested yielded amplicon of the expected size (720 bp), however, there was no amplification in the healthy control samples. Gel electrophoresis result also indicated that the strain Tz 539 of RYMV was not well amplified in SDT-RT-PCR as in the IC-RT-PCR technique. Rice leaves infected with RYMV strain S4lm (Tz554), tested by virus extraction RNA using RNase kit as a positive control was also amplified.

DISCUSSION

These results show that the IC- and SDT RT-PCR protocols optimized in this study can be used for rapid characterization of RYMV strains and are useful for

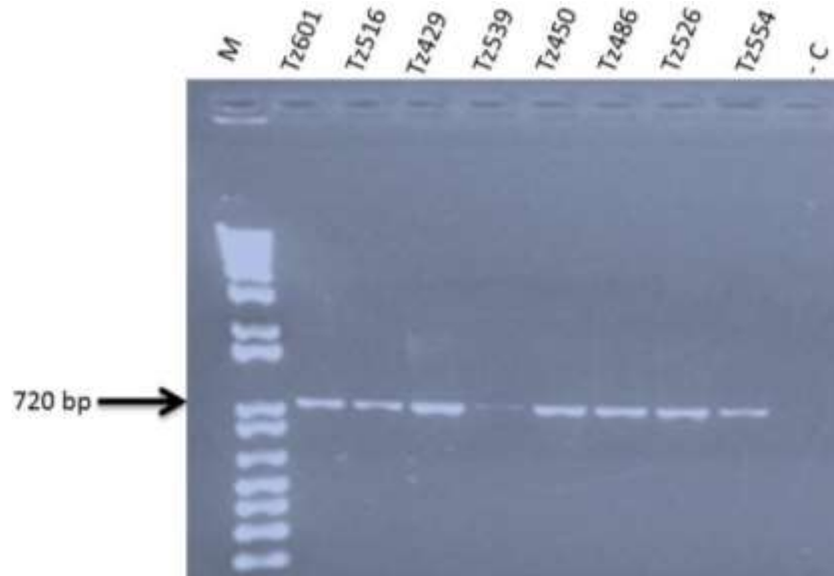


Figure 2. Simple-direct-tube-RT-PCR amplification products obtained from *Rice yellow mottle virus* isolates of different strains S4ug (Tz601), S4lv (Tz516), S4lm (Tz526), S5 (Tz429, Tz450), S6c (Tz486), S6w (Tz539), virus extraction RNA S4lm (Tz554) and healthy plant (- C). M = DNA molecular marker (100-bp ladder). The PCR products were analyzed in 1% agarose gel.

periodical surveys of RYMV. In the RT-PCR, the expected band size of 720 bp was observed in all infected rice leaves tested implying that the methods were useful and reliable for detection of a wide range of RYMV strains. This is the first report on the use SDT- and IC-RT-PCR for detection and assay of RYMV. This method has been used for detection of other plant viruses (Kobori et al., 2005; Suehiro et al., 2005; Temaja et al., 2012). The results indicate that SDT-RT-PCR and IC-RT-PCR detected RYMV in infected leaf samples similar to the virus extraction RNA-RT PCR technique.

In immunocapture, virus particles from the infected leaf isolates were trapped on the wall of an antibody coated tube and inhibitory plant extracts were removed by washing. The viral RNA released from the particles was then used as a template for cDNA synthesis using reverse transcriptase. The resulting cDNA was amplified in PCR with the RYMV conserved primers, antisense primer RYMV II and sense primer RYMV III at 100 µM (Fargette et al., 2002). Immunocapture RT-PCR technique has been utilized widely for detection of other plant viruses (Wetzel et al., 1992; Harper et al., 1999; Hema et al., 2003; Mansilla et al., 2003; Zein et al., 2008; Silva et al., 2011; Kiliç, and Yardimci; 2012; Sarovar et al., 2010; Mallik et al., 2012; Uehara-Ichiki et al., 2013). This technique has been reported to have higher sensitivity than Passive Haemagglutination Assay (PHA) and Latex Agglutination Reaction (LAR) and could detect the target virus in leaf extracts diluted from 10^{-3} to 10^{-5} (Uehara-Ichiki et al., 2013). The sensitivity of using ELISA coupled with RT-PCR has been demonstrated to be greater than just total nucleic acid extraction due to the immunocapture enrichment of samples prior to RT-PCR (Kogovsek et al., 2008; Yang et al., 2012). These findings support those of Ptacek et al. (2002) that immunocapture followed by the detection of viruses using RT-PCR is a versatile and sensitive diagnostic technique. The technique has also been used for detection of the virus in plant species or tissues that inhibit the PCR reaction and molecular detection (Ptacek et al., 2002).

In this study, the amount of sap added to the RT reaction play an important role in amplification, thus it was necessary to determine the amount of template needed for optimal RNA amplification. For instance, in this study, two scenarios were tested, one plant sample in which high amount (7 µl) of final solution containing RNA occurs and the second included plant samples with low amount (4 µl) of final solution containing RNA. This was done to determine a range of template that can be added to obtain optimal amplification. The results showed that, the plant sample containing high amount (7 µl) of final solution yielded amplicon at the size of 720 bp in electrophoresis analysis compared to low amount of final volumes, implying that amplification can be improved with high volumes as used in this study.

Gel electrophoresis results indicate that the fragments of all tested RYMV strains were amplified except Tz 539 RYMV strain band was faint in SDT-RT-PCR as compared to IC-RT-PCR technique, implying that the target amount of template may probably contained low virus concentration. The delay in fragment amplification may also possibly caused by the failure of primers to

anneal due to sequence variability. Chandler et al. (1998) suggest that the inhibitory effect of RT on PCR is mediated through the RT interactions with the specific messenger RNA (mRNA) or complementary DNA (cDNA) and that the inhibitory effect is dependent upon template concentration (or copy number). One of the problems encountered in the detection of *Grapevine leafroll-associated virus 3* (GLRaV-3) was the low concentration of virus in grapevine tissue (Acheche et al., 1999), which increased probability of inhibition from the RT enzyme on the PCR (Chandler et al., 1998).

Conclusion

The results of this study reveal that immunocapture (IC) and simple-direct-tube (SDT) -reverse transcriptase-polymerase chain reaction (RT-PCR) techniques for detection of *Rice yellow mottle virus* (RYMV) are rapid methods for characterization of RYMV strains and may be recommended for use soon after periodical surveys to quickly identify new strains for breeding purposes at low cost. With these simple techniques, large numbers of plant samples detected using RT-PCR assays would be managed. Immunocapture and SDT methods may also reduce risks from cross-contaminations in sample process as the need short time in RNA preparation. The SDT protocol seemed to be easier, faster and cost-effective than IC-RT-PCR in terms of reagents for the detection of RYMV. However, the SDT-RT-PCR technique was not able to detect RYMV samples with low RNA concentration as compared to IC-RT-PCR. Studies may also be focused on detection of RYMV in insect vectors and other viruses using these techniques.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors would like to thank the Department of Veterinary Medicine and Public Health, Sokoine University of Agriculture (SUA) for providing reagents for simple-direct-tube (SDT) technique. We are grateful for the technical assistance from Ms. Agnes Pinel-Galzi, Institut de Recherche pour le Developement (IRD), France. This work was supported by funds from the Innovative Agricultural Research Initiative (iAGRI).

REFERENCES

Abo ME, Sy AA, Alegbejo SM (1998). *Rice yellow mottle virus* (RYMV) in Africa: evolution, distribution, economic significance and sustainable rice production and management strategies. J. Sustain. Agric. 11:85-111.

Abubakar Z, Ali F, Pinel A, Traoré O, N'Guessan P, Notteghem J, Kimmins F, Konaté G, Fargette D (2003). Phylogeography of *Rice yellow mottle virus* in Africa. J. Gen. Virol. 84:733-743.

Acheche H, Fattouch S, M'hirsi S, Marzouki N, Marrakchi M (1999). Use of optimized PCR methods for the detection of GLRaV3: Closterovirus associated with Grapevine leafroll in Tunisian grapevine plants. Plant Mol. Biol. Rep. 17:31-42.

Bakker W (1970). Rice yellow mottle, a mechanically transmissible virus disease of rice in Kenya. Neth. J. Plant Pathol. 76:53-63.

Banwo OO, Alegbejo MD, Abo ME (2004). *Rice yellow mottle virus* genus Sobemovirus: a continental problem in Africa. J. Plant Prot. Sci. 40:26-36.

Chandler DP, Wagnon CA, Bolton H (1998). Reverse transcriptase inhibition of PCR at low concentrations of template and its implications for quantitative RT-PCR. Appl. Environ. Microbiol. 64:669-677.

Fargette D, Pinel A, Halimi H, Brugidou C, Fauquet C, Van RM (2002). Comparison of molecular and immunological typing of isolates of *Rice yellow mottle virus*. Arch. Virol. 147:583-596.

Fauquet C, Mayo M, Maniloff J, Desselberger U, Ball L (2005). Sobemovirus, Virus Taxonomy: Classification and Nomenclature of Viruses, 5th Ed. Eight report of the International Committee on Taxonomy of Viruses, Elsevier Academic Press, New York. pp. 888-890

Gouy M, Guindon S, Gascuel O (2010). SeaView version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. Mol. Biol. Evol. 27(2):221-224.

Grieco F, Gallitelli D (1999). Multiplex reverse transcriptase-polymerase chain reaction applied to virus detection in globe artichoke. Phytopathology 147:183-185.

Ha KJ, Yoo HN, Bae EH, Jung Y (2012). Development of an Indirect ELISA and Immunocapture RT-PCR for Lily Virus Detection. J. Microbiol. Biotechnol. 22(12):1776-1781.

Harper G, Dahal G, Thottappilly G, Hull R (1999). Detection of episomal *Banana streak badnavirus* by IC–PCR. J. Virol. Methods 79:1-8.

Hébrard E, Pinel-Galzi A, Bersoult A, Siré C, Fargette D (2006). Emergence of a resistance-breaking isolate of *Rice yellow mottle virus* during serial inoculations is due to a single substitution in the genome-linked vira l protein VPg. J. Gen. Virol. 87:1369-1373.

Hema M, Kirthi N, Sreenivasulu P, Savithri HS (2003). Development of recombinant coat protein antibody based IC–PT–PCR for detection and discrimination of *Sugarcane streak mosaic virus* isolates from Southern India. Arch. Virol. 148:1185-1193.

Hubert J, Luzi-Kihupi A, Hébrard E, Lyimo HJF (2016). Farmers' knowledge and perceptions of *Rice yellow mottle virus* in selected rice growing areas in Tanzania. Int. J. Sci. Res. 5(2):549-559.

Hubert J, Lyimo HJF, Luzi-Kihupi A (2017). Geographical Variation, Distribution and Diversity of *Rice yellow mottle virus* Phylotypes in Tanzania. Am. J. Plant Sci. 8:1264-1284.

Kanyeka ZL, Sangu E, Fargette D, Pinel-Galzi A, Hébrard E (2007). Distribution and diversity of local strains of *rice yellow mottle virus* in Tanzania. Afr. J. Crop Sci. 15(4):201-209.

Kiliç HC, Yardimci N (2012). Nested RT-PCR and immunocapture RT-PCR for detection of *Beet necrotic yellow vein virus* on sugar beet in Lake District of Turkey. Rom. Agric. Res. 29:333-337.

Kobori T, Ryang BS, Natsuaki T, Kosaka Y (2005). A new technique to select mild strains of *Cucumber mosaic virus*. Plant Dis. 89:879-882.

Kogovsek P, Gow L, Pompe-Novak M, Gruden K, Foster GD, Boonham N, Ravnikar M (2008). Single-step RT real-time PCR for sensitive detection and discrimination of Potato virus Y isolates. J. Virol. Methods 149:1-11.

Kouassi NK, N'Guessan P, Albar L, Fauquet CM, Brugidou C (2005). Distribution and Characterization of *Rice yellow mottle virus*: A Threat to African Farmers. Plant Dis. 89(2):124-133.

Lamo L, Cho G, Jane I, Dartey PKA, James E, Ekobu M, Alibu S, Okanya S, Oloka B, Otim M, Asea G, Kang K (2015). Developing Lowland Rice Germplasm with Resistance to Multiple Biotic Stresses through Anther Culture in Uganda. J. Korean Soc. Int. Agric. 27:415-420.

Ling K, Wechter WP, Jordan R (2007). Development of a one-step immunocapture real-time TaqMan RT-PCR assay for the broad spectrum detection of *Pepino mosaic virus*. J. Virol. Methods 144:65-72.

Lopez MM, Bertolini E, Olmos A, Caruso P, Gorris MT, Llop P, Penyaler R, Cambra M (2003). Innovative tools for detection of plant pathogenic viruses and bacteria. Int. J. Microbiol. 6:233-243.

Luzi-Kihupi A, Zakayo JA, Tusekelege H, Mkuya M, Kibanda NJM, Khatib KJ, Maerere A (2009). Mutation Breeding for Rice Improvement in Tanzania. In. Induced Plant Mutations in the Genomics Era. Food and Agriculture Organization of the United Nations, Rome, pp. 385-387.

Mallik I, Anderson NR, Gudmestad NC (2012). Detection and Differentiation of Potato Virus Y Strains from Potato Using Immunocapture Multiplex RT-PCR. Am. J. Potato Res. 89:184-191.

Mansilla C, S´anchez F, Ponz F (2003). The diagnosis of the tomato variant of *Pepino mosaic virus*: An IC-RT-PCR approach. Eur. J. Plant Pathol. 109:139-146.

N'Guessan P, Pinel A, Caruana ML, Frutos R, Sy A, Ghesqui`ere A, Fargette D (2000). Evidence of the presence of two serotypes of rice yellow mottle sobemovirus in Cˆoted'Ivoire. Eur. J. Plant Pathol. 106:167-178.

N'guessan P, Pinel A, Sy AA, Ghesquiere A, Fargette D (2001). Distribution, pathogenicity, and interactions of two strains of *Rice yellow mottle virus* in forested and savannah zones of West Africa. Plant Dis. 85:59-64.

Ochola D, Issaka S, Rakotomalala M, Pinel-Galzi A, Ndikumana I, Hubert J, Hébrard E, Séré Y, Tusiime G, Fargette D (2015). Emergence of *rice yellow mottle virus* in eastern Uganda: Recent and singular interplay between strains in East Africa and in Madagascar. Virus Res. 195:64-72.

Osman F, Leutenegger C, Golino D, Rowahni A (2007). Real-time RT-PCR (TaqMan®) assay for the detection of Grapevine leafroll associated viruses 1–5 and 9. J. Virol. Methods 141:22-29.

Pinel A, N'Guessan P, Bousalem M, Fargette D (2000). Molecular variability of geographically distinct isolates of *Rice yellow mottle virus* in Africa. Arch. Virol. 145:1621-1638.

Pinel-Galzi A, Rakotomalala M, Sangu E, Sorho F, Kanyeka Z, Traoré O, Sérémé D, Poulicard N, Rabenantoandro Y, Séré Y, Konaté G, Ghesquiere A, Hébrard E, Fargette D (2007). Theme and variations in the evolutionary pathways to virulence of an RNA plant virus species. PloS Pathog. 3(11):1761-1770.

Ptacek J, Kopek J, Dedic P, Matousek J (2002). Immunocapture RT-PCR probing of potato virus Y isolates. Acta Virol. 46:63-68.

Rowhani A, Uyemto JK, Golino DA, Martelli GP (2004). Pathogen testing and certification of Vitis and Prunus Species. Ann. Rev. Phytopathol. 43:261-278.

Sarovar B, Prasad YS, Gopal DVRS (2010). Detection of Tobacco streak virus by immunocapture-reverse transcriptase-polymerase chain reaction and molecular variability analysis of a part of RNA3 of sunflower, gherkin, and pumpkin from Andhra Pradesh, India. J. Agric. Sci. 36:194-198.

Séré Y, Onasanya A, Mwilene FE, Abo ME, Akator K (2008). Potential of insect vector screening method for development of durable resistant cultivars to *Rice yellow mottle virus* disease. Int. J. Virol. 4:41-47.

Silva JM, Carnelossi PR, Bijora T, Facco CU, Picoli MHS, Souto ER, Oliveira AJB, Almeida ÁMR (2011). Immunocapture-RT-PCR detection of *Cassava common mosaic virus* in cassava obtained from meristem-tip culture in Paraná state. Trop. Plant Pathol. 36(5):271-275.

Suehiro N, Matsuda K, Okuda S, Natsuaki T (2005). A simplified method for obtaining plant viral RNA for RT-PCR. J. Virol. Methods 125:67-73.

Tamm T, Truve E (2000). Sobemovirus. J. Virol. 74(14):6231-6241.

Temaja GRM, Puspawati NM, Mayadewi NNA (2012). Utilization of SDT- RT-PCR for plant virus detection. J. Agric. Sci. Biotechnol. 1(1):24-29.

Traoré O, Pinel-Galzi A, Sorho F, Sarra S, Rakotomalala M, Sangu E, Kanyeka Z, Séré Y, Konaté G, Fargette D (2009). A reassessment of the epidemiology of *Rice yellow mottle virus* following recent advances in field and molecular studies. Virus Res. 141:258-267.

Uehara-Ichiki T, Shiba T, Matsukura K, Ueno T, Hirae M, Sasaya T (2013). Detection and diagnosis of rice-infecting viruses. Front. Microbiol. 289(4):1-7.

Uga H, Tsuda S (2005). A one-step reverse transcription-polymerase chain reaction system for the simultaneous detection and identification of multiple tospovirus infections. Phytopathology 95:166-171.

Wetzel T, Condresse T, Macquaire G, Ravelonandro M, Dunez J (1992). A highly sensitive immunocapture polymerase chain reaction method for *Plum pox potyvirus* detection. J. Virol. Methods 39:27-37.

Wilson RT, Lewis I (2015). The Rice Value Chain in Tanzania. A Report from the Southern Highlands Food Systems Programme, FAO. pp. 9.

Wu J, Ni Y, Liu H, Ding M, Zhou X (2014). Monoclonal antibody-based serological assays and immunocapture-RT-PCR for detecting *Rice dwarf virus* in field rice plants and leafhopper vectors. J. Virol. Methods 195:134-140.

Yang J, Wang F, Chen D, Shen L, Qian Y, Liang Z, Zhou W, Yan T (2012). Development of a One-Step Immunocapture Real-Time RT-PCR Assay for Detection of *Tobacco Mosaic Virus* in Soil. Sensors 12(12):16685-16694.

Zein HS, Hussein MH, Hussein HA, Miyatake K **(**2008). The utilization of monoclonal antibodies in immunocapture RT-PCR and dot blotting immunobinding assays for the detection of *Cucumber mosaic virus.* Arab J. Biotechnol. 11(1):95-106.

academicJournals

Vol. 16(24), pp. 1338-1344, 14 June, 2017
DOI: 10.5897/AJB2017.15998
Article Number: 8A1F24464719
ISSN 1684-5315
Copyright © 2017
Author(s) retain the copyright of this article
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Phytochemical screening and amino acids analysis of mushrooms from Burkina Faso

Elise Sanon[1]*, Jean Claude W. Ouédraogo[2,3], Sylvain Ilboudo[3], Marie K. Laure Guissou[4], Pierre I. Guissou[3] and Philippe Sankara[1]

[1]Laboratoire Biosciences, équipe de Phytopathologie, Département de Biologie et Physiologie Végétales, UFR/SVT, Université Ouaga 1 Pr Joseph KI-ZERBO, 03 BP: 7021 Ouagadougou 03, Burkina Faso.
[2]Département de Chimie, UFR/SEA, Université Ouaga 1 Pr Joseph KI-ZERBO, 03 BP 7021 Ouagadougou 03, Burkina Faso.
[3]Institut de Recherche en Sciences de la Santé (IRSS/CNRST), 03, BP 7192, Ouagadougou, Burkina Faso.
[4]École Normale Supérieure, Université de Koudougou, BP 376, Koudougou, Burkina Faso.

