ABOUT IJBMBR

The International Journal for Biotechnology and Molecular Biology Research (IJBMBR) (ISSN 2141-2154) is published Monthly (one volume per year) by Academic Journals.

International Journal for Biotechnology and Molecular Biology Research (IJBMBR) provides rapid publication (monthly) of articles in all areas of the subject such as Green energy from chemicals and bio-wastes, Studies in the graft copolymerization of acrylonitrile onto cassava starch by ceric ion induced initiation, Antimutagenic activity of aqueous extract of Momordica charantia, Ethnomedicinal plants and other natural products with anti-HIV active compounds and their putative modes of action etc.

The Journal welcomes the submission of manuscripts that meet the general criteria of significance and scientific excellence. Papers will be published shortly after acceptance. All articles published in IJBMBR are peer-reviewed.

Submission of Manuscript

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

Click here to Submit manuscripts online

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

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

Prof Atagana, Harrison
Institute for Science and Technology Education
University of South Africa

Prof. UC Banerjee
Department of Pharmaceutical Technology
(Biotechnology)
National Institute of Pharmaceutical Education and Research
Punjab, INDIA

Dr. Y. Omidi
Faculty of Pharmacy,
Research Center for Pharmaceutical Nanotechnology,
School of Advanced Biomedical Sciences,
Tabriz University of Medical Sciences,
Tabriz, Iran.

Prof. Mohamed E. Wagih
University of New Brunswick (UNB-SJ),
Saint John College, NB,
E2L 4L5, Canada

Dr. Sripatha M. Udupa
ICARDA-INRA Cooperative Research Project,
International Center for Agricultural Research in the
Dry Areas (ICARDA), B.P. 6299,
Rabat Institutes, Rabat, Morocco

Dr. Amjad Masood Husaini
Sheh-e-Kashmir University of Agricultural Sciences & Technology
Bohlochipora, Dr. Ali Jan Road,
Nowshera, Srinagar, J&K-190011, India

Dr. Om Prakash Gupta
Directorate of Wheat Research (ICAR)
Post Box-158, Agrasain Marg, Karnal-132001,
Haryana, India

Editorial Board

Dr. Amro Hanora
Suez Canal University, Department of Microbiology and Immunology,
Faculty of Pharmacy, Suez Canal University, Box 41522 Ismailia, Egypt

Dr. C. Rajasekaran
VIT University
School of Bio-Sciences & Technology (SBST)

Dr. Yasar Karadag
Gaziosmanpasa University
Faculty of Agriculture,
Department of Field Crops, Tokat-Turkey

Dr. Ahmet Tutus
KSU (Kahramanmaras Sutcu Imam University)
Faculty of Forestry,
Department of Forest Industrial Engineering,
Kahramanmaras 46100 Turkey

Dr. Vinod Joshi
Desert Medicine Research Centre,
Indian Council of Medical Research
New Pali Road, Jodhpur, India

Dr. Eshrat Gharaei Fathabad
K.M.18 Khazarabad road,
Sari, Mazandaran, Iran

Dr. Shashideep Singhal
121 Dekalb Ave, Brooklyn,
NY 11201, New York, USA

Dr. Masayoshi Yamaguchi
101 Woodruff Circle, 1305 WMRB,
Atlanta, Georgia 30322-0001, USA

Dr. Okonko Iheanyi Omezuruike
Department of Virology,
Faculty of Basic Medical Sciences,
College of Medicine,
University College Hospital,
Ibadan, Nigeria

Dr. S. M. Shahid
University of Karachi,
Karachi-75270, Pakistan
<table>
<thead>
<tr>
<th>Name</th>
<th>Institution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dr. Chethan Kumar M</td>
<td>Post Graduate Departments of Bio-technology and Biochemistry, Ooty Road, Mysore - 570 025, Karnataka, India</td>
</tr>
<tr>
<td>Dr. M. Sattari</td>
<td>Rice Research Inst. of Iran, Iran</td>
</tr>
<tr>
<td>Dr. Zaved Ahmed Khan</td>
<td>VIT University, India</td>
</tr>
<tr>
<td>Dr. Subbiah Poopathi</td>
<td>Vector Control Research Centre, Indian Council of Medical Research (Ministry of Health &amp; Family Welfare, Govt. of India), Medical Complex, Indira Nagar, India</td>
</tr>
<tr>
<td>Dr. Reyazul Rouf Mir</td>
<td>International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru - 502 324, Greater Hyderabad, India</td>
</tr>
<tr>
<td>Dr. Prasanna Kumar S</td>
<td>Virginia Commonwealth University, USA</td>
</tr>
<tr>
<td>Dr. Naseem Ahmad</td>
<td>Plant Biotechnology Laboratory, Department of Botany, Aligarh Muslim University, Aligarh- 202 002, (UP), India</td>
</tr>
<tr>
<td>Dr. Zhen-Xing Tang</td>
<td>Food Bioengineering institute, Hangzhou Wahaha Co. Ltd, Hangzhou, Zhejiang, China</td>
</tr>
<tr>
<td>Dr. Jayanthi Abraham</td>
<td>VIT (Vellore Institute of Technology) University, Tamilnadu, India</td>
</tr>
<tr>
<td>Dr. Gobianand Kuppannan</td>
<td>National Institute of Animal Science, South Korea</td>
</tr>
<tr>
<td>Dr. R. Harikrishnan</td>
<td>Jeju National University, South Korea</td>
</tr>
<tr>
<td>Dr. Asit Ranjan Ghosh</td>
<td>Vellore Institute of Technology (VIT) University, School of Bio Sciences &amp; Technology, Medical Biotechnology Division, Vellore-632014, India</td>
</tr>
<tr>
<td>Dr. Kamal Dev</td>
<td>Shoolini University of Biotechnology and Management Sciences (SUBMS), India</td>
</tr>
<tr>
<td>Dr. Wichian Sittiprapaporn</td>
<td>Mahasarakham University, Thailand</td>
</tr>
<tr>
<td>Dr. Vijai Kumar Gupta</td>
<td>Molecular Glycobiochemistry Group, Department of Biochemistry, School of Natural Sciences, National University of Ireland, Galway, Ireland</td>
</tr>
<tr>
<td>Dr. Jeffy George</td>
<td>Department of Microbiology and Immunology, F. Edward Hébert School of Medicine, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD 20814, USA.</td>
</tr>
<tr>
<td>Dr. Gyanendra Singh</td>
<td>Stanley S. Scott Cancer Center, School of Medicine, Louisiana State University Health Sciences Center, New Orleans, LA 70112, USA.</td>
</tr>
<tr>
<td>Dr. Anupreet Kour</td>
<td>1620 Chevy Chase Dr., Champaign, IL 61821, USA.</td>
</tr>
<tr>
<td>Name</td>
<td>Institution</td>
</tr>
<tr>
<td>---------------------------------</td>
<td>------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Dr. Arun Sharma</td>
<td>Institute for Plant Genomics and Biotechnology (IPGB)</td>
</tr>
<tr>
<td></td>
<td>Borlaug Center, TAMU 2123 Texas A&amp;M University College Station, TX 77843 USA.</td>
</tr>
<tr>
<td>Dr. Mohsen Asker</td>
<td>Microbial Biotechnology Dept. National Research Centre Cairo, Egypt.</td>
</tr>
<tr>
<td>Dr. Elijah Miinda Ateka</td>
<td>Department of Horticulture, Jomo Kenyatta University of Agriculture and Technology (JKUAT) Kenya</td>
</tr>
<tr>
<td>Dr. Jozélio Freire De Carvalho</td>
<td>Faculdade de Medicina Da USP, Reumatologia Av. Dr. Arnaldo, 455 - 3º andar – Sala 3133. São Paulo - SP Brazil</td>
</tr>
<tr>
<td>Dr. Premendra Dhar Dwivedi</td>
<td>Food Toxicology Division Industrial Institute of Toxicology Research, Post Box No: 80, Mahatma Gandhi Marg, Lucknow 226001, India</td>
</tr>
<tr>
<td>Dr. Muhammad Abd El-Moez El-Saadani</td>
<td>Universities and Research Center District, New Borg El-Arab, P.O.Box: 21934 Alexandria, Egypt.</td>
</tr>
<tr>
<td>Dr. Donald J. Ferguson</td>
<td>Advanced Orthodontic Training Program, Nicolas &amp; Asp University College Dubai, UAE</td>
</tr>
<tr>
<td>Dr. Kalyan Goswami</td>
<td>Department of Biochemistry &amp; JB Tropical Disease Research Centre, Mahatma Gandhi Institute of Medical Sciences, Sevagram, Wardha-442102</td>
</tr>
<tr>
<td>Dr. A.K. Handa</td>
<td>National Research Centre for Agroforestry, Gwalior Road, JHANSI-284003 UP India.</td>
</tr>
<tr>
<td>Dr. Amjad M. Husaini</td>
<td>Metabolic Engineering &amp; Biotechnology Laboratory Division of Plant Breeding &amp; Genetics Sher-e-Kashmir University of Agricultural Sciences &amp; Technology of Kashmir J&amp;K-191121, India</td>
</tr>
<tr>
<td>Dr. Vinod Joshi</td>
<td>Laboratory of Virology &amp; Molecular Biology, Desert Medicine Research Centre, Pali Road, Jodhpur-342 005, India</td>
</tr>
<tr>
<td>Dr. T. Kalaivani</td>
<td>D/O S. Thiagarajan B-43, Rajaram Nagar, Salem - 636 007, Tamil Nadu, India</td>
</tr>
<tr>
<td>Dr. Priya Kalia</td>
<td>Orthopaedic Research Unit, Department of Surgery, Cambridge University, Cambridge, UK</td>
</tr>
<tr>
<td>Dr. Patricia Khashayar</td>
<td>Tehran University of Medical Sciences Endocrinology and Metabolism Research Center Shariati Hospital</td>
</tr>
<tr>
<td>Dr. Zaringhalam Moghadam</td>
<td>Shahid Beheshti Medical University (M.C) Tehran, Iran</td>
</tr>
<tr>
<td>Dr. Okeke Ikechukwu Linus</td>
<td>Department of Surgery, University of Ibadan Nigeria.</td>
</tr>
<tr>
<td>Dr. Rajesh Kumar Patel</td>
<td>Centre for Analysis and Learning in Livestock and Food (CALF) National Dairy Development Board (NDDB) Anand- 388 001 (Gujarat) INDIA</td>
</tr>
</tbody>
</table>
Dr. Pooja Ralli-Jain
Department of Pathology and Laboratory Medicine
University of California Irvine, Irvine, California, U.S.A.

Dr. Meltem Sesli
College of Tobacco Expertise, Turkish Republic, Celal Bayar University 45210, Akhisar, Manisa, Turkey

Dr. Reda H. Sammour
Tanta University, Faculty of Science, Tanta, Egypt

Dr. Seyed Soheil Saeedi Saravi
Mazandaran University of Medical sciences, Sari, Iran

Dr. R. Senthil Kumar
St. Matthew’s University, School of Medicine
Grand Cayman
Cayman Islands

Dr. Mohammad Reza Shakibaie
Kerman University of Medical Sciences, Kerman, Iran

Dr. Srividya Shivakumar
Dept of Microbiology, CPGS, Jain university, Bangalore

Dr. Shashideep Singhal
The Brooklyn Hospital Center
NewYork-Presbyterian Healthcare System
Brooklyn, NY.

