ABOUT AJB

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

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

Contact Us

Editorial Office: ajb@academicjournals.org
Help Desk: helpdesk@academicjournals.org
Website: http://www.academicjournals.org/journal/AJB
Submit manuscript online http://ms.academicjournals.me/
<table>
<thead>
<tr>
<th>Name</th>
<th>Address</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Editorial Board</strong></td>
<td></td>
</tr>
<tr>
<td>Prof. Sagadevan G. Mundree</td>
<td>Department of Molecular and Cell Biology, University of Cape Town, Private Bag Rondebosch 7701, South Africa</td>
</tr>
<tr>
<td>Dr. Martin Fregene</td>
<td>Centro Internacional de Agricultura Tropical (CIAT), Km 17 Cali-Palmira Recta, AA6713, Cali, Colombia</td>
</tr>
<tr>
<td>Prof. O. A. Ogunseitan</td>
<td>Laboratory for Molecular Ecology, Department of Environmental Analysis and Design, University of California, Irvine, CA 92697-7070, USA</td>
</tr>
<tr>
<td>Dr. Ibrahima Ndoye</td>
<td>UCAD, Faculte des Sciences et Techniques, Departement de Biologie Vegetale, BP 5005, Dakar, Senegal, Laboratoire Commun de Microbiologie, IRD/ISRA/UCAD, BP 1386, Dakar</td>
</tr>
<tr>
<td>Dr. Bamidele A. Iwalokun</td>
<td>Biochemistry Department, Lagos State University, P.M.B. 1087, Apapa – Lagos, Nigeria</td>
</tr>
<tr>
<td>Dr. Jacob Hodeba Mignouna</td>
<td>Associate Professor, Biotechnology, Virginia State University, Agricultural Research Station Box 9061, Petersburg, VA 23806, USA</td>
</tr>
<tr>
<td>Dr. Bright Ogheneovo Agindotan</td>
<td>Plant, Soil and Entomological Sciences Dept, University of Idaho, Moscow, ID 83843, USA</td>
</tr>
<tr>
<td>Dr. A.P. Njukeng</td>
<td>Département de Biologie Végétale, Faculté des Sciences, B.P. 67 Dschang, Université de Dschang, Rep. du CAMEROUN</td>
</tr>
<tr>
<td>Dr. E. Olatunde Farombi</td>
<td>Drug Metabolism and Toxicology Unit, Department of Biochemistry, University of Ibadan, Ibadan, Nigeria</td>
</tr>
<tr>
<td>Dr. Stephen Bakiamoh</td>
<td>Michigan Biotechnology Institute International, 3900 Collins Road, Lansing, MI 48909, USA</td>
</tr>
<tr>
<td>Dr. N. A. Amusa</td>
<td>Institute of Agricultural Research and Training, Obafemi Awolowo University, Moor Plantation, P.M.B 5029, Ibadan, Nigeria</td>
</tr>
<tr>
<td>Dr. Desouky Abd-El-Haleem</td>
<td>Environmental Biotechnology Department &amp; Bioprocess Development Department, Genetic Engineering and Biotechnology Research Institute (GEBRI), Mubarak City for Scientific Research and Technology Applications, New Burg-Elarab City, Alexandria, Egypt.</td>
</tr>
<tr>
<td>Dr. Simeon Oloni Kotchoni</td>
<td>Department of Plant Molecular Biology, Institute of Botany, Kirschallee 1, University of Bonn, D-53115 Germany.</td>
</tr>
<tr>
<td>Dr. Eriola Betiku</td>
<td>German Research Centre for Biotechnology, Biochemical Engineering Division, Mascheroder Weg 1, D-38124, Braunschweig, Germany</td>
</tr>
<tr>
<td>Dr. Daniel Masiga</td>
<td>International Centre of Insect Physiology and Ecology, Nairobi, Kenya</td>
</tr>
<tr>
<td>Dr. Essam A. Zaki</td>
<td>Genetic Engineering and Biotechnology Research Institute, GEBRI, Research Area, Borg El Arab, Post Code 21934, Alexandria, Egypt</td>
</tr>
</tbody>
</table>
Dr. Alfred Dixon  
*International Institute of Tropical Agriculture (IITA)*  
PMB 5320, Ibadan  
Oyo State, Nigeria

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

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

Dr. Solomon Olawale Odemuyiwa  
*Pulmonary Research Group*  
*Department of Medicine*  
550 Heritage Medical Research Centre  
University of Alberta  
Edmonton  
Canada T6G 2Z2

Prof. Anna-Maria Botha-Oberholster  
*Plant Molecular Genetics*  
*Department of Genetics*  
*Forestry and Agricultural Biotechnology Institute*  
*Faculty of Agricultural and Natural Sciences*  
University of Pretoria  
ZA-0002 Pretoria, South Africa

Dr. O. U. Ezeronye  
*Department of Biological Science*  
*Michael Okpara University of Agriculture*  
Umudike, Abia State, Nigeria.

Dr. Joseph Hounhouigan  
*Maître de Conférence*  
*Sciences et technologies des aliments*  
*Faculté des Sciences Agronomiques*  
*Université d’Abomey-Calavi*  
01 BP 526 Cotonou  
République du Bénin

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

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

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

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

Dr. Deborah Rayfield  
*Physiology and Anatomy*  
*Bowie State University*  
*Department of Natural Sciences*  
*Crawford Building, Room 003C*  
*Bowie MD 20715, USA*
<table>
<thead>
<tr>
<th>Name</th>
<th>Institution and Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dr. Marlene Shehata</td>
<td>University of Ottawa Heart Institute&lt;br&gt;Genetics of Cardiovascular Diseases&lt;br&gt;40 Ruskin Street&lt;br&gt;K1Y-4W7, Ottawa, ON, CANADA</td>
</tr>
<tr>
<td>Dr. Hany Sayed Hafez</td>
<td>The American University in Cairo, Egypt</td>
</tr>
<tr>
<td>Dr. Clement O. Adebooye</td>
<td>Department of Plant Science&lt;br&gt;Obafemi Awolowo University, Ile-Ife&lt;br&gt;Nigeria</td>
</tr>
<tr>
<td>Dr. Ali Demir Sezer</td>
<td>Marmara Üniversitesi Eczacilik Fakültesi, Tıbbiye cad. No: 49, 34668, Haydarpasa, İstanbul, Turkey</td>
</tr>
<tr>
<td>Dr. Ali Gazanchain</td>
<td>P.O. Box: 91735-1148, Mashhad, Iran.</td>
</tr>
<tr>
<td>Dr. Anant B. Patel</td>
<td>Centre for Cellular and Molecular Biology&lt;br&gt;Uppal Road, Hyderabad 500007&lt;br&gt;India</td>
</tr>
<tr>
<td>Prof. Arne Elofsson</td>
<td>Department of Biophysics and Biochemistry&lt;br&gt;Bioinformatics at Stockholm University, Sweden</td>
</tr>
<tr>
<td>Prof. Bahram Goliaei</td>
<td>Departments of Biophysics and Bioinformatics&lt;br&gt;Laboratory of Biophysics and Molecular Biology&lt;br&gt;University of Tehran, Institute of Biochemistry and Biophysics&lt;br&gt;Iran</td>
</tr>
<tr>
<td>Dr. Nora Babudri</td>
<td>Dipartimento di Biologia cellulare e ambientale&lt;br&gt;Università di Perugia&lt;br&gt;Via Pascoli&lt;br&gt;Italy</td>
</tr>
<tr>
<td>Dr. S. Adesola Ajayi</td>
<td>Seed Science Laboratory&lt;br&gt;Department of Plant Science&lt;br&gt;Faculty of Agriculture&lt;br&gt;Obafemi Awolowo University&lt;br&gt;Ile-Ife 220005, Nigeria</td>
</tr>
<tr>
<td>Dr. Yee-Joo TAN</td>
<td>Department of Microbiology&lt;br&gt;Yong Loo Lin School of Medicine, National University Health System (NUHS), National University of Singapore&lt;br&gt;MD4, 5 Science Drive 2, Singapore 117597&lt;br&gt;Singapore</td>
</tr>
<tr>
<td>Prof. Hidetaka Hori</td>
<td>Laboratories of Food and Life Science, Graduate School of Science and Technology, Niigata University, Niigata 950-2181, Japan</td>
</tr>
<tr>
<td>Prof. Thomas R. DeGregori</td>
<td>University of Houston, Texas 77204 5019, USA</td>
</tr>
<tr>
<td>Dr. Wolfgang Ernst Bernhard Jelkmann</td>
<td>Medical Faculty, University of Lübeck, Germany</td>
</tr>
<tr>
<td>Dr. Moktar Hamdi</td>
<td>Department of Biochemical Engineering, Laboratory of Ecology and Microbial Technology&lt;br&gt;National Institute of Applied Sciences and Technology. BP: 676. 1080, Tunisia</td>
</tr>
<tr>
<td>Dr. Salvador Ventura</td>
<td>Department de Bioquimica i Biologia Molecular&lt;br&gt;Institut de Biotecnologia i de Biomedicina&lt;br&gt;Universitat Autònoma de Barcelona&lt;br&gt;Bellaterra-08193&lt;br&gt;Spain</td>
</tr>
<tr>
<td>Dr. Claudio A. Hetz</td>
<td>Faculty of Medicine, University of Chile&lt;br&gt;Independencia 1027&lt;br&gt;Santiago, Chile</td>
</tr>
<tr>
<td>Prof. Felix Dapare Dakora</td>
<td>Research Development and Technology Promotion&lt;br&gt;Cape Peninsula University of Technology, Room 2.8 Admin. Bldg. Keizersgracht, P.O. 652, Cape Town 8000, South Africa</td>
</tr>
</tbody>
</table>
Dr. Geremew Bultosa  
*Department of Food Science and Post harvest Technology*  
Haramaya University  
*Personal Box 22, Haramaya University Campus*  
Dire Dawa,  
Ethiopia

Dr. José Eduardo Garcia  
*Londrina State University*  
Brazil

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

Prof. M. A. Awal  
*Department of Anatomy and Histplogy, Bangladesh Agricultural University,*  
Mymensingh-2202,  
Bangladesh

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

Prof. Danilo López-Hernández  
*Istituto de Zoología Tropical, Facultad de Ciencias, Universidad Central de Venezuela.*  
*Institute of Research for the Development (IRD), Montpellier,*  
France

Prof. Donald Arthur Cowan  
*Department of Biotechnology,*  
*University of the Western Cape Bellville 7535 Cape Town, South Africa*

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

Dr. Luísa Maria de Sousa Mesquita Pereira  
*IPATIMUP R. Dr. Roberto Frias, s/n 4200-465 Porto Portugal*

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

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

Dr. Adewunmi Babatunde Idowu  
*Department of Biological Sciences*  
*University of Agriculture Abia*  
Abia State,  
Nigeria

Dr. Yifan Dai  
*Associate Director of Research*  
*Revivicor Inc.*  
100 Technology Drive, Suite 414  
Pittsburgh, PA 15219  
USA

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

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

Dr. Moji Mohammadi  
*402-28 Upper Canada Drive*  
*Toronto, ON, M2P 1R9 (416) 512-7795*  
Canada
Prof. Jean-Marc Sabatier  
Directeur de Recherche Laboratoire ERT-62  
Ingénierie des Peptides à Visée Thérapeutique,  
Université de la Méditerranée-Ambrilia Biopharma inc.,  
Faculté de Médecine Nord, Bd Pierre Dramard, 13916,  
Marseille cédex 20.  
France

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

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

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

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

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

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

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

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

Dr. G. Reza Balali  
Molecular Mycology and Plant Pathology  
Department of Biology  
University of Isfahan  
Isfahan  
Iran

Dr. Beatrice Kilel  
P.O Box 1413  
Manassas, VA 20108  
USA

Prof. H. Sunny Sun  
Institute of Molecular Medicine  
National Cheng Kung University Medical College  
1 University road Tainan 70101,  
Taiwan

Prof. Ima Nirwana Soelaiman  
Department of Pharmacology  
Faculty of Medicine  
Universiti Kebangsaan Malaysia  
Jalan Raja Muda Abdul Aziz  
50300 Kuala Lumpur,  
Malaysia

Prof. Tunde Ogunsanwo  
Faculty of Science,  
Olabisi Onabanjo University,  
Ago-Iwoye.  
Nigeria