Received 21 March, 2017; Accepted 31 May, 2017

Mushrooms play an important social, economic and ecological role in the world. In this study, the proximate compositions, minerals and amino acids contents of *Russula alveolata*, *Russula* cf. *compressa*, *Russula flavobrunnea* var. *aurantioflava* and *Russula ochrocephala* from Burkina Faso were investigated. The chemical analysis showed the presence of volatile oil, sterols and triterpenes, carotenoids, saponosides, reducing compounds and cardenolides. Several amino acids including aspartate, glutamic acid, serine, glycine, arginine, threonine, alanine, proline, tyrosine, valine, methionine, isoleucine, leucine, phenylalanine and lysine were identified in mushroom species. Among the identified amino acids, seven are essential for human body: Phenylalanine, valine, threonine, isoleucine, methionine, leucine and lysine.

Key words: Chemical composition, amino acids, *Russula*, mushrooms, Burkina Faso.

INTRODUCTION

Mushrooms are of fungus type with a large variety of them. Each component is closely linked to its physiological nature. Their lifestyle shows their identity: no pigment (chlorophyll) assimilation comparable to higher plants, no opportunity to draw their energy from the carbonic acid of the air. Mushroom has necessarily one of these lifestyles: saprotrophism, parasitism and symbiotic parasitism (Heim, 1984). The russules are macromycetes of the genus *Russula* Pers. Fr., in the family Russulaceae Lotsy and order Russulales Kreisel. This family consists primarily of symbiotic species of ectomycorrhizal types (ECM), involving mainly the roots of woody plants in wooded regions (Bâ et al., 2012). This group of macroscopic fungi differs from the others of Russulaceae family by the absence of latex when broken.

The literature shows that several taxonomic studies

*Corresponding author. E-mail: wonnadia@yahoo.fr. Tel: (00226) 74457272 or 72822035.

Author(s) agree that this article remains permanently open access under the terms of the Creative Commons Attribution License 4.0 International License

were carried out on the *Russula* genus in Africa (Buyck, 1993, 1994, 1997). More recently, new taxa were described in Zimbabwe (Buyck and Sharp, 2007), Tanzania (Buyck, 1995, 2005; Härkönen et al., 2003), Zambia (Buyck, 1999, 2004), Burundi (Buyck, 1995, 1999), Cameroon (Douanla-Meli and Langer, 2009), Madagascar (Heim, 1936; Buyck, 2008) and Burkina Faso (Sanon et al., 2014; Sanon, 2015).

Nowadays, up to 197 taxa of *Russula* are described in Africa (Sanon et al., 2014). Despite the importance of taxonomic studies on russules, it should be noted that very few chemical and biochemical studies have been conducted on them and their food value is not evaluated. Indeed, determining the nutritional value of russules requires measuring the rate and analyzing the composition and spectrum of amino acids, fatty acids, vitamins and minerals present.

The present study was aimed to investigate the nutritional value of four species of russules namely *Russula alveolata*, *Russula compressa*, *Russula flavobrunnea var. aurantioflava* and *Russula ochrocephala* by analyzing the composition of the different spectra of the amino acids contained in these fungal samples using high performance liquid chromatography (HPLC) method.

MATERIALS AND METHODS

Fungal material/biological material

The fungal material was composed of the carpophores of four mushroom species: *R. flavobrunnea var. aurantioflava, R. ochrocephala, R.* cf. *compressa* and *R. alveolata*. The mushroom samples were collected during each rainy season (2012-2014) following a standard protocol (Sanon, 2015). The first three mushrooms were collected in the forest of Dan which is a forest gallery at about fifteen kilometer from the city of Orodara at 10°52'28''N and 04°50'10''W, while the last one was collected in the forest gallery of Kou (western part of Burkina Faso). Morphological features were recorded using fresh materials that were dried afterward using a propane gas field dryer (De Kesel, 2001).

The species were then identified and voucher materials were stored at the herbarium of the University of Ouagadougou (Thiers, 2013) under the following reference numbers: SE009 (holotype OUA, MNHN Paris); 014b; 015; 145; 166; 167; 174; 178 (paratype OUA, MNHN Paris) for *R. flavobrunnea var. aurantioflava*, SE002 (holotype OUA, MNHN Paris); SE003a, SE005, SE146 (paratype OUA, MNHN Paris) *for Russula alveolata ;* SE144 (holotype OUA, MNHN Paris); SE147, 148, 151, 165, 173 (paratype OUA, MNHN Paris) for *R.* cf. *compressa* and SE 155 (holotype OUA, MNHN Paris) ; SE 157 ; SE 158 ; SE 162 ; SE 163 ; and SE 175 (paratype OUA, MNHN Paris) for *R. ochrocephala*.

Technical material

Phytochemical screening

The extracts from individual mushroom powders were prepared by maceration of 10 g of fungal powder in 50 mL of dichloromethane (DCM) for 24 h in a sealed pyrex bottle at room temperature. After filtration, each mark was extracted with methanol 80% (v/v) for 24 h and filtered. The mark was then extracted by infusion with 50 mL of distilled water. The organic extracts and the aqueous one were dried by evaporation under reduced pressure. Aliquots (1 g) of the aqueous extracts were redissolved in 30 mL of distilled water, and hydrolyzed with chlorhydric acid (10%) under reflux for 30 min and then extracted (liquid/liquid extraction) with dichloromethane. All these extracts were used for the phytochemical screening. The phytochemical screening was performed according to Cieuli (1982). Water, ethanol and dichloromethane extracts were used to assess the presence of: volatile oils, emodols, alkaloid bases, salt alkaloids, carotenoids, coumarins, flavonoid aglycons, oses and polysaccharides, reducing compounds, polyphenolic compounds, saponosids and sterols and triterpenes.

HPLC conditions for amino acids analyses

The profile and the amount of total amino acids were determined by reverse phase HPLC, using the Pico-Tag system described by Bidingmeyer et al. (1984). Samples were first defatted and hydrolyzed. For hydrolysis, 0.4 g of defatted powder of mushrooms was introduced into a hydrolysis pyrex bottle and 15 mL of 6 M chlorhydric acid were added. The bottle was flushed for 30 s with nitrogen and sealed immediately with cap. The bottle was then placed in an electric oven for 24 h at 110°C for sample hydrolysis. The sample solution was cooled down until room temperature and transferred into a 50 mL volumetric flask. Some mili-Q water was added until the 50 mL level was reached, and mixed (Hagen, 1989). Approximately, 1 mL of the diluted solution is homogenized and filtered through a filter of 0.45 µm. An aliquot of 10 µL of the solution is placed in a reaction tube and dried for 15 min with the Picotag Workstation.

The sample was then re-dissolved in 10 µL of re-drying solution ethanol-water triethylamine (2:2:1). They were dried again for 15 min and finally derivatized with 20 µL phenylisothiocyanate reagent (ethanol, water : triethylamine : phenylisothiocyanate 7:1:1:1) for 20 min at room temperature. Excess reagent was removed using a vacuum for 45 min in Pico-Tag Work station. Derivatized samples were dissolved in 100 µL Pico-Tag Sample diluent solution (WAT088119).

The HPLC analyses (identification and quantification) of the amino acids were performed under the conditions described by Bidlingmeyer et al. (1984). The system consists of Waters HPLC including degasser, controller - model 600, pump and detector - model 2487. Sample volume of 4 µL was injected. Chromatographic separation was performed on a PICO•TAG Column (3.9 × 150 mm), previously equilibrated for at least 8 min with Eluent A which consists of sodium acetate and triethylamine in water at pH 6.4-Acetonitrile (94:6 v/v). Solvent gradients were formed by varying the proportion of eluent A to eluent B (acetonitrile-water 60:40 v/v). Eluent B was increased to 46% in 10 min and subsequently increased to 100% in 10.5 min at a flow rate of 1 mL/min. The elution was performed at 254 nm with the column conditions set at 38°C. The areas under the peaks were used to calculate the concentrations of the amino acids using a Pierce Standard H amino acid calibration mixture (Rockford, IL).

RESULTS AND DISCUSSION

Phytochemical screening

The phytochemical screening was performed for the water, methanol and dichloromethane extracts. The results are summarized in Table 1. The phytochemical analysis revealed (Table 1) the presence of volatile oils,

Table 1. Phytochemical analyses extracts from mushroom species.

Extracts	Chemical groups	*R. alveolata*	*R. compressa*	*R. flavobrunnea var. aurantioflava*	*R. ochrocephala*
DCM	Volatile oil	++	++	+++	+++
	Sterols and triterpenes	+	+	++	++
	Emodols	-	-	-	-
	Carotenoids	+	+	++	++
Methanol	Polyphenols	-	-	-	-
	Saponosides	+++	+++	++	+++
	Reducing sugars	+	+	+	+
	Gallic tannins	-	-	-	-
Water	Anthracenosides	-	-	-	-
	Cardenolides	+	+	+	+

- = Absence; + = present in small amount (concentration); ++ = moderately present; +++ = present in large amount.

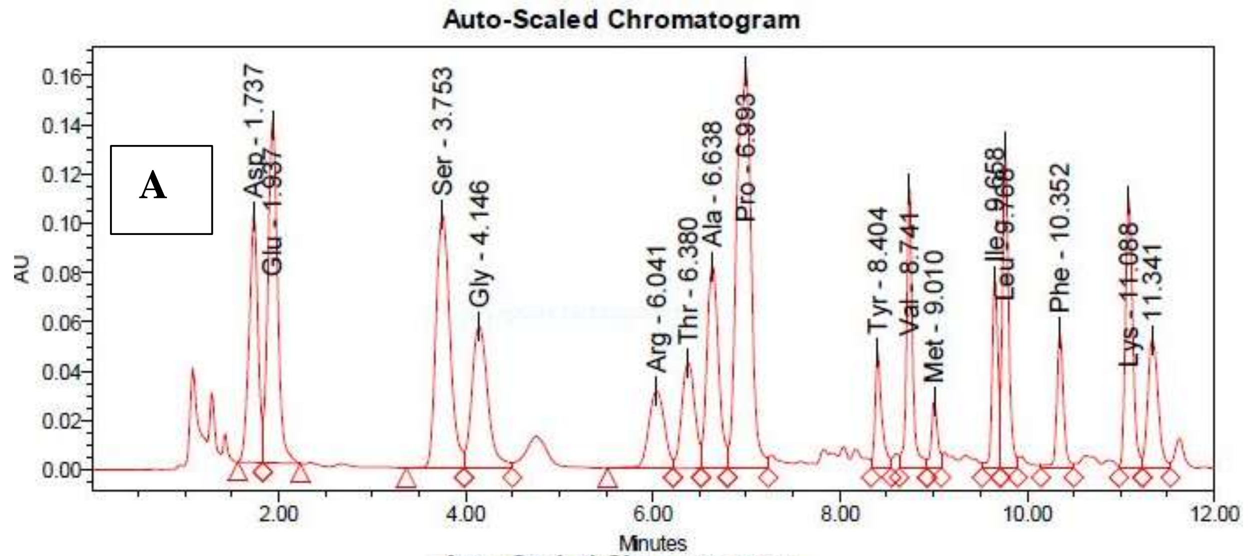
sterols and triterpenes, carotenoids, saponosides, reducing sugars and cardenolids. In the dichloromethane extract, volatile oils were in large amount in *R. flavobrunnea var. aurantioflava* and *R. ochrocephala* whilst. But in *R. alveolata* and *R. compressa*, they were moderately present. Sterols and triterpenes, and carotenoids are moderately found in *R. flavobrunnea var. aurantioflava* and *R. ochrocephala*, while they were in small amount in *R. alveolata* and *R. compressa.* In the ethanol extracts, reducing sugars were detected in the four species in small amount. It can be noticed that saponosides were in large amount in *R. alveolata*, *R. compressa* and *R. ochrocephala* but moderately present in *R. flavobrunnea var. aurantioflava*. In the aqueous extract, cardenolides were present in small amount in all the samples. The phytoconstituents such as emodols, polyphenols, gallic tannins and anthracenosides were absents in all the samples. The literature revealed that mushrooms contain saponins, polyphenols and terpenoids (Wandati et al., 2013) as found in the present study. In addition to the cited phytoconstituants, the mushrooms in the present study contain carotenoids, reducing sugars and trace of cardenolids. In a phytochemical study, Sanjay et al. (2015) found that edible mushrooms can also contain alkaloids and flavonoids.

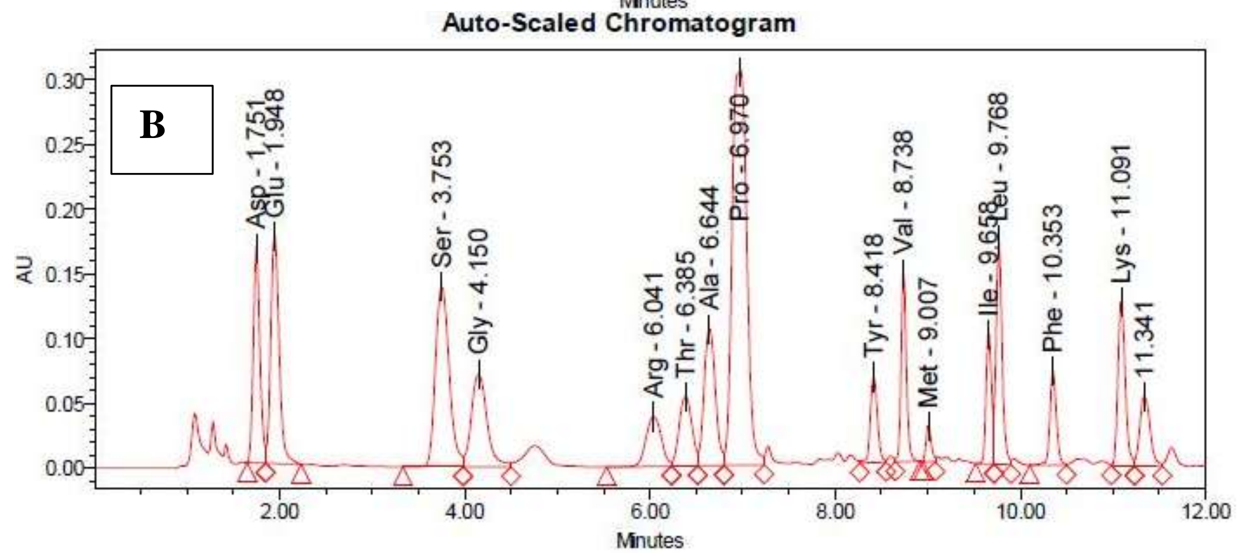
Amino acids analysis by HPLC

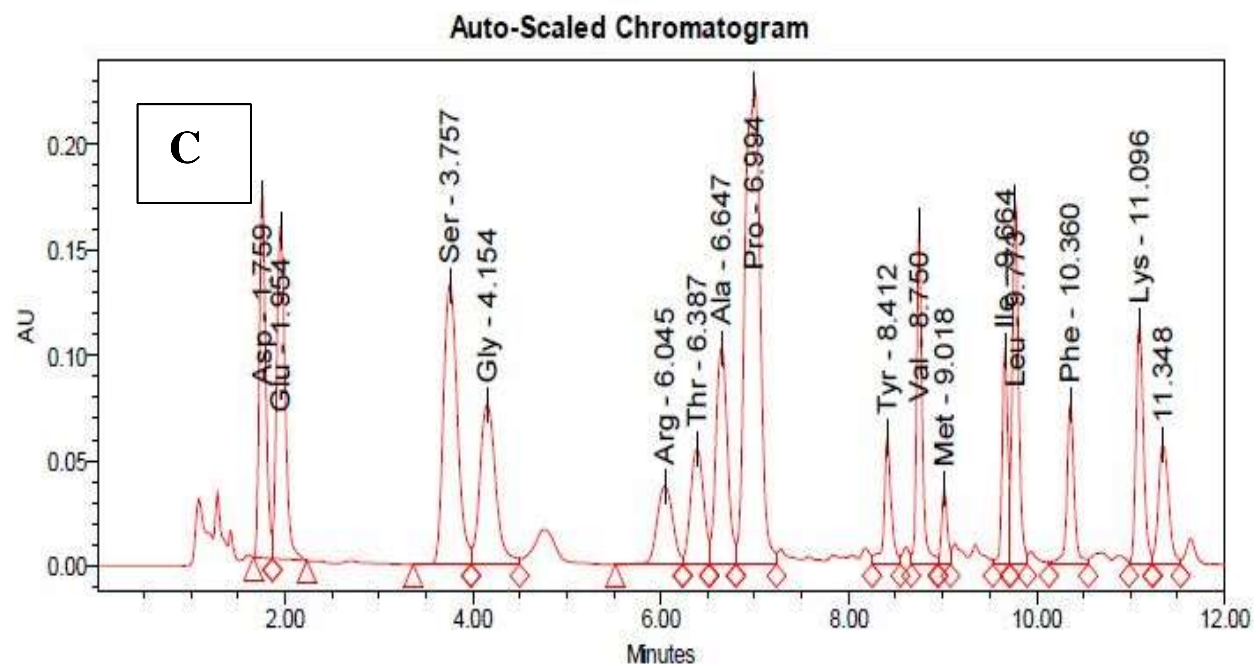
The HPLC chromatograms of amino acids are presented in Figure 1A to D and the summary in Table 2. Figure 1 and Table 2 indicate that the mushrooms contain various amino acids. A total of fifteen amino acids have been detected in all the mushrooms species: aspartate, glutamic acid, serine, glycine, arginine, threonine, alanine, proline, tyrosine, valine, methionine, isoleucine, leucine, phenylalanine and lysine. Among these amino acids, seven are regarded as essential to human body: phenylalanine, valine, threonine, isoleucine, methionine, leucine and lysine. Histidine and cysteine were absent in all the species.

Chemical analysis showed that the four species of russules namely *R. alveolata*, *R. compressa* aff., *R. flavobrunnea var. aurantioflava* and *R. ochrocephala* contain steroid and/or triterpene compounds (sterols and triterpenes, saponins), carotenoids, reducing compounds, cardenolides in trace and fifteen amino acids. Among the fifteen amino acids, it was observed that the proline had the highest concentration with a value of 4.082 g/100 g in *R. compressa*, followed by glutamic acid with the highest concentration in general and particularly in R. *ochrocephala* (2.202 g/100 g). The less concentrated amino acid is methionine in all the samples. The literature showed that mushrooms are deficient in cysteine and methionine (Agrahar-Murugkar and Subbulakshmi, 2005; Mdachi et al., 2004), isoleucine, leucine and lysine (Nakalembe and Kabasa, 2013), tryptophan (Agrahar-Murugkar and Subbulakshmi, 2005) and phenylalanine (Nakalembe and Kabasa, 2013). On the contrary, leucine was reported as the most abundant amino acid in wild mushrooms (Agrahar-Murugkar and Subbulakshmi, 2005). In the present study, cysteine and histidine were not detected and methionine was the less concentrated amino acid. Within the 7 essential amino acids, leucine had the highest concentration (1.062 g/100 g) in R. *flavobrunnea* var. *Aurantioflava*, while methionine remains the less concentrated one. The retention time of the amino acids ranged from about 1.7 to 11 min. Lysine has the maximal retention time and the smaller one is obtain for Asp.