Dr. Sripada M. Udupa
International Center for Agricultural Research in the Dry Areas (ICARDA), B.P. 6299, Rabat Instituts, Rabat, Morocco.

Dr. Wei Wu
Institute for Biocomplexity and Informatics
Department of Bio Science
The University of Calgary
Canada

Dr. Xiao-Bing Zhang
Molecular Regeneration Laboratory, MC1528B
11234 Anderson Street
Loma Linda, CA  92350

Prof. Dr. Ozfer Yesilada
Inonu University
Faculty of Arts and Sciences
Department of Biology
44280 Malatya
Turkey

Dr. Edson Boasquevisque
Universidade do Estado do Rio de Janeiro- UERJ
Av 28 de setembro, 87, fundos (LMMC-IBRAG).
Vila Isabel, city: Rio de Janeiro/ RJ
Brasil

Dr. Abhilash M.
The Oxford College of Engineering
Hosur Road, Bangalore - 560068

Dr. Nasar Uddin Ahmed
Department of Genetics and Plant Breeding
Patuakhali Science and Technology University
Dumki, Patuakhali-8602
Bangladesh

Dr. Mervat Morsy EL-Gendy
Chemistry of Natural and Microbial Products Department,
National Research Center, Dokki, Cairo, Egypt

Dr. Gjumrakch Aliev
Health Science and Healthcare Administration Program,
University of Atlanta, Atlanta, Georgia, USA

Dr. Muhammad Asgher
Department of Chemistry and Biochemistry,
University of Agriculture, Faisalabad, Pakistan

Dr. Anand Bharatkumar
Parul Institute of Pharmacy, Limda, Waghodia, Vadodara
Dr. Chinmoy Kumar Bose,
Netaji Subhash Chandra Bose Cancer Research Institute
16A, Park Lane, Park Street, Kolkata 700 016, India.

Dr. Mousumi Debnath
Jaipur Engineering College and Research Centre (JECRC) Department of Biotechnology,
Shri Ram ki Nangal, Via Vatika, Tonk Road, Jaipur-303905, India

Dr. Dolan C. Saha
Dept. of Biochemistry and Molecular Biology,
Faculty of Medicine, University of Calgary, Canada

Dr. Ramasamy Harikrishnan
Department of Aquatic Biomedical Sciences
School of Marine Biomedical Science
College of Ocean Sciences
Jeju National University
Jeju city, Jeju 690 756, South Korea

Dr. Abdul Haque
Health Biotechnology division, nibge, Faisalabad, Pakistan

Dr. Kuvalekar Aniket Arun
Interactive Research School for Health Affairs (IRHSA), Bharati Vidyapeeth University, Pune, Maharashtra, India

Dr. Asit Ranjan Ghosh
School of Bio Science & Technology, Division of Medical Biotechnology, Vellore Institute of Technology (VIT) University, Vellore-632014, India

Dr. Prasanna Kumar Santhekadur
Department of Human and Molecular Genetics
Virginia Commonwealth University Richmond, VA

Dr. Majid Sattari
Rice Research Institute of Iran
Iran

Dr. Mihael Cristin Ichim
National Institute Research and Development for Biological Sciences / “Stejarul” Research Centre for Biological Sciences
Alexandru cel Bun St., 6, Piatra Neamt, 610004, Romania

Dr. Sailas Benjamin
Enzyme Technology Laboratory
Biotechnology Division
Department of Botany
University of Calicut
Kerala - 673 635 India

Dr. Sreeramanan Subramaniam
School of Biological Sciences, Universiti Sains Malaysia (USM), Minden Heights, 11800, Penang, Malaysia

Dr. Vijai Kumar Gupta,
Department of Biochemistry, NUI, Galway, Ireland

Dr. Vitor Engrácia Valenti
Universidade Federal de São Paulo
Rua Napoleão de Barros, 715, Térreso São Paulo, SP Brazil.

Dr. Ravindra Pogaku
School of Engineering and IT
Universiti Malaysia Sabah
88999 Kota Kinabalu Sabah, Malaysia

Dr. Ahmed Eid Abdel-Hamid Eweis Fazary
School of Pharmacy, College of Medicine, National Taiwan University, Taipei 100, Taiwan.

Dr. Mohammad Hashemi
Dept. of Clinical Biochemistry, School of Medicine, Zahedan University of Medical Sciences, Zahedan, Iran
Dr. Hesham, Abd El-Latif
Genetics Department,
Assiut University, Assiut 71516,
Egypt.

Prof. Jia-ying Xin
College of Food Engineering
Harbin University of Commerce
138 Tongda Road
Daoli District
Harbin 150076, Heilongjiang
P.R. China

Dr. Kabir Mohammad Humayun
Plant Molecular Biotech Lab
Department of Medical Biotechnology
College of Biomedical Science
Kangwon National University
Kangwon-do, Chuncheon, 200-701
South Korea

Dr. Kalpesh Gaur
Geetanjali College of Pharmaceutical Studies Manwa Khera,
Udaipur- 313002. Rajasthan,
India

Dr. Meganathan, Kannan
Center for Biologics Evaluation and Research (CBER),
U.S. Food and Drug Administration (FDA),
Bldg. NIH 29A, Room 2C-10,
8800 Rockville Pike,
Bethesda, MD 20892.
USA.

Assist. Prof. Ali Karadeniz
Department of Physiology,
Faculty of Veterinary Medicine,
University of Atatürk 25240 ERZURUM
Turkey

Dr. Matthew Kostek
Department of Kinesiology
University of Connecticut
Storrs CT

Dr. Tansu Kucuk
Gulhane School of Medicine
Department of Obstetrics and Gynecology
Etilk 06018 Ankara,
Turkey

Dr. Kuo-Sheng Hung
Department of Neurosurgery
Taipei Medical University - Wan Fang Medical Center
111 Section 3, Hsing-Long Rd,
Taipei 116,
Taiwan

Dr. V. Manju
Department of Biochemistry,
Periyar University,
Salem -11.

Dr. Mbagwu Ferdinand Nkem
Department of Plant science and Biotechnology,
Faculty of Science,
Imo State University
Nigeria.

Dr. Anand Pithadia
Parul Institute of Pharmacy
Vadodara, Gujarat,
India

Dr. Radhakrishnan Ramaraj
Department of Internal Medicine
University of Arizona
Tucson 85724
AZ

Dr. M. Rasool
School of Bio Sciences and Technology,
VIT University,
Vellore-632104, Tamil Nadu,
India

Dr. Reda A.I. Abou-Shanab
Genetic Engineering & Biotechnology Research Institute (GEBRI)
Mubarak City for Scientific Research and Technology Applications
New Burg El-Arab City, Universities and Research Institutes
Zone, P.O. 21934, Alexandria,
Egypt.

Dr. MR. Pravin Babarao Suruse
Department of Pharmaceutics
Sharad Pawar College of Pharmacy
Wanadongri, Hingna Road
Nagpur- 441 110. (M. S.)
Dr. Jan Woraratanadharm  
*GenPhar, Inc.,*  
*Mount Pleasant, SC*

Dr. Serap Yalin  
*Mersin University Pharmacy Faculty*  
*Department of Biochemistry, Mersin*  
*Turkey*

Dr. YongYong Shi  
*Bio-X Center,*  
*Shanghai Jiao Tong University,*  
*Hao Ran Building, 1954 Hua Shan Road,*  
*Shanghai 200030,*  
*PR China*

Dr. Jyotdeep Kaur  
*Department of Biochemistry,*  
*Post Graduate Institute of Medical Education and Research (PGIMER),*  
*Chandigarh*

Dr. Rajkumar  
*Dept. Of Radiation Biosciences,*  
*Institute of Nuclear Medicine and Allied Sciences*  
*Brig. S.K. Mazumdar Road, Timarpur,*  
*Delhi-110054*  
*India*

Dr. Meera Sumanth  
*Visveswarapura Institute of Pharmaceutical Sciences,*  
*22nd Main, 24th Cross, B.S.K II stage,*  
*Bangalore-560070*  
*Karnataka,*  
*India.*

Dr. Jai S. Ghosh  
*Department of Microbiology,*  
*Shivaji University,*  
*Kolhapur 416004,*  
*India*

Prof. Dr. Alaa H. Al-Charrakh  
*Babylon University, College of Medicine.*  
*Dept. of Microbiology*  
*Hilla, Iraq*
Instructions for Author

Electronic submission of manuscripts is strongly encouraged, provided that the text, tables, and figures are included in a single Microsoft Word file (preferably in Arial font).

The cover letter should include the corresponding author’s full address and telephone/fax numbers and should be in an e-mail message sent to the Editor, with the file, whose name should begin with the first author’s surname, as an attachment.

Article Types
Three types of manuscripts may be submitted:

Regular articles: These should describe new and carefully confirmed findings, and experimental procedures should be given in sufficient detail for others to verify the work. The length of a full paper should be the minimum required to describe and interpret the work clearly.

Short Communications: A Short Communication is suitable for recording the results of complete small investigations or giving details of new models or hypotheses, innovative methods, techniques or apparatus. The style of main sections need not conform to that of full-length papers. Short communications are 2 to 4 printed pages (about 6 to 12 manuscript pages) in length.

Reviews: Submissions of reviews and perspectives covering topics of current interest are welcome and encouraged. Reviews should be concise and no longer than 4-6 printed pages (about 12 to 18 manuscript pages). Reviews are also peer-reviewed.

Review Process
All manuscripts are reviewed by an editor and members of the Editorial Board or qualified outside reviewers. Authors cannot nominate reviewers. Only reviewers randomly selected from our database with specialization in the subject area will be contacted to evaluate the manuscripts. The process will be blind review.

Decisions will be made as rapidly as possible, and the Journal strives to return reviewers’ comments to authors as fast as possible. The editorial board will re-review manuscripts that are accepted pending revision. It is the goal of the AJMR to publish manuscripts within weeks after submission.

Regular articles
All portions of the manuscript must be typed double-spaced and all pages numbered starting from the title page.

The Title should be a brief phrase describing the contents of the paper. The Title Page should include the authors’ full names and affiliations, the name of the corresponding author along with phone, fax and E-mail information. Present addresses of authors should appear as a footnote.

The Abstract should be informative and completely self-explanatory, briefly present the topic, state the scope of the experiments, indicate significant data, and point out major findings and conclusions. The Abstract should be 100 to 200 words in length. Complete sentences, active verbs, and the third person should be used, and the abstract should be written in the past tense. Standard nomenclature should be used and abbreviations should be avoided. No literature should be cited. Following the abstract, about 3 to 10 key words that will provide indexing references should be listed.

A list of non-standard Abbreviations should be added. In general, non-standard abbreviations should be used only when the full term is very long and used often. Each abbreviation should be spelled out and introduced in parentheses the first time it is used in the text. Only recommended SI units should be used. Authors should use the solidus presentation (mg/ml). Standard abbreviations (such as ATP and DNA) need not be defined.

The Introduction should provide a clear statement of the problem, the relevant literature on the subject, and the proposed approach or solution. It should be understandable to colleagues from a broad range of scientific disciplines.