Dr. Evans C. Egwim  
Federal Polytechnic,  
Bida Science Laboratory Technology Department,  
PMB 55, Bida, Niger State,  
Nigeria
<table>
<thead>
<tr>
<th>Name</th>
<th>Institution</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Prof. George N. Goulielmos</strong></td>
<td>Medical School, University of Crete, Voutes, 715 00 Heraklion, Crete, Greece</td>
</tr>
<tr>
<td><strong>Dr. Uttam Krishna</strong></td>
<td>Cadila Pharmaceuticals limited, India 1389, Tarsad Road, Dholka, Dist: Ahmedabad, Gujarat, India</td>
</tr>
<tr>
<td><strong>Prof. Mohamed Attia El-Tayeb Ibrahim</strong></td>
<td>Botany Department, Faculty of Science at Qena, South Valley University, Qena 83523, Egypt</td>
</tr>
<tr>
<td><strong>Dr. Nelson K. Ojijo Olang’o</strong></td>
<td>Department of Food Science &amp; Technology, JKUAT P. O. Box 62000, 00200, Nairobi, Kenya</td>
</tr>
<tr>
<td><strong>Dr. Pablo Marco Veras Peixoto</strong></td>
<td>University of New York NYU College of Dentistry, 345 E. 24th Street, New York, NY 10010 USA</td>
</tr>
<tr>
<td><strong>Prof. T E Cloete</strong></td>
<td>University of Pretoria Department of Microbiology and Plant Pathology, University of Pretoria, Pretoria, South Africa</td>
</tr>
<tr>
<td><strong>Prof. Djamel Saidi</strong></td>
<td>Laboratoire de Physiologie de la Nutrition et de Sécurité, Alimentaire Département de Biologie, Faculté des Sciences, Université d’Oran, 31000 - Algérie, Algeria</td>
</tr>
<tr>
<td><strong>Dr. Tomohide Uno</strong></td>
<td>Department of Biofunctional chemistry, Faculty of Agriculture Nada-ku, Kobe., Hyogo, 657-8501, Japan</td>
</tr>
<tr>
<td><strong>Dr. Ulises Uruzúa</strong></td>
<td>Faculty of Medicine, University of Chile Independencia 1027, Santiago, Chile</td>
</tr>
<tr>
<td><strong>Dr. Aritua Valentine</strong></td>
<td>National Agricultural Biotechnology Center, Kawanda Agricultural Research Institute (KARI), P.O. Box, 7065, Kampala, Uganda</td>
</tr>
<tr>
<td><strong>Prof. Yee-Joo Tan</strong></td>
<td>Institute of Molecular and Cell Biology 61 Biopolis Drive, Proteos, Singapore 138673 Singapore</td>
</tr>
<tr>
<td><strong>Prof. Viroj Wiwanitkit</strong></td>
<td>Department of Laboratory Medicine, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand</td>
</tr>
<tr>
<td><strong>Dr. Thomas Silou</strong></td>
<td>Universit of Brazzaville BP 389 Congo</td>
</tr>
<tr>
<td><strong>Prof. Burtram Clinton Fielding</strong></td>
<td>University of the Western Cape, Western Cape, South Africa</td>
</tr>
<tr>
<td><strong>Dr. Brncić (Brncic) Mladen</strong></td>
<td>Faculty of Food Technology and Biotechnology, Pierottijeva 6, 10000 Zagreb, Croatia.</td>
</tr>
<tr>
<td><strong>Dr. Meltem Sesli</strong></td>
<td>College of Tobacco Expertise, Turkish Republic, Celal Bayar University 45210, Akhisar, Manisa, Turkey.</td>
</tr>
<tr>
<td><strong>Dr. Idress Hamad Attitalla</strong></td>
<td>Omar El-Mukhtar University, Faculty of Science, Botany Department, El-Beida, Libya.</td>
</tr>
<tr>
<td><strong>Dr. Linga R. Gutha</strong></td>
<td>Washington State University at Prosser, 24106 N Bunn Road, Prosser WA 99350-8694</td>
</tr>
<tr>
<td>Name</td>
<td>Institution and Address</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Dr Helal Ragab Moussa</td>
<td>Bahnay, Al-bagour, Menoufia, Egypt.</td>
</tr>
<tr>
<td>Dr VIPUL GOHEL</td>
<td>DuPont Industrial Biosciences, Danisco (India) Pvt Ltd, 5th Floor, Block 4B, DLF Corporate Park, DLF Phase III, Gurgaon 122 002, Haryana (India)</td>
</tr>
<tr>
<td>Dr. Sang-Han Lee</td>
<td>Department of Food Science &amp; Biotechnology, Kyungpook National University, Daegu 702-701, Korea.</td>
</tr>
<tr>
<td>Dr. Bhaskar Dutta</td>
<td>DoD Biotechnology High Performance Computing Software Applications Institute (BHSAI), U.S. Army Medical Research and Materiel Command, 2405 Whittier Drive, Frederick, MD 21702</td>
</tr>
<tr>
<td>Dr. Muhammad Akram</td>
<td>Faculty of Eastern Medicine and Surgery, Hamdard Al-Majeed College of Eastern Medicine, Hamdard University, Karachi.</td>
</tr>
<tr>
<td>Dr. M. Muruganandam</td>
<td>Department of Biotechnology, St. Michael College of Engineering &amp; Technology, Kalayarkoil, India.</td>
</tr>
<tr>
<td>Dr. Gökhan Aydin</td>
<td>Suleyman Demirel University, Atabey Vocational School, Isparta-Türkiye, Turkey.</td>
</tr>
<tr>
<td>Dr. Rajib Roychowdhury</td>
<td>Centre for Biotechnology (CBT), Visva Bharati, West-Bengal, India.</td>
</tr>
<tr>
<td>Dr Takuji Ohyama</td>
<td>Faculty of Agriculture, Niigata University</td>
</tr>
<tr>
<td>Dr Mehdi Vasfi Marandi</td>
<td>University of Tehran</td>
</tr>
<tr>
<td>Dr Fügen DURLU-ÖZKAYA</td>
<td>Gazi University, Tourism Faculty, Dept. of Gastronomy and Culinary Art</td>
</tr>
<tr>
<td>Dr. Reza Yari</td>
<td>Islamic Azad University, Boroujerd Branch</td>
</tr>
<tr>
<td>Dr Zahra Tahmasebi Fard</td>
<td>Roudehen branche, Islamic Azad University</td>
</tr>
<tr>
<td>Dr Albert Magí</td>
<td>Giro Technological Centre</td>
</tr>
<tr>
<td>Dr Ping ZHENG</td>
<td>Zhejiang University, Hangzhou, China</td>
</tr>
<tr>
<td>Dr. Kgomotso P. Sibeko</td>
<td>University of Pretoria</td>
</tr>
<tr>
<td>Dr Jian Wu</td>
<td>Harbin medical university, China</td>
</tr>
<tr>
<td>Dr Hsiu-Chi Cheng</td>
<td>National Cheng Kung University and Hospital</td>
</tr>
<tr>
<td>Prof. Pavel Kalac</td>
<td>University of South Bohemia, Czech Republic</td>
</tr>
<tr>
<td>Dr Kürsat Korkmaz</td>
<td>Ordu University, Faculty of Agriculture, Department of Soil Science and Plant Nutrition</td>
</tr>
<tr>
<td>Dr. Shuyang Yu</td>
<td>Department of Microbiology, University of Iowa, Address: 51 newton road, 3-730B BSB bldg. Iowa City, IA, 52246, USA</td>
</tr>
</tbody>
</table>
Dr. Mousavi Khaneghah
*College of Applied Science and Technology - Applied Food Science, Tehran, Iran.*

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

Dr Legeesse Adane Bahiru
*Department of Chemistry, Jimma University, Ethiopia.*

Dr James John
*School Of Life Sciences, Pondicherry University, Kalapet, Pondicherry*
### ARTICLES

<table>
<thead>
<tr>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Investigating the potential of <em>Aucoumea klaineana</em> Pierre sapwood and heartwood wastes to produce cellulosic ethanol</td>
<td>2587</td>
</tr>
<tr>
<td>Rodrigue Safou-Tchiama, Sébastien Ngwa Obame, Nicolas Brosse, Patrice Soulounganga and Timoléon Andzi Barhé</td>
<td></td>
</tr>
<tr>
<td>Comparison of seed priming techniques with regards to germination and growth of watermelon seedlings in laboratory condition</td>
<td>2596</td>
</tr>
<tr>
<td>Williams Ferreira Souza Barbosa, Fábio Steiner, Leandro Castro Matias de Oliveira and Paulo Henrique and Menezes das Chagas</td>
<td></td>
</tr>
<tr>
<td>Linkage disequilibrium and association mapping of drought tolerance in cotton (<em>Gossypium hirsutum</em> L.) germplasm population from diverse regions of Pakistan</td>
<td>2603</td>
</tr>
<tr>
<td>Abdelhafiz Adam Dahab, Muhammad Saeed, Nada Babiker Hamza, Bahaeldeen Babiker Mohamed and Tayyab Husnain</td>
<td></td>
</tr>
<tr>
<td>Inbreeding depression in crosses of <em>coerulea</em> clones of Walker’s Cattleya (<em>Cattleya walkeriana</em> Gardner)</td>
<td>2613</td>
</tr>
<tr>
<td>Miléia Ricci Picolo, Ceci Castilho Custódio, Nelson Barbosa Machado-Neto</td>
<td></td>
</tr>
<tr>
<td>The protective role of honey against cytotoxicity of cadmium chloride in mice</td>
<td>2620</td>
</tr>
<tr>
<td>Asmaa Shahrour, Mohamed Zowail and Khaled Sharafeldin</td>
<td></td>
</tr>
<tr>
<td>Shelf life improvement of sorghum beer (pito) through the addition of <em>Moringa oleifera</em> and pasteurization</td>
<td>2627</td>
</tr>
<tr>
<td>Florence Adwoa Ayirezang, Courage Kosi Setsoafia Saba, Francis Kweku Amagloh and Hellie Gonu</td>
<td></td>
</tr>
</tbody>
</table>
Investigating the potential of *Aucoumea klaineana* Pierre sapwood and heartwood wastes to produce cellulosic ethanol

Rodrigue Safou-Tchiama1,2*, Sébastien Ngwa Obame1, Nicolas Brosse3, Patrice Soulounganga4 and Timoléon Andzi Barhé2

2Laboratoire des Substances Naturelles et de Synthèse Organométalliques. Unité de Recherche en Chimie. Université des Sciences et Techniques de Masuku. BP. 941 Franceville, Gabon.
3Laboratoire d’Etudes et de Recherche sur le Matériau Bois, Faculté des Sciences et Techniques, Nancy-Université, Vandœuvre-lès-Nancy, France.

Received 7 June, 2016; Accepted 24 October, 2016

This work investigates for the first time the different reactivity exhibited by sapwood and heartwood wastes under two conditions: They were pretreated with catalyzed ethanol organosolv in the presence of sulfuric acid and their pulps underwent enzymatic hydrolysis by *Trichoderma reesei* which causes them to release neutral sugars for the production of cellulosic ethanol. *Aucoumea klaineana Pierre* (Okoumé) wood wastes were used for the experiment. Organosolv pretreatment was performed to investigate how to harness the benefits of fermentable sugars and lignin, which were reacted at varying defined severity levels as follows: 160°C≤T≤210°C and 0%≤[H₂SO₄]≤2% (w/w). The highest ethanol organosolv lignin content was obtained at T=160°C and [H₂SO₄]=1% for sapwood (18.10%) and heartwood (19.46%) (w/w). Enzymatic hydrolysis of the pretreated wood samples displayed that sapwood and heartwood pulps released their highest sugars content under free acid pretreatment conditions. The maximum neutral sugar released by heartwood pulps pretreated at T=160°C and [H₂SO₄]=0% was 0.126 g/L while that from sapwood pretreated at T=185°C and [H₂SO₄]=0% was 0.125 g/L. It is noteworthy that, the neutral sugars from sapwood and heartwood do not have the same sensitivity to temperature increase. When pretreated at T=160°C and [H₂SO₄]=0%, sapwood pulps yielded the following neutral sugars: Xyl< Ara≈Gal < Man < Glu, while heartwood was: Ara < Gal < Xyl < Man < Glu. However, with increased temperature ( T=185°C and [H₂SO₄]=0%), sapwood yielded the following neutral sugars: Ara < Gal < Xyl < Man < Glu, while heartwood yielded the following: Xyl < Ara≈Gal < Man < Glu. Similar trend was observed in both sapwood and heartwood pulps when sulfuric acid concentration was increased.