Amino acids are very important for the regular


Auto-Scaled Chromatogram
A
AU
Minutes
Asp - 1.737
Glu - 1.937
Ser - 3.753
Gly - 4.146
Arg - 6.041
Thr - 6.380
Ala - 6.638
Pro - 6.993
Tyr - 8.404
Val - 8.741
Met - 9.010
Ile - 9.658
Leu - 9.768
Phe - 10.352
Lys - 11.088
11.341


Auto-Scaled Chromatogram
B
AU
Minutes
Asp - 1.751
Glu - 1.948
Ser - 3.753
Gly - 4.150
Arg - 6.041
Thr - 6.385
Ala - 6.644
Pro - 6.970
Tyr - 8.418
Val - 8.738
Met - 9.007
Ile - 9.658
Leu - 9.768
Phe - 10.353
Lys - 11.091
11.341


Auto-Scaled Chromatogram
C
AU
Minutes
Asp - 1.759
Glu - 1.954
Ser - 3.757
Gly - 4.154
Arg - 6.045
Thr - 6.387
Ala - 6.647
Pro - 6.994
Tyr - 8.412
Val - 8.750
Met - 9.018
Ile - 9.664
Leu - 9.775
Phe - 10.360
Lys - 11.096
11.348

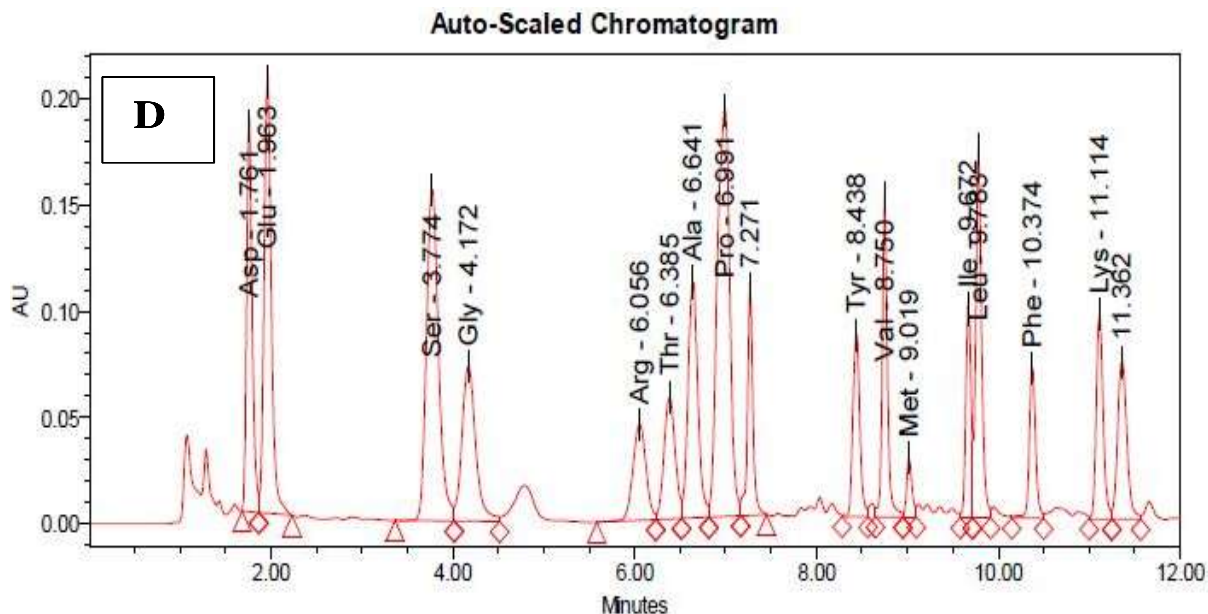


Figure 1. Peaks of amino acids, (A) *Russula alveolata*, (B) *Russula compressa*, (C) *Russula flavobrunnea var. aurantioflava* AND (D) *Russula ochrocephala* using HPLC.

biological activities of human body. They are the building blocks of the body. Besides building cells and repairing tissue, they constitute an antibody to combat bacteria and viruses; they are part of enzyme and hormonal system. Amino acids are very important for building nucleoproteins (RNA & DNA) (Imura and Okada, 1998). Eight amino acids are regarded as essential to human body: Phenylalanine, valine, threonine, tryptophan, isoleucine, methionine, leucine and lysine. Non synthesized amino acids are called " essential " and must be ingested by the body. The functions of the essential amino acids are numerous. They can give rise to other types of molecules. In addition, they can serve as precursors to the biosynthesis of non-essential amino acids. For example, histidine that may be converted to glutamic acid phenylalanine can be oxidized to give tyrosine. The obtained results are consistent with the assertions of Mau et al. (2002) that: "proteins that represent 4 to 35% of the dry weight of mushrooms contain many essential amino acids (from 32.9 to 48.5 g/100g protein), such as leucine, isoleucine, valine, lysine and threonine". In addition, cysteine (or sulphur containing amino acids), tyrosine (or aromatic amino acids), histidine and arginine are required by infants and growth (FAO/WHO/UNU, 2007; Sheil et al., 1986). Individual living with phenylketonuria (PKU) must have tyrosine intake in their food because the person living with PKU cannot convert the phenylalanine into tyrosine (Sheil et al., 1986).

As mentioned above, the phytochemical screening showed the presence of many phytoconstituents: Sterols and triterpenes, carotenoids, saponosides, reducing sugars and cardenolides. The sterols are lipids which help maintain the structural and functional integrity of the cell membranes in humans. They also play a role of cholesterol (Katan et al., 2003). Terpenoids are secondary metabolites synthesized by plants, marine organisms, etc. The saponosides (or saponins) are high-molecular-weight glycosides, consisting of a sugarunit(s) linked to a triterpene or a steroid aglycone. Many saponins are from plants and have detergent properties, capable of acting on the permeability of cell membranes. It would play an anti-inflammatory role with triterpenes. Carotenoids play an important role in nutrition and health. They are able to trap free radicals generated constantly by our organism (antioxidant properties) and many are provitamin A. Some of them have also anti-cancer properties. They also stimulate antibody synthesis (World Cancer Research Fund/American Institute for Cancer Research, 2007).

Finally, cardenolides or cardiotonic glycosides have the same structure as the steroidal saponins and also have detergent properties. They have a therapeutic effect: cardiotonic at very controlled dose (narrow therapeutic index), strengthens the heartbeat and reduces the side effects such as pericardial toxicity (Malcolm, 1991). With all these many nutritional virtues in these mushrooms, it can be said that they are good edible russules. Previous studies show that the nutritional value of mushrooms are based on their protein and essential amino acids content because they are good source of essential amino acids (Chang and Miles, 2004). The results showed that they are comparable to foods such as milk, beans, spinach and soybeans (Boa, 2006). Their content in vitamin C is

Table 2. Retention time (RT) and values of amino acids of different mushrooms.

S/N	Amino acids	RT (min)				Amount (g/100g)			
		R. alveolata	*R. compressa*	*R. flavobrunnea var. aurantioflava*	*R. ochrocephala*	*R. alveolata*	*R. compressa*	*R. flavobrunnea var. aurantioflava*	*R. ochrocephala*
1	Asp	1.737	1.751	1.759	1.761	1.208	1.507	1.454	1.382
2	Glu	1.937	1.948	1.954	1.963	2.000	2.268	1.928	2.202
3	Ser	3.753	3.753	3.757	3.774	1.472	1.956	1.799	1.877
4	Gly	4.146	4.150	4.154	4.172	0.652	0.811	0.828	0.697
5	His	4.789	4.789	4.789	4.789	0	0	0	0
6	Arg	6.041	6.041	6.045	6.056	0.815	1.005	0.091	0.944
7	Thr	6.380	6.385	6.387	6.385	0.601	0.777	0.743	0.671
8	Ala	6.646	6.644	6.647	6.641	0.769	0.973	0.908	0.857
9	Pro	6.993	6.970	6.994	6.991	2.073	4.082	2.918	2.081
10	Tyr	8.404	8.418	8.412	8.438	0.479	0.947	0.749	0.902
11	Val	8.741	8.738	8.750	8.750	0.752	0.975	0.925	0.832
12	Met	9.010	9.007	9.018	9.019	0.217	0.262	0.247	0.196
13	Cys	9.323	9.323	9.323	9.323	0	0	0	0
14	Ile	9.658	9.658	9.664	9.672	0.525	0.683	0.625	0.572
15	Leu	9.768	9.768	9.773	9.783	0.798	1.127	1.062	1.018
16	Phe	10.352	10.353	10.360	10.374	0.508	0.722	0.659	0.607
17	Lys	11.088	11.091	11.096	11.114	0.574	0.693	0.576	0.465

also higher than in carrot, celery and cucumber and are comparable to spinach (Chu et al., 2002). The content of riboflavin (vitamin B2) in mushrooms is generally greater than in vegetables, approaching in some cases those in eggs and cheese (Mattila et al., 2001). In addition, mushrooms are rich in fiber and minerals (Boa, 2006). Finally, although they have low fat content, their lipid profile is highly unsaturated; so mushrooms are also some sources of essential fatty acids.

Nowadays, over 80% people consider mushrooms as healthy food and do not hesitate to include them in their diet for cardiovascular health (Chu et al., 2002). In view of all these nutritional qualities of mushrooms in general and the four species of russules in particular, one can say that the four studied mushrooms are edible and their consumption needs to be popularized. But caution is needed because each species contains traces of cardenolides which, in high doses, can cause cardiac toxicity.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors are grateful the head of the Chemistry Laboratory of IRSS for the phytochemical screening and Dr. Sawadogo, Lingani Hagretou and her team at IRSAT for the Fungal amino acid analysis.

REFERENCES

Agrahar-Murugkar D, Subbulakshmi G (2005). Nutritional value of edible wild mushrooms collected from the Khasi hills of Meghalaya. Food Chem. 89:599-603.

Bâ AM, Duponnois R, Moyersoen B, Diedhiou AG (2012). Ectomycorrhizal symbiosis of tropical African trees. Mycorrhiza 22:1-29.

Bidlingmeyer BA, Cohen SA, Tarvin TL (1984). Rapid analysis of amino acids using pre-column derivitization. J. Chromatogr. 336:93-104.

Boa ER (2006). Champignons comestibles sauvages: vue d'ensemble sur leur utilisation et leur importance pour les

populations. Produits forestiers non ligneux. FAO, Rome. 17:157.
Bidlingmeyer BC (1984). Rapid analysis of amino acids using Pre-column derivatization. J. Chrom. 336:93-104.
Buyck B (1993). *Russula I (Russulaceae).* In. Rammeloo J, Heinemann P. (Éds.) Flore illustrée des Champignons d'Afrique Centrale. 15:335-408.
Buyck B (1994). *Russula II (Russulaceae).* In. Rammeloo J, Heinemann P. (Éds.). Flore illustrée des Champignons d'Afrique Centrale. 16:411-542.
Buyck B (1995*). Russula* subsection Amoeninae in tropical African miombo woodland. Doc. Mycol. 98-100:103-112.
Buyck B (1997). *Russula III (Russulaceae).* In. Rammeloo J. & Heinemann P. (Éd.). Flore illustrée des Champignons d'Afrique Centrale. 1:545-598.
Buyck B (1999). Two new remarkable *African Russula* with reddening context. Kew Bull. 54:663-673.
Buyck B (2005). New African woodland taxa for *Russula* subsection *Mamillatinae*. Crypt. Mycol. 26(2):85-95.
Buyck B (2008). Wild edible mushrooms in Madagascar: an evolving enigma. Econ. Bot. 62(3):509-520.
Buyck B, Sharp C (2007). Two new species and first records for 12 other *Russula (Russulales)* from Zimbabwe. Crypt. Mycol. 28(1):13-27.
Buyck B (2004). Short diagnoses and descriptions for some exotic *Russula (Basidiomycotina)*. Crypt. Mycol. 25(2):127-128.
Chang ST, Miles PG (2004). The nutritional attributes of edible mushrooms. In. Chang, ST, Miles, PG. (eds), Mushrooms, Cultivation, Nutritional Value, Medicinal Effect, and Environmental Impact. Second edition. CRC Press, New York. pp. 27-37.
Chu YF, Sun J, Wu X, Hai Liu R (2002). Antioxidant and antiproliferative activities of common vegetables. J. Agric. Food Chem. 50:6910-6916.
Cieuli I (1982). Methodology for analysis of vegetable drug. Pratical manual on industrial utilization of Medecine an aromatic plants. Bucharest Ministry of Chemical Industry, pp. 16-27.
De Kesel A (2001). A mushroom dryer for the travelling mycologist. Field Mycol. 2(4):131-133.
Douanla-Meli C, Langer E (2009). Fungi of Cameroon II. Two new Russulales species. (Basidiomycota). Nova Hedwigia 88:491-502.
FAO/WHO/UNU (2007). Protein and amino acid requirements in human nutrition, WHO Press. 150.
Hagen WR (1989). Direct electron transfer of redox proteins at the bare glassy carbon electrode. Eur. J. Biochem. 189:523-530.
Härkönen M, Niemelä T, Mwasumbi L (2003). Tanzanian mushrooms. Edible, harmful and other fungi, Norrlinia 10. Helsinki, Botanical Museum, Finish Museum of Natural History. 200.
Heim R (1936). Aperçu sur la flore mycologique malgache III. Trois bolets gigantesques d'Afrique et de Madagascar. Rev. Mycol. (Paris) 1:1-18.
Heim R (1984). Champignons d'Europe: généralités - *Ascomycètes - Basidiomycètes*. Nouvelle édition, Société Nouvelle des éditions Boubée, 680 p.
Imura K, Okada A (1998). Amino acid metabolism in pediatric patients. Nutrition 14(1):143-148.
Katan MB, Grundy SM., Jones P, Law M, Miettinen T, Paoletti R (2003). Efficacy and safety of plant stanols and sterols in the management of blood cholesterol levels. Mayo Clin. Proc. 78(8):965-978.
Malcolm SB (1991). Cardenolide mediated interaction between plants and herbivores. In. G.A. Rosenthal. M. R. Berenbaum (eds). Herbivores: Their Interactions with Secondary Metabolites. Vol. I. The chemical participants. 2nd ed. San Diego. Academic Press. pp. 251-296.
Mattila P, Kônkô K, Eurola M, Pihlava JM, Astola, Vahteristo L, Hietaniemi V, Kumpulainen, Valtonen JM, Piironen V. (2001). Contents of vitamins, minerai elements and some phenolic compounds in cultivated mushrooms. J. Agric. Food. Chem. 49:2343-2348.
Mau JL, Lin HC, Chen CC (2002). Antioxidant properties of several medicinal mushrooms. J. Agric. Food. Chem. 50:6072-6077.
Mdachi SJM, Nkunya MHH, Nyigo VA, IT Urasa (2004). Amino acid composition of some Tanzanian wild mushrooms. Food Chem. 86:179-182.
Nakalembe I, Kabasa JD (2013). Fatty and amino acids composition of selected wild edible mushrooms of bunyoro sub-region, Uganda. Afr. J. Food Agric. Nutr. Dev. 13(1):7225-7241.
Sanjay P, Kartik DV, Pithawala EA, Shukla MD, Lahiri SK, Jain NK, Modi HA (2015). Phytochemical screening, Total Phenolic Content, Antibacterial and antioxidant activity of wild edible Mushroom *Pleurotus ostreatus*. Int. Res. J. Pharm. 6(1):65-69.
Sanon E (2015). Diversité, morphologies et études moléculaires des champignons macroscopiques du genre *Russula* des forêts du Kou et de Dan à l'ouest du Burkina Faso. Thèse de Doctorat Unique. Université de Ouagadougou. 227.
Sanon E, Guissou KML, Nana A, Hama O, Sankara P (2014). Diversité des champignons macroscopiques de la forêt classée du Kou et étude ethnomycologique chez les bobos de Kokorowé et de Nasso au Burkina Faso. Annale de l'Université de Ouagadougou. 10:69-95
Sheil N, Duignan IP, Saul, Naughten ER (1986). Maternal phenylketonuria with increased tyrosine supplements. Inherit. Metab. Dis. 9(2):231-233.
Thiers B (2013). Index Herbariorum: a global directory of public herbaria and associated staff. New York Garden's Virtual Herbarium.
Wandati GM, Kenji, Onguso JM (2013). Phytochemicals in Edible Wild Mushrooms From Selected Areas in Kenya. J. Food Res. 2(3):137-144.
World Cancer Research Fund / American Institute for Cancer Research (2007). Food, Nutrition, Physical Activity, and the Prevention of Cancer: a Global Perspective. Washington DC, AICR. 76.

academicJournals

Vol. 16(24), pp. 1345-1353, 14 June, 2017
DOI: 10.5897/AJB2016.15733
Article Number: CFACE1664721
ISSN 1684-5315
Copyright © 2017
Author(s) retain the copyright of this article
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Establishment, multiplication, rooting and acclimatization of *Cabralea canjerana* (Vell.) Martius

Eliseo Salvatierra Gimenes[1], Nathalia Pimentel[2], James Hancock[3] and Dilson Antônio Bisognin[1*]

[1]Department of Plant Science, Federal University of Santa Maria, Av. Roraima 1000, Camobi, 97105-900, Santa Maria, Rio Grande do Sul, Brazil.
[2]Postgraduate Program of Forest Engineering, Department of Forestry Science, Federal University of Santa Maria, Av. Roraima 1000, Camobi, 97105-900, Santa Maria, Rio Grande do Sul, Brazil.
[3]Department of Horticulture, Michigan State University, Plant and Soil Bldg. 48824-1325, East Lansing, MI, USA.

Received 13 October, 2016; Accepted 28 April, 2017

Seedling production of canjerana (*Cabralea canjerana* (Vell.) Martius) has been limited by difficulty in germination, caused by recalcitrant behavior of their seeds. The objective of this study was to develop an efficient protocol for conservation and production of canjerana micropropagated plantlets. Seeds of canjerana were disinfected with 0, 2.5, 5.0, 7.5 and 10% of NaOCl solution, at immersion times of 10, 20 and 30 min to produce aseptic seedlings, which were cultivated on Murashige and Skoog (MS) and Woody Plant Medium (WPM) media. Nodal segments were cultivated in WPM medium supplemented with 0 and 2.5 µM of 6-benzylamine purine (BAP), kinetin (KIN) and thidiazuron (TDZ) or with 0, 1, 3, 6, 9 and 12 µM of BAP. Micro-cuttings were cultivated in MS and WPM media with 0 and 5.0 µM of indole-3-butyric acid (IBA) and naphthaleneacetic acid (NAA). The rooted micro-cuttings were acclimatized in a humid chamber in a greenhouse. The highest percentage of aseptic seedlings was produced with a solution of 7.5% of NaOCl and immersion times of 20 and 30 min. Adding BAP, KIN and TDZ or increasing concentrations of BAP to the WPM medium did not increase shoot number and length. Neither the base medium nor the auxin (IBA and NAA) had a significant effect on the survival of micro-cuttings after 60 days of cultivation, but the addition of 5.0 µM of NAA did increase the percentage of rooting and survival during the acclimatization. Both nodal segments and micro-stumps of canjerana have showed a low rate of multiplication. The addition of 5.0 µM of NAA to the basal medium increased the percentage of *in vitro* rooting and the percentage of survival during acclimatization of canjerana plantlets.

Key words: *In vitro* propagation, *in vitro* multiplication, *in vitro* rooting, acclimatization, microclonal hedge.

INTRODUCTION

To complement conventional tree breeding programs, three specific biotechnological techniques have been successfully used, namely, tissue culture, for *in vitro* propagation and conservation; molecular markers, to assess the genetic diversity within and among populations; and encapsulation and cryopreservation, for long-term conservation (Varshney and Anis, 2014). Among these techniques, tissue culture is an appealing approach for conservation, because it aids in the propagation of species with limited number of explants,

low seed yield due to inbreeding depression (Hendrix and Kyhl, 2000), complex and unresolved seed dormancy mechanisms (Merritt and Dixon, 2003; Merritt et al., 2007), and poor seed set and viability due to environmental stresses, such as drought, predation, and/or disease (Varshney and Anis, 2014). Tissue culture is an alternative for canjerana (*Cabralea canjerana* (Vell.) Martius), an important native tree species of many countries of South America. Canjerana seeds are difficult to store, having a drastic reduction in germination viability after 15 days of seed processing (Grunennvaldt et al., 2014). This species has an excellent quality of wood, being used in construction, recovery of degraded areas and by the timber industry (Carvalho, 2003; Pereira et al., 2011). Moreover, the fruit is used in popular medicine due to his biochemical properties against *Trypanosoma cruzi*, which causes chagasic disease (Fournet et al., 1996).