Materials and methods should be complete enough to allow experiments to be reproduced. However, only truly new procedures should be described in detail; previously published procedures should be cited, and important modifications of published procedures should be mentioned briefly. Capitalize trade names and include the manufacturer’s name and address. Subheadings should be used. Methods in general use need not be described in detail.
Results should be presented with clarity and precision. The results should be written in the past tense when describing findings in the authors' experiments. Previously published findings should be written in the present tense. Results should be explained, but largely without referring to the literature. Discussion, speculation and detailed interpretation of data should not be included in the Results but should be put into the Discussion section.

The Discussion should interpret the findings in view of the results obtained in this and in past studies on this topic. State the conclusions in a few sentences at the end of the paper. The Results and Discussion sections can include subheadings, and when appropriate, both sections can be combined.

The Acknowledgments of people, grants, funds, etc should be brief.

Tables should be kept to a minimum and be designed to be as simple as possible. Tables are to be typed double-spaced throughout, including headings and footnotes. Each table should be on a separate page, numbered consecutively in Arabic numerals and supplied with a heading and a legend. Tables should be self-explanatory without reference to the text. The details of the methods used in the experiments should preferably be described in the legend instead of in the text. The same data should not be presented in both table and graph form or repeated in the text.

Figure legends should be typed in numerical order on a separate sheet. Graphics should be prepared using applications capable of generating high resolution GIF, TIFF, JPEG or Powerpoint before pasting in the Microsoft Word manuscript file. Tables should be prepared in Microsoft Word. Use Arabic numerals to designate figures and upper case letters for their parts (Figure 1). Begin each legend with a title and include sufficient description so that the figure is understandable without reading the text of the manuscript. Information given in legends should not be repeated in the text.

References: In the text, a reference identified by means of an author’s name should be followed by the date of the reference in parentheses. When there are more than two authors, only the first author’s name should be mentioned, followed by ‘et al’. In the event that an author cited has had two or more works published during the same year, the reference, both in the text and in the reference list, should be identified by a lower case letter like ‘a’ and ‘b’ after the date to distinguish the works.

Examples:
Abayomi (2000), Agindotan et al. (2003), (Kelebeni, 1983), (Usman and Smith, 1992), (Chege, 1998; 1987a,b; Tijani, 1993,1995), (Kumasi et al., 2001)

References should be listed at the end of the paper in alphabetical order. Articles in preparation or articles submitted for publication, unpublished observations, personal communications, etc. should not be included in the reference list but should only be mentioned in the article text (e.g., A. Kingori, University of Nairobi, Kenya, personal communication). Journal names are abbreviated according to Chemical Abstracts. Authors are fully responsible for the accuracy of the references.

Examples:

Short Communications

Short Communications are limited to a maximum of two figures and one table. They should present a complete study that is more limited in scope than is found in full-length papers. The items of manuscript preparation listed above apply to Short Communications with the following differences: (1) Abstracts are limited to 100 words; (2) instead of a separate Materials and Methods section, experimental procedures may be incorporated into Figure Legends and Table footnotes; (3) Results and Discussion should be combined into a single section.

Proofs and Reprints: Electronic proofs will be sent (email attachment) to the corresponding author as a PDF file. Page proofs are considered to be the final version of the manuscript. With the exception of typographical or minor clerical errors, no changes will be made in the manuscript at the proof stage.
Fees and Charges: Authors are required to pay a $650 handling fee. Publication of an article in the International Journal for Biotechnology and Molecular Biology Research is not contingent upon the author's ability to pay the charges. Neither is acceptance to pay the handling fee a guarantee that the paper will be accepted for publication. Authors may still request (in advance) that the editorial office waive some of the handling fee under special circumstances.

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

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

Disclaimer of Warranties
In no event shall Academic Journals be liable for any special, incidental, indirect, or consequential damages of any kind arising out of or in connection with the use of the articles or other material derived from the UBMBR, whether or not advised of the possibility of damage, and on any theory of liability.
This publication is provided "as is" without warranty of any kind, either expressed or implied, including, but not limited to, the implied warranties of merchantability, fitness for a particular purpose, or non-infringement. Descriptions of, or references to, products or publications does not imply endorsement of that product or publication. While every effort is made by Academic Journals to see that no inaccurate or misleading data, opinion or statements appear in this publication, they wish to make it clear that the data and opinions appearing in the articles and advertisements herein are the responsibility of the contributor or advertiser concerned. Academic Journals makes no warranty of any kind, either express or implied, regarding the quality, accuracy, availability, or validity of the data or information in this publication or of any other publication to which it may be linked.
ARTICLES

Molecular Identification Of *Lactobacillus Plantarum* Isolated From Fermenting Cereals
Adeyemo, S. M. and Onilude, A. A.

Molecular Characterization Of Resistance To Russian Wheat Aphid (*Diuraphis Noxia* Kurdjumov) In Bread Wheat (*Triticum Aestivum* L.) Line KRWA9
E. A. Masinde, J. N. Malinga, W. A. Ngenya, R.M. S Mulwa and M. Cakir
Molecular identification of *Lactobacillus plantarum* isolated from fermenting cereals

Adeyemo, S. M.¹* and Onilude, A. A.²

¹Department of Microbiology, Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria.
²Department of Microbiology, University of Ibadan, Oyo State, Nigeria.

Received 9 January, 2014; Accepted 26 November, 2014

The identification of a microbial isolate to genus level only amounts to a partial characterization of the isolate, but this can tell us a lot about that organism. Knowing the species allows the laboratory access to the body of knowledge that exists on that species. Identification schemes using phenotypic characteristics such as colony and cell morphology, Gram reaction and other staining characteristics, nutritional and physiological requirements for growth and metabolic characteristics have been developed and improved over many decades to a point where laboratories are able to identify isolates to species level using simple conventional methods. This phenotypic method however have some limitations apart from being laborious and time consuming, some organisms may however be misidentified either at genus or species level. This work aims at looking directly at the genome of lactic acid bacteria (LAB) and from this identifies some species using its genotypic and phenotypic characteristics. These bacteria species were identified by sequencing specific sections of ribosomal DNA - the 16S rRNA gene, after amplification by PCR, and then comparing the results to sequences stored on a related database. The results from both conventional and molecular methods were then compared. Twenty (20) *Lactobacillus plantarum* were isolated from spontaneously fermented cereals made into “Ogi” and identified using classical methods. They were further characterized using molecular methods by polymerase chain reaction (PCR) amplification of 16S rDNA genes to confirm their identities. The genotypic characterization however showed that 85% of the organisms identified using conventional method as *L. plantarum* correlated, while 15% did not correlate; 2 were identified as *Lactobacillus pentosus* and one unidentified *Lactobacillus* sp. The method is a rapid and reliable way of producing a large number of copies of a specific DNA sequence for the identification of LAB. This method is however, able to solve the problem of poor identification that is usually associated with the identification of this fastidious organism that is regularly used as probiotics, starter culture and biopreservatives in fermented foods that are consumed and in biotechnology because they are generally regarded as safe.

**Key words:** Molecular methods, conventional, *Lactobacillus plantarum* identification, fermented foods, species and genera level, rapid, reliable.

**INTRODUCTION**

Microorganisms have been isolated from different sources especially from different food samples and grown in pure cultures over the centuries. A major aspect of microbiology and the work of food microbiologists and various microbiology laboratories is the ability to identify and characterize various isolates so that they can be
differentiated from one another. Different schemes that can be used to describe the characteristics and properties of microbial isolates are essential in every branch of microbiology. These schemes have been undergoing different forms of development and refinement over the years. The various methods are not static; but have been improved from time to time and proper identification is very essential when it has to do with foods that are consumed (Lucke, 2000; Olaoye and Onilude, 2009). The advent of molecular biology in the 1980s contributed a set of powerful new tools that have helped microbiologists to detect the smallest variations within microbial species and even within individual strains (Olaoye and Onilude, 2009). This is because different organisms have different genetic combination.

In fact, the technology has progressed far beyond the level needed by most routine laboratories, where identifying the species of any isolate is likely to be sufficient. Distinguishing between different strains of the same species (typing) is more likely to be of value in a research laboratory. Nevertheless, methods and equipment designed to help with both species identification and typing are commercially available for a range of applications (Lucke, 2000).

There are different molecular characterization techniques namely genotyping, multilocus sequence typing (MLST), pulsed-field gel electrophoresis (PFGE), ribotyping, repetitive sequence-based PCR (rep-PCR) and the use of 16S rDNA genes which relies on the relative stability of the 16S and 23S rRNA genes coding for ribosomal-RNA and so on (Ogier et al., 2002; Gomes et al., 2008; Paula et al., 2012).

Molecular characterization of microorganisms however has some distinct advantages over the known conventional methods. The molecular method of identification and characterization of microorganisms have been preferred over the classical ones which make use of the biochemical reactions and proteolytic activities of the organisms (Morgan et al., 2009). The classical and conventional method of identification is slow, laborious, time consuming and may not be 100% specific and accurate. It is also problematic and subjective due to ambiguous biochemical or physiological traits.

Bulut et al. (2005) reported that identification of lactic acid bacteria (LAB) by phenotypic methods such as sugar fermentation may be uncertain and complicated owing to the increase in species that vary with few characters. The commercially available system based on this technology is a valuable complementary tool to other routine identification technologies. However, identification based on the 16S rRNA gene is by no means infallible as the sequence stretch analysed is a reduced section of the full genome and the variability of this marker is low.

The development of molecular typing methods has offered the possibility of accelerating a great deal of bacterial identification which avoid so many biases that are related to the classical methods. The polymerase chain reaction (PCR) has however provided a method to detect DNA sequences with high speed and sensitivity. This technique is emerging as a new tool in identifying and selecting bacteria with specific and desirable functions (Bulut et al., 2005). A combination of different approaches in the identification of different organisms offer a solution to the use of the conventional method that makes use of the ability of LAB to produce acid from carbohydrate and other metabolic activities only (Morgan et al., 2009).

According to Merien et al. (2013), the nucleotide base sequences of Lactobacillus spp. 16S ribosomal DNA also provides accurate basis for phylogenetic identification of organisms that are slow growing, fastidious and are therefore poorly identified by conventional methods. These small ribosomal units exist universally among bacteria and include regions with species-specific variability which makes it possible to identify bacteria to species level.

The use of Lactobacillus sp. as probiotics in man has been found to enhance their immunity and increase their ability to fight and survive against food related pathogens. Also, nursing mothers prefer natural products with fewer artificial preservatives in foods that are used for weaning infants with natural fortification or supplements. They have also been found to be consumed in fermented foods that contain them for their health benefits (Adeyemo and Onilude, 2013).

Lactobacillus plantarum particularly has also been implicated in the reduction of raffinose-family of oligosaccharide content of soybeans used in the formulation of a weaning food blend by their ability to hydrolyse the raffinose to simple sugars and hence improve the weaning food (Adeyemo and Onilude, 2014). Fermentation with cultures containing LAB is able to produce healthy, safe, high quality and nutritious beneficial food products such as fermented milk, meat, vegetables, grains, cereals, legumes, meat, beverages, etc. These organisms produce lactic acid which has a way of preserving such fermented foods and also improve the flavour, texture and nutritional compounds of such foods through the metabolic activities of LAB during fermentation. Also, the metabolism and physiology of LAB is used in different biotechnological processes in industries to formulate LAB starters with useful metabolic activities and capabilities so as to ensure a wide range of quality fermented products with consistent characteristics (Adeyemo and Onilude, 2013).