**Key words:** *Aucoumea klaineana Pierre*, wood wastes, ethanol organosolv lignin, enzymatic hydrolysis, bioethanol.
INTRODUCTION

Biofuels help to overcome the global warming concern because they use carbon from the atmosphere and give it back to it, while fossil energies increase the carbon content in the atmosphere. Biofuel was first mentioned in 1903 and 1926, which corresponds to the beginning of the automotive era (Payen, 1982). Nowadays, first generation bioethanol from sugarcane in Brazil or corn in USA (Wheals et al., 1999) is blended with gasoline and used in all petrol engines without modifications (Miller, 2003; Steven and Verhe, 2004).

Considering that the fossil fuels reservoirs will serve man’s needs for a very limited time period, scientific search for alternatives has already been initiated. Amongst the renewable resources for emerging biotechnological strategies used to produce high energy-less volume fuels, lignocellulose like cellulose which is the most abundantly synthesized and stable carbohydrate of the biosphere has been identified. However, the stable nature of the substrate and some fermentation difficulties of its monomeric products have been the major hurdles in using cellulose. But due to its ubiquitous nature and abundant availability as renewable resource, research on the utilization of cellulose for obtaining bioethanol continues in diverse fields.

There are still many issues to be addressed in considering the future of ethanol fuels. Many critics have charged that the energy balance of ethanol fuels is flawed, arguing that the energy inputs exceed the energy content of the final product. However, a study released by the United States Department of Agriculture in July of 2002 refutes these claims and finds that ethanol has an output: input energy ratio of 1.34:1 (Shapouri et al., 2002). Furthermore, another study found that the energy balance of gasoline is actually negative, giving ethanol a 1.42:1 output energy ratio compared to gasoline (Alternative Fuels Data Center, 2003). On the other hand, converting the entire feedstock plant to useful end products allows ethanol costs to decrease as manufacturing becomes more profitable. Besides being a renewable fuel made from plants, with high octane at low cost, ethanol is a much cleaner fuel than petrol. Ethanol blends dramatically reduce emissions of hydrocarbons, (major sources of ground level ozone formation), cancer-causing benzene and butadiene, sulphur dioxide and particulate matter.

However, the use of ethanol for fuel will almost certainly require cellulose technology. Likewise, Taherzadeh and Kairimi (2007) have recently indicated that lignocelluloses can be expected to be major feedstocks for ethanol production in the future. Evans (2005) has explained that, being 50% of the total dry matter of plant, cellulose is potentially a huge renewable energy store and vast amounts of this material are routinely thrown away. Thus, ethanol cellulose from the wood waste timber industries is an opportunity for equatorial oil-producing developing countries of Congo basin like Gabon to receive transfer of technologies. This will allow those countries to produce much ethanol at cheaper rates to obtain fuels at a reduced cost. A prospective study has stated that ethanol (from sugar cane, maize, sorghum and manioc fibers) or biodiesel (from palm oil and Jatropha) demand in Gabon will be 12 and 46 millions of liters respectively, in the year 2020 (Sielhorst et al., 2008). However, the first boards of trunk wastes from sawmill and the inner heartwoods from the rotary cutting logs of Okoumé’s plywood industry which remain undervalorized can be suitable source for ethanol cellulose production. Previous studies have shown that ethanol can be obtained from wood specie like Eucalyptus (Ferrari et al., 1992, Romani et al., 2012, Yanez-S et al., 2014).

Despite that hydrolysis of lignocelluloses by sulfuric acids is relatively old, it remains the most investigated process (Harris et al., 1945). Advantages and disadvantages of lignocellulosic pretreatment process for ethanol cellulose production with sulfuric acid have been extensively discussed by Taherzadeh and Karimi (2007). Nevertheless, Organosolv pretreatment released a cellulose residue which can be used for ethanol production, high neutral sugars yield in aqueous phase, better delignified fibers and relatively pure lignin for which interest grows in various industrials (Brosse et al., 2010; El Hage, 2010).

However, the differences in the reactivity of sapwood and heartwood pretreated with sulfuric acid and the behavior of their partially delignified fibers through enzymatic hydrolysis have not been investigated. The aim of this study is to investigate the potential of sapwood and inner heartwood of Okoumé as biomass candidates treated with ethanol organosolv lignin in an experimental design to find better conditions for lignin extraction. The neutral sugar contents obtained from enzymatic hydrolysis of the partially delignified pulps shall be used to discriminate between the two wood wastes. The relative sensitivity of the cellulose-hemicellulose matrix sugars from sapwood and heartwood to temperature and sulfuric acid concentration increase will be investigated.

MATERIALS AND METHODS

The raw material of Okoumé was collected in February 2016 at SNBG (Société Nationale des Bois du Gabon). The air-dry material was ground and milled to a particle size of 0.25 mm (for Klason lignin) and 0.6 mm (for Ethanol Organosolv Lignin) using a Willey
mill. It was stored at room temperature during the course of this study. All chemical reagents used in this study were purchased from Aldrich and VWR (France) and used as received. *Trichoderma reesel* (product number C2730) was supplied by Sigma Aldrich. Other chemicals used include absolute ethanol, sulfuric acid, sodium hydroxide, potassium sodium tartrate, acetic acid glacial.

**Extractives and ash content**

The extractive contents were obtained as follows: Triplicates of 1 g of unextracted heartwood or sapwood sawdust were subjected to Soxhlet extraction with acetone of 70% (v/v) for 24 h to remove the extracts. The solid to liquid ratio was 2% (w/v). Then, the wood was oven-dried at 105°C for 24 h and cooled to ambient temperature in a desiccator containing phosphorus pentoxide (P₂O₅).

The ash content of the wood samples was obtained at Tappi T 211 cm-86 (2003) adapted as follows: 1 g of unextracted and dried heartwood or sapwood sawdust was weighted with an analytical and accurate balance of 0.1 mg. The samples were deposited in a ceramic ashing crucible, and introduced in a muffle furnace equipped with a thermostat set at 575±25°C equipped with optional ramping program. The wood biomass was ignited in a first step at 250°C for 2 h, and then a second step was carried out at 500°C for 24 h. At the termination of the pyrolysis, the ceramic ashing crucible was removed to the furnace directly into a desiccator containing P₂O₅ for cooling at room temperature until constant weight was obtained. The ash content was calculated as follows:

\[
Ash(\%) = \frac{M_{ash}}{M_b} \times 100
\]

\(M_{ash}\) is the weight of ash obtained after pyrolysis and \(M_b\) is the unextracted moisten-free dry wood samples weight.

**Klason lignin measurement**

175 mg extractive-free and dried wood material were hydrolyzed with 72% (w/v) sulfuric acid and stirred for 1 h before autoclaving at Tappi T 222 om-11 (2006). The autoclaved samples were filtered with Buchner and the dried residue was weighed to give the Klason lignin (KL) content; while the liquid fraction was keep at -5°C for the neutral sugar content determination.

**Ethanol organosolv lignin extraction**

15 g of unextracted and dried material samples were treated with 65% (v/v) ethanol in the presence of sulfuric acid as catalyst and different levels of temperature (Table 1), using a published protocol (Pan et al., 2006, 2007; El Hage, 2010). The solid to liquid ratio was 1/7 (w/v). The process was optimized by an experiment design run with the statistical JMP® 11 Découver JMP. Cary, North Carolina: SAS Institute Inc.2013 software. The pre-treatments were carried out in a 1.0 L glasslined pressure Parr reactor equipped with a 4842 temperature controller (Parr Instrument Company, Mole, IL) for 1 h (manually controlled). The reactor was cooled until 30°C, and the pre-treated wood pulps were washed with warm (60°C) ethanol/water (2:1, 3x50.00mL). The washes were combined and 3 volumes of water were added to precipitate the Ethanol Organosolv Lignin (EOL), which was collected by centrifugation (3000 rpm, 15 min and 10°C) and dried. The EOL content is calculated as follows:

\[
EOL(\%) = \frac{M_{EOL}}{M_d} \times 100
\]

\(M_{EOL}\) is the weight of dried EOL and \(M_d\) is the moisten-free dry wood samples weight.

**Enzymatic hydrolysis of the pretreated wood samples**

Enzymatic hydrolysis of the pulps obtained after EOL extraction was carried out using a published procedure with slight modifications (El Hage, 2010; Brosse et al., 2010). The sapwood and heartwood pulps obtained after EOL treatment were grinded to pass 0.5 mm, oven-dried at 103°C for 24 h and cooled at room temperature in a desiccator which contained P₂O₅. 2.0 g of the pretreated and dried pulps was poured into an Erlenmeyer flask with 98 mL of sodium acetate buffer (0.05 M; pH=4.8). Then, 0.38 mL of the enzymatic solution of 15 FPU per gram of substrate (2%) (w/v) was added to the mixture and stirred. It was incubated at 50°C for 48 h in an air-bath shaking incubator at 150 rpm. About 0.2 mL samples were drawn periodically, diluted in 1 mL distilled water, and placed in 1.5 mL Eppendorf safe-lock tube. In order to arrest enzymatic activity, the samples in the Eppendorf tube were placed in boiling water at 97°C for 5 min, cooled and kept in a refrigerator at -5°C until further analysis.

**Monosaccharides and uronic acids reducing measurement**

An aliquot (2.5 mL) from the filtrates obtained after enzymatic hydrolysis was freeze-dried and diluted to 10%, then the monosaccharide and uronic acids content in the filtrate were quantified by the High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) procedure. A Dionex ICS-3000 HPLC system was used to separate and quantify neutral sugar and uronic acids. The system consisted of a SP gradient pump, an AS auto-sampler, an ED electrochemical detector with gold working electrode, an Ag/AgCl reference electrode,

<table>
<thead>
<tr>
<th>Number of reactions</th>
<th>Temperature (°C)</th>
<th>[H₂SO₄] (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>-1</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>-1</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>0</td>
<td>-1</td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>-1</td>
</tr>
<tr>
<td>11</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>12</td>
<td>-1</td>
<td>-1</td>
</tr>
</tbody>
</table>

Table 1. Experimental matrix of the face centered composite design conditions used for the pretreatments of Okoumé's heartwood and sapwood.

Experimental conditions: 160°C≤T≤210°C such as -1=160°C; 0=185°C and +1=210°C. 1%≤[H₂SO₄]≤5% (w/w); -1=0%; 0=1% and 1=2%. The reaction time is 1 hour. Ethanol (65%)/H₂O=1v/2v and the solid to liquid ratio =1/7. The central point was R1; R5; R7 and R R11.
and Chromeleon software version 6.8 (Dionex Corp, USA). The stationary phase used was a Carbopac PA20 (3 × 150 mm), Dionex column with a guard column (3 × 50 mm, Dionex), while mobile phase was water, 250 mM NaOAc, and 1 M NaOH/20 mM NaOH. Monomers and uronic acids were separated using isocratic conditions and linear gradient elution. All eluents were degassed before use by flushing with helium for 20 min with 250 mM NaOH solution and re-equilibrated for 10 min in the starting conditions. Samples were injected through 25L full loop at a discharge rate of 0.4 mL/min and separations effected at 35°C column temperature. The pulse sequence for pulsed amperometric detection consisted of potential +100 mV (0 to 200 ms), +100 mV integration (200 to 400 ms), -2000 mV (410 to 420 ms) +600 mV (430 ms), and -100 mV (440 to 500 ms).

**Statistical analysis**

The data were analyzed by a one-way analysis of variance (ANOVA) followed by the Fischer’s LSD (last significant difference) test at α=0.05 level of significance with Rr643.0.2 software.

**RESULTS AND DISCUSSION**

**Dry density, ash, extractives and Klason lignin content**

The analysis of the biomass (Table 2) pointed out a significant difference (P<0.05) between the sapwood and heartwood dry density. The former displayed the highest dry density. However, the heartwood was more abundant in the extracts than the sapwood. Similar trend was previously observed for lodgepole pine (Campbell et al., 1990) and loblolly pine (Thompson et al., 2006). Furthermore, the extractive content released by the heartwood is close to those previously reported by Safou-Tchiama (2005) and Medzegue (2007) who obtained 2.1±0.1 and 2.0±0.4% respectively. No significant difference was found (P>0.05) between the ash content of sapwood and heartwood (Table 2) which is lower than those reported by previous works (Table 3). In addition, no statistical difference (P>0.05) was found in the KL content of the selected samples. The lack of variability in the lignin content released by the sapwood and heartwood was observed for other wood species (Thompson et al., 2006). However, the KL content obtained was in the same range with those published by Brunck et al. (1990) and Minkué ’Eny (2000) who found 30 and 30.9% (g/dry weight), respectively.