Micropropagation allows the *in vitro* conservation and plantlet production of selected genotypes at any time from a microclonal hedge, that is composed of the root system and the rest of the aerial part of excised plants (micro-stumps), from micropropagated plantlets produced by *in vitro* subcultures and the miniclonal hedge that consists of rooted mini-cuttings from conventional cutting method-derived sprouts (Sivarajan et al., 2014), and can be derived from seedlings, cuttings or micropropagation (micro-stumps) (Titon et al., 2006). Indeed, mini-cutting is one of the latest vegetative propagation techniques used for mass production of woody species plantlets (Hartmann et al., 2011).

Micropropagated plantlets are highly desirable as microclonal hedge material, because they have the advantages of high genetic and phytosanitary quality and physiological juvenility, which allows for the production of mini-cuttings with high competence for rooting (Varshney and Anis, 2014). Therefore, combining conventional breeding with biotechnological techniques enables the identification and multiplication of superior genotypes, necessary for rapid and uniform forest production (Haggman et al., 2014).

The *in vitro* cultivation process of forest species has different phases, emphasizing the initial establishment of *in vitro* cultures and the acclimatization of plants after rooting, which may be the major obstacles of micropropagation (Grattapaglia and Machado, 1998). *In vitro* establishment of forest species has several difficulties in obtaining tissues free from contamination caused by fungi and bacteria (Thorpe et al., 1991), while the major problem of acclimatization is the high mortality due to many species do not have the ability to convert from heterotrophic to autotrophic growth (Paiva and Oliveira, 2006).

Micropropagation of native trees has been accomplished in Brazil with *Handroanthus heptaphyllus* (Vell.) Mattos (Duarte et al., 2016; Pimentel et al., 2016), *Apuleia leiocarpa* (Vogel J. F. Macbride) (Lencina et al., 2016), *Ilex paraguariensis* (Saint Hilaire) (Tronco et al., 2015) and *Handroanthus impetiginous* (Mart. ex DC Mattos) (Martins et al., 2011), while in the USA with *Liquidambar styraciflua* (L.) (Durkovic and Lux, 2010), *Sequoia sempervirens* (D Don Endl.) (Ozudogru et al., 2011), and *Quercus alba* (L.) (Vieitez et al., 2012), among other species. Besides this, more studies on micropropagation of native trees species, including those with great quality of timber as canjerana, are needed, since only one study has been developed with micropropagation of this species as well as, the authors reported that this protocol needs to be optimized (Rocha et al., 2007).

Thus, the objective of this study was to develop a protocol for the micropropagation of canjerana from seeds of selected individuals, as a tool for conserving and producing plantlets of superior genotypes for field evaluations and breeding new cultivars.

MATERIALS AND METHODS

The study was carried out in the laboratories and greenhouses of the Center for Plant Breeding and Asexual Propagation, Department of Plant Science, Federal University of Santa Maria, Brazil. Eight experiments were carried out, including seed disinfection and germination, *in vitro* multiplication and rooting, and acclimatization in the greenhouse. To start the process, fruits of canjerana were collected from selected stock plants (Figure 1A), packed in a sealed plastic bag and stored in a cool and dry environment of the laboratory until they dehisced (Rocha et al., 2007). Seeds were extracted when the fruits first opened, and immediately pre-disinfected with a solution of 2.5% of sodium hypochlorite (NaOCl) for 10 min and rinsed three times with distilled water (Figure 1B).

For *in vitro* establishment of canjerana seedlings (Experiment 1), seeds were first washed with distilled H_2O in a sterilized metallic sieve before removing the aril. To obtain aseptic canjerana seedling, seeds were immersed in a 0, 2.5, 5.0, 7.5, or 10% solution of NaOCl, with one drop of Tween® 20 per 100 ml of distilled water for 10, 20 and 30 min and rinsed three times with sterile-distilled water. The 0% concentration contained only one drop of Tween® 20 per 100 ml of distilled water. All these procedures were accomplished in a laminar flow chamber under aseptic conditions. After disinfection, one seed was cultivated in glass culture tubes (15 ml) containing 5 ml of culture medium with 30 g L^{-1} of sucrose and 6 g L^{-1} of agar, with pH adjusted to 5.8, capped with aluminum foil and autoclaved for 20 min at 1 atmosphere.

The experiment was set up as a factorial, 5 × 3 (NaOCl concentrations and immersion times, respectively) in a complete random design with eight replications of five seeds. Germinated seeds (seeds with visible radicles) (Figure 1C) were scored every three days; to calculate the mean germination time (MGT) and the

*Corresponding author. E-mail: dilson.bisognin@ufsm.br. Tel/Fax: 0xx55 32208900.

Author(s) agree that this article remains permanently open access under the terms of the Creative Commons Attribution License 4.0 International License

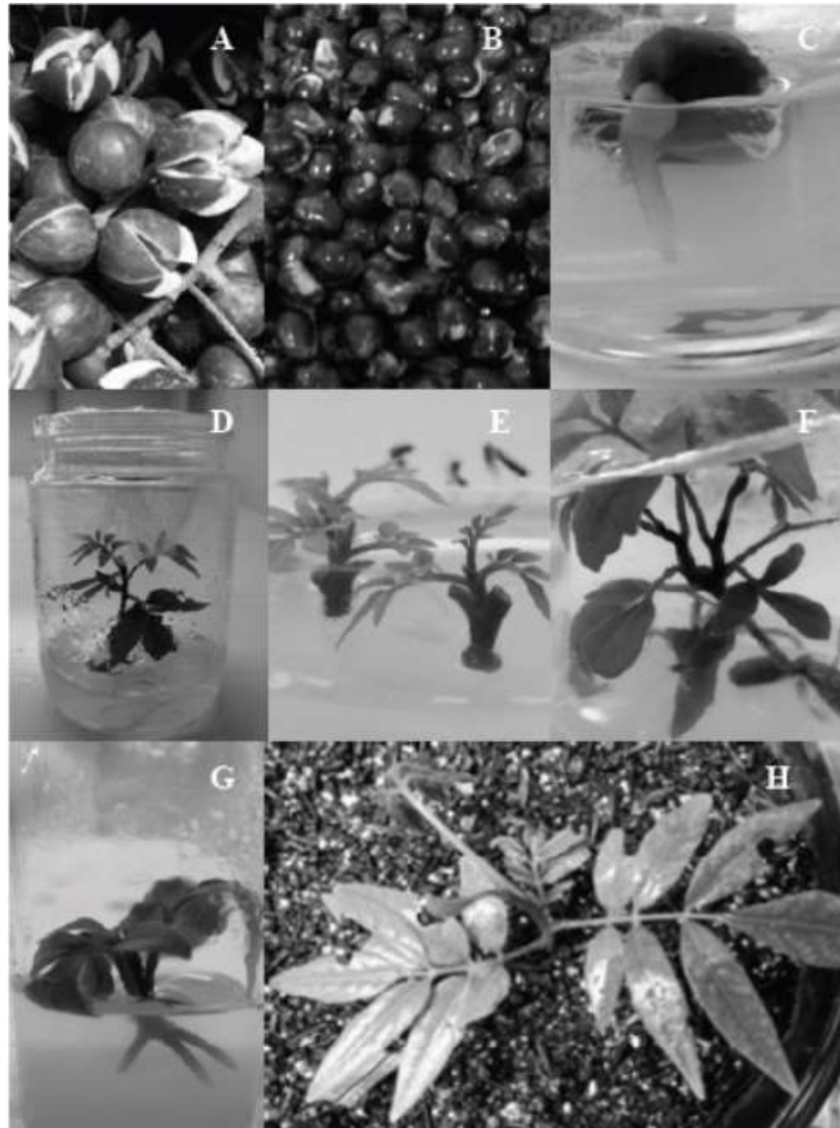


Figure 1. Process of plantlet production from selected canjerana stock plants: dehiscence of fruits of canjerana (A) disinfected seeds (B), *in vitro* germination (C). Aseptic seedlings of canjerana obtained from in vitro establishment and cultivated in different culture media for in vitro growth (D), which provided the explants used for in vitro multiplication (E). The microstump with new shoots (F), micro-cutting rooted in culture medium and ready for acclimatization (G) and micro-cutting acclimatizated in greenhouse (H).

index of germination rate (IGR).

MGT = (N1T1+N2T2+...+NnTn) / (N1+N2+...+Nn), and IGR = (N1/T1+N1/T2+...+Nn/Tn),

where N is the number of germinated seeds on a particular day and T is the time in days (Fick et al., 2007).

Thirty days after inoculation, the percentage of seeds that were free of fungi and/or bacteria, the germination rate and the proportion of normal seedlings were also evaluated.

To evaluate the effect of culture medium on *in vitro* growth (Experiment 2), aseptic seedlings of canjerana obtained from *in vitro* establishment were cultivated in three different culture media, MS (Murashige and Skoog, 1962), ½ MS (MS with half concentration of minerals and vitamins), and Woody Plant Medium (WPM) (Lloyd and McCow, 1981), with 30 g L^{-1} of sucrose and 6 g L^{-1} of agar (Figure 1D), with pH adjusted to 5.8, capped with aluminium foil and autoclaved for 20 min at 1 atmosphere.

The experiment was set up as a complete random design with four replications of four seedlings. The plant height, number of leaves and internodes and total length of roots were evaluated after 60 days of seedling cultivation.

For *in vitro* multiplication (Experiment 3), aseptic seedlings of canjerana were also used as source of explants. The explants were separated into basal, intermediate and apical segments that were 0.5 to 1.0 cm in length and contained one axillary bud. The explants were planted in flask (150 ml) containing 40 ml of WPM medium with 0 or 2.5 μM of 6-benzylamine purine (BAP), 30 g L^{-1} of sucrose

and 6 g L^{-1} of agar. The pH was adjusted to 5.8, capped with aluminium foil and autoclaved for 20 min at 1 atmosphere.

The experiment was set up as a factorial 2 × 3 (BAP concentration and type of segments) in a complete random design with five replications of four segments. Shoot number and length (cm) were evaluated after 60 days of cultivation (Figure 1E).

Basal and intermediate segments of canjerana aseptic seedlings were used to study the effect of different cytokines and BAP concentrations on *in vitro* multiplication. For experiment 4, segments of canjerana were planted in WPM medium containing 2.5 µM of BAP, kinetin (KIN), or thidiazuron (TDZ) added with 30 g L^{-1} of sucrose and 6 g L^{-1} of agar. For experiment 5, segments of canjerana were planted in WPM medium containing 0, 1.0, 3.0, 6.0, 9.0, or 12 µM of BAP added with 30 g L^{-1} of sucrose and 6 g L^{-1} of agar. The pH adjusted to 5.8, capped with aluminum foil and autoclaved for 20 min at 1 atmosphere. Both experiments were carried out in complete random design, with eight replications of six segments. Shoot number and length (cm) were evaluated after 60 days of cultivation.

After excising the nodal segments used as explants for the previous experiments, the root system and the rest of the aerial part of the aseptic seedlings (micro-stumps) were transferred to MS or WPM medium added with 30 g L^{-1} of sucrose and 6 g L^{-1} of agar (Figure 1F) (Experiment 6). The pH adjusted to 5.8, capped with aluminum foil and autoclaved for 20 min at 1 atmosphere. The experiment was set up in a complete random design, with eight replications of six micro-stumps. The percentage of micro-stump survival and the number of micro-cuttings produced per micro-stump were evaluated after 60 days of cultivation.

For *in vitro* rooting (Experiment 7), micro-cuttings were grown in MS or WPM basal media, with 0 and 5.0 µM of indolebutyric acid (IBA) or naphthalene acetic acid (NAA), added with 30 g L^{-1} of sucrose and 6 g L^{-1} of agar. The pH adjusted to 5.8, capped with aluminum foil and autoclaved for 20 min at 1 atmosphere. The micro-cuttings were 1 to 2 cm in length and had two leaves.

The experiment was a 2 × 3 factorial (base media and auxin concentrations) in a complete random design, with five replications of four micro-cuttings. The percentage of rooting was evaluated after 60 days of cultivation (Figure 1G).

For the acclimatization of canjerana plantlets (Experiment 8), the rooted micro-cuttings from the three culture media were planted in plastic pots containing 150 cm^3 of a commercial substrate (organic pine bark base, H.Decker Company, Santa Catarina, Brazil) for acclimatization. The pots were placed in the shade under a bench for a week and irrigated twice a day. The pots were then placed on the top of the bench and irrigated once a day, until the substrate exceeds the field capacity, and kept in a greenhouse with a mean temperature of 22°C and 50% of shade checked automatically through a control board with a thermohygrometer. The experimental was a complete random design, with five replications of four rooted micro-cuttings. The survival percentage was evaluated after 30 days of acclimatization (Figure 1H).

Data were subjected to analysis of variance and for those variables with significant differences ($p \leq 0.05$), treatment means were compared by Tukey test or polynomial regression, as appropriate. The percentage data were transformed to arcsin $\sqrt{x/100}$ and the counting data to $\sqrt{x+0.5}$ to meet the statistical presuppositions, especially variance homogeneity. All analysis were done with software Estat (UNESP, Jaboticabal) program (Estat., 1994).

RESULTS AND DISCUSSION

The analysis of variance in experiment 1 showed a significant interaction ($p \leq 0.05$) between concentrations of NaOCl and immersion times for the percentages of disinfection and germination. Seeds treated with 7.5% of NaOCl for 30 min had more than 80% of disinfection and more than 90% of these seeds germinated (Figure 2A and B). The percentage of disinfection and vigor (time to germination) were more strongly associated with the concentration of NaOCl than the immersion time (Figure 2A). The highest percentages of disinfection and germination were observed with the concentration of 7.5% of NaOCl for the three immersion times (Figure 2B).

In addition, the highest percentage of disinfection was found when seeds were treated with 7.5% of NaOCl for 20 or 30 min (Figure 2A), which was also associated with the highest vigor, quantified by the lowest mean germination time (Figure 2D), the highest index of germination rate (IGR) (Figure 2E) and the highest percentage of normal seedlings (Figure 2C). Therefore, the immersion times of 20 and 30 min where the most effective seed disinfection treatments without affecting germination and vigor. Seeds treated with 7.5 and 10% of NaOCl may have increased the degree of seed moisture (Sofiatti et al., 2008) and the permeability of seed tissues, reducing their germination time (Figure 2D) and increasing their germination rate. It probably occurred, because sodium hypochlorite is a strong oxidant and its action may change the properties of cell membranes of the integument or provide additional oxygen to the seed (Rocha et al., 2007).

In this study, a higher concentration of NaOCl and longer time of immersion than Rocha et al. (2007) was used without significantly affecting seed germination (Figure 2A) and vigor (Figure 2D). In canjerana seeds, concentrations of up to 10% of NaOCl for 30 min could be used, since it had a similar effect on seed germination and vigor as the 7.5% solution. After this work, this strategy is being used for *in vitro* establishment of aseptic seedlings of canjerana.

In the experiment 2, the base culture medium affected significantly seedling growth (Table 1). The WPM and MS medium resulted in taller seedlings, with more leaves and internodes and total length of roots than the ½ MS medium. Similar results were observed in explants of seedling from *Hagenia abyssinica* (Bruce J.F. Gmel), which presented either a similar *in vitro* growth, when cultivated in MS or WPM media (Feyissa et al., 2005). In contrast, plantlets of *Punica granatum* (L.) cultivated in WPM medium were apparently more vigorous and with longer shoots when compared with MS culture medium, which is an important feature in micropropagation (Valizadeh et al., 2013). Although, the most appropriate culture medium depends upon the species and type of explant; in the present study, aseptic seedlings of canjerana had a similar growth in both MS and WPM media. For this reason, WPM medium was hereinafter used on *in vitro* multiplication of canjerana, due to the fact that this culture medium developed by Lloyd and McCow (1981) has been the most widely used in studies

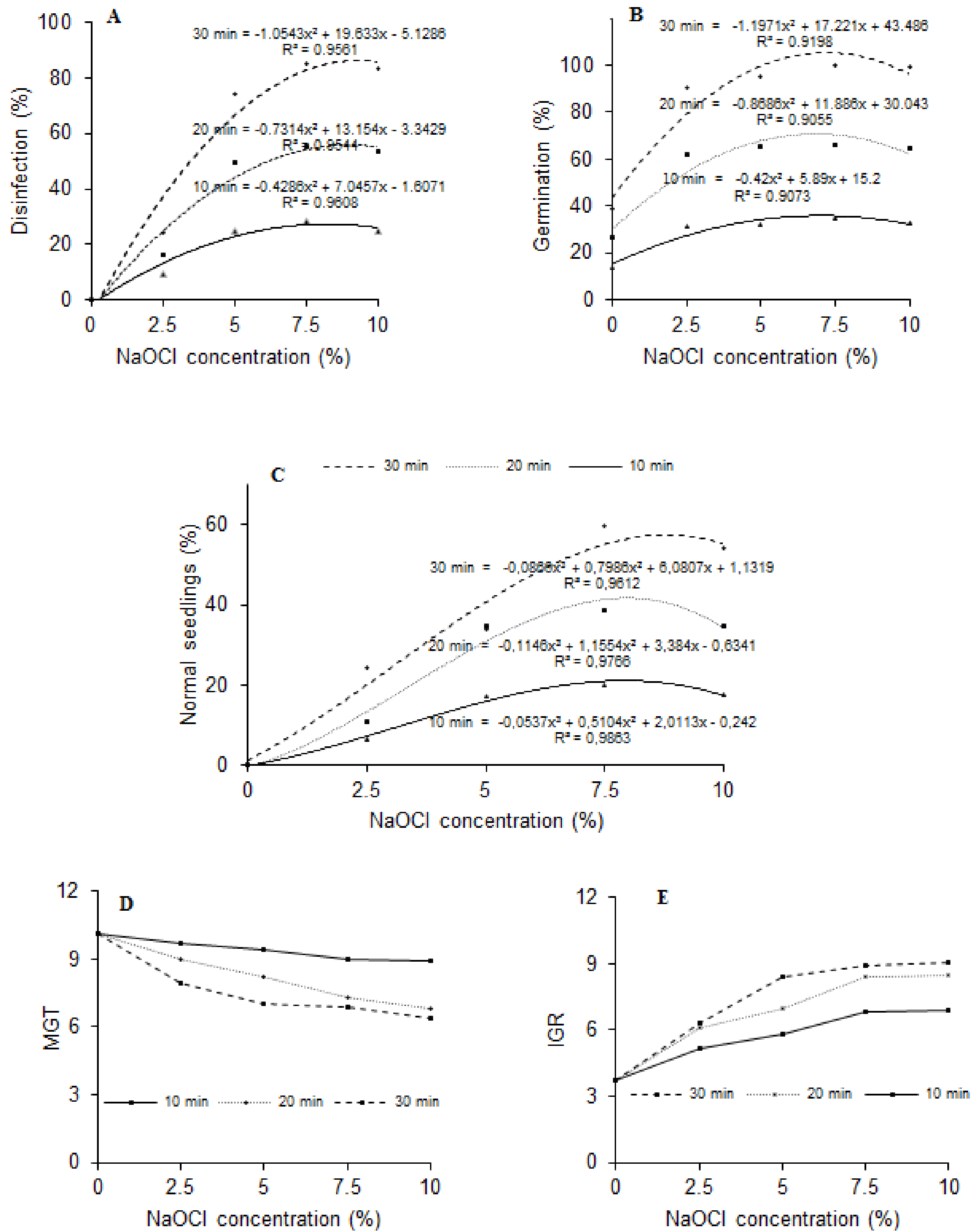


Figure 2. Percentages of not infected seeds (A), rates of germination (B), percentages of normal seedlings (C), mean germination time (MGT) (D), and the index of germination rate (IGR) (E) of canjerana seeds immersed in five different concentrations of NaOCl at different times.

involving propagation of woody species, as is the case of canjerana.