Being used as probiotics and starter culture in many

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

Author(s) agree that this article remain permanently open access under the terms of the Creative Commons Attribution License 4.0 International License.
food industries and in fermentation technology, a prompt and rapid identification of *L. plantarum* is of utmost importance so as not to confuse this very important organism with other organisms of the same genus or species that are closely related. As a result of this, there is need for accurate identification of this organism, the importance of which cannot be over emphasized.

MATERIALS AND METHODS

Sample collection

Local varieties (LV) of sorghum (*Sorghum bicolor*) were obtained from a market and typed varieties (TV) from Institute of Agricultural Research & Training, Ibadan, Nigeria. They were all processed to ogi in the laboratory using the traditional method of Banigo and Muller (1972). Ogi was also obtained from traditional sellers within Ibadan (CO) and used for comparative studies. The samples were collected in clean polythene bags and transported to the laboratory.

Isolation of lactic acid bacteria

One gram each of the samples listed above was subjected to tenfold serial dilutions using the method of Harrigan and MacCance (1976). Isolation of organisms was done with the pour plate method using molten MRS agar. After solidification, they were incubated anaerobically in an anaerobic jar at 30°C for 48-72 h. Pure cultures were selected and stored on slant overlaid with sterile glycerol.

Identification of the isolates

**Morphological and macroscopic characteristics**

For proper identification of the isolates, the cultural, morphological, biochemical and physiological characterization including microscopic and macroscopic examinations of the various isolates were carried out according to Sneath et al. (2009). Gram positive and catalase negative organisms were subjected to further biochemical tests.

**Biochemical characteristics**

Isolates were identified phenotypically on the basis of the following biochemical test after Gram’s staining, catalase, oxidase, methyl red test, Voges Proskauer, nitrate reduction, starch, casein and gelatin hydrolysis, growth at different pH and temperature and NaCl ranges and the ability to produce CO₂ from glucose and production of acid from carbohydrates such as fructose, lactose, maltose, galactose, arabinose, mannose, xylose, dulcitol, inositol, mannitol, raffinose, trehalose, rhamnose, etc. (Sneath et al., 2009).

**Genetic characterization of isolates**

**Extraction of genomic DNA of LAB isolates**

DNA extraction from the LAB isolates was carried out using a modified GES (5M guanidine thiocyanate (Fisher scientific, England), 0.1 N EDTA (Sigma, England) and 0.5% N-lauroyl sarcosine sodium salt (Sigma, England) (w/v) DNA extraction method (Pitcher et al., 1989). Aliquots of 1.5 ml of overnight cultures grown in appropriate broth were centrifuged (Biofuge, Heraeus, Germany) in Eppendraf tubes at 13,000 g for 1 min. Pellets obtained were washed in 1 ml of ice cold lysis buffer (25 mM Tris-HCl (Sigma, England), 10 mM EDTA, 50 mM sucrose (BOH GPR 3039397), pH 8). The pellets were re-suspended in 100 µl of lysis buffer in addition to 50 mg/ml lysozyme (Sigma, England.) and incubated at 37°C for 30 min. 0.5 ml of the GES solution were added and mixed thoroughly. This was incubated at room temperature for 15 min. The lysate was then placed on ice for 2 min and 0.25 ml of 7.5 M ammonium acetate (Fisher scientific, England). Cooled ice was also added, vortexed and incubated on ice for 10 min. Aliquots (0.5 ml) of 24:1 chloroform : isoamylalcohol (Sigma, England) were added, vortexed and centrifuged for 10 min at 13,000 g. Aliquots of 800 ml of the upper phase were removed quantitively and placed in a clean Eppendraf tube. Cold isopropanol (Fisher scientific, England) was added and mixed for 1 min. This was then centrifuged at 13,000 g for 5 min and the supernatant removed from the pellet. The pellet was washed three times in 500 µl of 70% ethanol and dried at 37°C for 15 min. Aliquots (50 µl) of TE buffer were added and 5 µl of the DNA were checked on 1% agarose (Biogene, Kimbolton, UK) gels in 200 ml 1X TAE buffer and the DNA samples were then stored at -20°C for future use.

**Polymerase chain reaction (PCR) amplification of 16S rDNA gene**

The method of Bulut et al. (2005) was used. Amplification of 16s rDNA gene - ITS region, was performed by using the following primer pairs. Forward (16S ITS For), 5'-AGAGTTTGATCCTGCCTCAG-3' and reverse (16S - ITS Rev), 5'-CAAGGATATCAGCGGT-3', 16s rDNA V3, forward 5'- CTTAGGGAGGGCGACAG-3' and reverse, 5'-ARRACCGCGCTGTGCG-3' The forward 5'-CCTACCGCCGCGCTGTGCG-3' and reverse, 5'-ATTCCCGCGCTGTGCG-3', primers used occupied positions 341-358 and 518-534, respectively of the V3 region in the 16S ribosomal DNA of *Escherichia coli*. The primers specify about 200 bp of the PCR products (as could be seen on the gel after electrophoresis).

The V3 primer pair was used for ease of sequencing of the gene, using the variable region 3 (V3), for the genetic identification of the isolates.

Each of the polymerase chain reactions (PCR) was performed in a 50 µl reaction volume containing 50 µg genomic DNA as the template. 10 µl of 0.2 mM deoxynucleoside triphosphates, dNTPs (Promega U20A - U23A, Madison, WI, USA), 10 µl of 2.5 mM MgCl₂, 10 pmol each (0.1 µl volume) of the DNA primer in PCR buffer (Promega, UK), and 10 µl of 1.25 units Taq DNA polymerase (Promega, UK) and 18.9 µl distilled water. Amplification conditions were as follows: an initial denaturation step of 5 min at 94°C, 40 amplification cycles, each consisting of 1 min denaturation at 94°C, 1 min annealing at 42°C, and 1 min elongation at 72°C. Reactions were terminated with a final extension step for 10 min at 72°C. PCR amplification was performed in a Thermocycler (Techne- Progene, Cambridge, UK).

**Gel electrophoresis of 16S rDNA PCR Products**

Electrophoresis of the amplified 16s rDNA PCR products were performed on the Bio-Rad contour - clamped homogenous electric field (CHEF) DRII electrophoresis cell. This was done through 1.5% (w/v) agarose gel (Biogene, Germany) in 0.5 X TAE buffer at 84 V for 1.5-2 h. This was prepared by boiling 1.5 g of agarose powder in 100 ml of 0.5X TAE buffer. A 100 bp ladder (Promega, U.K) and 1 Kb DNA ladder (Promega, U.K) were used as molecular size markers.
Sequencing and analysis of 16S rDNA gene

Purification of PCR 16S rDNA gene

75 µl of the PCR 16S rDNA amplified products (obtained above) were resolved in 1% agarose gels with the conditions earlier described. PCR products were resolved by gel electrophoresis, using an agarose gel (1.5%; Biogene) that was stained with of 0.5 µg/ml ethidium bromide, in 1x TAE buffer at 84 V for 1.5 - 2 h.

The DNA bands were then visualised using a UV transilluminator (Amersham Pharmacia Biotech, UK) with 313 nm emission and pictures were taken using Fuji Film Imaging system FT1-500 (Amersham Pharmacia Biotech, UK).

The resulting bands in agarose gel were carefully excised with sterile scalpels and then purified the Wizard PCR preps DNA purification kit (Promega, USA). The purified DNA was kept at 4°C until used.

Drying of the purified 16S rDNA genes

To a 50 µl of the purified DNA, 0.1 µl of sodium acetate buffer (3M, pH 5.0) and 2.0 µl of 100% ethanol were added. This was then incubated at -20°C for 1 h. It was brought out and left to stand at room temperature for 5 min, and then centrifuged at 13,000 g at 4°C for 45 min. The liquid was removed, leaving only the DNA in the Eppendorf tubes. The DNA was dried in an incubator at 37°C for 30 min.

Sequencing of 16S rDNA gene

The dry DNA samples (obtained using V3 primers) were sequenced using a computer analytical sequencer (MGW - Biotech, Germany) with the V3 and V5 primer Rev, acting as the basis according to manufacturer’s instructions. The generated nucleotide sequences were subjected to analysis. Sequencing of the purified 16S rDNA DNA products was performed using the sequencing unit of the University of Nottingham; a 373 DNA sequence (Perkin-Elmer Applied Biosystems) was used with the Taq Dye Deoxy terminator cycle sequencing kit (Perkin-Elmer Applied Biosystems). The full identities of the isolates were then obtained by subjecting the nucleotide sequences to searches in the Gene Bank (http://www.ncbi.nlm.nih.gov/blast/) with the Blast search program.

Analysis of the 16S rDNA gene sequence

The generated sequences of the 16s rDNA genes were subjected to alignment in the databases at the BLAST, Basic Local Alignment and Search Tool, Website: http://www.ncbi.nih.gov/blast/. The isolates were then identified based on the result of the analysis.

RESULTS

Table 1 shows the result of the conventional method of identification of LAB, the carbohydrate utilization pattern and biochemical characteristics of the isolates. All the 20 isolates were identified as L. plantarum. The result obtained agrees with the characterization pattern of other authors (Sneath et al., 2009).

Table 2 shows the comparison between the phenotypic method and the genotypic method using the 16S rDNA gene sequence of the 20 isolates that were initially identified as L. plantarum. The topmost sequences producing significant alignments when the nucleotide sequences were subjected to Basic Local Alignment Search Tool (BLAST) in the gene bank Database (http://www.ncbi.nlm.nih.gov/blast/) for L. plantarum isolates.

Altogether, seventeen L. plantarum isolates that have been identified before showed a significant alignment in the gene database. The result of the PCR sequencing correlated in 17 out of 20 isolates while there was no correlation in 3 out of 20. The names and accession numbers of these seventeen isolates have significant alignments with the L. plantarum. All the seventeen topmost species was shown to produce significant alignment with the marker and have expected value (E value) of between 1e - 73 and 5e - 7 and maximum identification (Max identity) of between 95 and 100%. They were all L. plantarum. Three out of the twenty isolates did not have significant alignment with the others. They were identified as L. pentosus and one unidentified Lactobacillus sp. There was significant difference in the molecular method and the conventional methods. The result however did not correlate but a divergent view was presented which shows a difference in their gene sequence.

Table 3 shows the qualities and quantities of the 16S rDNA genes of the L. plantarum obtained by PCR using V3 primer, after purification.

The 16S rDNA of the 17 species after amplification with primers was found to belong to the L. plantarum group as they were identified as L. plantarum by partial gene sequencing. The 16S rDNA genes of the other of the three organisms were not shown because a different gene sequence was presented.

Figure 1 shows the L. plantarum strain 16S ribosomal RNA gene, the partial sequence alignment of 16S rDNA after amplification of the gene by PCR in the gene bank data base. Molecular characterisation of the isolates was done by extracting the DNA gene sequence using universal primers and when compared, it was identified as L. plantarum with alignment.

Figure 2 shows the nucleotide sizes in base pairs (bp) of the plasmids of the selected seventeen L. plantarum isolate that were used for further work after their identities have been confirmed by 16S rDNA. This base sequence provides significant information on the 16S rDNA gene sequence of the L. plantarum. The nucleotide base sequence of the 16S rDNA has provided a basis for phylogenetic identification and analysis.