The holocellulose deducted from the KL pointed out that the sapwood should be richer (70.04%) in cellulose-hemicelluloses complex than the heartwood (68.18%) as observed in lodgepole pine (Campbell et al., 1990). Nevertheless, the holocellulose content obtained from the inner heartwood is accordingly lower than that published previously by Lal et al. (1977), Minkué M’Eny (2000), Medzegue, (2007); Safou-Tchiama, 2005 and Safou-Tchiama et al. (2007) who investigated the whole heartwood including mature wood assumed to be richer in fibers than the inner heartwood which is mainly composed of juvenile wood. The latter is poor in pulps (Campbell et al., 1990). The holocellulose content

### Table 2. Dry density, ash, extractives and Klason lignin content of Okoumé’s sapwood and heartwood.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Dry density (g/cm³)</th>
<th>Ash (%)</th>
<th>Extractives (%)</th>
<th>Klason lignin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sapwood</td>
<td>0.484±0.034d</td>
<td>0.232d</td>
<td>0.530±0.100d</td>
<td>29.43±1.00d</td>
</tr>
<tr>
<td>Heartwood</td>
<td>0.429±0.046d</td>
<td>0.224d</td>
<td>1.912±0.090d</td>
<td>29.91±1.19d</td>
</tr>
</tbody>
</table>

Table 2. Dry density, ash, extractives and Klason lignin content of Okoumé’s sapwood and heartwood. (Means ith the same letters are not statistically different at the 0.05 level of significance. # Mean±S.D

### Table 3. Chemical composition of Okoumé as published by various authors.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Ash (%)</td>
<td>0.4</td>
<td>1.8</td>
<td>0.33</td>
<td>0.43</td>
<td>80.0±0.30</td>
</tr>
<tr>
<td>Holocellulose (%)</td>
<td></td>
<td>-</td>
<td>77.20</td>
<td>75.60</td>
<td>32.3±0.50*</td>
</tr>
<tr>
<td>Hemicelluloses (%)</td>
<td></td>
<td>-</td>
<td>-</td>
<td>47.3±1.00*</td>
<td>-</td>
</tr>
<tr>
<td>Glucose (%)</td>
<td></td>
<td>-</td>
<td>-</td>
<td>55.70±3.70</td>
<td>-</td>
</tr>
<tr>
<td>Xylose (%)</td>
<td></td>
<td>-</td>
<td>-</td>
<td>10.30±0.70</td>
<td>-</td>
</tr>
<tr>
<td>Arabinose (%)</td>
<td></td>
<td>-</td>
<td>-</td>
<td>0.30±0.05</td>
<td>-</td>
</tr>
<tr>
<td>Galactose (%)</td>
<td></td>
<td>-</td>
<td>-</td>
<td>0.30±0.04</td>
<td>-</td>
</tr>
<tr>
<td>Mannose (%)</td>
<td></td>
<td>-</td>
<td>-</td>
<td>4.10±0.30</td>
<td>-</td>
</tr>
</tbody>
</table>

The ash content was based on percentage of dried wood sawdust. The sugar content was based on percentage of dried holocellulose.

and Chromelion software version 6.8 (Dionex Corp, USA). The stationary phase used was a Carbopac PA20 (3 × 150 mm), Dionex column with a guard column (3 × 50 mm, Dionex), while mobile phase was water, 250 mM NaOAc, and 1 M NaOH/20 mM NaOH. Monomers and uronic acids were separated using isocratic conditions and linear gradient elution. All eluents were degassed before use by flushing with helium for 20 min with 250 mM NaOH solution and re-equilibrated for 10 min in the starting conditions. Samples were injected through 25L full loop at a discharge rate of 0.4 mL/min and separations effected at 35°C column temperature. The pulse sequence for pulsed amperometric detection consisted of potential +100 mV (0 to 200 ms), +100 mV integration (200 to 400 ms), -2000 mV (410 to 420 ms) +600 mV (430 ms), and -100 mV (440 to 500 ms).

**Statistical analysis**

The data were analyzed by a one-way analysis of variance (ANOVA) followed by the Fischer’s LSD (last significant difference) test at α=0.05 level of significance with Rr643.0.2 software.
displayed by the sapwood and the inner heartwood of Okoumé is very close to that found for promising lignocellulose biomass like Miscanthus x Giganteus which displayed sugar content of 70% (El Hage, 2010).

### Ethanol organosolv lignin

The EOL extracted from the wood samples is presented in Table 4. Reactions R9 and R10 suggest that EOL could be obtained from the sapwood and heartwood of Okoumé by heating free acid at T=185°C and T=210°C, respectively, and the highest acid free EOL content was yielded by sapwood heated at 210°C. Nevertheless, the KL released by R10 is very weak compared to the untreated wood samples (Table 2). This suggests that a mass was lost when the sapwood and heartwood were subjected to high temperatures. The polysaccharide matrix of the sapwood was more sensitive to temperature increase than the heartwood at [H₂SO₄]=0% (Table 4).

Increasing acid concentration shows that both sapwood and heartwood released their highest EOL in R2 pretreatment (Table 4). Their respective content is very close to that obtained for a cellulose ethanol candidate like Pinus taeda which yielded 19% of EOL (Sannigrahi et al., 2010). Nevertheless, the pretreatment conditions used for that wood specie were energy and acid consuming (T=170°C; [H₂SO₄]=1.1%). On the other hand, both the wood wastes from Okoumé exhibited higher EOL than that extracted from Miscanthus (16%) (El Hage, 2010).

When both temperature (T>160°C) and sulfuric acid concentration ([H₂SO₄]>1%) increased, the residual KL content of the sapwood and heartwood pulps increased as well (Table 4). Both the wood samples exhibited similar trend and their highest KL content was displayed in R4 conditions which corresponds to the maximum process severity. That result suggests a strong susceptibility of the sapwood and heartwood sugars to degradation, both at high temperature and acid concentration as observed for other lignocelluloses biomass (El Hage, 2010; Audu et al., 2012). This results in a pseudo-lignin formation which would increase strongly the KL content above the average value of untreated sapwood and heartwood as observed for other lignocellulosic materials (Hu et al., 2012; Li et al., 2007).

### Enzymatic hydrolysis and sugar content

The pretreated sapwood and heartwood pulps were subjected to enzymatic hydrolysis and the total sugar based on dried weight per L (g/L) is presented in Figure 1. Reactions R2 (T=160°C; [H₂SO₄]=1%), R6 (T=160°C; [H₂SO₄]=2%) and R9 (T=185°C; [H₂SO₄]=0%) were the most promising for the sapwood while the heartwood exhibited its highest neutral sugars content under R2, R10 (T=210°C; [H₂SO₄]=0%) and R12 (T=160°C; [H₂SO₄]=0%) pretreatment conditions. Those results have pointed out the variability between the sapwood and heartwood of Okoumé under different temperature and acid concentrations.

### Effect of acid concentration on neutral sugar content

The total sugar content released through enzymatic hydrolysis of the pulps is presented in Figure 1. The impact of acid concentration on enzymatic hydrolysis of the pulps was studied in comparison with the acid free reaction R12 (T=160°C; [H₂SO₄]=0%). It is obvious that all the neutral sugars content released by the sapwood are lower than those from heartwood. On the other hand, the sugar yield order from the sapwood pretreated in R12
is as follows: Xyl < Ara = Gal < Man < Glu while that from the heartwood pulps is: Ara < Gal < Xyl < Man < Glu (Table 5). That result has pointed out that there is strong variability between the sapwood and heartwood pulps in enzymatic hydrolysis. The enzymatic hydrolysis of the heartwood led to a release of a high mannose content, confirming the abundance of mannose within Okoumé cell wall fibers (Safou-Tchiama et al., 2007).

Within the sapwood, Figure 1 shows that increasing acid concentration of the pretreatment leads to significant increase of the neutral sugar released by enzymatic hydrolysis of the pulps in R2 (T=160°C; [H₂SO₄]=1%) and R6 (T=160°C; [H₂SO₄]=2%) compared to R12 (T=160°C; [H₂SO₄]=0%). The neutral sugars yielded from R2 were: Gal < Man < Ara < Xyl < Glu (Table 5). The relative neutral sugars increase (Figure 2) shows clearly that increasing acid concentration under the pretreatment conditions of R2 hydrolyzed strongly mannose of the sapwood pulps, while the enzymatic hydrolysis of all the other neutral sugars was facilitated. The highest increase

![Figure 1](image_url)

**Figure 1.** Total neutral total sugars yield obtained after enzymatic hydrolysis of pretreated heartwood (HW) and sapwood (SW) pulps at 160°C≤T≤210°C and 1%≤[H₂SO₄]≤2%. R0 (T=25°C; [H₂SO₄]=0%) is the reaction from which enzymatic hydrolysis was performed with unpretreated heartwood and sapwood.

**Table 5.** Monosaccharaides and uronic acids reducing content (mg/L) of the liquid fraction of Ethanol Organosolv Lignin pulps from Okoumé sapwood (SW) and heartwood (HW) enzymatically hydrolyzed according to the pretreatment conditions.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Rha</th>
<th>Ara</th>
<th>Gal</th>
<th>Glu</th>
<th>Xyl</th>
<th>Man</th>
<th>GalAc</th>
<th>GluAc</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SW</td>
<td>HW</td>
<td>SW</td>
<td>HW</td>
<td>SW</td>
<td>HW</td>
<td>SW</td>
<td>HW</td>
</tr>
<tr>
<td>R2</td>
<td>0.00</td>
<td>0.00</td>
<td>1.90</td>
<td>1.33</td>
<td>0.44</td>
<td>0.34</td>
<td>39.28</td>
<td>85.97</td>
</tr>
<tr>
<td>R3</td>
<td>0.00</td>
<td>0.00</td>
<td>3.80</td>
<td>2.49</td>
<td>0.29</td>
<td>0.33</td>
<td>13.14</td>
<td>18.34</td>
</tr>
<tr>
<td>R4</td>
<td>0.00</td>
<td>0.00</td>
<td>0.90</td>
<td>0.37</td>
<td>0.30</td>
<td>0.40</td>
<td>7.62</td>
<td>8.12</td>
</tr>
<tr>
<td>R5</td>
<td>0.00</td>
<td>0.00</td>
<td>1.54</td>
<td>0.78</td>
<td>0.35</td>
<td>0.24</td>
<td>9.94</td>
<td>9.53</td>
</tr>
<tr>
<td>R6</td>
<td>0.00</td>
<td>0.00</td>
<td>0.93</td>
<td>0.38</td>
<td>0.32</td>
<td>0.39</td>
<td>66.93</td>
<td>8.24</td>
</tr>
<tr>
<td>R7</td>
<td>0.00</td>
<td>0.00</td>
<td>1.25</td>
<td>0.38</td>
<td>0.36</td>
<td>0.40</td>
<td>11.71</td>
<td>8.32</td>
</tr>
<tr>
<td>R8</td>
<td>0.00</td>
<td>0.00</td>
<td>1.80</td>
<td>0.37</td>
<td>0.49</td>
<td>0.39</td>
<td>17.66</td>
<td>8.08</td>
</tr>
<tr>
<td>R9</td>
<td>0.17</td>
<td>0.00</td>
<td>0.42</td>
<td>0.37</td>
<td>1.17</td>
<td>0.38</td>
<td>94.97</td>
<td>8.13</td>
</tr>
<tr>
<td>R10</td>
<td>0.00</td>
<td>0.00</td>
<td>0.38</td>
<td>0.12</td>
<td>0.41</td>
<td>0.46</td>
<td>8.32</td>
<td>99.97</td>
</tr>
<tr>
<td>R11</td>
<td>0.00</td>
<td>0.00</td>
<td>0.38</td>
<td>1.00</td>
<td>0.41</td>
<td>0.37</td>
<td>8.30</td>
<td>18.92</td>
</tr>
<tr>
<td>R12</td>
<td>0.22</td>
<td>0.00</td>
<td>0.27</td>
<td>0.73</td>
<td>0.37</td>
<td>0.93</td>
<td>8.15</td>
<td>96.63</td>
</tr>
<tr>
<td>R0</td>
<td>0.00</td>
<td>0.06</td>
<td>0.37</td>
<td>0.38</td>
<td>0.40</td>
<td>0.56</td>
<td>8.18</td>
<td>80.38</td>
</tr>
</tbody>
</table>

R0 (T=25°C; [H₂SO₄]=0%). Sulfuric acid concentration is based on (w/w). Rha, rhamnose; Ara, arabinose; Gal, galactose; Glu, glucose; Xyl, xylose; Man: mannose; GalAc, galacto-uronic acid; GluAc, glucuronic acid.
was observed for arabinose, glucose and xylose. Furthermore, increasing acid concentration for +2% in R6 leads to an increase in the neutral sugars yields by the enzymatic hydrolysis of sapwood pulps (Figure 1). The corresponding sugars were released as follows: Gal < Xyl < Ara < Man < Glu (Table 5). Increasing acid concentration under R6 conditions was favourable to the enzymatic hydrolysis of glucose. Arabinose and xylose yields remained higher in R12 (Figure 2) whereas they were lower in R2 (Table 5). This suggests the inhibiting effect of sulfuric acid concentration on enzymatic hydrolysis of arabinose and xylose within the sapwood pulps pretreated in R6 (T=160°C; [H₂SO₄]=+2%). That inhibiting effect was more marked for galactose (Figure 2).