With regards to cytokines (Experiment 3), there were no significant interactions ($p \geq 0.05$) between BAP concentrations and the source of explants for the number and length of shoots after 60 days (Table 2). The largest number and length of shoots were verified in WPM medium supplemented with 6 μM of BAP, but with no statistically significant differences between the treatments (Supplemental Figure 1). It is likely that the greatest

Table 1. Length of aerial parts, number of leaves and internodes, and total length of roots of canjerana seedlings cultivated in three culture media for 60 days.

Treatment	Length of aerial parts (cm)	Number of leaves	Number of internodes	Total length of roots (cm)
WPM	3.7[a]*	5.1[a]	2.5[a]	10.3[a]
MS	3.6[a]	4.5[a]	2.5[a]	10.2[a]
½ MS	2.4[b]	2.5[b]	2.1[b]	6.6[b]

*Means values followed by the same letter are not significantly different by Tukey's test at 5% of probability.

Table 2. Shoot number and length of apical, intermediate and basal segments of canjerana cultivated in two concentrations of 6-benzylamine purine (BAP) for 60 days.

Treatments	Shoot number	Shoot length (cm)
Concentration		
BAP (0 µM)	0.3[a]*	0.2[a]
BAP (2.5 µM)	0.5[a]	0.5[a]
Type of segment		
Basal	0.9[a]	0.6[a]
Intermediate	0.4[ab]	0.4[ab]
Apical	0.0[b]	0.0[b]

*Means values followed by the same letter are not significantly different by Tukey's test at 5% of probability.

growth of shoots was observed in the basal and intermediate segments, independent of BAP concentration in the media. Thus, the lowest multiplication rate observed on apical explants of canjerana, might have occurred probably due to the adventitious shoots regenerated from the apical portion, which presented less vigor and therefore is being more sensitive to ethylene accumulation (Sá et al., 2012). Similar results were reported by Anis and Faisal (2005), who observed a higher rate multiplication in nodal segments of *Psoralea corylifolia*.

In the experiment 4, the addition of different cytokines in the media did not increase significantly the shoot number and length, and in fact TDZ significantly reduced the number of shoots (Table 3). In addition, in experiment 5, increasing the concentration of BAP in the media did not significantly affect shoot number and length (Supplemental Figure 1). Regardless of treatment, the number of shoots of canjerana was low. A low number of shoots were also observed with *Balanites aegyptiaca* Del. Varshney and Anis (2014) and it is in agreement with previous studies of canjerana (Rocha et al., 2007). According to these authors, the low number of shoots is because these species have erect stems and a small tree crown, which may affect the rate of *in vitro* multiplication and even the sprouting of vegetative material from the microclonal hedge. Another possibility would be that the physiology of canjerana segments results in a monopodial development. According to Nicoloso and Erig (2002), in *Pfaffia glomerata* (Spreng Pedersen), the physiological condition of the apical segment induced the expression of monopodial development, which is an expression of the relationship between the indoleacetic acid and cytokines (Hartmann et al., 2011).

Table 3. Shoot number and length of nodal segments of canjerana cultivated in different cytokinins for 60 days.

Cytokinins	Shoot number	Shoot length (cm)
BAP (2.5 µM)	0.8[a]*	0.5[a]
KIN (2.5 µM)	0.6[a]	0.5[a]
Control	0.6[a]	0.4[a]
TDZ (2.5 µM)	0.2[b]	0.2[a]

*Means values followed by the same letter are not significantly different by Tukey's test at 5% of probability.

In this study, there was no difference in shoot number and length even with the application of 12 µM of BAP. This inability to establish stabilized shoot cultures as well as a slow growth rate in microculture indicated recalcitrance of this species. This might be due to the exposure of explants to higher cytokine concentrations during the induction phase may have led to the accumulation of cytokines, which inhibited further shoot multiplication and growth (Malik et al., 2005).

Table 4. Percentage of survival of micro-stumps and number of micro-cutting per micro-stump of canjerana cultivated in two culture media for 60 days.

Culture media	Survival (%)	Number of micro-cuttings
WPM	70.0[a]*	1.40[a]
MS	40.0[b]	0.95[a]

*Means values followed by the same letter are not significantly different by Tukey's test at 5% of probability.

Table 5. Percentages of survival and rooting of canjerana micro-cuttings cultivated on two base medium, with or without two auxins after 60 days and the percentage of survival of rooted micro-cuttings after 30 days of acclimatization in a humid chamber in the greenhouse.

Treatments	Survival (%)	Rooting (%)	Survival (%)
Base medium			
WPM	75.0[a]*	20.0[a]	-
MS	65.0[a]	31.6[a]	-
Auxins			
NAA (5.0 µM)**	70.0[a]	57.5[a]	62.5[a]
IBA (5.0 µM)***	67.5[a]	20.0[b]	37.5[b]
Control (0 µM)	72.5[a]	0.0[c]	0.0[c]

*Means followed by the same letter are not significantly different by Tukey's test at 5% of probability. **NAA: Naphtalene acetic acid. ***IBA: indolebutyric acid.

In the experiment 6, the culture media did not affect significantly the production of micro-cuttings per micro-stump, but the WPM medium resulted in a higher percentage of micro-stump survival than the MS medium (Table 4). As observed with nodal segments, micro-stumps also showed a small number of shoots resulting in a low yield of micro-cuttings, with an average of 1.1 micro-cutting per micro-stump, compared to *Eucalyptus* species hybrids with a production between 1.5 and 2.3 micro-cuttings per micro-stump (Wendling at al., 2000). According to Titon et al. (2003), few shoots emitted by micro-stump is a normal response after decapitation of the apex, possibly due to the persistence of apical dominance. However, it is expected that through a proper management of micro-stumps by performing successive collections of micro-cuttings, causes a loss of apical dominance and increase the number of vegetative propagules of canjerana.

In the experiment 7, the base media and type of auxin had no significant effect on micro-cutting survival after 60 days of cultivation, but the addition of 5.0 µM of NAA increased the percentage of rooting and also the percentage of survival during acclimatization (Table 5). The addition of NAA to the base medium resulted in a higher percentage of rooting (57.5%) than the addition of IBA (20%) (Table 5). This percentage of rooting is not as high as the 87.5% found with segments of canjerana cultivated in ½ MS medium added with 5.0 µM of IBA reported by Rocha et al. (2007). This similar result reported by Rocha et al. (2007), was achieved with a tropical medicinal tree species, *Garcinia indica* (Thouars) Choisy in 66.6 to 91.66% of shoots cultivated on half-strength MS medium supplemented with 10 µM of IBA, while 52.77 to 77.77% shoots developed roots on NAA supplemented media (Malik et al., 2005).

In the experiment 8, the addition of 5.0 µM NAA to the base medium also increased significantly the percentage of survival during acclimatization from 37.5 to 62.5% (Table 5), although these values were not very high. Based on preliminary studies with different compositions of substrates, it is likely that higher percentages of survival during acclimatization can be achieved by selecting appropriate combination of substrate composition and keeping high air humidity in the wet chamber, with a relative humidity of approximately 85%, supplied automatically by a climate control.

Conclusion

To obtain vigorous and aseptic seedlings, seeds of canjerana can be treated with a 7.5% of sodium hypochlorite solution for 30 min and inoculated in a 30 g L^{-1} of sucrose and 6 g L^{-1} of agar, with pH adjusted to 5.8.

These seedlings can be grown in either WPM or MS media to increase the number of propagules produced per genotype. Nodal segments will not root in MS and WPM media; however, shoots produced from micro-stumps do root when 5.0 µM of NAA is added to the basal media. Nodal segments of canjerana and micro-stumps have a low rate of multiplication; therefore, tissue culture will be most valuable in maintaining superior genotypes of canjerana *in vitro* and as a source of stock plants for the microclonal hedge. The micropropagation of canjerana, as other native tree species, has a high potential for maintaining germoplasm *in vitro*, accelerating the breeding scheme for the identification of desirable genotypes.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors are grateful to Coordination for the Improvement of Higher Level Personnel (Capes) and the National Council for Scientific and Technological Development (CNPq) for the research fellowships.

REFERENCES

Anis M, Faisal M (2005) *In vitro* regeneration and mass multiplication of *Psoralea corliyfolia*- an endangered medicinal plant. Indian J. Biotechnol. 4:261-264.

Carvalho PER (2003). Espécies arbóreas brasileiras, 644 p.

Duarte E, Sansberro P, Luna, C (2016) Micropropagation of *Handroanthus heptaphyllus* (Vell.) Mattos from seedling explants. Afr. J. Biotechnol. 15(25):1292-1298.

Durkovic J, Lux A (2010). Micropropagation with a novel pattern of adventitious rooting in American sweetgum. Trees 24(3):491-497.

Estat. (1994). Sistema para análises estatísticas (v.02). Jaboticabal: Departamento de Ciências Exatas, FCAV-UNESP.

Feyissa T, Welander M, Negash L (2005). Micropropagation of *Hagenia abyssinica*: A multipurpose tree. Plant Cell Tissue Organ Cult. 80:119-127.

Fick TA, Bisognin DA, Quadros, KM, Horbach M, Reiniger LRS (2007). Estabelecimento e crescimento in vitro de plântulas de Louro Pardo. Ciência Florestal 17(4):343-349.

Fournet A, Rojas AA, Charles B, Bruneton J (1996). Chemical constituents of essential oils of Muña, Bolivian plants traditionally used as pesticides, and their insecticidal properties against Chagas disease vectors. J. Ethnopharmacol. 52:145-149.

Grattapaglia D, Machado MA (1998). Micropropagação. In. Torres AC, Caldas LS, Buso JA. Cultura de tecidos e transformação genética de plantas 1:43-76.

Grunennvaldt RL, Cantarelli EB, Salamoni AT (2014). Armazenamento e viabilidade de sementes de *Cabralea canjerana*. Comunicata Scientiae 5(1):98-105.

Haggman H, Sutela S, Walter C, Fladung M (2014). Challenges and Opportunities for the World's Forests in the 21st Century. In. Biosafety Considerations in the Context of Deployment of GE. Trees Pp. 491-525.

Hartmann HT, Kester DE, Davies FT, Geneve RL (2011). Plant propagation: principles and practices. 928 p.

Hendrix SD, Kyhl JF (2000). Population size and reproduction in *Phlox pilosa*. Conserv. Biol. 14(1):304-313.

Lencina KH, Bisognin DA, Pimentel N, Kielse P, Mello US (2016). *In vitro* multiplication of apuleia (*Apuleia leiocarpa* (Vogel) J. F.Macbride). Aust. J. Basic Appl. Sci. 10(10):185-191.

Lloyd G, McCow B (1981). Commercially feasible micropropagation of Mountain laurel, *Kalmia latifolia*, by use of shoot tip culture. Combined Proceedings of the International Plant Propagators Society 30:421-427.

Malik SK, Chaudhury R, Kalia RK (2005). Rapid *in vitro* multiplication and conservation of *Garcinia indica*: A tropical medicinal tree species. Sci. Hortic. 106:539-553.

Martins JPR, Santos BR, Barbosa S, Máximo WPF, Beijo LA, Paiva R (2011). Crescimento e Aspectos Sintomatológicos na Aclimatização de Ipê Roxo. Cerne 17(4):435-442.

Merritt DJ, Dixon KW (2003). Seed storage characteristics and dormancy of Australian indigenous plant species In. *Seed conservation:* turning science into practice. Smith RD, Dickie JB, Linington SH, Pritchard HW and Probert RJ Royal Botanical Gardens Kew, Pp. 809-823.

Merritt DJ, Turner SR, Clarke S, Dixon KW (2007). Seed dormancy and germination stimulation syndromes for Australian temperate species. Aust. J. Bot. 55(3):336-344.

Murashige T, Skoog, F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15(3):473-497.

Nicoloso FT, Erig AC (2002). Efeito do tipo de segmento nodal e tamanho do recipiente no crescimento de plantas de *Pfaffia glomerata in vitro*. Ciênc. Agrotec. 26:1499-1506.

Ozudogru EA, Kirdok E, Kaya E, Capuana M, Carlo AD, Engelmann F (2011). Medium-Term conservation of Redwood in vitro shoots cultures and encapsulated buds. Sci. Hortic. 127(3):431-435.

Paiva R, Oliveira LM (2006). Fisiologia e Produção Vegetal. 104p.

Pereira MF, Bandeira LF, Blanco AJV, Coelho ASG, Ciampi AY, Franceschinelli EV (2011). Isolation anda characterization of microsatellite loci in *Cabralea canjera*na (Meliaceae). Am. J. Bot. 98(1):10-12.

Pimentel N, Bisognin DA, Rodrigues MB, Kielse P, Lencina KH (2016). Micropropagation of *Handroanthus heptaphyllus* (Vellosso Mattos). Aust. J. Basic Appl. Sci. 10(16):79-87.

Rocha SC, Quorim M, Ribas, LLF, Koehler, HS (2007). Micropropagação de *Cabralea canjerana*. Rev. Árvore 31(1):43-50.

Sá AJ, Lédo AS, Lédo CAS, Pasqual M, Silva AVC, Silva Junior JF (2012). Sealing and explant types on the mangaba micropropagation. Ciênc. Agrotec. *36*(4):406-414.

Sivarajan A, Anbazhagan M, Arumugam K (2014). Clonal propagation of *Eucalyptus grandis* using different techniques. Int. J. Res. Plant Sci. 4(2):42-45.

Sofiatti V, Araujo EF, Araujo RF, Reis MS, Silva LVBD, Cargnin A (2008). Uso do hipoclorito de sódio para degradação do endocarpo de sementes de cafeeiro com diferentes graus de umidade. Rev. Bras. Sementes 30(1):150-160.

Thorpe TA, Harry IS, Kumar PP (1991). Application of micropropagation to forestry. In. Micropropagation. Eds. Debergh PC, Zimmerman RH. pp. 311-336.

Titon M, Xavier A, Otoni WC (2006). Clonal propagation of *Eucalyptus grandis* using the mini-cutting and micro-cutting techniques. Scientia Forestalis 71: 109-117.

Titon M, Xavier A, Otoni WC, Reis GG (2003). Efeito do AIB no enraizamento de miniestacas e microestacas de clones de *Eucalyptus grandis* W. Hill ex Maiden. Rev. Árvore 27(1):1-7.

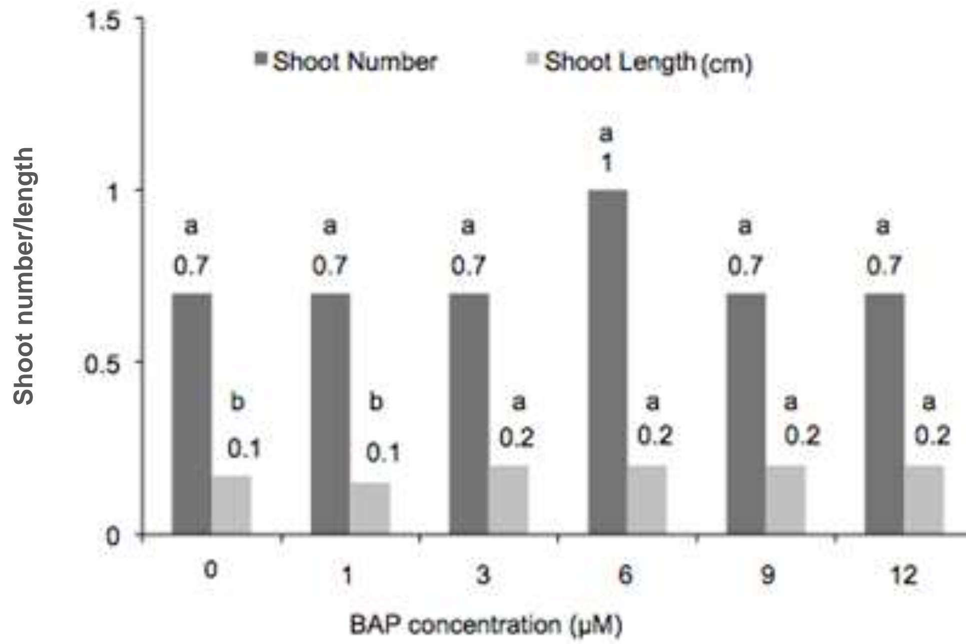
Tronco KMQ, Bisognin DA, Fleig FD, Horbach MA (2015). Enraizamento *ex vitro* e aclimatização de microestacas de *Ilex paraguariensis* A. St Hil. Cerne 21(3):371-378.

Valizadeh BK, Ershadi A, Tohidfar M (2013). *In vitro* propagation of pomegranate (*Punica granatum* l.) Cv. 'Males Yazdi'. Albanian J. Agric. Sci. 12(1):1-5.

Varshney A, Anis M (2014). Trees: propagation and conservation. 116p.

Vieitez AM, Corredoira E, Martínez MT, San José MC, Sánchez C, Valladares S, Vidal N, Ballester A (2012). Application of Biotechnological tools to Quercus improvement. Eur. J. Forest Res. 131:519-539.

Wendling I, Xavier A, Gomes JM, Pires IE, Andrade HB (2000). Vegetative propagation of *Eucalyptus* spp. Hybrids by micro-cutting. Rev. Árvore 24(2):181-186.



Supplemental Figure 1. Shoot number and length of nodal segments of canjerana cultivated in different concentrations of 6-benzylamine purine (BAP) for 60 days.

academicJournals

Vol. 16(24), pp. 1354-1365, 14 June, 2017
DOI: 10.5897/AJB2017.15942
Article Number: D4548B964723
ISSN 1684-5315
Copyright © 2017
Author(s) retain the copyright of this article
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Optimised cetyltrimethylammonium bromide (CTAB) DNA extraction method of plant leaf with high polysaccharide and polyphenolic compounds for downstream reliable molecular analyses

Turaki A. A.[1,2,3]*, Ahmad B.[3], Magaji U. F.[3], Abdulrazak U. K.[3], Yusuf B. A.[3] and Hamza A. B.[1,2]

[1]Natural Resources Institute, University of Greenwich, UK.
[2]Kebbi State University of Science and Technology, Aliero, Kebbi State, Nigeria.
[3]Federal University of Birnin Kebbi, Kebbi State, Nigeria.

Received 14 February, 2017; Accepted 28 April, 2017

Yam (*Dioscorea* species) and banana (*Musa* species) leaf sample contain high levels of polysaccharide and polyphenolic compounds. Extraction of good quality DNA from such leaves is usually problematic. Therefore, there is a need to extract good quality DNA in order to perform downstream DNA analysis, especially for the detection of viruses due to their low titration in some infected leaves. The cetyltrimethylammonium bromide (CTAB) DNA extraction method was selected in order to optimise DNA extraction from plant material with high level of polysaccharides. Four steps of the Lodhi et al. (1994) method were modified to be user friendly in less resource laboratories of developing countries. Yield and purity of the extracted DNAs were quantified using a NanoDrop 2000 spectrophotometer and by migration on an agarose gel after polymerase chain reaction (PCR) amplification. The results of the DNA yields and purity were in the range of 287.40±2.23 to 424.95±1.85 ng/ul and 2.10±0.05 to 2.19±0.04, respectively. Modification was found to yield DNA of reasonable quantity, quality and purity. Furthermore, the method can be used in a laboratory with less sophisticated waste disposal system.

Key words: Deoxyribonucleic acid (DNA), cetyltrimethyl ammonium bromide (CTAB), yam, banana, tomato.

INTRODUCTION

Yam (*Dioscorea* species) leaf sample contain high levels of polysaccharide and polyphenolic compounds. The presences of these compounds in yam leaf tissue often affect the isolation of good quality DNA (Kenyon et al., 2008). This has presented a problem for the downstream molecular analyses of extracted DNAs from yam samples especially in the detection and analysis of yam viruses such as badnaviruses. Moreso, the low virus titre in

*Corresponding author. E-mail: turakialiyu@yahoo.com. Tel: +234(0) 8148534433.