DISCUSSION

Accurate and definitive microorganism identification is essential for a wide variety of application including biotechnological, industrial, biomedical, pharmaceutical and environmental studies. The 16S rDNA sequence based analysis is a central method to understand not only
Table 1. Physiological and biochemical characteristics of isolates.

| Isolate code | Gram reaction | Cell morphology | Catalase | Oxidase | Gelatin hydrolysis | M.R | V.P | H₂S | Propanol | Growth at 15°C | pH at 3.9 | pH at 9.2 | pH at 5 | 4% NaCl | CIT | U.TI | Glucose | Xylose | Rhamnose | Triaminon citrate | Raffinose | Rafinose | Sucrose | Lactose | Galactose | Fructose | Arabinose | Mannose | Maltose | Manno | Inositol | Molality | Indole | Ni-H₂ | Nitr Nitrate red | Probable identity |
|--------------|---------------|-----------------|----------|---------|-------------------|-----|-----|-----|----------|----------------|---------|-----------|----------|---------|-----|------|---------|-------|--------|----------------|--------|--------|---------|---------|----------|----------|----------|--------|-------|--------|---------|--------|--------|
| 1            | +             | R               | -        | -       | -                  | -   | -   | -   | +        | +            | +       | +         | -        | +       | -   | -    | +       | +     | +      | +               | +      | -      | +        | +       | +       | +       | +        | +      | +      | +       | +       | +       | +       | +       | +      | +      | +       | Lactobacillus plantarum |
| 2            | +             | R               | -        | -       | -                  | -   | -   | -   | +        | +            | +       | +         | -        | +       | -   | -    | +       | +     | -      | +               | +      | -      | +        | +       | +       | +       | +        | +      | +      | +       | +       | +       | +       | +       | +      | +      | +       | L. plantarum |
| 3            | +             | R               | -        | -       | -                  | -   | -   | -   | +        | +            | +       | +         | -        | +       | -   | -    | +       | +     | -      | +               | +      | -      | +        | +       | +       | +       | +        | +      | +      | +       | +       | +       | +       | +       | +      | +      | +       | L. plantarum |
| 4            | +             | R               | -        | -       | -                  | -   | -   | -   | +        | +            | +       | +         | -        | +       | -   | -    | +       | +     | -      | +               | +      | -      | +        | +       | +       | +       | +        | +      | +      | +       | +       | +       | +       | +       | +      | +      | +       | L. plantarum |
| 5            | +             | R               | -        | -       | -                  | -   | -   | -   | +        | +            | +       | +         | -        | +       | -   | -    | +       | +     | -      | +               | +      | -      | +        | +       | +       | +       | +        | +      | +      | +       | +       | +       | +       | +       | +      | +      | +       | L. plantarum |
| 6            | +             | R               | -        | -       | -                  | -   | -   | -   | +        | +            | +       | +         | -        | +       | -   | -    | +       | +     | -      | +               | +      | -      | +        | +       | +       | +       | +        | +      | +      | +       | +       | +       | +       | +       | +      | +      | +       | L. plantarum |
| 7            | +             | R               | -        | -       | -                  | -   | -   | -   | +        | +            | +       | +         | -        | +       | -   | -    | +       | +     | -      | +               | +      | -      | +        | +       | +       | +       | +        | +      | +      | +       | +       | +       | +       | +       | +      | +      | +       | L. plantarum |
| 8            | +             | R               | -        | -       | -                  | -   | -   | -   | +        | +            | +       | +         | -        | +       | -   | -    | +       | +     | -      | +               | +      | -      | +        | +       | +       | +       | +        | +      | +      | +       | +       | +       | +       | +       | +      | +      | +       | L. plantarum |
| 9            | +             | R               | -        | -       | -                  | -   | -   | -   | +        | +            | +       | +         | -        | +       | -   | -    | +       | +     | -      | +               | +      | -      | +        | +       | +       | +       | +        | +      | +      | +       | +       | +       | +       | +       | +      | +      | +       | L. plantarum |
| 10           | +             | R               | -        | -       | -                  | -   | -   | -   | +        | +            | +       | +         | -        | +       | -   | -    | +       | +     | -      | +               | +      | -      | +        | +       | +       | +       | +        | +      | +      | +       | +       | +       | +       | +       | +      | +      | +       | L. plantarum |
| 11           | +             | R               | -        | -       | -                  | -   | -   | -   | +        | +            | +       | +         | -        | +       | -   | -    | +       | +     | -      | +               | +      | -      | +        | +       | +       | +       | +        | +      | +      | +       | +       | +       | +       | +       | +      | +      | +       | L. plantarum |
| 12           | +             | R               | -        | -       | -                  | -   | -   | -   | +        | +            | +       | +         | -        | +       | -   | -    | +       | +     | -      | +               | +      | -      | +        | +       | +       | +       | +        | +      | +      | +       | +       | +       | +       | +       | +      | +      | +       | L. plantarum |
| 13           | +             | R               | -        | -       | -                  | -   | -   | -   | +        | +            | +       | +         | -        | +       | -   | -    | +       | +     | -      | +               | +      | -      | +        | +       | +       | +       | +        | +      | +      | +       | +       | +       | +       | +       | +      | +      | +       | L. plantarum |
| 14           | +             | R               | -        | -       | -                  | -   | -   | -   | +        | +            | +       | +         | -        | +       | -   | -    | +       | +     | -      | +               | +      | -      | +        | +       | +       | +       | +        | +      | +      | +       | +       | +       | +       | +       | +      | +      | +       | L. plantarum |
| 15           | +             | R               | -        | -       | -                  | -   | -   | -   | +        | +            | +       | +         | -        | +       | -   | -    | +       | +     | -      | +               | +      | -      | +        | +       | +       | +       | +        | +      | +      | +       | +       | +       | +       | +       | +      | +      | +       | L. plantarum |
| 16           | +             | R               | -        | -       | -                  | -   | -   | -   | +        | +            | +       | +         | -        | +       | -   | -    | +       | +     | -      | +               | +      | -      | +        | +       | +       | +       | +        | +      | +      | +       | +       | +       | +       | +       | +      | +      | +       | L. plantarum |
| 17           | +             | R               | -        | -       | -                  | -   | -   | -   | +        | +            | +       | +         | -        | +       | -   | -    | +       | +     | -      | +               | +      | -      | +        | +       | +       | +       | +        | +      | +      | +       | +       | +       | +       | +       | +      | +      | +       | L. plantarum |
| 18           | +             | R               | -        | -       | -                  | -   | -   | -   | +        | +            | +       | +         | -        | +       | -   | -    | +       | +     | -      | +               | +      | -      | +        | +       | +       | +       | +        | +      | +      | +       | +       | +       | +       | +       | +      | +      | +       | L. plantarum |
| 19           | +             | R               | -        | -       | -                  | -   | -   | -   | +        | +            | +       | +         | -        | +       | -   | -    | +       | +     | -      | +               | +      | -      | +        | +       | +       | +       | +        | +      | +      | +       | +       | +       | +       | +       | +      | +      | +       | L. plantarum |
| 20           | +             | R               | -        | -       | -                  | -   | -   | -   | +        | +            | +       | +         | -        | +       | -   | -    | +       | +     | -      | +               | +      | -      | +        | +       | +       | +       | +        | +      | +      | +       | +       | +       | +       | +       | +      | +      | +       | L. plantarum |

R = Rod; + = A positive reaction; = A negative reaction; D = A delayed reaction; W = A weakly positive reaction; M.R = methyl red test, V.P = Voges Proskauer
the microbial diversity within and across the group but also to identify new strains. Bacterial species have at least one copy of the 16S rDNA gene containing highly conserved regions together with hyper variable regions, which is used for identification of new strains. However, a considerable variation can occur between species in both the length and the sequence of 16S rDNA ITS region, therefore this region is useful in characterization of bacterial species (Mohammed et al., 2011). The 16S rDNA gene is very useful because the genome of all bacteria contains this conserved gene and any small variability in this region is unique and specific to each species. This characteristic is usually harnessed in their identification (Mohania et al., 2008).

Considering the conventional method for identifying LAB isolates, the objective of this study was to compare the phenotypic method and the 16S rDNA sequencing which is a species-specific PCR reaction for the proper identification of the twenty Lactobacillus sp. The genus level was however the same for all the isolates, they were further characterized using PCR reactions to perform complete identification. The results obtained with 95% reliability and higher were considered; those lower than this were not considered because their gene sequences were identified as different organisms. Considering that species-specific PCR reactions target specific genes of genera and species, the molecular method was considered reliable. Molecular bacteria identification is based on the full length of 16S rDNA gene sequence by several studies have shown that the initial few base pair sequence provides sufficient discrimination between strains because this region shows a high genetic diversity.

Of the 20 isolates used in this work, three presented divergent results as compared to 16S rDNA sequencing and species-specific PCR reaction. This confirmed the result of 17 out of 20 isolates tested (17/20), that is, 85% and divergent result were obtained in 3 out of 20 (15%) isolates that were screened (3/20). Out of these, 2 were identified as L. pentosus while the last was a

### Table 2. Comparison of phenotypic and genotypic methods of identification of L. plantarum.

<table>
<thead>
<tr>
<th>Isolate code</th>
<th>Conventional identity</th>
<th>Closest relative (using 16srDNA gene sequencing)</th>
<th>Identity</th>
<th>Gene bank accession no</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. plantarum CO1</td>
<td>L. plantarum</td>
<td>L. plantarum</td>
<td>95%</td>
<td>GQ180906.1</td>
</tr>
<tr>
<td>L. plantarum CO2</td>
<td>L. plantarum</td>
<td>L. plantarum</td>
<td>95%</td>
<td>GQ166683.1</td>
</tr>
<tr>
<td>L. plantarum CO3</td>
<td>L. plantarum</td>
<td>L. plantarum</td>
<td>95%</td>
<td>GQ166682.1</td>
</tr>
<tr>
<td>L. plantarum CO4</td>
<td>L. plantarum</td>
<td>L. plantarum</td>
<td>95%</td>
<td>GQ166681.1</td>
</tr>
<tr>
<td>L. plantarum CO5</td>
<td>L. plantarum</td>
<td>L. pentosus</td>
<td>91%</td>
<td>GQ180915.1</td>
</tr>
<tr>
<td>L. plantarum CO6</td>
<td>L. plantarum</td>
<td>L. plantarum</td>
<td>95%</td>
<td>GQ180902.1</td>
</tr>
<tr>
<td>L. plantarum CO7</td>
<td>L. plantarum</td>
<td>L. plantarum</td>
<td>95%</td>
<td>FJ861114.1</td>
</tr>
<tr>
<td>L. plantarum CO8</td>
<td>L. plantarum</td>
<td>L. plantarum</td>
<td>95%</td>
<td>FJ861113.1</td>
</tr>
<tr>
<td>L. plantarum CO9</td>
<td>L. plantarum</td>
<td>L. plantarum</td>
<td>95%</td>
<td>FJ861112.1</td>
</tr>
<tr>
<td>L. plantarum CO10</td>
<td>L. plantarum</td>
<td>L. plantarum</td>
<td>95%</td>
<td>FJ861111.1</td>
</tr>
<tr>
<td>L. plantarum CO11</td>
<td>L. plantarum</td>
<td>L. plantarum</td>
<td>95%</td>
<td>FJ851116.1</td>
</tr>
<tr>
<td>L. plantarum CO12</td>
<td>L. plantarum</td>
<td>L. plantarum</td>
<td>95%</td>
<td>FJ851115.1</td>
</tr>
<tr>
<td>L. plantarum CO13</td>
<td>L. plantarum</td>
<td>L. plantarum</td>
<td>95%</td>
<td>FJ851113.1</td>
</tr>
<tr>
<td>L. plantarum CO14</td>
<td>L. plantarum</td>
<td>L. plantarum</td>
<td>95%</td>
<td>FJ84495.1</td>
</tr>
<tr>
<td>L. plantarum CO15</td>
<td>L. plantarum</td>
<td>L. plantarum</td>
<td>95%</td>
<td>FJ844955.1</td>
</tr>
<tr>
<td>L. plantarum CO16</td>
<td>L. plantarum</td>
<td>L. plantarum</td>
<td>95%</td>
<td>FJ844949.1</td>
</tr>
<tr>
<td>L. plantarum CO17</td>
<td>L. plantarum</td>
<td>L. plantarum</td>
<td>95%</td>
<td>FJ844994.1</td>
</tr>
<tr>
<td>L. plantarum CO18</td>
<td>L. plantarum</td>
<td>L. plantarum</td>
<td>95%</td>
<td>FJ844945.4</td>
</tr>
<tr>
<td>L. plantarum CO19</td>
<td>L. plantarum</td>
<td>L. plantarum</td>
<td>90%</td>
<td>FJ843956.1</td>
</tr>
<tr>
<td>L. plantarum CO20</td>
<td>L. plantarum</td>
<td>L. plantarum</td>
<td>95%</td>
<td>FJ844953.1</td>
</tr>
</tbody>
</table>