Raising acid concentration either in R2 or R6 reduced the total sugar yielded from the heartwood fibers in R12 (Figure 1). Nevertheless, in R2 reaction, the total sugar yielded from enzymatic hydrolysis of heartwood pulps is higher than that of sapwood (Figure 1); glucose and mannose remained the major neutral sugars within the heartwood pulps (Table 5). With the exception of arabinose for which raising acid concentration according to R2 increased its yield after enzymatic hydrolysis of the heartwood pulps, the other neutral sugars content decreased dramatically (Figure 2). That decrease was observed for all the neutral sugars released by the enzymatic hydrolysis of the heartwood pulps from R6 pretreatment; and the following sugar yields order was obtained: Xyl < Ara < Gal < Man < Glu, while that from R2 was: Gal < Xyl < Ara < Man < Glu (Table 5). It is noteworthy that xylose remained strongly sensitive to acid concentration at high temperature as previously found in other biomass (Xiang et al., 2004). In addition, glucose and mannose have pointed out a strong sensitivity towards increased acid concentration within the heartwood pretreated either in R2 (T=160°C; [H₂SO₄]=1%) or R6 (T=160°C; [H₂SO₄]=2%) as shown in Figure 2. Those results have pointed out that heartwood exhibited an opposite response towards acid sulfuric increase compared to sapwood. That should be explained by the difference in chemical composition between Aucoumea klaineana heartwood and sapwood. The latter was found to be rich in terpenes, furans or phenol compounds bearing esterified 4-hydroxybenzoic acid (unpublished data) described as inhibitors for enzymatic hydrolysis of wood pulps (Taherzadeh and Karimi, 2007).

**Effect of temperature on sugar content**

The total neutral sugars yield (Table 5) shows that sapwood and heartwood pulps do not have the same sensitivity towards temperature increase. Enzymatic hydrolysis of the sapwood pulps pretreated under R9 (T=185°C; [H₂SO₄]=0%) conditions released the highest total sugars content (Figure 1), and the following sugar yield order was obtained: Ara < Gal < Xyl < Man < Glu (Table 5). Raising temperature in R9 reaction increased strongly the galactose, glucose, xylose and mannose content released from the enzymatic hydrolysis of

![Figure 2. Relative neutral total sugar yield increase after enzymatic hydrolysis of sapwood (SW) and heartwood (HW) pulps from the promising pretreatments R2 (T=160°C; [H₂SO₄]=1%); R6 (T=160°C; [H₂SO₄]=2%); R9 (T=185°C; [H₂SO₄]=0%) and R10 (T=210°C; [H₂SO₄]=0%) in comparison with the free acid pretreated heartwood and sapwood pulps from reaction R12 (T=160°C; [H₂SO₄]=0%).](image)
apa wood pulps when compared to heartwood which released one of the lowest neutral sugars yielded as follows: Xyl < Ara = Gal < Man < Glu (Table 5). The results above have pointed out a strong sensitivity of all the heartwood neutral sugars to temperature increase. They decreased dramatically under the pretreatment conditions of R12 (T=160°C; [H2SO4]=0%) (Figure 2).

Nevertheless, enzymatic hydrolysis of the wood pulps pretreated under R10 (T=210°C; [H2SO4]=0%) conditions was favourable to heartwood. The latter exhibited one of the highest total sugar content (Figure 1) and the same sugars yield order like R12 pretreatment (Table 5). But raising temperature from 160 to 210°C hydrolyzed dramatically the arabinose, galactose, xylose and mannose of the heartwood pulps while enzymatic hydrolysis of glucose was favored (Figure 2); confirming that pretreating the heartwood at T=185 or 210°C with [H2SO4]=0% hydrolyzed the neutral sugars in the same extent. Major neutral sugars like glucose and xylose were more sensitive to temperature hydrolysis when the heartwood was pretreated at T=185°C.

Finally, comparing the sensitivity of the wood samples to temperature or sulfuric acid concentration increase has pointed out that sapwood and heartwood do not display the same behavior (Figure 2). The heartwood’s galactose, xylose and mannose yielded from the enzymatic hydrolysis decreased systematically when temperature and sulfuric acid concentration increased in R12. The same trend was observed not only for arabinose from R6, R9 and R10 pretreatment conditions; but also for the heartwood’s glucose from R2, R6 and R9 (Figure 2). However in Figure 2, it is noteworthy that increasing temperature and acid concentration point out an inverse trend for the sapwood. Arabinose, glucose and xylose yielded by enzymatic hydrolysis of the sapwood pulps from R2, R6, R9 and R10 increased indeed, and the galactose yielded from R6, R9 and R10 increased in R12 as well; whereas mannose content from the R6, R9 and R10 pretreatment conditions increased compared to the heartwood.

**Conclusion**

*Aucoumea klaineana Pierre* sapwood and heartwood have pointed out a strong potential for EOL extracted under middle conditions. Assuming that lignin is the second most abundant polymeric material on earth, and it is still underutilized, the high content of EOL displayed by *A. klaineana Pierre* can offer opportunities for producing sustainable alternatives to non-renewable products such as polyurethanes, thermoplastic polymers, epoxy and phenolic resins, as well as corrosion inhibitors. On the other hand, enzymatic hydrolysis by *Trichoderma reesei* has pointed out that the highest sugar contents are obtained from the acid free pretreated sapwood (T=185°C) and heartwood (T=160°C) pulps. However, increasing temperature and sulfuric acid concentration in the pretreatment process of the sapwood pulps leads to an increase in the total neutral sugars content released by the enzymatic hydrolysis. Nevertheless, the heartwood exhibited a strong sensitivity to temperature and sulfuric acid concentration increase; most of the neutral sugars tend to be hydrolyzed during the heartwood pretreatment process. But further investigations are necessary for a better understanding of the molecular composition and supramolecular organization of the two EOL. The lignin-hemicellulose linkages as well as cellulose-hemicellulose interactions which control the access of chemicals and enzymatic hydrolysis shall be performed. The yield of the fermentation process of the neutral sugars from the promising reactions will discriminate between the potential of sapwood and heartwood of Okoumé to be used as source for cellulosic ethanol production.

**Conflicts of Interests**

The authors have not declared any conflict of interests.

**ACKNOWLEDGEMENT**

This research was possible as a result of the RIFFEAC (Réseau des Institutions de Formation Forestière et Environnementale en Afrique Centrale) Master of Science degree in Wood Sciences scholarship program. The authors would like to thank ENEF Gabon (École Nationale des Eaux et Forêts) for his funding and all the supports he brought to this research. The study was also supported by a fellowship granted by the ANBG (Agence Nationale des Bourses du Gabon). They thank the LERMAB (Laboratoire d’Etude et de Recherche sur le Matériaux Bois, Université de Loraine) for the scientific and technical support.

**REFERENCES**


Ferrari MD, Neirotti E, Albornoz C, Saucedo E (1992). Ethanol production from eucalyptus wood hemicellulose hydrolysate by *Pichia*


Miller Jr GT (2003). Environmental science working with the earth. Brooks/Cole-Thomson Learning 511 Forest Lodge Road Pacific Grove, CA 93950, USA.


Full Length Research Paper

Comparison of seed priming techniques with regards to germination and growth of watermelon seedlings in laboratory condition

Willams Ferreira Souza Barbosa, Fábio Steiner*, Leandro Castro Matias de Oliveira and Paulo Henrique and Menezes das Chagas

Mato Grosso do Sul State University - UEMS, Department of Agronomy, Rodovia MS 306, km 6.4, Cassilândia, Mato Grosso do Sul, Brazil.

Received 15 February, 2016; Accepted 11 November, 2016

Seeds of watermelon [Citrullus lanatus (Thunb.) Matsum and Nakai, cv. Crimson Sweet] were used to investigate the effects of different priming techniques on seed germination and early seedling growth. The seeds were soaked in solutions of 0.2% gibberellin (50 mg L⁻¹ gibberellic acid), 0.2% cytokinin (90 mg L⁻¹ kinetin), 0.2% potassium nitrate (2 g L⁻¹ KNO₃), 0.2% calcium nitrate [2 g L⁻¹ Ca(NO₃)₂] or water (hydropriming) for 6 h at 25°C. After drying, five replicates of 25 seeds were distributed in plastic boxes with blotter paper and kept into a seed germinator at 26°C for 12 days. The different priming treatments significantly affect the measurements of the seed germination and growth of watermelon seedlings. The germination of watermelon seeds ranged from 0 to 100%, and was significantly greater when seeds were subjected to priming with GA₃, KNO₃, Ca(NO₃)₂ and water (control), and lower under cytokinin (CK) priming. The seed priming with 0.2% solution of CK resulted in 100% of abnormal seedlings, and therefore should not be used by watermelon growers. Seed priming with GA₃, KNO₃, Ca(NO₃)₂ and water (hydropriming) increased the shoot length, whereas GA₃, Ca(NO₃)₂ and water priming improved the radicle length, as well as shoot dry matter watermelon seedlings. The KNO₃ and water priming increased the root dry matter of watermelon seedlings. Seed priming with KNO₃ and Ca(NO₃)₂ improved the germination rate and seedling vigor index. The results of this study show that seed priming with GA₃, KNO₃, Ca(NO₃)₂ and water (hydropriming) may be useful tools due to their positive effects on germination percentage and growth characteristics of watermelon seedlings.

Key words: Citrullus lanatus, cytokinin, gibberellin, hydropriming.

INTRODUCTION

Watermelon [Citrullus lanatus (Thunb.) Matsum and Nakai] is an annual vine of the cucurbit family (Cucurbitaceae). It is a native species from warm and dry regions of Africa, and now widely cultivated throughout...
the world for its edible fruits (Filgueira, 2008). Among the cucurbits, watermelon is the most popular in Brazil and in the world. Optimum seed germination and seedling emergence in watermelon occur at relatively high temperatures (25-28°C). Poor seed germination is a common phenomenon at sub-optimal temperatures (Demir and Mavi, 2004), which causes a great concern for growers that growing watermelon seedlings in late winter and early spring in the southern and south eastern regions of the Brazil. These two regions were responsible for nearly 50% of Brazilian production of watermelon in the 2014/2015 season (AGRIANUAL, 2015). Delayed and reduced seedling emergence cause non-uniform stand establishment, which result in yield reductions (Singh et al., 2001) and impairs the early watermelon markets in the cool regions of Brazil.

Many treatment techniques have been developed to improve the germination of watermelon seeds, especially under improper conditions. There is no universal technique for improving seed germination. Among the methods used, pretreatment of seeds with plant growth regulators and salts are considered the most appropriate and promising because of ease of application, scale of economies, and labor-saving attributes compared with methods in which the environment must be controlled for prolonged periods of time (Demir and Mavi, 2004; Nascimento, 2005; Ghassemi-Golezani and Esmaeipour, 2008). Indeed, seed priming treatments using salts such as potassium nitrate (KNO₃) have been effective in improving watermelon germination under improper conditions (Demir and Mavi, 2004; Nascimento, 2005). Hydropriming treatment has also been successfully applied to improve germination performance of watermelon (Huang et al., 2002) and cucumber seeds (Gurgel et al., 2009). However, few reports were documented on priming treatments using plant growth regulators (PGRs) such as gibberellin and cytokinin in watermelon seeds.