Author(s) agree that this article remains permanently open access under the terms of the Creative Commons Attribution License 4.0 International License

infected yams does not allow the expression of a well-defined symptoms on yams leaves (Seal and Muller, 2007; Seal et al., 2014). Therefore, the need for extracting reasonably pure DNA of both good quality and quantity for the downstream successful DNA-based diagnostic techniques is required. Hence, the aim of this study was to optimise a DNA extraction methodology for plants with high levels of polysaccharide and polyphenolic compounds. Cetyltrimethylammonium bromide (CTAB) DNA extraction method was selected as the method to optimise, as it is generally the method of choice for plant material with high polysaccharides and other inhibitory substances; it has been used successfully for yam in the past (Lebas, 2002; Kenyon et al., 2008) and for other recalcitrant plant species (Csaikl et al., 1998; Michiels et al., 2002; Sharma et al., 2008; Tiwari et al., 2012). Extracted DNA that is pure give good polymerase chain reaction (PCR) products compared to DNA with lower purity which requires several dilutions before it will give a good PCR product. In this study, polymerase chain reaction (PCR) methods were used to evaluate DNA purity by detecting the presence of yam badnavirus sequences on samples known to be badnavirus positive. PCR techniques provide greater sensitivity compared to other methods such as symptom observation and serological methods (Kenyon et al., 2008; Seal et al., 2014).

MATERIALS AND METHODS

Plant

Plant samples used for the modification of the CTAB method and detection of badnaviruses consisted of fresh yam leaf samples obtained from the International Institute of Tropical Agriculture (IITA) (n = 231), dried yam leaf samples collected by Dr. Ed Canning in 1997 (n = 44) during Cameroon survey and Professor Susan Seal in 2001 (n = 7) from CIRAD France, as well as fresh and etiolated leaves of *Dioscorea rotundata*, *Dioscorea alata,* banana and tomato grown in the quarantine glasshouse at Natural Resources Institution, UK. A total of 275 plant samples were used for DNA extraction.

Optimisation of the DNA extraction protocol

The Lodhi et al. (1994) DNA extraction method was modified for the extraction of total DNA from yam leaf material. Banana and tomato leaf tissues were included in the optimisation experiments in order to determine if modifications made were specific improvements for yam tissue alone or modifications to other plant species as well. Banana and yam samples represented plant leaf tissue with high levels of polysaccharides and polyphenols, whereas tomato contains much lower levels of these PCR-inhibitory compounds (Peterson et al., 1997). DNAs extracted from three replicate leaf homogenate samples were used for the comparison of each of the modification steps. These modification steps were incorporated into the Lodhi et al. (1994) method in a stepwise manner and at each step the yield and purity of the extracted DNAs were quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, UK). The results of the DNA yields and purity were expressed in mean values ± standard error (SEM) of DNA in a total elution volume of 100 µl TE buffer represented in column charts.

Modification steps evaluated

Sodium sulphite and PVP-40 to prevent oxidation of polyphenolic compounds

Lodhi et al. (1994) used PVP-40 (1%) and β-mercaptoethanol (0.2%) in CTAB buffer for the removal of polyphenols. In this study, β-mercaptoethanol (0.2%) was replaced with 1% sodium sulphite (Na_2SO_3) and the PVP-40 concentration was increased to 2%. This replacement was because of the toxicity and difficulty associated with disposing of β-mercaptoethanol waste in developing countries compared to sodium sulphite and PVP-40 waste. Furthermore, the use of sodium sulphite and PVP-40 is more cheaper compared to the use of β-mercaptoethanol. These modification steps were denoted as NS (for the use of 1% Na_2SO_3 with 2% PVP-40) and BM (for the use of 0.2% β-mercaptoethanol with 1% PVP-40).

Cell lysis incubation time for the release of genetic material

The incubation times at elevated temperature are fundamental for the lysis of the cell walls and membranes by detergents (2% (w/v) CTAB, 100 mM Tris-HCl (pH 8.0), 20 mM EDTA, 1.4 M NaCl)) and hence this step was optimised. Lodhi et al. (1994) incubated the homogenate for 25 min at 60°C. In this study, homogenates were incubated at 60°C for a range of times (between 25 and 40 min). This variation in time was to find the optimum time required to obtain high yields of extracted DNAs from samples.

Phenol:Chloroform:Isoamyl alcohol step for the removal of proteins

Extraction of DNA involves adding an equal volume of organic solvent to an aqueous solution of tissue homogenate. Centrifugation of the mixture yields a lower organic phase that contains the extracted proteins, and an upper aqueous phase containing DNA. The Lodhi et al. (1994) method uses a chloroform:isoamyl alcohol (CAA 24:1,v/v) extraction step for removal of proteins. A modification step was evaluated by replacing CAA with a phenol:chloroform:isoamyl alcohol (PCAA, 25:24:1, v/v/v) extraction. The mixture of PCAA dissolve more organic compound from the mixture compared to CAA.

Cold isopropanol and sodium chloride for the removal of polysaccharide

Yam contains high levels of polysaccharides (Mumford and Seal, 1997). The Lodhi et al. (1994) method used ethanol with 5 M NaCl to selectively precipitate the DNA away from the polysaccharides. A modification was made in which ethanol was replaced with isopropanol. This was because DNA is less soluble in isopropanol and at low concentration of isopropanol DNA falls out of the solution faster compared to ethanol. The precipitation was performed at -20°C with varying incubation period time intervals (20 to 90 min) in order to select the optimum incubation time in terms of quantity and purity.

Detail steps of the modified Lodhi et al. (1994) CTAB method

(1) Weigh leaf tissues (~100 mg per sample) and placed in individual polythene bags or in a grinding motor.
(2) Add 1 ml of warm extraction buffer (2% (w/v) CTAB, 100 mM

Tris-HCl (pH 8.0), 20 mM EDTA, 1.4 M NaCl) containing freshly added 2% (w/v) polyvinylpyrrolidone-40 (PVP-40) and 1% (w/v) sodium sulfite (Na_2SO_3).
(3) Ground the leaf materials using a small hand roller or pistol
(4) Transfer crude extract (700 µl) into 1.5 ml microcentrifuge tubes
(5) Vortex and incubate in a water bath (60°C, 30 min) for cell lysis.
(6) Add 700 µl phenol:chloroform:isoamylalcohol 25:24:1 (v/v/v) into the incubated extract, vortexed and centrifuged (15000 *g*, 10 min) in a microcentrifuge tube for first clarification.
(7) Tranfer about 700 µl of the aqueous layer carefully into a fresh microcentrifuge tube then add 700 µl chloroform:isoamylalcohol 24:1 (v/v), vortexed and centrifuged (15000 *g*, 10 min) in a microcentrifuge tube for second clarification.
(8) Transfer about 700 µl of the aqueous layer carefully into a fresh microcentrifuge tube then add 75 µl of 5 M NaCl and 450 µl of cold isopropanol.
(9) Invert the mixture 4 to 5 times then incubate (-20°C, 1 h)
(10) Centrifuge the mixture (4°C, 15000 *g*, 10 min) to pellet the nucleic acids.
(11) Decanted the supernatant off carefully not to throw out the pellet.
(12) Wash the pellet by adding 500 µl of 70% (v/v) ethanol, and centrifuge (15000 *g*, 5 min).
(13) Repeat step 12.
(14) Dried the pellet and then re-suspended in 100 µl 1x TE buffer (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA) and stored at -20°C until use.
(15) Dilute 1:10 (v/v) extracted DNA in nuclease-free sterile distilled water prior to PCR.

Statistical analyses

The statistical tools used for the analyses of the DNA concentrations and purity results obtained from the DNAs extracted using the CTAB methods were as the following.

t-test

In this study, *t*-test comparing two samples assuming equal variances was used to find out if the null hypothesis is supported.

Analysis of variance (ANOVA)

ANOVA single factor was carried out to determine whether there were significant differences between the DNA concentration and purity obtained in the methods.

The null hypothesis

To determine if there is any significant difference between the DNA concentrations or purity obtained in the DNAs extracted using the modified CTAB method versus those obtained in the Lodhi et al. (1994) method and Lebas (2002).

Comparison of the modified CTAB to other DNA extraction methods

Once all the favoured modifications to the selected Lodhi et al. (1994) method had been made, the resulting revised method was termed the 'modified CTAB' method. DNA yields and purity of this method was then compared with the original method on various leaf materials. DNA extracted from yams (*D. alata* and *D. rotundata*), were compared to DNA extracted from banana and tomato leaves using the three methods of DNA isolation (Lodhi et al., 1994; Lebas, 2002), and newly modified CTAB method. The new modified CTAB method was also compared to the original for the extraction of DNA from leaf tissues that had either been freshly harvested, or had been stored for 24 h at 4°C or room temperature (RT).

Evaluation of extracted DNA as template for restriction digestion PCR and RCA amplification

To assess the effectiveness of the modified method, DNA (5 ng) were digested with restriction enzymes (Figure 4A) and electrophoresed alongside undigested DNA. Furthermore, badnavirus partial RT-RNaseH region (Badna-FP/-RP) primers were used at various DNA dilutions to determine if the expected PCR product (~ 579 bp) could be amplified.

PCR reaction

The PCR protocol was adapted from Yang et al. (2003) and 25 µl reactions were set up containing 1x PCR buffer (75 mM Tris-HCl, pH 8.8, 20 mM $(NH_4)_2SO_4$, 0.01% (v/v) Tween 20) (Thermo Scientific, UK), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP (Promega, UK), 0.4 µM of each primer, 1.25 U Super *Taq* polymerase (Thermo Scientific, UK) and 5 µl DNA template. PCR cycling was performed in a GeneAmp® 9700 cycler (Applied Biosystems, UK) programmed as follows: 94°C/2 min initial denaturation, 40 cycles of 94°C/20 s, 55°C/30 s and 72°C/1 min and final cycle of 72°C/10 min.

Gel staining

Agarose gels were stained using 5 µl 'Red Safe™ 20,000x' nucleic acid staining solution (iNtRON Biotechnology Inc., Korea) for 100 ml of warm agarose solution.

HEPES treatment for polysaccharides

A HEPES initial washing step of ground leaf tissue was tried to determine if it was effective at removing polysaccharides from the leaf tissue before extracting total DNAs by the modified CTAB extraction method. Ground fresh leaf tissue (100 mg) was thoroughly mixed with 1 ml of 0.1 M HEPES-buffer pH 8.0 containing (1% (w/v) PVP-40, 1% (w/v) L-ascorbic acid, 2% (v/v) β-mercaptoethanol) and centrifuged (10000 *g* for 5 min). The supernatant was then discarded before addition of CTAB extraction buffer.

Genomic DNA purification

For the purification of large scale CTAB DNA extractions, pelleted nucleic acids were re-suspended in 1x TE buffer containing 0.7 M NaCl. Once re-suspended, extracts were mixed with an equal volume of equilibration QBT buffer (750 mM NaCl; 50 mM MOPS pH 7.0; 15% (v/v) isopropanol, 0.15% (v/v) Triton X-100; Qiagen, UK). QBT buffer (5 ml) was added to each column (Tip-100\G, Qiagen, UK) and allowed to stand for 5 min for column equilibration. The sample mixtures were then applied to the columns and allowed to stand for 15 to 20 min depending on the follow of the sample through the column. The genomic DNA in the mixture binds to the column resin as the sample passes through the column allowing the RNA, proteins, polysaccharide and low molecular-weight impurities to be removed by a medium-salt wash QC buffer (1 M NaCl; 50 mM MOPS pH 7.0; 15% (v/v) isopropanol, Qiagen, UK). Genomic DNA was then eluted in a high-salt elution QF buffer (1.25 M NaCl; 50 mM Tris-HCl pH 8.5; 15% (v/v) isopropanol, Qiagen, UK) and then

concentrated and desalted by isopropanol precipitation.

Determination of quantity and purity of the extracted DNA

The quantity and purity of the extracted DNAs were analysed using a NanoDrop 2000 spectrophotometer (Thermo Scientific, UK). DNA purity is the ratio of spectrophotometric absorbance of DNA at λ=260 nm and protein at λ=280 nm.

RESULTS

Optimisation of DNA extraction protocols

The results obtained with different modification steps are described as the following.

Separation of DNA from polyphenols

The NS step was incorporated into the Lodhi et al. (1994) method to reduce the oxidation of phenolic plant metabolites during DNA extraction. The separation of DNA from phenolic compounds was historically reported as difficult (Ghaffari et al., 2011); however, the inclusion of antioxidants in the extraction buffer assists in overcoming downstream problems associated with the presence of oxidised phenolic compounds in DNA extracts. Results of DNA concentration and purity obtained from the inclusion of NS and BM step in Lodhi et al. (1994) were recorded and their mean values were compared (Figure 1A and B). Although there was a difference between the mean values obtained from both NS and BM inclusions (Figure 1A and B), statistically the *t*-test demonstrated that there was no significant difference (P>0.05) between the Lodhi et al. (1994) and modified methods including the NS and BM modification steps. Nevertheless, the utilisation of NS method is considered favourable compared to β-mercaptoethanol because of its toxicity and problems associated the waste disposal of β-mercaptoethanol especially in a less equip laboratory set up.

Release of genetic materials from the cell

To release nuclear material (DNA/RNA) into an extraction buffer is achieved through detergents (CTAB) to lyse cell walls and membranes. In this study, different incubation times (25 to 40 min) at 60°C were evaluated, and the optimum incubation time that yielded the greatest DNA yield was at 60°C for 30 min. The mean values of DNA concentration and purity obtained from the inclusion of NS and BM step and homogenate incubation time for 30 min and 25 min in Lodhi et al. (1994) were recorded and compared (Figure 1A to B). There was a significant difference (P<0.05) between the DNA concentrations achieved using the modified method and those by the Lodhi et al. (1994) method. In contrast, there was no significant difference (P>0.05) between the DNA purity of the modified method compared to the Lodhi et al. (1994) method. It was observed that incubation for 40 min and above and incubation below 25 min generated lower quantities of DNA and purity (data not shown).

Separation of DNA from proteins

Incubated homogenates were deproteinized using an organic solvent, which reduces the formation of insoluble protein-nucleic acid complex thereby separating the DNA in the aqueous phase and proteins in the organic phase. It is also important to make sure the pH of the organic solvent is >7.8 as a decrease in pH (that is, more acidic conditions) results in the absence of DNA in the aqueous phase. In this study, CAA was replaced by PCAA as the first clarification in the deproteinization step of the modified method. The results have indicated an increase in the mean values of both DNA purity and quantity as shown in Figure 1A and B. Statistically by the *t*-test, the null hypothesis is therefore rejected as there is a significant difference (P<0.05) between the DNA concentration/ purity of the modified method and those of Lodhi et al. (1994) method at this modification step. The increase in the mean values of DNA purity for the modified method compared with Lodhi et al. (1994) after PCAA step indicates that PCAA is better at removing proteins from DNA than CAA alone.

Separation of DNA from polysaccharides

Contamination of DNA with polysaccharides is problematic and difficult to eliminate. In this study, extracted DNAs were precipitated through different incubation lengths (20 to 90 min) at -20°C in cold isopropanol together with 5 M NaCl (data not shown). The optimum incubation time for the DNA precipitation was recorded at 1 h producing mean value ratios of $A_{260}/A_{230\ nm}$ above 1.6.

Comparison of the modified CTAB to other DNA extraction methods

The effectiveness of the modified CTAB method (including all favourable modification steps) was compared to Lodhi et al. (1994) using leaf tissues from three different species (banana, *D. rotundata* and tomato) stored at three different conditions (fresh, 4°C/24 h and RT/24 h). From the results, DNAs of high quantities were obtained from fresh leaf samples compared to etiolated leaves, and from the modified method compared to the Lodhi et al. (1994) method (Figure 2A). A significant difference (P<0.05) was observed between the DNA concentrations of fresh and etiolated leaf samples of *D. rotundata* and banana (and not for tomato; where P>0.05) using both modified method and Lodhi et al. (1994) method (Figure 2B).

Furthermore, the mean values of DNA concentration and purity for the extracted DNAs from four leaf tissues (banana, *D. alata*, *D. rotundata* and tomato) using the modified method were compared with those of the Lodhi et al. (1994) and Lebas (2002) methods as shown in Table 1 and Figure 3A and B. The DNA concentrations obtained from these methods were analysed. Statistically, there was a significant difference (P<0.05) between the results obtained with the three different methods for yam *D. rotundata* (P = 0.0021), *D. alata* (P = 0.0035) and banana (P = 0.0127) there was no significant difference (P>0.05) for tomato leaves (P = 0.0701). The mean value ratios obtained for $A_{260}/A_{230\ nm}$ were above 1.6 indicating low levels of polysaccharide contamination in the extracted DNA, and the $A_{260}/A_{280\ nm}$ ratio were above 1.8 (Table 1) representing low amounts of protein contamination in the extracted DNA.

PCR detection of badnaviruses

The quality of the extracted DNAs were also determined by running undigested and digested total DNA extracts. A perfect migration pattern was observed without any signs of degradation (Figure 4A). PCR of known badnavirus-positive *D. rotundata* leaf samples (fresh, etiolated and dried) were tested for badnavirus in the following dilutions: undiluted, 1:100 dilution and 1:1000 dilution as shown in Figure 4B. The results show that dilution of the DNA sample does affect the outcome of PCR reaction. Diluted DNAs gave intensive PCR bands compared to undiluted DNAs (Figure 4B). Furthermore, ~65% badnavirus PCR success was recorded in undiluted

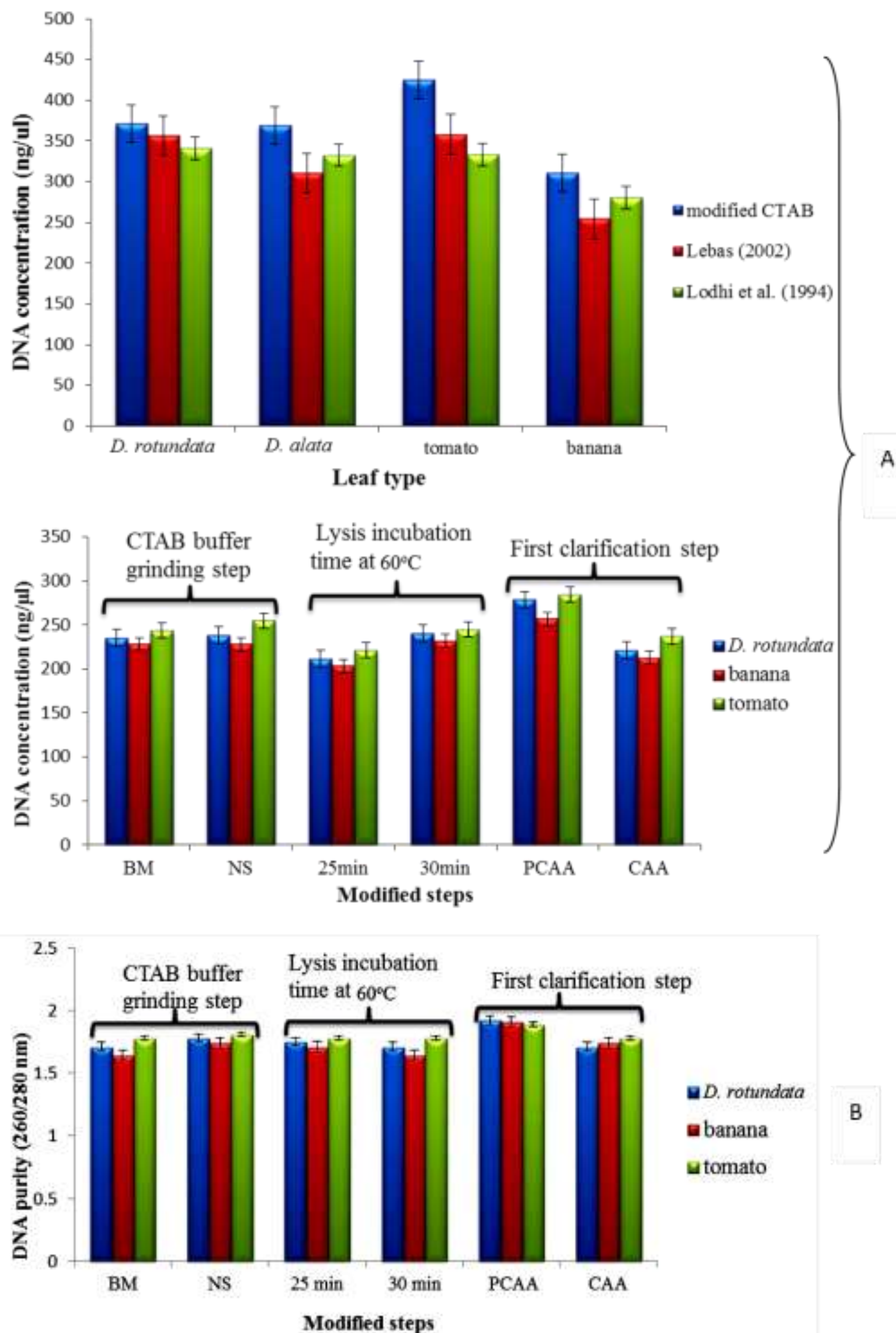


Figure 1. (A) Comparison of DNA concentration (mean ± SEM) of Lodhi et al. (1994) method and modifications steps of Lodhi et al. (1994) method using three different plant species (banana, *D. rotundata* and tomato). Statistically (using the *t*-test) the null hypothesis is rejected as $P<0.05$ between the DNA concentration of modified method and those of Lodhi et al. (1994) method at the modification step of 25 min and 30 min and PCAA and CAA, accepted ($P>0.05$) for NS and BM. (B) Comparison of DNA purity (mean ± SEM) of Lodhi et al. (1994) method and modifications steps of Lodhi et al. (1994) method using three different plant species (banana, *D. rotundata* and tomato). Statistically (using the *t*-test) the null hypothesis is accepted ($P>0.05$) between the DNA purity of modified method and those of Lodhi et al. (1994) method at the modification step of 25 and 30 min and NS and BM rejected ($P<0.05$) for PCAA and CAA.