### Table 3. Qualities and quantities of the 16S rDNA genes of the L. plantarum obtained by PCR using V3 primer, after purification.

<table>
<thead>
<tr>
<th>S/N</th>
<th>Sample ID</th>
<th>Conc. (ug/L)</th>
<th>A260nm</th>
<th>A260/280</th>
<th>0.0260/230</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>H101</td>
<td>18.34</td>
<td>0.367</td>
<td>1.82</td>
<td>0.02</td>
</tr>
</tbody>
</table>
**GQ180905.1** *Lactobacillus plantarum* strain TJ2 16S ribosomal RNA gene, partial sequence, Length = 182, Score = 283 bits (153), Expect = 1e-73, Identities = 153/153 (100%), Gaps = 0/153 (0%) Strand = Plus/Plus

**Figure 1.** Alignment of 16S rDNA nucleotide sequences of *L. plantarum* against *L. plantarum* strain LpT2 (accession no GQ166663.1) and *L. plantarum* strain LpT1 (accession no GQ166662.1) in the gene bank data base.

**Figure 2.** The nucleotide sizes of the plasmids of the selected nine *L. plantarum* isolate.
**Lactobacillus** sp. that could not be identified. This result agrees with the report of Marroki et al. (2011) who reported a similar view stating that *L. plantarum* and *L. pentosus* have very similar 16S rDNA sequences that only differ by 2 base pair. Other authors also reported that both organisms belong to the same phylogenetic group and they can only be differentiated when analysis of 16S-23S larger spacer is done (Ennahar et al., 2003). This may also be the same reason for the other *Lactobacillus* sp. that was not identified by this method presenting a result that did not correlate with those obtained earlier by phenotypic method. However, the result of the conventional method cannot be discarded completely but it can be regarded as giving a clue or presumptive result which can then be confirmed by molecular method.

Differences between genotypic and phenotypic tests have been identified previously not just for LAB but also for many other bacteria (Gomes et al., 2008; Paula et al., 2012). They also noted that this tool is useful for identifying microorganisms at sub species level which cannot easily be identified by other common technique. Phenotypic method may also have poor reproducibility as a result of changes that occur during the growth and metabolism of different organisms. This also agrees with the report of Mohania et al. (2008) who reported that bacterial isolates do not express their genes at the same time or they may lose some characteristics such as plasmids during culturing. This may however be responsible for the inconsistencies that are usually identified in sugar fermentation patterns and other biochemical tests that rely on physiological characteristics of different organisms for identification.

Gill et al. (2006) also stressed another importance of this molecular method being a desirable advantage of 16S rDNA over the conventional one. Apart from being rapid, the sequence could also be performed not only on bacterial culture but also on the sample so as to study the diversity of the organisms without culturing. The efficacy and efficiency of this method was clearly demonstrated in this work by differentiating strains belonging to the same species and it has been clearly identified by various authors such as Gill et al. (2006) and Morgan et al. (2009) because the results are not subjective.

The molecular method used in this work further confirmed the real identities of *L. plantarum* that were used for further work in the fermentation pattern for the formulation of a weaning food blend as earlier reported by Adeyemo and Onilude (2013). The real identities of the organisms are usually revealed by molecular methods and the results can be reproduced at any time and in different places without environmental variations. Based on the result of this study, the 16S rDNA sequencing method is specific for the gene of target and broader strategies that can characterize lactic acid bacteria without prior knowledge of genetic targets, this is however a desirable characteristics of this method, it is thus recommended for proper identification of organisms to be used in fermented foods as starter culture or bio-preservation.

The result obtained in this work agrees with the result obtained by Parker et al. (2001). They opined that several PCR methods have subsequently been developed to overcome difficulties experienced with phenotypic methods. The method described in this work allows the amplification of specific PCR products. This enables direct sequencing of unknown regions without the need for DNA cloning but makes use of analysis of microbial genetic elements. Shittu et al. (2006) also noted the accuracy of the molecular diagnostic method in the ability to rapidly identify microorganisms isolated from clinical samples from genus level to species level using automated systems. Reduction of analysis time and reproducibility would be advantageous, especially for organisms that are fastidious, slow-growing and of medical and industrial importance.

The result obtained also solves the problem of misidentification. This agrees with the work of Woo et al. (2008) who reported that some LAB species are closely related to *Lactobacillus* sp. The importance of accurate identification need to be emphasized in LAB obtained from fermented foods that are used as probiotics or starter cultures. This is because some LAB are also involved in clinical infections such as *Leuconostoc* sp., *Pediococcus* sp. and *Enterococcus* sp. These organisms are of medical importance and should not be misidentified with other *Lactobacillus* sp. The use of 16S rDNA will lower the risk of inaccurate or poor identification of these pathogens that are also similar to other *Lactobacillus* sp.

However, in industrial microbiology for example, there are various importance of rapid methods of identification of microorganisms. First, it is of paramount importance to food/industrial microbiologists for screening and identification of organisms that are of great industrial and biotechnological purposes. Rapid detection and identification of microorganisms also allows for continuous monitoring of microbial growth in relation to various metabolites that are produced by them especially in pharmaceutical industries such as enzymes, vaccines, antibiotics, organic acids etc. Also, the ease of producing a large number of copies of a specific DNA sequence can be applied in the industry for the production of many important products from microorganisms using some specific genes from them.

Finally, the advantage of genotyping is that it is an accurate method for the identification of *L. plantarum* in that the genome is stable; the genetic composition of the organism is independent of cultural conditions and method of isolation; it can easily be subjected to automation and the results can be analysed statistically with ease. LAB are referred to as “probiotics” and it belongs to the group of organisms that are generally regarded as safe (GRAS). Its prompt and quick identification is a
useful tool in distinguishing between these probiotics and other opportunistic pathogens that may also be present as contaminant in fermented foods.

Conflict of Interests

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

ACKNOWLEDGEMENT

The author wishes to acknowledge the contribution of Dr. Olusegun Olaoye of Michael Okpara University of Agriculture, Umudike, Abia State, Nigeria towards the molecular aspect of this work.

REFERENCES


Molecular characterization of resistance to Russian wheat aphid (*Diuraphis noxia* Kurdjumov) in bread wheat (*Triticum aestivum* L.) line KRWA9

E. A. Masinde1,2*, J. N. Malinga1, W. A. Ngenya1,2, R.M.S Mulwa2 and M. Cakir3

1Kenya Agricultural Research Institute, P.O Box Private Bag - 20107 Njoro, Kenya.
2Crops Horticulture and Soils Department, Egerton University P.O Box 523, Egerton, Kenya.
3WA State Agricultural Biotechnology Center, School of Veterinary and Life Sciences, Murdoch University 90 South St., Murdoch WA 6150, Australia

Received 11 August, 2014; Accepted 12 September, 2014

The Russian wheat aphid (RWA), *Diuraphis noxia* (Kurdjumov) causes extensive economic damage to wheat (*Triticum aestivum* L.) in most wheat growing regions of the world. Control of RWA using systemic insecticides is expensive and pollutes the environment therefore the most effective method of RWA control is the development of RWA resistant cultivars. This study was initiated to determine inheritance of RWA resistance in a wheat resistance source KRWA9, and identify the chromosome location of the resistance gene. Inheritance was studied in parent materials, F1 populations, F2 populations and F2:3 families of a cross between resistant line KRWA9 and a susceptible variety NjoroBW2. Seedlings were infested with RWA then scored for damage on a visual scale of 1 to 9 after 21 days of infestation. The segregation data from NjoroBW2 × KRWA9 population depicted monogenic dominant inheritance of the resistance gene with phenotypic ratios of 3:1 in F2 populations and 1:2:1 in F2:3 families. Bulk segregant analysis approach was used for the mapping of resistance. Nine simple sequence repeat (SSR) primers were tested between parental lines and bulks, and only chromosome 7DS SSR marker *Xgwm111* produced clear polymorphism between the parental lines and the resistant and susceptible bulks. Detailed analysis of this marker with the full population revealed very close linkage to resistance with a coefficient of determination (R2) value of 85%. This marker provides good opportunities for the marker-assisted breeding towards improving Russian wheat aphid resistance.

**Key words:** Russian wheat aphid, resistance, susceptibility, simple sequence repeat (SSR) markers.

INTRODUCTION

The Russian wheat aphid (RWA), *Diuraphis noxia* (Kurdjumov), a pest of wheat and barley, is indigenous to southern Russia, Iran, Afghanistan and countries bordering the Mediterranean Sea (Hewitt et al., 1984). The pest has spread widely and is now found in all the continents except Australia (Ennahli et al., 2009), and...
causes economic damage to wheat in many parts of the world. In Ethiopia, Miller and Haile (1988) reported 68% yield loss in wheat. In South Africa, 21–92% yield losses were reported (Du Toit and Walters, 1984). In Kenya, it can cause losses of up to 90% in wheat (Malinga, 2007) and sometimes up to 100% due to prolonged drought conditions. RWA attacks the plant by infesting the young growing tip, deep in the leaf whorls where it feeds from the phloem of longitudinal veins. Symptoms of RWA attack appear as chlorotic spots that coalesce to form white, yellow or purple streaks running parallel to the mid rib of leaves (Botha and Matsiliza, 2006). In young plants, heavy infestation leads to prostate tillers while adult plants show trapped ears within the flag leaf looking like a fish hook. Severe infestation may lead to head sterility and death of host plant.

Insecticide use and particularly contact foliar applications are ineffective because of the feeding nature of the aphid. The aphid feeds within the rolled leaf whorl so cannot be easily reached by contact foliar sprays. This necessitates the use of more expensive systemic insecticides which apart from being harmful to the environment promote development of resistant biotypes and destroys biological agents. RWA resistant cultivars have been observed to have a yield advantage as compared to susceptible cultivars (Tolmay et al., 2000) and resistant cultivars have low cost as seed is usually the least expensive component in the production system besides being environment friendly. Host plant resistance is therefore, the most desirable alternative that could form part of an integrated pest management programme (IPM).