Cytokinin (CK) and gibberellin (GA₃) are key hormones controlling plant development. These plant hormones have an important role on several physiological and developmental processes, control of the cell cycle, apical dominance, including morphogenesis of shoots and roots, lateral root initiation, stem elongation, leaf and cotyledon expansion, and regulation of senescence (Al-Al-Khassawneh et al., 2006; Taiz and Zeiger, 2010; Kerbauy, 2012). Seed priming with optimal concentrations of CK and GA₃ has been shown to have beneficial effects on germination, growth and yield of a wide range of plant species (Jamal and Rha, 2007; Alonso-Ramirez et al., 2009; Nasri et al., 2012; Kandil et al., 2014).

Gibberellin at 200 mg L⁻¹ enhanced the seed germination and seedling growth of papaya (Lopes and Souza, 2008). Nasri et al. (2012) reported GA₃ increased germination percentage of lettuce under salt stress conditions. Albuquerque et al. (2009) reported GA₃ increased the growth characteristic in sweet pepper. Batista et al. (2015) studied the effect of different priming techniques on germination and growth of pepper and reported that GA₃ at 200 mg L⁻¹ enhanced the germination and seedling growth when compared to unprimed seeds. Iqbal et al. (2006) showed that application of CK at 100 or 200 mg L⁻¹ increased the germination rate and early seedling growth of wheat when compared with hydropriming treatment. Cytokinins at 10 or 100 mg L⁻¹ significantly increased the germination rate of pigeon pea seeds compared to unprimed seeds (Sneideris et al., 2015). However, seed priming with CK at 50 or 100 mg L⁻¹ inhibited the primary root development of maize seedlings compared to control. These and other contradictory results seem to indicate an inherent differential response among different species or genotypes; therefore, justifying the need of conducting more research in order to investigate the effects of seed priming with CK on germination and early growth of watermelon.

This research was carried out to investigate the effects of different priming techniques on seed germination and initial growth of watermelon seedlings (C. lanatus (Thunb.) Matsum and Nakai).

MATERIALS AND METHODS

Plant material and priming treatments

The experiment was conducted in the Plant Propagation Laboratory of the Mato Grosso do Sul State University (UEMS), in Cassilândia, MS, Brazil (Latitude: 19°05’20” S, Longitude: 51°48’24” W), during the month November 2015.

Seeds of watermelon [Citrullus lanatus (Thunb.) Matsum and Nakai; cv. Crimson Sweet] were surface sterilized with 1% (v/v) of sodium hypochlorite solution for 5 min and washed immediately with distilled water many times. The sterilized seeds were then subjected to priming by direct immersion in solutions of 0.2% gibberellin (50 mg L⁻¹ gibberellic acid - GA₃), 0.2% cytokinin (90 mg L⁻¹ kinetin - CK), 0.2% potassium nitrate (2 g L⁻¹ KNO₃), and 0.2% calcium nitrate [2 g L⁻¹ Ca(NO₃)₂] for 6 h at 25°C. A set of seeds subjected to direct immersion in distilled water was taken as control. After priming period, seeds were put to dry in plastic boxes (11.0 x 11.0 x 3.5 cm, type Gerbox) with germitest paper at room temperature (24-28°C) for 48 h (Eira and Marcos-Filho, 1990).

Germination and growth conditions

Five replicates of 25 seeds were evenly distributed in plastic boxes with blotter paper, properly moistened with distilled water, in a volume equivalent to 2.5 times the mass of dry paper. The boxes were then closed with lids to prevent evaporation and maintain the relative humidity close to 100%. Germination was carried out in a germination chamber under 12/12 h photoperiod (light/darkness), light fluence of 40 μmol m⁻² s⁻¹ photosynthetic photon flux density (PPFD), relative humidity of 90% (± 5%) and temperature of 28°C (± 2°C) for 12 days. Germinated seeds were recorded every 24 h for 12 days.

Measurements of germination and seedling growth

The germination (GC), germination rate index (GRI), early growth
and vigor index of watermelon seedlings were measured. At 12 days were also measured the percentage of abnormal seedlings and dead seeds. The Equations 1 and 2 and the parameters therein were employed to express the process of seed germination:

\[ G(\%) = \frac{S_{N0}}{S_{N0} \times 100} \]  

Where G is germination percentage, \( S_{N0} \) is the number of germinated seeds, and \( S_{N0} \) is the number of experimental seeds with viability (25 seeds):

\[ \text{GRI} = \Sigma \left( \frac{n_i}{t} \right) \]  

Where, GRI is the germination rate index (seed day\(^{-1}\)), \( n_i \) is the number of germinated seeds on a given day, and \( t \) is the time in days from the starting/sowing day (0) (Maguire, 1962).

The shoot and radicle length was measured in 15 normal seedlings randomly obtained after count of the total germination (12th day) using meter scale. The results were expressed in centimeter (cm). For the determination of dry matter of shoot and roots, all normal seedlings obtained at the end of the germination period were separated into shoot and roots, dried in a forced air circulation oven for three days at 60°C, and then weighed. The results were expressed in mg seedling\(^{-1}\).

Vigor index of seedlings was calculated according to Zhang et al. (2007) as shown in Equation 3:

\[ \text{SVI} = S_l \times \Sigma \left( \frac{n_i}{t} \right) \]  

Where SVI is seedling vigor index, \( S_l \) is the shoot length in the twelfth day (cm), \( n_i \) is the number of germinated seeds on a given day, and \( t \) is the time in days from the starting/sowing day (0).

### Statistical analyses

The normality of data was previously tested by the Kolmogorov-Smirnov test and then data were submitted to analysis of variance (ANOVA), and means of five priming treatments were compared by the Tukey test at the 0.05 level of confidence. For statistical analysis, the data expressed in percentage were previously transformed into arc sin (\( x/100 \)). The analyses were performed using Sisvar version 5.3 software for Windows (Statistical Analysis Software, UFLA, Lavras, MG, BRA).

### RESULTS AND DISCUSSION

**Effects of priming techniques on seed germination**

The different priming treatment significantly affects the measurements of the germination process and rate of watermelon seeds (Figure 1). The germination percentage values in the control treatment (Figure 1A) were higher than the standard values (that is, 80%) for commercial watermelon seeds in Brazil (BRASIL, 2012), indicating that the seeds used in this study had high physiological quality.

The germination percentage of watermelon seeds ranged from 0 to 100%, and was significantly greater when seeds were subjected to priming with \( \text{GA}_3 \), \( \text{KNO}_3 \), \( \text{Ca(NO}_3)_2 \) and water (control), and lower under CK priming (Figure 1A). The high efficiency of seed priming with PGRs and salts in improving the germination and growth of the seedlings have been reported by other authors. Alonso-Ramirez et al. (2009) showed that \( \text{GA}_3 \) have strong stimulatory effect on seed germination, of which their exogenous application was repeatedly found to promote germination of \( \text{Arabidopsis} \) seeds even under unfavorable stress conditions. Seed priming with \( \text{GA}_3 \) caused an increase in seed germination and seedling growth of sweet pepper (Albuquerque et al., 2009), lettuce (Nasri et al., 2012) and sugar beet (Jamil and Rha, 2007; Kandil et al., 2014). Batista et al. (2015) showed that all the priming methods tested (that is, \( \text{GA}_3 \), \( \text{KNO}_3 \), \( \text{Ca(NO}_3)_2 \) and hydropriming) resulted in the improvement of germination rate of pepper seeds when compared to unprimed seeds (control). Huang et al. (2002) and Gurgel et al. (2009) reported that hydropriming treatment can be successfully applied on watermelon and cucumber seeds to improve germination performance, respectively.

The germination rate index ranged from 0 to 6.4 seed day\(^{-1}\), and was significantly greater under \( \text{KNO}_3 \) and \( \text{Ca(NO}_3)_2 \) priming, followed by \( \text{GA}_3 \) priming and hydropriming (control), and lower under CK priming (Figure 1B). High values obtained for germination rate index indicate mean higher seed vigor of one treatment in relation to another. Marcos-Filho (2015) reported that the uniformity and speed of seedling emergence are important components of seed performance, thus directly affecting stand establishment.

The seed priming with 0.2% solution of CK resulted in 100% of abnormal seedlings (Figure 2), and was significantly greater than the other priming techniques used (Figure 1C). Although cytokinins are required for many growth and developmental processes in plants such as cell division, morphogenesis of shoots and roots, apical dominance, chloroplast maturation, leaf and cotyledon expansion, and seed germination (Hirose et al., 2008; Taiz and Zeiger, 2010; Kerbauy, 2012), the exogenous application of supra-optimal cytokinin concentrations has remarkable effect on the inhibition of cell elongation process in both shoots and roots (Taiz and Zeiger, 2010). The inhibition of internode and root elongation induced by excess cytokinin is due to the production of ethylene triggered by the enzyme 1-aminocyclopropane-1-carboxylic acid synthase (ACS) (Kerbauy, 2012). These results indicate that chances in endogenous CK concentration may negatively regulate elongation of shoots and roots, as seen in Figure 2. Aragao et al. (2001) studied the effect of seed priming with CK on germination and growth of maize and reported that benzyl aminopurine (BAP) at 50 or 100 mg L\(^{-1}\) inhibited the development of the primary root compared to control. However, seed priming with optimal concentrations of CK has been shown to have beneficial effects on germination and early growth of wheat (Iqbal et al., 2006) and pigeon pea (Sneiders et al., 2015).

Another factor that may be related to inhibiting the growth of watermelon seedlings is that the action of CK is
light-dependent. Changes in fluence rate of white light were shown to have effect on the action of CK and therefore one elongation of the stem and root. Under conditions of low light fluence, as in this study (40 μmol m⁻² s⁻¹ PPFD), the cytokinin inhibits elongation of shoots and roots (Kerbauy, 2012).

The percentage dead seed varied from 0 to 5%, and was significantly greater under GA₃ priming, and lower under CK and KNO₃ priming (Figure 1D). The low percentage of dead seeds is indicative of the high initial viability of watermelon seeds used.

**Effects of priming techniques on initial seedling growth**

The growth of watermelon seedlings was significantly affected by different priming treatments (Figure 3). Seed priming with GA₃, KNO₃, Ca(NO₃)₂ and water (hydropriming) resulted in higher shoot length of watermelon seedlings (Figure 3A). These results indicate that the seed priming with gibberellic acid, salts or water were adequate to promote the shoot growth of watermelon. Batista et al. (2015) also reported the efficiency of GA₃, KNO₃, Ca(NO₃)₂ and water priming to enhance the shoot growth of per seedlings compared to unprimed seeds.

Radicle length of the watermelon seedlings was favored under hydropriming (3.95 cm), followed by Ca(NO₃)₂ and GA₃ priming, whereas the KNO₃ priming had the lowest effect (2.62 cm) (Figure 3B). Shoot dry matter of watermelon seedlings was significantly higher under GA₃, Ca(NO₃)₂ and water priming (Figure 3C), whereas the higher dry matter of the roots was obtained with KNO₃ and water priming (Figure 3D). Demir and Mavi (2004) and Nascimento (2005) reported that seed priming with KNO₃ enhanced the seed germination and growth of watermelon under improper conditions. The high efficiency of hydropriming treatments in improving the early seedling growth was also reported previously in

**Figure 1.** Effect of different priming treatments on the germination percentage; 12 days (A), germination rate index (B), abnormal seedlings (C), and dead seeds (D) of watermelon seeds [*Citrullus lanatus* (Thunb.) Matsum and Nakai, cv. Crimson Sweet]. Bars followed by the same lower case letters are not significantly different by Tukey test at the 0.05 level of confidence. Data refer to mean values (n = 4) ± standard error.
Figure 2. Abnormal seedlings proceeding from watermelon seeds of the cultivar Crimson Sweet subjected to priming by direct immersion in 0.2% cytokinin solution (90 mg L⁻¹ of kinetin) at 12 days after sowing. The illustration shows seedlings with malformed roots, thickening of the hypocotyl, and without the formation of shoot.

watermelon (Huang et al., 2002), lentil (Ghassemi-Golezani et al., 2008) and cucumber (Gurgel et al., 2009). Seed priming with GA₃ has been shown to have beneficial effects on germination and growth of a wide range of plant species (Jamil and Rha, 2007; Lopes and Souza, 2008; Albuquerque et al., 2009; Alonso-Ramirez et al., 2009; Nasri et al., 2012; Kandil et al., 2014). The seedling vigor index ranged from 0 to 72.2, and was significantly greater under KNO₃ and Ca(NO₃)₂ priming, followed by GA₃ priming and hydropriming (control), and lower under CK priming (Figure 4). The vigor tests allow identifying the seeds with higher or lower probability to show better performance in field conditions. Vigorous seeds more efficiently mobilize reserves from storage tissues to the embryo axis and this capacity is reflected in higher seedling growth (Marcos-Filho, 2015). Therefore, vigor tests are important tools as an aid to germination test in research on physiological conditioning of seeds.