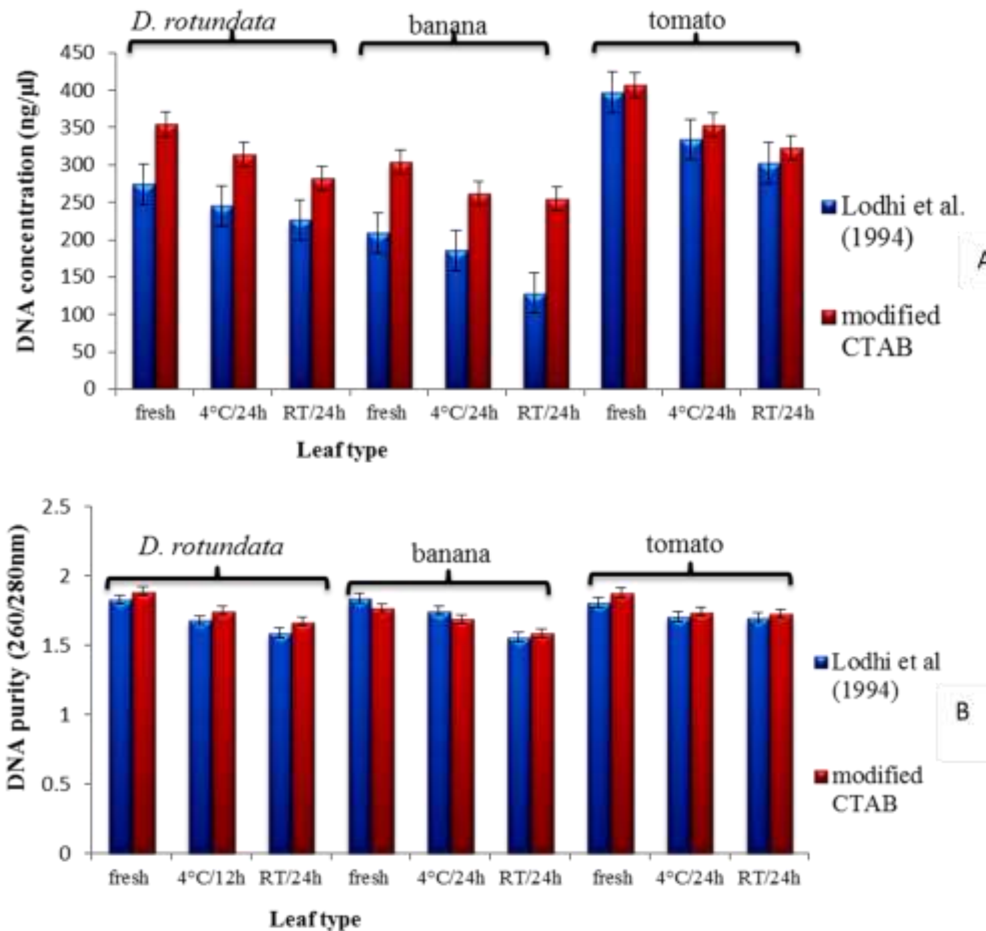


Figure 2. (A) Comparison of DNA concentration (mean ± SEM) of Lodhi et al. (1994) and modified CTAB method on three different plant species (banana, *D. rotundata* and tomato) at three different storage conditions. Statistically (using the ANOVA test) there is a significant difference ($P<0.05$) between the DNA concentrations of fresh and etiolated leaf samples of *D. rotundata* and banana, for tomato no significant difference ($P>0.05$) was found. (B) Comparison of DNA purity (mean ± SEM) of Lodhi et al. (1994) and modified CTAB method on three different plant species (banana, *D. rotundata* and tomato) at three different storage conditions. Statistically (using the ANOVA test) there is a significant difference ($P<0.05$) between the DNA purity of fresh and etiolated leaf samples of *D. rotundata* and banana, for tomato no significant difference ($P>0.05$) between the methods.

compared to the 100% success in diluted samples, indicating that dilution is vital to generate meaningful PCR results, presumably as it dilutes the concentration of PCR inhibitory substances (Figure 5).

After the method modification, a total of 231 DNAs were extracted from yam leaf samples obtained from IITA, and the DNAs were screened for the presence of badnavirus sequences by the PCR method using degenerate primers designed by Yang et al. (2003). The samples containing a band of the expected size (~579 bp) after agarose electrophoresis were scored as positive. The percentage PCR successes were evaluated (Figure 5) and this justified the effectiveness of the modified method in extracting sufficiently pure DNA. The method was also found to be suitable for extracting total DNA from dried, fresh or etiolated yam leaf tissue (Figure 5).

Purification of CTAB extracted DNA for max preps

Despite the optimized CTAB method able to extract high quality DNA from 100 mg leaf tissue, however, this modified method failed to yield DNA of sufficient quality on the 'maxi'-preps (~3 g of leaf tissue) needed for further analysis such as Southern blot hybridisation. Therefore, further optimisation of the CTAB method had to be carried out. Yields from two additional steps were compared to the modified CTAB method. The two additional steps tried were the inclusion of a polysaccharide pre-treatment step using HEPES, and a Qiagen Tip100/G column purification of the DNA generated with the modified CTAB method. Figure 6 shows that Qiagen Tip100/G column purification of extracted DNA (QC1-QC4) yielded DNA of better quality compared to the modified CTAB method (CT1-CT4) developed and the modified CTAB method on HEPES treated ground leaf (HP1-HP4). From the result in Figure 6, it can be seen that the low molecular weight DNA/RNAs were successfully removed from the DNAs of *D. rotundata* samples (lane QC1-2) using Qiagen Tip100/G column purification. In contrast, the low molecular weight DNA/RNA was not completely removed from *D. alata* sample (lane QC3-4). This could be due to large quantities of mucilaginous substances that were observed to drop through the

Table 1. Comparison of DNA quantity and purity extracted using three different CTAB DNA extraction methods.

Plant species	DNA concentration (ng/ul) obtained using method			DNA purity (A_{260}/A_{280nm}) obtained using method		
	Modified CTAB	Lebas (2002)	Lodhi et al. (1994)	Modified CTAB	Lebas (2002)	Lodhi et al. (1994)
D. alata	369.08 ±2.05	310.68 ±2.12	332.52 ±2.05	2.10 ±0.05	2.07 ±0.07	1.98 ±0.02
D. rotuntada	424.95 ±1.85	358.12 ±1.95	333.02±1.95	2.10 ±0.07	1.86 ±0.01	1.97 ±0.06
Banana	287.40 ±2.23	272.50 ±2.97	285.80 ±3.12	2.15 ±0.01	2.07 ±0.07	2.13 ±0.09
Tomato	371.10 ±2.15	356.60 ±2.45	340.70 ±1.60	2.19 ±0.04	2.11 ±0.02	2.14 ±0.08

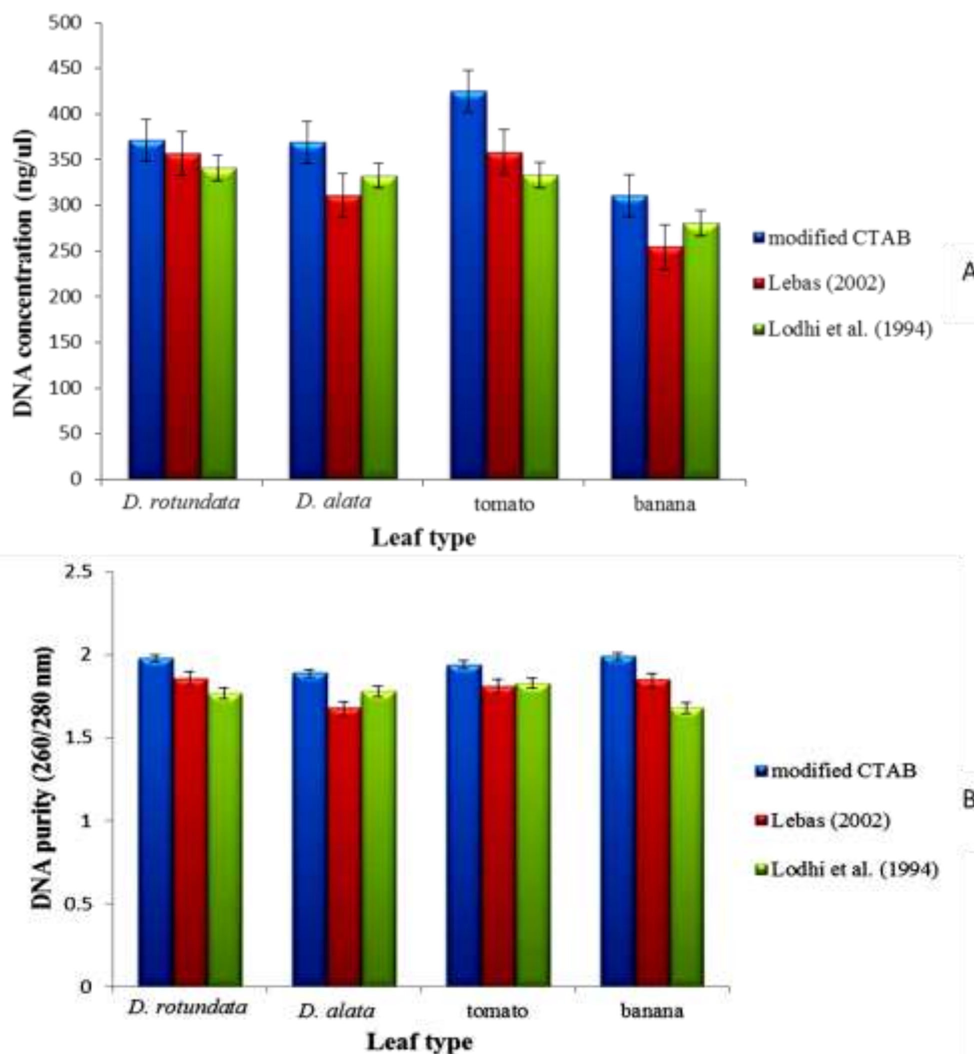


Figure 3. (A) Comparison of DNA concentration (mean ± SEM) of three CTAB methods of DNA extraction: the modified CTAB method, the Lodhi et al. (1994) method and the Lebas (2002) method using four different plant species. Statistically (using the ANOVA test), there was a significant difference ($P<0.05$) between the results obtained with the three different methods for yam *D. rotundata* ($P = 0.0055$), *D. alata* ($P = 0.0391$) and banana ($P = 0.00672$) no significant difference ($P>0.05$) for tomato ($P = 0.1819$). (B) Comparison of DNA purity (mean ± SEM) of three CTAB methods of DNA extraction namely the modified CTAB method, the Lodhi et al. (1994) method and the Lebas (2002) method using four different plant species. Statistically (using the ANOVA test), there is a significant difference ($P<0.05$) between the results obtained with the three different methods for yam *D. rotundata* ($P = 0.0021$), *D. alata* ($P = 0.0035$) and banana ($P = 0.0127$) there is no significant difference ($P>0.05$) for tomato ($P = 0.0701$).

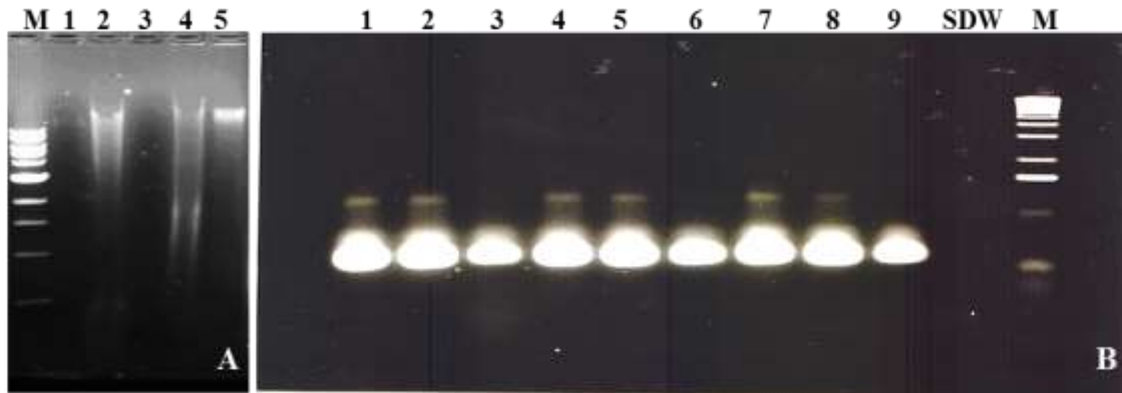


Figure 4. (A) Electrophoresis of digested and undigested extracted DNA from a *D. rotundata* leaf sample by the modified CTAB method. Lane M = 1 kb DNA ladder (NEB, UK), lane 1 = negative control (SDW as template), lane 2 = DNA digested with *Eco*RI, lane 3 = negative control (SDW as template), lane 4 = DNA digested with *Taq*I and lane 5 = undigested DNA. (B) PCR on known yam badnavirus PCR-positive samples using three different dilutions. Lane 1 = dried leaf diluted 1:1000, lane 2 = dried leaf diluted 1:100, lane 3 = dried leaf undiluted sample, lane 4 = etiolated leaf diluted 1:1000, lane 5 = etiolated leaf diluted 1:100, lane 6 = etiolated leaf undiluted sample, lane 7 = fresh leaf diluted 1:1000, lane 8 = fresh leaf diluted 1:100, lane 9 = fresh leaf undiluted sample, lane 10 = negative control (SDW as template) and lane M = 1 kb DNA ladder (NEB, UK).

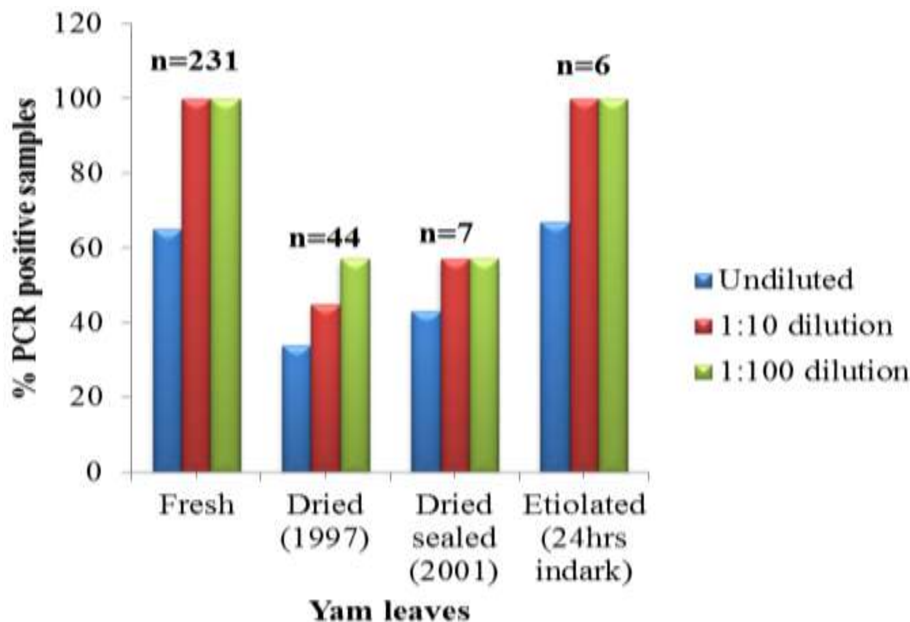


Figure 5. Percentage PCR successes of badnavirus sequences using Badna-FP and Badna-RP primers at different dilution factors for extracted DNA of *Dioscorea* species (*D. alata, D. bulbifera, D. cayenensis, D. dumetorum, D. esculenta* and *D. rotundata*) leaf samples (fresh, dried, etiolated) using the modified CTAB method.

columns for *D. alata* compared to *D. rotundata* samples during purification. This led to a slow drop of the buffers through the columns in *D. alata* samples until a gentle force was applied to enable the flow of the solutions as suggested by the manufacturers of the columns (Qiagen, UK). This additional force might have led to unwanted products passing through.

DISCUSSION

DNA extraction

Isolation of genomic DNA from yam leaves and

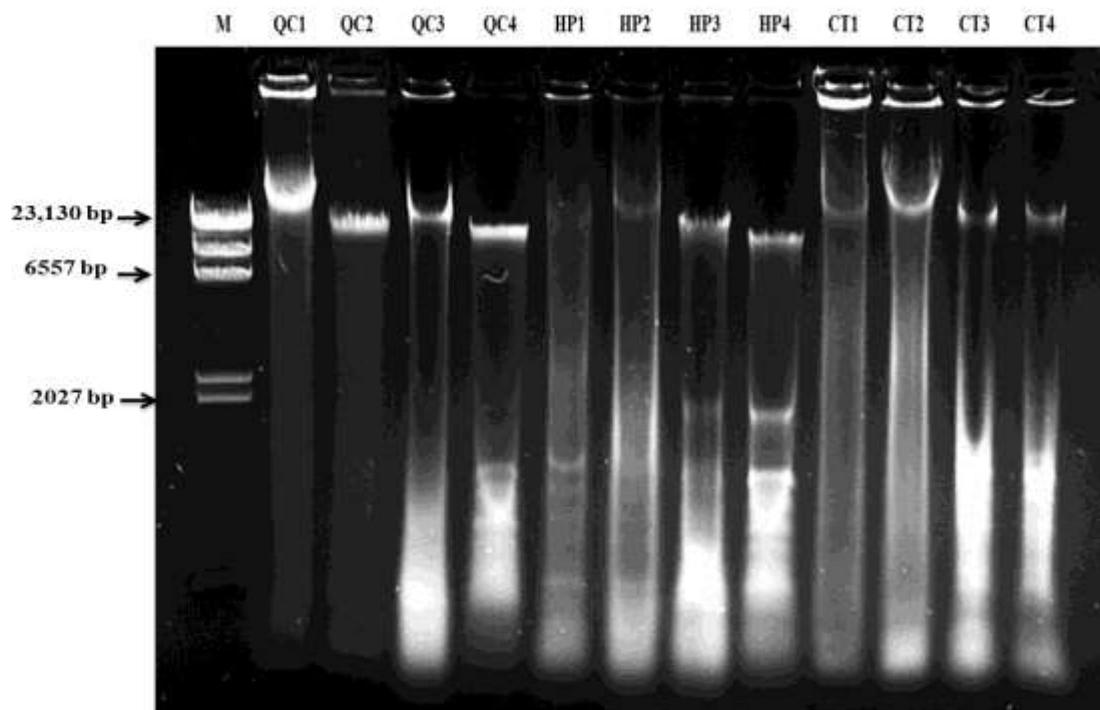


Figure 6. Total genomic yam DNAs extracted from *D. alata* and *D. rotundata* using three treatments, namely Qiagen Tip100/G column purification of extracted DNA (QC), modified CTAB method on HEPES-treated leaf (HP) and modified CTAB method (CT), all electrophoresed through a 0.8% (w/v) agarose in 0.5x TBE gel. Lane M = lambda *Hind*III ladder (NEB, UK), lane QC1, HP1 and CT1 = *D. rotundata* sample (G9), lane QC2, HP2 and CT2 = *D. rotundata* sample (G12), lane QC3, HP3 and CT3 = *D. alata* sample (G5) and lane QC4, HP4 and CT4 = *D. alata* sample (G16).

subsequent analysis such as PCR amplification, restriction digestions are complicated due to the co-purification of polyphenols and polysaccharides (Mumford and Seal, 1997). Moreover, when cells are lysed polyphenolic compounds bind to the DNA, which can cause damage to the DNA and also inhibit the activity of DNA manipulation enzymes such as polymerases or restriction enzymes. Thus, separation of these compounds from the DNA is necessary for good quality DNA extraction (Michiels et al., 2002; Sharma et al., 2008; Tiwari et al., 2012). Several methods exist for the separation of these compounds from DNA, among which is the CTAB method which was first introduced by Taylor and Powell (1982). Since then the method has been widely used for plant DNA extraction often with slight modifications (Lodhi et al., 1994; Barnwell et al., 1998; Michiels et al., 2002; Sharma et al., 2008; Abarshi et al., 2010; Attitalla, 2011; Adeyemi and Ogundipe, 2012; Tiwari et al., 2012).