The first RWA resistant cultivar, TugelaDn (containing resistance gene Dn1), was released in South Africa in 1992 (Van Niekerk, 2001). A new biotype-designated RWASA2 was identified in 2005 virulent to Dn1, Dn2, Dn3 and Dn9 (Jankielsohn, 2011). Most of the RWA resistant cultivars available for commercial production in South Africa (Tolmay et al., 2007) were overcome by RWASA2. Similarly, resistant cultivar Halt (containing Dn4) was released in the United States in 1994 (Quick et al., 1996), but a new biotype, USARWA2 with virulence to resistance genes Dn4 and Dny was reported in 2004 (Haley et al., 2004), also overcoming the majority of commercially available resistant cultivars. Although RWA resistance expression is known to be influenced by genetic background (Randolph et al., 2005; Tolmay and Van Deventer, 2005), it is nonetheless assumed to function on a gene-for-gene basis in terms of the resistance/biotype interaction (Ricciardi et al., 2010). Recently a third biotype, RWASA3 virulent to Dn1, Dn2, Dn3, Dn4 and Dn9 was reported in South Africa by Jankielsohn (2011). Notably, neither Dn4 nor Dny had been deployed against RWA in South Africa. In Kenya, two biotypes with genetic differences have been discovered in the major wheat growing areas, that is, Njoro and Timau (Malinga et al., 2007a). Amplified fragment length polymorphism markers used to detect genetic differences showed that the Njoro biotype may contain more virulent populations as compared to Timau biotype (Malinga et al., 2007a). This was the first confirmatory report on biotypes in Kenya and it raised great challenges to resistance breeding programs for Russian wheat aphid.

Breeding for RWA resistant cultivars requires a reliable method of selecting plants containing a resistant gene. While phenotype based selection method is straightforward, it has several limitations like the environmental influence on symptoms of damage expression. It is therefore highly desirable to employ a screening technique that is based on molecular markers linked to the resistance genes. Aside from overcoming the problems associated with phenotypic screening, marker-assisted selection (MAS) would enable gene pyramiding which is the combination of two or more resistance genes efficiently. This will expedite the process of breeding for multiple and durable resistance.

Most of the known wheat genes conferring resistance to RWA, have been mapped using microsatellite markers. Nine of these resistance genes are located on the D genome of wheat and one on the 1RS/1BL translocation (McIntosh et al., 2003). A study by Liu et al. (2001) revealed that the locus for wheat microsatellite GWM111 (Xgwm111), located on wheat chromosome 7DS (short arm), is tightly linked to RWA resistance genes Dn1, Dn2 and Dn5, as well as Dnx in wheat resistance source PI 220127. The segregation data indicated that RWA resistance in PI 220127 is also conferred by a single dominant resistance gene (Dnx) (Liu et al., 2001). These results by Liu et al. (2001) confirmed that Dn1, Dn2 and Dn5 are tightly linked to each other, and this provided new information about their location, being 7DS, near the centromere, instead of as previously reported on 7DL. According to Miller et al. (2001), the marker Xgwm437 is closely linked to Dn2 at 2.8cM. Xgwm106 and Xgwm337 flanked Dn4 on chromosome 1DS at 7.4 and 12.9 cM, respectively (Liu et al., 2002). Nkongolo et al. (1991a) reported RWA resistance gene Dn3 in Triticum tauschii. Dn5 is located on wheat chromosome 7DS rather than 7DL and microsatellite marker Xgwm635 shows close linkage to the gene (Liu et al., 2001). The markers Xgwm44 and Xgwm111 are linked to Dn6 near the centromere on chromosome 7DS at 14.6 and 3.0 cM, respectively (Liu et al., 2002). This was the first report of the chromosome location of Dn6, which is either allelic or tightly linked to Dn1, Dn2, Dn5 and Dnx. Xgwm635 (near the distal end of 7DS) clearly marked the location of a previously suggested resistance gene in PI 294994, which was designated as Dn8 (Liu et al., 2001). Xgwm642, in a defense gene-rich region of chromosome 1DL, marked another new gene Dn9 from PI 294994 (Liu et al., 2001). A third new gene Dny from the Chinese wheat Lin-Yuan207 was localized on chromosome 1DL between Xgwm111 and Xgwm337 (Liu et al., 2001).
study was carried out with PCR markers for Russian Wheat Aphid Resistance Gene Dn7 on Chromosome 1RS/1BL and two markers which amplified rye-specific fragments proved to be useful for MAS. Xrems1303 amplified a 320-bp band only in cultivars with high-level resistance to USA biotype 2 and was effective for MAS of Dn7. Xlb267 was found to be linked to the susceptible locus and amplified a fragment specific for rye Petkus 1RS (Lapitan et al., 2007).

Most of the Kenyan commercial wheat varieties are susceptible to RWA (Kiplagat, 2005) and since breeding of RWA resistant cultivars is further complicated due to presence of RWA biotypes, rapid breeding for and deployment of additional wheat cultivars resistant to RWA is urgently needed to reduce further losses from RWA outbreaks. This study was carried out to determine the inheritance and chromosome location of RWA resistance gene in the wheat source KRWA9.

MATERIALS AND METHODS

Plant materials and population development

Seeds were obtained from the Kenya Agricultural Research Institute, Njoro and planted in the crossing block in a row spacing of 30 cm. Crossing was carried out between resistant line ‘KRWA9’ and susceptible commercial variety ‘Njoro BW2’ to obtain F1 progeny. The F1 progeny was planted the following season and selfed to obtain F2 seeds. F2 families were obtained by planting seeds harvested from individual F2 plants. Plants grew under normal rainfall regime with occasional irrigation supplement.

Phenotyping

The parents, F1 plants, 100 F2 plants and F2:3 families were screened for RWA resistance under greenhouse conditions. Parents, F1 and F2 seedlings were grown in 20-cm-diameter pots containing sterilized forest soil and sand at a ratio of 3:1 mixed with 5 g Di-ammonium phosphate (18-46-0) fertilizer. Each pot contained two to four seedlings. Fifteen (15) seeds from each F2:3 family were planted on evaluation flats (1.5 × 1.0 × 0.75 m) containing two to four seedlings. Fifteen seedlings were grown in 20 cm pots and were caged with a 60 cm high wire cage and covered with a nylon ester screen mesh (68 meshes per square cm) to prevent aphids from getting in or escaping. Five adult aphids (3 - 5 instar stage) were used for each plant to ensure maximum infestation pressure was achieved. RWA infestation was rated at twenty one days after infestation and scoring done according to a modified 1 - 9 visual scale (Malinga, 2007). Plants showing damage scale of 1 - 5 were grouped as resistant and 6 - 9 susceptible.

Statistical analysis

The data of RWA reaction for individual F2 plants was tested against an expected phenotypic segregation ratio of 3:1 using the Chi square ($\chi^2$) goodness of fit test, to confirm the mode of inheritance at probability level of $P = 0.05$. The data on RWA reaction for individual F2 families was tested against an expected phenotypic segregation ratio of 1:2:1 using the Chi square ($\chi^2$) test to also confirm the mode of inheritance at probability level of $P = 0.05$. The segregation of F2:3 families was expected to confirm the segregation ratios observed in F2 populations and aid in the classification of F2 lines for the bulk segregant analysis.

Genotyping using microsatellite markers

DNA was isolated from parents and 100 F2 plants following the protocol by Dellaporta and Woods (1983) with some modifications. Approximately 500 mg of leaf tissue was ground with liquid nitrogen before adding and mixing with 500 μl of extraction buffer (0.1 M Tris-HCl pH 8.0, 0.05 M ethylenediaminetetraacetic acid (EDTA), 0.5 M NaCl, 1% polyvinylpyrrolidone, 1.6% sodium dodecyl sulphate (SDS). This was followed by the addition of 50 μl of 20% SDS, and after mixing by inversion the tubes were incubated for 15 min at 65°C. The samples were removed from incubator and 250 ml of potassium acetate (-20°C) followed by incubation in freezer for 10 min at -20°C. The samples were then centrifuged at 13,000 rpm for 5 min and 500 μl of isopropanol (at -20°C) was added to the supernatant in new tubes. The mixture was incubated for 10 min at -20°C followed by centrifugation at 13,000 rpm for 5 min. The supernatant was discarded, DNA pellet washed with 500 μl of 70% ethanol (at -20°C) followed by air-drying. The DNA pellet was resuspended in 100 ul of 10:1 TE (10 mM Tris:1 mM EDTA) buffer. The samples were RNase treated by adding 2.25 μl of 10 mg ml$^{-1}$ RNase and incubating for 30 min at 65°C followed by storing at -20°C till further use.

DNA was quantified spectrophotometrically and quality checked by 1% agarose gel electrophoresis, against lambda DNA of known quantity. Presence of DNA was confirmed by visualizing the bands on the gel under a UV transilluminator (Alpha Innotech, Taiwan). Comparison of the concentration of DNA was done against known standards of 100, 125, 250 500 and 1000 ng/μl lambda DNA to determine quantity. DNA was diluted to a working stock of 30 ng/μl for PCR reactions. Bulk segregant analysis (BSA) with microsatellite markers was used to identify DNA markers associated with RWA resistance. Nine primers for Xgwm microsatellites were used in this study. These microsatellite markers have been mapped in wheat chromosome 7D. They included Xgwm30, Xgwm44, Xgwm46, Xgwm56, Xgwm111, Xgwm297, Xgwm333, Xgwm437 and Xgwm644 (Roder et al., 1998). BSA was done using DNA from KRWA9, NjoroBW2, resistant homoygous plants, resistant heterozygous (segmenting) plants, homozygous susceptible plants and control resistance sources Pl 137739 (Dn1), Pl 262660 (Dn2), USA9 (Dn7) and Pl 294994 (Dn5, Dn8 and Dn9). DNA solution was bulked into their respective resistant and susceptible bulks. The resistant bulk consisted of equal amounts of DNA 10 μl from eight homozygous resistant plants. The susceptible bulk contained DNA from eight susceptible plants. The third bulk contained DNA from segregating plants. There were two more bulks with equal amounts of DNA 10 μl from each parent NjoroBW2 and KRWA9. All PCR reactions were performed in 13 μl reaction volumes containing 1.25 μl of 10X PCR buffer, 8.5 μl of ddH2O, 0.5 μl of 10 mM dNTPs, 0.75 μl of 50 mM MgCl2, 0.25 μl of 10 mM each of forward and reverse primer and 0.05 μl of Invitrogen Taq DNA polymerase recombinant.
Marker analysis

Informative bands were scored as present (+) or absent (−) and since SSRs are co-dominant markers, it was expected that alleles from both parents would be observed in some samples. Single marker analysis was done using the JoinMap software (Stam and Van Ooijen 1995) to detect QTL associated with Xgwm111. Linear regression was done to obtain coefficient of determination ($R^2$) that explains the phenotypic variation arising from QTL linked to a marker. Chi-square goodness-of-fit test was carried out to test conformity to Mendelian segregation patterns. The Chi square ($\chi^2$) value and segregation ratios from gel data were later compared against Chi square ($\chi^2$) value and phenotypic segregation ratios resulting from RWA reactions of individual F2 populations and F2:3 families.