In general, the results presented here indicate that seed priming with GA₃, KNO₃, Ca(NO₃)₂ and water (hydropriming) may be successfully applied to improve the germination and initial growth of watermelon seedlings. Germination and seedling emergence stages are critical for crop production; rapid and uniform field emergence is essential to achieve high yield and uniform plant stands, resulted in early maturity and reduced disease attack (Singh et al., 2001; Subedi and Ma, 2005).

Conclusions

Seed priming with GA₃, KNO₃, Ca(NO₃)₂ and water (hydropriming) may be successfully applied on watermelon seeds to improve germination performance and growth characteristics of seedlings. Seed priming with KNO₃ and Ca(NO₃)₂ improved the germination rate and seedling vigor index. The seed priming with 0.2% solution of CK (90 mg L⁻¹ kinetin) inhibited the germination and cell elongation process of the seedlings.
Figure 3. Effect of different priming treatments on shoot length (A), radicle length (B), shoot dry matter (C) and root dry matter (D) of watermelon seedlings [Citrullus lanatus (Thunb.) Matsum and Nakai, cv. Crimson Sweet]. Bars followed by the same lower case letters are not significantly different by Tukey test at the 0.05 level of confidence. Data refer to mean values (n = 4) ± standard error.

Conflict of interests

The authors have not declared any conflict of interests.

REFERENCES

Instrução Normativa que estabelece os padrões de identidade e qualidade para a produção e a comercialização de sementes de espécies olerícolas, condimentares, medicinais e aromáticas. Portaria SDA n° 155 de 27 de novembro de 2012.


Linkage disequilibrium and association mapping of drought tolerance in cotton (*Gossypium hirsutum* L.) germplasm population from diverse regions of Pakistan

Abdelhafiz Adam Dahab1*, Muhammad Saeed2, Nada Babiker Hamza3, Bahaeldeen Babiker Mohamed1 and Tayyab Husnain4

1Environmental and Natural Resources Research Institute (ENRRI), National Centre for Research (NCR), Khartoum, Sudan.
2Department of Botany, Government College University, Faisalabad, Pakistan.
3Commission for Biotechnology and Genetic Engineering, National Centre for Research, Khartoum, Sudan.
4Centre of Excellence in Molecular Biology (CEMB), University of the Punjab, Lahore, Pakistan.

Received 22 November, 2015; Accepted 1 February, 2016

Drought stress is a major abiotic stress that limits crop production. Molecular association mapping techniques through linkage disequilibrium (LD) can be effectively used to tag genomic regions involved in drought stress tolerance. With the association mapping approach, 90 genotypes of cotton *Gossypium hirsutum*, from diverse regions of Pakistan were used. The morpho-physiological traits of all genotypes were evaluated in greenhouse under well-watered and drought stress conditions. Mean squares from analysis of variance for all morpho-physiological traits revealed highly significant variations (P≤0.05) between water levels and genotypes. Cotton varieties were screened for polymorphism with 180 simple sequence repeat (SSR) markers. Out of these 180 SSR markers, 95 were polymorphic. Genotyping of the selected 95 SSR primer pairs generated 57.5% polymorphism, and the number of polymorphic alleles per primer was 2.10. Population structure, linkage disequilibrium, and association mapping between pairs of SSR marker loci were studied. The significance of pairwise LD (P≤0.005) among all possible SSR loci was evaluated at significant threshold values (R²≥0.05); 7.1% of the SSR marker pairs showed significant pairwise LD in 90 accessions of *G. hirsutum*. Also we observed a significant (R²) LD between 13 pairs of SSR loci; each pair within the same chromosome in a range of 180 cm between NAU1230 and NAU3095 loci in chromosome (D5) and 1.612 cm between NAU462 and NAU3414 in chromosome A9. This indicates tight linkage between two alleles on the same chromosome. Markers, NAU3414, NAU2691, NAU1141 and NAU1190 were associated with more than single traits under drought treatments. Highest phenotypic variance explaining (R²) was ascribed to NAU3011 chromosome D13 significantly (p≤0.001) associated with root length under drought treatment.

**Key words:** Cotton *Gossypium hirsutum*, drought stress, association mapping, linkage disequilibrium (LD), simple sequence repeats (SSRs).

**INTRODUCTION**

Global climate change poses serious problems for sustained crop production. Due to the continuous water deficit for agricultural production, development of drought tolerant crop to meet the food and fibre demand has
become a necessity (Saeed et al., 2012). Increasing aridity of semi-arid regions and limited water resources have led to a crucial necessity for improving crop drought resistance (Passiourea, 2007). There is extensive research on genetic and breeding programs of cotton and it has a long history of improvement through conventional breeding and selection with frequent long-term yield achievements. The identification of drought-related QTLs plays an essential role in crop improvement through marker assisted selection. DNA marker studies have laid basis for revealing the molecular basis for the traits related to drought tolerance (YongSheng et al., 2009). Among the variety of genetic markers, SSR markers have shown high potential to detect polymorphism (Dongre et al., 2011; Dahab et al., 2013) and have been used extensively for cotton genome mapping and marker assisted selection (He et al., 2007). Researchers have mapped QTLs for morphological traits (Liang et al., 2014), physiological traits (Saeed et al., 2011), earliness (Li et al., 2013), yield (LiFang et al., 2010) and fibre traits (Islam et al., 2014). Abdelraheem et al. (2015) assessed a backcross inbred line (BILs) population derived from a cross between Pima cotton, ‘Pima S-7’ (Gossypium barbadense) and Upland cotton ‘Sure- Grow 747’ (Gossypium hirsutum) for their drought tolerance morphological traits using PEG induced osmotic stress. A total of six QTLs were detected for plant height (PH), fresh shoot and root weight (RW), explaining 10.9 to 19.2% of the phenotypic variation (PV). Rodriguez-Uribe et al. (2014), studying drought tolerance of cotton, stated that, of a total of 110 drought-responsive genes identified through a microarray analysis, there was 79% expression. Saeed et al. (2011) evaluated F2, F2:3 and F3:4 populations derived from an intraspecific cross between two G. hirsutum lines for morphological, physiological and yield traits. Seven QTLs were detected for osmotic potential, osmotic adjustment, plant height, and seed cotton yield. Association mapping, based on linkage disequilibrium (LD), is a new methodology which examines thousands of polymorphisms for assessing QTL effect. It is more effective compared to linkage analysis since it does not require generation of segregating populations/large numbers of progeny. Association mapping has three advantages: increased mapping resolution, reduced research time, and greater allele numbers. It is a powerful technique used to identify genomic regions linked to specific variants of a phenotypic trait (Saeed et al., 2014). Genome-wide association in plants has wide range of use and there are many reports of association studies on many crops such as barley (Gutiérrez et al., 2011; Cockram et al., 2010), rice (Shao et al., 2011), bread wheat (Yu et al., 2012), maize (Poland et al., 2011), triticale (Niedziela et al., 2012), bean (Shi et al., 2011), sugar beet (Würschum et al., 2011), cotton fiber quality traits (Abdurakhmonov et al., 2009) and cotton salinity stress tolerance (Saeed et al., 2014). Cotton Gossypium spp. is widely used for natural fiber in the textile industry. The worldwide commercial effect of cotton production is ~$500 billion per annum with consumption of ~115-million bales or ~27-million metric tons (MT) of fibre (Chen et al., 2007). Cotton is one of the main warm-season cultivars, grown during summer in arid and semiarid regions where water is limited (Singh et al., 2007). The adaptive traits of plants to unfavourable environmental conditions include numbers of physiological, morphological and biochemical features of whole plant (Saeed et al., 2012). Genes involved in molecular mechanisms can be tagged with the help of molecular mapping approaches. In our present study, we assessed extent of LD in the G. hirsutum germplasm from diverse regions of Pakistan. The aims of this study were to assess the population structure, linkage disequilibrium (LD), and association of molecular markers with drought stress tolerance of cotton (G. hirsutum L.) in a collection of 90 elite cotton germplasm accessions.

MATERIALS AND METHODS

Plant

The plant material consisted of 90 genotypes of cotton G. hirsutum (Table 1) collected from diverse regions of Pakistan. The cotton varieties were grown in green house during March-April 2010. Four seeds of each genotype were sown after soaking overnight at field capacity of 2-3 cm$^3$ depth in 8 polythene bags of 25×5 cm$^3$, filled with ~ 1.15 kg of compost soil (peat, sand, soil, 1:1:1). They were arranged in a randomized complete block design with three replications and two treatments. One set consists of 4 bags kept as a control (W1) and the other as water stressed (W2). After germination, only one plant/bag was kept for data recording. Standard pH (6.5), temperature (25 ± 2°C), humidity (50%) and light requirements (13 h photoperiod) for cotton growth were maintained throughout the total duration of experiment. Seedlings grown under both stress and non-stress conditions were irrigated and fertilized till the development of the first true leaf, and thereafter, seedlings grown under control condition (W1) were watered daily to keep the soil at field capacity. Water stress condition was developed by withholding water supply to the seedlings grown under water stress condition (W2), and the effect of water stress was monitored visually and with soil moisture meter (HH2 Theta Probe Type, Delta-T device, Cambridge, England). At initial wilting stage (observed visually), when soil had 14 to 16% soil moisture content, the stressed plants were watered to relieve the sign of wilting but not enough to reach field capacity. The experiment was continued for 45 days from the date of emergence till the 3rd main stem leaf was fully expanded. The plants grown under normal water supply and stressed conditions were measured for morphological and physiological parameters.
The DNA concentration was determined by the method described (Zhang et al., 2000). The DNA samples were stored at -20°C until further use. The DNA quality was evaluated with 1% agarose gel electrophoresis prepared using 0.5X TAE buffer, and ethidium bromide (10 ng/100 ml) was added to the gel to stain the DNA bands. The samples were electrophoresed for approximately 30 min after which the products were viewed using an ultraviolet transilluminator and photographed using the Syngene Gel Documentation System. The DNA concentration was estimated by the absorbance at 260 nm. The working DNA samples (containing 50 ng μL⁻¹) were stored at 4°C for genotyping. SSR primer pairs used were from different sources: NAU from Nanjing Agricultural University, Nanjing, China (Han et al., 2006); BNL primers from Research Genetics Co. (Huntsville, AL, USA, http://www.resgen.com); JESPR from sequences of Reddy et al. (2001); TM from Dr. John Yu, USDA-ARS, Crops Germplasm Research Unit, TE, USA; CIR from Nguyen et al. (2004). Details about these markers can be found at www.cottonmarker.org. All 90 accessions were genotyped using a 180 core set of SSR marker primers. These chromosome-specific primer pairs were selected using the results of different laboratories and published papers (Siu et al., 2000; Han et al., 2006; Shen et al., 2005; Abdurakhonov et al., 2007). They were based on information related to important QTLs and chromosome distribution.

**Phenotyping of plant materials**

On 26th April, 2010 green-house experiment was completed and the following parameters were measured. First, plant length (PH) and plant fresh weight (PFW) were recorded after the plants were separated into shoot and root parts, and data were recorded for shoot length (SL), root length (RL), fresh shoot weight (FSW) and fresh root weight (FRW). The respective shoots and roots of all plants were then oven-dried at 70°C till a constant dry weight was reached. The dry weight of shoot and root of respective plants were recorded and summed up to get the dry plant weight (DPW). The root shoot ratio (RSR) was calculated using the formula: RSR= DRW/DSW. Relative water contents (RWC) were calculated using the following formula: RWC = [(Fresh weight - Dry weight) / (Turgid weight – Dry weight)] × 100.