In this study, the extraction method was based on the CTAB method reported by Lodhi et al. (1994). The method used was optimised by comparison of Lodhi et al. (1994), with that used by Lebas (2002). Leaf tissues used for the optimisation were banana and yam known to contain relatively high amounts of polyphenols and polysaccharides (Sharma et al., 2008) and tomato with a lower amount of polysaccharides and high polyphenols (Peterson et al., 1997).

Lodhi et al. (1994) reported as their CTAB method was favoured over other methods based on the fact that the method had few protocol steps, was less expensive and above all the method offered DNA of good yield and quality from grapevine plant tissue that contained high amount of polyphenol and polysaccharides (Lodhi et al., 1994; Ghaffari et al., 2011). The high polyphenolic compound and polysaccharides in yam leaves were successfully removed from the extracted DNA using the optimised CTAB method, through the addition of increased concentration of PVP-40, sodium sulphite and subsequent precipitation with sodium chloride. The concentration of PVP-40 used in the modified CTAB method was similar to that used by Fang et al. (1992) and Shankar et al. (2011). The DNA values ratio of $A_{260}/A_{230\ nm}$ obtained were ≥1.8 indicating that the extracted DNAs contained low polysaccharides (Fang et al., 1992). When the DNAs give ratios of $A_{260}/A_{230\ nm} \leq 1.6$, this indicates the presence of high levels of polysaccharides in the DNA (http://www.nanodrop.com/Library/T009-NanoDrop%201000-&-NanoDrop%208000-Nucleic-Acid-Purity-Ratios.pdf).

Although, there was a difference between the mean values obtained in the purity and quantity of DNA obtained between the use of PVP-40 or β-mercaptoethanol in removing polyphenols (Figure 1A and B), these were not significant as P>0.05. However, PVP-40 was chosen because it is less toxic and hence easier to dispose of its waste compared to β-mercaptoethanol. An additional advantage to the method is that samples can be processed at this initial extraction stage on a clean bench top without the need for a fume hood compared to the methods that use β-mercaptoethanol.

Another step that was optimised in the protocol was the cell lysis incubation time for which the optimum condition in this study was 60°C for 30 min. One critical step found in achieving good quality DNA from yam leaves was minimizing the time taken from grinding step to this cell lysis incubation step. The increased amounts of PVP-40 together with sodium sulphite in the CTAB buffer and increased speed of grinding to the incubation stage helped in reducing the brownish colouring of the DNA extracted.

Incorporation of a PCAA step not only yielded DNA of good purity as reported by several authors (Porebski et al., 1997; Barnwell et al., 1998; Padmalatha and Prasad, 2006), it also increased mean values of DNA yields (Figure 1A). The result highlighted the efficiency of PCAA in deproteinization compared to CAA. Two steps of washing the pellet with 70% ethanol were found to provide an increase in DNA purity in the range of 1.89 ± 0.04 to 1.92 ± 0.02 compared to the range of 1.71 ± 0.03 to 1.78 ± 0.05 obtained, when CAA was used (Figure 1B). Pure DNA preparations have expected $A_{260}/A_{280\ nm}$ ratios ≥1.8 (William et al., 1997). Ratios of $A_{260}/A_{280\ nm}$ below 1.6 indicate that the DNA contains large amount of proteins (http://www.nanodrop.com/Library/T009-NanoDrop%201000-&-NanoDrop%208000-Nucleic-Acid-Purity-Ratios.pdf).

Although spectrophotometric method is less accurate for estimation of quantity and purity of DNA mix with proteins, nucleotide and primers because the method cannot differentiate between the contaminants and the DNA. This leads to false overestimate of DNA concentration (Sambrook et al., 1989). However, it is still the simplest and fastest method of determining DNA concentration and purity (Teare et al., 1997). However, from the results, it can be seen that the modification steps were helpful in obtaining DNA of reasonable good yield and quality.

Results from DNA concentration obtained from the comparison of three methods (Figure 3A) reveal a statistically significant difference (P<0.05) among yields obtained from yam and banana leaves. However, there is no significant difference (P>0.05) among the results found from tomato DNAs. This could be attributed to the lower polysaccharide and protein concentrations in the tomato leaf tissue. This indicated that both methods are suitable for extraction of DNA from the tomato leaf.

The efficiency of the modification was tested by comparing the mean concentrations of the extracted DNAs from samples stored at different conditions. The results obtained (Figure 2A and B) showed that storage has an effect on DNA quantity and purity. The lower ratio values obtained for leaves stored at 4°C and in dark at RT from the results (Figure 2A to B) indicate the presence of contaminants such as proteins and aromatic ring structure compounds in the DNA mixture that absorbed at λ 280 nm; it appears that such compounds might have been generated under these storage conditions further validation of this is necessary.

The adequate quality of the DNAs was verified by electrophoresing the extracted DNAs alongside digested DNAs. DNA migration and PCR results recorded (Figure 4A and B) indicated that the extracted DNA samples contained fewer inhibitors that interfered with restriction reaction and PCR.

PCR amplification results were obtained using different dilutions of known badnavirus PCR-positive samples of fresh, etiolated and dried yam leaf. The increased band intensity observed from the diluted samples compared to undiluted could be due to PCR inhibitors present in the undiluted samples (Figure 4B). Upon further dilution, the effect of the inhibitors was reduced. A reduced PCR success rate was recorded on dried undiluted 34% (15/44) compared to the fresh undiluted sample 69% (159/231) (Figure 5).

Cost-per-sample analysis revealed that the use of modified CTAB method for DNA extraction is cost efficient compared to the use of the Qiagen DNeasy plant kit. The lower cost of extracting DNA with the modified CTAB method could be of advantage over the kit method considering that yams are produced in developing countries where resources to purchase reagents and kits are more restricted, yet labour is relatively inexpensive. However, the cost saving should only be realised if the quality of the DNA is better and downstream applications are not affected as the cost of PCR, cloning, sequencing and restriction digestion of the extracted DNAs far outweigh the cost of DNA extraction. Furthermore, considering the poor waste management systems in many sub-Sahara African laboratories, the disposal of PCAA from the modified CTAB method will pose a problem and therefore in these situations the Qiagen DNeasy plant kit method could be favoured over the modified CTAB method.

For laboratories with adequate waste disposal systems, an additional advantage of the modified CTAB method over the kit method is that it can be performed at any desired scale, whereas the kit method can only be performed at the scales supplied by the kit manufacturer due to the DNA-binding capacity of the columns supplied. Although a comparison of the success rate of PCR screening between the optimised CTAB method and the kit method was not undertaken in this study, similar modifications of a CTAB method have shown efficient

PCR amplification of DNAs from yam samples compared to the Qiagen DNeasy plant kit extraction method (Lebas, 2002).

Purification of CTAB extracted DNA

Separation of polysaccharide from DNA is one of the major challenges of DNA extraction from plants material containing high levels of polysaccharide (Sharma et al., 2002; Ghaffari et al., 2011). Separation of these two polymers is usually achieved by the high salt concentrations used in the extraction buffers and the DNA precipitation step (Fang et al., 1992). Evidence arising from this study indicates that the modified CTAB extraction yielded good quality DNA for a 'mini prep'. The method could not yield sufficient DNA of high quality for a 'maxi prep'. To obtain higher quality DNA of sufficient quantity DNA from 'max prep' further purification of the 'maxi prep' extracted DNAs had to be carried out. The purification of extracted DNAs using a Qiagen Tip100\G column was found to yield DNA of much better quality compared to adding a HEPES-treatment to leaves (Figure 6), a suggestion made by IITA to overcome this problem (R. Bhattacharjee and G. Gezagegh, personal communication). However, the problem experienced with incorporating a Qiagen Tip100/G columns purification step was that about two-thirds of the DNA was lost during purification.

PCR detection of yam badnavirus sequences

In this study, the PCR-based diagnostic assay used for the detection of badnavirus sequences was similar to those used successfully for detection of many badnavirus sequences in different tropical food crops such as banana (Geering et al., 2000), cacao (Muller et al., 2001), pineapple (Thomson et al., 1996), sugarcane (Braithwaite et al., 1995), taro (Yang et al., 2003) and yam (Eni et al., 2008; Kenyon et al., 2008; Lima et al., 2013). All the PCR tested samples collected from IITA were badnavirus positive. This was in agreement with the Seal et al. (2014) that all West African *D. rotundata* samples are badnavirus positive. Dilution of extracted yam DNAs before PCR was critical as this was found to increase the success of PCR amplification (Figure 5).

The 100% PCR-positives obtained from diluted DNAs of the 151 West African native yams (*D. cayenensis* to *D. rotundata* complex) was not surprising considering that earlier studies also reported 100% PCR-positives from *D. cayenensis* to *D. rotundata* samples from West Africa (Seal et al., 2014). The latter and Kenyon et al. (2008) also reported PCR-positives from ELISA-negative samples. Therefore, these results suggest that some of the PCR-positives might originate from EPRVs. Furthermore, this also reflect the high incidence of badnavirus infection rates as reported from several West African field surveys, recording up to 100% badnavirus incidences (Eni et al., 2008; Asala et al., 2012; Yeyeh et al., 2014; Kumar, unpublished).

Several factors are known to affect the efficiency of badnavirus detection by PCR, among which is the choice of primers. A high degeneracy of primers may lead to non-specific amplification of targeted DNA, which will result in the generation of non-specific PCR products (Linhart and Shamir, 2005). Lebas (2002) reported that non-specific PCR products were obtained using degenerate primers designed from BSV (Lockhart and Olszewski, 1993) on South Pacific badnavirus isolates infecting yam. However, degenerate primers (Badna-FP and Badna-RP) used for this study were designed by Yang et al. (2003), and were used successfully for the detection of badnaviruses from yam breeding lines and landraces collected from IITA. The primers have also been used for the amplifications of DBSNV (Seal and Muller, 2007), detection of yam-infecting badnaviruses from the South Pacific (Lebas, 2002) and West Africa (Eni et al., 2009).

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

The authors would like to thank Professor Susan E. Seal, professor in Molecular Biology at the Department of Agriculture, Health and Environment Natural Resources Institute University of Greenwich UK for her guidance.

REFERENCES

Abarshi MM, Mohammed IU, Wasswa P, Hillocks RJ, Holt J, Legg JP, Seal SE, Maruthi MN (2010). Optimization of diagnostic RT-PCR protocols and sampling procedures for the reliable and cost-effective detection of Cassava brown streak virus. J. Virol. Methods 163:353-359.

Adeyemi TO, Ogundipe OT (2012). Preliminary studies on isolation of genomic DNA suitable for PCR from some African *Sapindaceae juss*. Biotechnology 11:172-177.

Asala S, Alegbejo MD, Kashina B, Banwo OO, Asiedu R, Kumar PL (2012). Distribution and incidence of viruses infecting yam (*Dioscorea* spp.) in Nigeria. Glob. J. Biosci. Biotechnol. 2:163-167.

Attitalla IH (2011). Modified CTAB method for high genomic DNA extraction from medicinal plants. Pak. J Biol. Sci. 14(2):998-999

Barnwell P, Blanchard AN, Bryant JA, Nicholas S, Fred WA (1998) Isolation of DNA from the highly mucilaginous succulent plant Sedum telephium. Plant Mol. Biol. Rep. 16:133-138.

Braithwaite KS, Egeskov NM, Smith GR (1995). Detection of sugarcane bacilliform virus using the polymerase chain reaction. Plant Dis. 79:792-796.

Csaikl UM, Bastian H, Brettschneider R, Gauch S, Meir A, Schauerte M, Scholz F, Sperisen C, Vornam B, Ziegenhagen B (1998). Comparative analysis of different DNA extraction protocols: A fast, universal maxi-preparation of high quality plant DNA for genetic evaluation and phylogenetic studies. Plant Mol. Biol. Rep. 16:69-86.

Eni AO, Hughes JDA, Asiedu R, Rey MEC (2009). Survey of the incidence and distribution of viruses infecting yam (*Dioscorea* spp.) in

Ghana and Togo. Ann. Appl. Biol. 156:243-251.
Eni AO, Hughes JDA, Rey MEC (2008). Survey of the incidence and distribution of five viruses infecting yams in the major yam-producing zones in Benin. Ann. Appl. Biol. 156:223-232.
Fang G, Hammar S, Grumet R (1992). A quick and inexpensive method for removing polysaccharides from plant genomic DNA. BioTechniques 13:52-54.
Geering ADW, McMichael LA, Dietzgen RG, Thomas JE (2000). Genetic diversity among *Banana streak virus* isolates from Australia. Am. Phytopathol. Soc. 90:921-927.
Ghaffari S, Hasnaoui N, Ferchichi A (2011). Rapid, High Quality DNA Isolation from Tunisian Grapevine (*Vitis vinifera* L.) Cultivars and Optimization of the RAPD Marker Technique. Rom. Biotechnol. Lett. 16:2881-2890.
Kenyon L, Lebas BSM, Seal SE (2008). Yams (*Dioscorea* spp.) from the South Pacific Islands contain many novel badnaviruses: implications for international movement of yam germplasm. Arch. Virol. 153:877-889.
Lebas BSM (2002). Diversity of viruses infecting yams Dioscorea species in the South Pacific. Ph.D. thesis, The University of Greenwich Chatham Maritime, UK. pp. 157-181.
Lima JS, Alison TML, Gloria PC, Sarah JCS, Iraildes PA, Sami JM, Zerbini FM, Lima GSA (2013). Variabilidade genética de isolados de badnavírus infectando inhame (*Dioscorea* spp.) no Nordeste do Brasil. Trop. Plant Pathol. 38:349-353.
Linhart C, Shamir R (2005). The degenerate primer design problem: theory and applications. J. Comput Biol. 12:431-456.
Lockhart BEL, Olszewski NE (1993). Serological and genomic heterogeneity of banana streak badnavirus: implications for virus detection in *Musa* germplasm pp.102-113. In. “Breeding Banana and Plantain for Resistance to Diseases and Pest” Ganry J. (eds.). CIRAD-INIBAP, Montpellier, France.
Lodhi MA, Guang-Ning Y, Norman FW, Bruce IR (1994). A simple and efficient method for DNA extraction from grapevine cultivars, *Vitis* species and *Ampelopsis*. Plant Mol. Biol. Rep. 12:6-13.
Michiels A, Wim VE, Tucker M, Liesbet VR, André VL (2002). Extraction of high-quality genomic DNA from latex-containing plants. Anal. Biochem. 315:85-89.
Muller E, Jacquot E, Yot P (2001). Early detection of *Cacao swollen shoot virus* using the polymerase chain reaction. J. Virol. Methods 93:15-22.
Mumford RA, Seal SE (1997). Rapid single-tube immunocapture RT-PCR for the detection of two yam potyviruses. J. Virol. Methods 69:73-79.
Padmalatha K, Prasad MNV (2006). Optimisation of DNA isolation and PCR protocol for RAPD analysis of selected medicinal and aromatic plants of conservation concern from peninsular India. Afr. J. Biotechnol. 5:230-234.
Peterson DG, Boehm KS, Stack SM (1997). Isolation of milligram quantities of nuclear DNA from tomato (*Lycopersicon esculentum*) a plant containing high amounts of polyphenols compounds. Plant Mol. Rep. 15:148-153.
Porebski S, Bailey LG, Baum BR (1997). Modification of CTAB DNA extraction protocol for plants containing high polysaccharide and polyphenol components. Plant Mol. Biol. Rep. 12:8-13.
Sambrook, J, Fritsch EF and Maniatis T (1989). Molecular cloning: a laboratory manual. 2rd edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbour, New York, USA. pp. 9.31-9.57
Seal SE, Muller E (2007). Molecular analysis of a full-length sequence of a new yam badnavirus from *Dioscorea sansibarensis*. Arch. Virol. 7:1-7.
Seal SE, Turaki A, Muller E, Kumar PL, Kenyon L, Filloux D, Galzi S, Lopez-Montes A, Iskra-Caruana ML (2014). The prevalence of badnaviruses in West African yams (Dioscorea cayenensis-rotundata) and evidence of endogenous pararetrovirus sequences in their genomes. Virus Res. 186:144-154.
Shankar K, Chavan V, Shinde S, Patil B. (2011). An improved DNA extraction protocol from four *in vitro* banana cultivars. Asian J. Biotechnol. 3:84-90.
Sharma AD, Gill PK, Singh P (2002). DNA isolation from dry and fresh samples of polysaccharide-rich plants. Plant Mol. Biol. Rep. 20:415-419.
Sharma K, Mishra AK, Misra RS (2008). A simple and efficient method for extraction of genomic DNA from tropical crops. Afr. J. Biotechnol. 8:1018-1022.
Taylor N, Powell A (1982). Isolation of plant DNA and RNA. Focus 4:4-6.
Teare JM, Islam R, Flanagan R, Gallagher S, Davies MG, Grabou C (1997). Measurement of nucleic acid concentration using the DyNA Quant^{TM} and Genequant^{TM}. BioTechniques 22:1170-1174.
Thomson KG, Dietzgen RG, Thomas JE, Teakle DS (1996). Detection of pineapple bacilliform virus using the polymerase chain reaction. Ann. Appl. Biol. 129:57-59.
Tiwari KL, Jadhav SK, Gupta S (2012). Modified CTAB technique for isolation of DNA from some medicinal plants. Res. J. Med. Plant 6:65-73.
William WW, Karol M, Piotr C (1997) Effect of pH and ionic strength on the spectrophotometric assessment of nucleic acid purity. *BioTechniques* 22:474-481.
Yang IC, Hafner GJ, Revill PA, Dale JL, Harding RM (2003). Sequence diversity of South Pacific isolates of taro bacilliform virus and the development of a PCR-based diagnostic test. Arch. Virol. 148:1957-1968.
Yeyeh TMN, Diallo HA, Akinbade SA, Séka K, Kumar PL (2014). Distribution, incidence and severity of viral diseases of yam (*Dioscorea* spp.) in Côte d’Ivoire. Afr. J. Biotechnol. 13:465-470.

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