RESULTS

Inheritance analysis

The resistant parent KRWA9 showed resistance reactions having minimal levels of chlorosis and rolling, with damage scores of 1 - 3. This indicated high levels of resistance in the resistant parent. The susceptible parent NjoroBW2 showed a susceptible reaction with damage scores of 7 - 9. Most NjoroBW2 seedlings had severe leaf chlorosis, streaking and rolling leading to death after 21 days of infestation. The F1 population of cross NjoroBW2 × KRWA9 showed resistance reaction with damage scores of 1 - 4. The resistance reaction of F1 population was not significantly different from the reaction of KRWA9 indicating that the resistance gene in KRWA9 is dominant. The $\chi^2$ statistics for NjoroBW2 × KRWA9 F1 population was significant at $P<0.05$ with a fit in ratio of 3:1 (Table 1). In NjoroBW2 × KRWA9 F2 generation, the hybrids segregated and were classified into their respective phenotypic classes. The F2 population showed both susceptible and resistant reactions with damage scores of 1 - 9. The $\chi^2$ statistics was significant at $P<0.05$ with a fit in ratio of 3:1 (Table 1). The F2:3 progenies were classified as homozygous resistant and heterozygous resistant (segregating) based on the seedling reactions to RWA. The F2:3 homozygous resistant progenies showed damage scores of 1 - 5, indicating resistance. Heterozygous resistant progenies showed damage scores of 1 - 9 indicating both resistance and susceptible reactions. The $\chi^2$ statistics for F2:3 population of NjoroBW2 × KRWA9 was significant at $P<0.05$ (Table 1) with a fit in ratio of 1:2:1. These results confirmed the model of 3:1 at F2 populations with a fit of 1:2:1 at F2:3 families for monohybrid inheritance.

**Table 1.** Chi-square values for seedling reaction to Russian wheat aphid in KRWA9, NjoroBW2, F1, F2 and F2:3 populations of KRWA9 × NjoroBW2 cross.

<table>
<thead>
<tr>
<th>Parents and crosses parents</th>
<th>Pop</th>
<th>Total</th>
<th>R</th>
<th>S</th>
<th>Observed R:S</th>
<th>Expected R:S</th>
<th>$\chi^2$</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>KRWA9</td>
<td>P1</td>
<td>44</td>
<td>44</td>
<td>0</td>
<td>44:0</td>
<td>44:0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NjoroBW2</td>
<td>P2</td>
<td>45</td>
<td>0</td>
<td>45</td>
<td>0:45</td>
<td>0:45</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crosses</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KRWA9 × NjoroBW2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F1</td>
<td>24</td>
<td>24</td>
<td>0</td>
<td>24:0</td>
<td>24:0 (1:0)</td>
<td>0.00</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>F2</td>
<td>100</td>
<td>77</td>
<td>23</td>
<td>77:23</td>
<td>75:25 (3:1)</td>
<td>0.21</td>
<td>0.644</td>
<td></td>
</tr>
<tr>
<td>KRWA9 × NjoroBW2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F2:3</td>
<td>100</td>
<td>28:49:23</td>
<td>28:49:23</td>
<td>25:50:25 (1:2:1)</td>
<td>0.53</td>
<td>0.767</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$R =$ Resistance, $S =$ Susceptible, $Pop =$ Population, $\chi^2 =$ Chi-square, Seg = Segregating, Significance at $P = 0.05$ level (df = 1, CV = 3.841 and df = 2, CV = 5.991).
Genotypic analysis

Nine primers (Xgwm30, Xgwm44, Xgwm46, Xgwm56, Xgwm111, Xgwm297, Xgwm333, Xgwm437 and Xgwm644) were screened for polymorphism and only chromosome 7DS primer Xgwm111 produced a distinguishing polymorphism. Primer Xgwm111 produced a band that clearly and consistently differentiated the parents, resistant and susceptible bulks (Figure 1). A band was produced on control resistance source PI 137739 which was similar to the one on resistance source KRWA9. The band was approximately 210 bp and was subsequently tested on F2 population individuals. Other bands were produced on resistance sources PI 262660 (Dn2), PI 294994 (Dn5, Dn8 and Dn9) and AUS9 (Dn7). Figure 1 shows the banding patterns for KRWA9, NjoroBW2, homozygous resistant plants, heterozygous resistant plants, homozygous susceptible plants and control resistance sources “R299”, “R278”, “R26” and “AU9”. KRWA9 showed two distinctive bands; one was 210 bp while the other was 160 bp. The susceptible parent NjoroBW2 showed two distinctive bands; one was 280 bp while the other was 160 bp (Figure 1). It was observed that both parents had a common 160 bp band. The 210 bp band was present in the resistant parent but absent in the susceptible parent. This band was designated as the band of interest. The inclusion of different resistant sources helped to accurately identify the DNA markers for gene of interest. The primer Xgwm111 also produced a 210 bp band that clearly and consistently differentiated the parents, resistant, heterozygous and susceptible plants in the F2 population (Figure 2). Based on the banding patterns observed in the F2 population, 28 plants were homozygous resistant, 49 heterozygous and 23 homozygous susceptible (Table 3). This ratio did not differ from the expected 1:2:1 segregation ratio ($\chi^2 = 5.991, df = 2, P \leq 0.05$).

Linkage analysis

The F2 population of NjoroBW2 × KRWA9 cross showed a wide range of segregation for response to infestation by RWA. The frequency distribution of RWA feeding damage on the F2 population was somewhat bimodal, indicating the presence of one major resistance gene in KRWA9 (Figure 3). Simple regression analysis identified marker Xgwm111 to be highly significantly associated with resistance in KRWA9. The marker had an LOD score of 40.1 and high $R^2$ value of 85% indicating that it is a very significant marker for the resistance in KRWA9 (Table 2). Genetic data for Xgwm111 marker showed a
Table 2. Statistical indicators for SSR marker Xgwm111.

<table>
<thead>
<tr>
<th>Marker</th>
<th>LOD*</th>
<th>R**</th>
<th>P***</th>
<th>Source of resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xgwm111</td>
<td>40.1</td>
<td>85%</td>
<td>0.000</td>
<td>KRWA9</td>
</tr>
</tbody>
</table>

* = p ≤ 0.1, ** = p ≤ 0.05, *** = p ≤ 0.01

Table 3. Summary of primer Xgwm111 F₂ gel data.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Observed values</th>
<th>Expected values</th>
<th>Chi square (χ²)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>28</td>
<td>25</td>
<td>0.53</td>
<td>0.767</td>
</tr>
<tr>
<td>B</td>
<td>23</td>
<td>25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>49</td>
<td>50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>100</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

A = Homozygous resistant, B = homozygous susceptible, H = heterozygous (Significance at P = 0.05 level, df = 2, CV = 5.991).

Figure 3. RWA damage distribution in F₂ population.

DISCUSSION

KRWA9 was selected for this study because visual observations of RWA feeding damage on it suggested that this source of resistance has high level resistance (Pathak et al., 2007; Malinga et al., 2008). This resistance could be transferred to NjoroBW2 a popular commercial wheat variety which is susceptible to RWA. The F₁ seedlings of the cross between NjoroBW2 and KRWA9 were all resistant indicating the resistance in KRWA9 is dominant. The segregation observed in the F₂ population and the F₂:3 families further confirmed the dominance of resistance in KRWA9. Most RWA resistant genotypes have single dominant genes located on chromosome 1D and 7D (Du toit, 1987; Nkongolo et al.,...
Resistance sources reported to have single dominant genes include PI137739 (Dn1), PI262660 (Dn2), PI372129 (Dn4) and PI243781 (Dn6) (Du Toit, 1989; Nkongolo et al., 1991b; Saidi and Quick, 1994). The dominant nature of RWA resistance gene could be easily identified in the segregating populations. However, the major problem with single gene inheritance is that insect can develop biotypes very fast if the resistant cultivar is grown on a large scale. Colorado State University has developed several commercially available RWA resistant varieties of winter wheat such as Halt, Prairie Red, Prowers 99 and Yuma (Thomas et al., 2002). All these varieties have the Dn4 resistance gene derived from PI 372129 (Turchikum 57). It was later reported that RWA resistant cultivars with the Dn4 gene were susceptible to a new biotype designated as “Biotype 2” (Haley et al., 2004). This led to sourcing of more resistant materials. Gene Dn7 that was previously transferred from rye to wheat background via a 1 RS/1BL translocation had been reported to be resistant biotype 1 and 2 and depicts high levels of resistance as compared to other Dn genes (Collins et al., 2005; Turanli et al., 2012). However, part of the rye chromosome containing Dn7 has detrimental genes resulting to poor bread making quality (Graybosch et al., 1990). Breeding for resistance with Dn7 gene is no longer a desirable strategy and identification of diverse sources of resistance would be a highly desirable to keep ahead of biotype development in RWA. Pyramiding two or more resistance genes in a single cultivar will also increase the longevity of resistance.

The marker Xgwm111 has previously been found to be linked to genes Dn1, Dn2 and Dn5 in resistance sources PI 137739, PI 262660 and PI 294994, respectively (Liu et al., 2005). In their study, the marker Xgwm111 produced band sizes 210 bp in PI 137739 for gene Dn1, 200 bp in PI 262660 for gene Dn2 and 200 bp in PI294994 for gene Dn5 (Liu et al., 2005). The results are in agreement with Liu et al. (2001, 2002), who reported that Xgwm111 amplifies functional fragments from DNA of RWA-resistant wheat sources with expected sizes of 200 to 225 bp that are associated with RWA resistance.

In the F2 population, marker Xgwm111 followed the expected Mendelian segregation ratio of 3:1 or 1:2:1 (Table 3). These findings are consistent with Pathak et al. (2007) on a single dominant gene controlling resistance in KRWA9. The marker also completely co-segregated with the disease data and it is believed that the resistance gene in KRWA9 must be tightly linked to the marker. This offers a good opportunity for breeders to use this marker to select for resistance to RWA.

**Conclusion**

The usage of host plant resistance at the low cost is environmentally safe and is an ideal method to control the Russian wheat aphid. KRWA9 is a good source of resistance to RWA biotypes in Kenya and marker Xgwm111 could be used for marker assisted selection of resistance associated with this line. Similarity exists between KRWA9 and PI 137739, therefore there is a need to screen more markers in order to find more polymorphic markers in this region of chromosome 7DS. Most RWA resistance sources are monogenic and the challenge is that insects can develop biotypes very fast which could overcome the resistant cultivars. Identification of many sources of RWA resistance would be highly desirable to keep ahead of biotype development in the RWA by way of deploying multiple resistance genes to new breeding lines.

**Conflict of Interests**

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

**ACKNOWLEDGEMENTS**

We would like to thank the Kenya Agricultural Research Institute for facilitating the development, screening and molecular analysis breeding populations. We are grateful to Egerton University for technical support in carrying out this study. Funding of this research was provided by Enhanced Agricultural Productivity Programme (EAPP) through the Kenya Agricultural Research Institute and Murdoch University, Australia.

**REFERENCES**


Haley SD, Pears FB, Walker CB, Rudolph JB, Randolph TL (2004). Occurrence of a new Russian wheat aphid biotype in Colorado...