**SSR genotyping**

For extraction of the genomic DNA from each accession group, 4 to 5 young fully expanded leaves from each plant were collected and stored at -80°C. The genomic DNA was isolated from the frozen leaf tissues using the cetyltrimethyl ammonium bromide (CTAB) method described (Zhang et al., 2000). The DNA samples were stored at -20°C until further use. The DNA quality was evaluated with 1% agarose gel electrophoresis prepared using 0.5X TAE buffer, and ethidium bromide (10 ng/100 ml) was added to the gel to stain the DNA bands. The samples were electrophoresed for approximately 30 min after which the products were viewed using an ultraviolet transilluminator and photographed using the Syngene Gel Documentation System. The DNA concentration was estimated by the absorbance at 260 nm. The working DNA samples (containing 50 ng μL⁻¹) were stored at 4°C for genotyping. SSR primer pairs used were from different sources: NAU from Nanjing Agricultural University, Nanjing, China (Han et al., 2006); BNL primers from Research Genetics Co. (Huntsville, AL, USA, http://www.resgen.com); JESPR from sequences of Reddy et al. (2001); TM from Dr. John Yu, USDA-ARS, Crops Germplasm Research Unit, TE, USA; CIR from Nguyen et al. (2004) (Table 2). Details about these markers can be found at www.cottonmarker.org. All 90 accessions were genotyped using a 180 core set of SSR marker primers. These chromosome-specific primer pairs were selected using the results of different laboratories and published papers (Siu et al., 2000; Han et al., 2006; Shen et al., 2005; Abdurakhonov et al., 2007). They were based on information related to important QTLs and chromosome distribution.

### Table 1. List of cultivars used in the study.

<table>
<thead>
<tr>
<th>S/N</th>
<th>Cultivar name</th>
<th>S/N</th>
<th>Cultivar name</th>
<th>S/N</th>
<th>Cultivar name</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CIM-496</td>
<td>31</td>
<td>MNH-806</td>
<td>61</td>
<td>XU-ZHOU-142</td>
</tr>
<tr>
<td>2</td>
<td>CIM-482</td>
<td>32</td>
<td>MNH-802</td>
<td>62</td>
<td>XING TAI-68-71</td>
</tr>
<tr>
<td>3</td>
<td>CIM-473</td>
<td>33</td>
<td>MNH-770</td>
<td>63</td>
<td>XIAO-VEMIAN</td>
</tr>
<tr>
<td>4</td>
<td>CIM-448</td>
<td>34</td>
<td>MNH-720</td>
<td>64</td>
<td>VH-148</td>
</tr>
<tr>
<td>5</td>
<td>CIM-446</td>
<td>35</td>
<td>MNH-636</td>
<td>65</td>
<td>UA-73</td>
</tr>
<tr>
<td>6</td>
<td>CIM-4/99</td>
<td>36</td>
<td>MNH-636</td>
<td>66</td>
<td>UA-7-25/46</td>
</tr>
<tr>
<td>7</td>
<td>CIM-1100</td>
<td>37</td>
<td>MNH-6070</td>
<td>67</td>
<td>UA-31-102</td>
</tr>
<tr>
<td>8</td>
<td>CIM-109</td>
<td>38</td>
<td>MNH-554</td>
<td>68</td>
<td>U-4(5143)</td>
</tr>
<tr>
<td>9</td>
<td>CHINA</td>
<td>39</td>
<td>MNH-552</td>
<td>69</td>
<td>U-4</td>
</tr>
<tr>
<td>10</td>
<td>BT-3701</td>
<td>40</td>
<td>MNH-147</td>
<td>70</td>
<td>U-276</td>
</tr>
<tr>
<td>11</td>
<td>BT-2009</td>
<td>41</td>
<td>MG-66</td>
<td>71</td>
<td>TH-35/99</td>
</tr>
<tr>
<td>12</td>
<td>BT-1573</td>
<td>42</td>
<td>M-944-0243</td>
<td>72</td>
<td>Stoneville-825</td>
</tr>
<tr>
<td>13</td>
<td>BS-1</td>
<td>43</td>
<td>M-944-00-0030</td>
<td>73</td>
<td>Stoneville-213</td>
</tr>
<tr>
<td>14</td>
<td>BH-118</td>
<td>44</td>
<td>L-S-S</td>
<td>74</td>
<td>Stoneville</td>
</tr>
<tr>
<td>15</td>
<td>B-557</td>
<td>45</td>
<td>LA-208</td>
<td>75</td>
<td>STAMP-82</td>
</tr>
<tr>
<td>16</td>
<td>AYT-85094</td>
<td>46</td>
<td>IS-7-F1</td>
<td>76</td>
<td>SLS-1</td>
</tr>
<tr>
<td>17</td>
<td>AYT-85094</td>
<td>47</td>
<td>IRMA-1480</td>
<td>77</td>
<td>SLH-284</td>
</tr>
<tr>
<td>18</td>
<td>Australia-407721</td>
<td>48</td>
<td>GR-156-</td>
<td>78</td>
<td>S-14</td>
</tr>
<tr>
<td>19</td>
<td>4 F</td>
<td>49</td>
<td>FH-901</td>
<td>79</td>
<td>S-12</td>
</tr>
<tr>
<td>20</td>
<td>362-F</td>
<td>50</td>
<td>FH-900</td>
<td>80</td>
<td>S-11</td>
</tr>
<tr>
<td>21</td>
<td>299F</td>
<td>51</td>
<td>FH-682</td>
<td>81</td>
<td>Rehmani</td>
</tr>
<tr>
<td>22</td>
<td>268-F</td>
<td>52</td>
<td>FH-125</td>
<td>82</td>
<td>N-KARSHOMG</td>
</tr>
<tr>
<td>23</td>
<td>268 F</td>
<td>53</td>
<td>FH-113</td>
<td>83</td>
<td>NIBGE-4</td>
</tr>
<tr>
<td>24</td>
<td>1027 ALF</td>
<td>54</td>
<td>FH-1000</td>
<td>84</td>
<td>NIBGE-4</td>
</tr>
<tr>
<td>25</td>
<td>1021 (KIVI)</td>
<td>55</td>
<td>841/52</td>
<td>85</td>
<td>NIAB-78</td>
</tr>
<tr>
<td>26</td>
<td>65090</td>
<td>56</td>
<td>CRSM-38</td>
<td>86</td>
<td>MS-93</td>
</tr>
<tr>
<td>27</td>
<td>6040</td>
<td>57</td>
<td>CRSM-38</td>
<td>87</td>
<td>MS-40</td>
</tr>
<tr>
<td>28</td>
<td>3996</td>
<td>58</td>
<td>CRS-2009</td>
<td>88</td>
<td>MNH-814</td>
</tr>
<tr>
<td>29</td>
<td>2616</td>
<td>59</td>
<td>CIM-534</td>
<td>89</td>
<td>MNH-812</td>
</tr>
<tr>
<td>30</td>
<td>814</td>
<td>60</td>
<td>CIM-506</td>
<td>90</td>
<td>MNH-807</td>
</tr>
</tbody>
</table>
List of SSR markers used in the study.

<table>
<thead>
<tr>
<th>S/N</th>
<th>Primers</th>
<th>chr.0</th>
<th>Position</th>
<th>S/N</th>
<th>Primers</th>
<th>chr.0</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>BNL1053</td>
<td>-</td>
<td>-</td>
<td>49</td>
<td>NAU2691-275</td>
<td>D3(ch.17)</td>
<td>81.063</td>
</tr>
<tr>
<td>2</td>
<td>BNL1421</td>
<td>A1(ch.1)</td>
<td>163.5</td>
<td>50</td>
<td>NAU2697-165</td>
<td>D13(ch.18)</td>
<td>87.399</td>
</tr>
<tr>
<td>3</td>
<td>BNL1606-180</td>
<td>D3(ch.17)</td>
<td>50.727</td>
<td>51</td>
<td>NAU2714-450</td>
<td>D6(ch.25)</td>
<td>112.068</td>
</tr>
<tr>
<td>4</td>
<td>BNL1672-110</td>
<td>A9(ch.9)</td>
<td>60.015</td>
<td>52</td>
<td>NAU2974_195</td>
<td>D7(ch.16)</td>
<td>101.7</td>
</tr>
<tr>
<td>5</td>
<td>BNL226</td>
<td>D2(ch.14)</td>
<td>120</td>
<td>53</td>
<td>NAU2980-270</td>
<td>D13(ch.18)</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>BNL2569-170</td>
<td>A6(ch.6)</td>
<td>111.001</td>
<td>54</td>
<td>NAU2995-195</td>
<td>A7(ch.7)</td>
<td>70.732</td>
</tr>
<tr>
<td>7</td>
<td>BNL3089-125</td>
<td>A4(ch.4)</td>
<td>3.399</td>
<td>55</td>
<td>NAU3011-330</td>
<td>D13(ch.18)</td>
<td>98.587</td>
</tr>
<tr>
<td>8</td>
<td>BNL3280-195</td>
<td>D10(ch.20)</td>
<td>107.214</td>
<td>56</td>
<td>NAU3053-190</td>
<td>D7(ch.16)</td>
<td>30.704</td>
</tr>
<tr>
<td>9</td>
<td>BNL3359-210</td>
<td>A6(ch.6)</td>
<td>134.565</td>
<td>57</td>
<td>NAU3084-235</td>
<td>D12(ch.26)</td>
<td>130.319</td>
</tr>
<tr>
<td>10</td>
<td>BNL3590-180</td>
<td>A2(ch.2)</td>
<td>61.01</td>
<td>58</td>
<td>NAU3092-245</td>
<td>D5(ch.19)</td>
<td>9.968</td>
</tr>
<tr>
<td>11</td>
<td>BNL409-100</td>
<td>A13(Chr.13)</td>
<td>127.092</td>
<td>59</td>
<td>NAU3095-225</td>
<td>D5(ch.19)</td>
<td>180.972</td>
</tr>
<tr>
<td>12</td>
<td>BNL448</td>
<td>D4(ch.22)</td>
<td>22.7</td>
<td>60</td>
<td>NAU3100-195</td>
<td>D9(Chr.23)</td>
<td>25.886</td>
</tr>
<tr>
<td>13</td>
<td>BNL496-335</td>
<td>D10(ch.20)</td>
<td>61.17</td>
<td>61</td>
<td>NAU3206-500</td>
<td>A6(ch.6)</td>
<td>36.353</td>
</tr>
<tr>
<td>14</td>
<td>JESPR153-130</td>
<td>A13(Chr.13)</td>
<td>62.641</td>
<td>62</td>
<td>NAU3207-275</td>
<td>A8(ch.8)</td>
<td>70.619</td>
</tr>
<tr>
<td>15</td>
<td>JESPR220-200</td>
<td>D4(ch.22)</td>
<td>102.872</td>
<td>63</td>
<td>NAU3209-265</td>
<td>D2(ch.14)</td>
<td>104.477</td>
</tr>
<tr>
<td>16</td>
<td>JESPR274-105</td>
<td>A9(ch.9)</td>
<td>42.802</td>
<td>64</td>
<td>NAU3254-1600</td>
<td>A1(ch.1)</td>
<td>102.873</td>
</tr>
<tr>
<td>17</td>
<td>JESPR291-175</td>
<td>D8(ch.24)</td>
<td>152.036</td>
<td>65</td>
<td>NAU3306-2400</td>
<td>D6(ch.25)</td>
<td>39.547</td>
</tr>
<tr>
<td>18</td>
<td>JESPR92</td>
<td>D12(ch.26)</td>
<td>132.1</td>
<td>66</td>
<td>NAU3385-500</td>
<td>A1(ch.1)</td>
<td>74.005</td>
</tr>
<tr>
<td>19</td>
<td>NAU1070-165</td>
<td>D2(ch.14)</td>
<td>13.512</td>
<td>67</td>
<td>NAU3414-225</td>
<td>A9(ch.9)</td>
<td>119.156</td>
</tr>
<tr>
<td>20</td>
<td>NAU1102</td>
<td>D5(ch.19)</td>
<td>0</td>
<td>68</td>
<td>NAU3424-210</td>
<td>D7(ch.16)</td>
<td>-</td>
</tr>
<tr>
<td>21</td>
<td>NAU1103</td>
<td>D11(ch.21)</td>
<td>7.5</td>
<td>69</td>
<td>NAU3519-215</td>
<td>A12(ch.12)</td>
<td>146.714</td>
</tr>
<tr>
<td>22</td>
<td>NAU1125</td>
<td>D8(ch.24)</td>
<td>47.7</td>
<td>70</td>
<td>NAU3529-295</td>
<td>A5(ch.5)</td>
<td>117.035</td>
</tr>
<tr>
<td>23</td>
<td>NAU1141-200</td>
<td>A13(ch.13)</td>
<td>31.193</td>
<td>71</td>
<td>NAU3606</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>NAU1167-200</td>
<td>A3(ch.3)</td>
<td>105.325</td>
<td>72</td>
<td>NAU3608-220</td>
<td>D7(ch.16)</td>
<td>121.531</td>
</tr>
<tr>
<td>25</td>
<td>NAU1190_400</td>
<td>A3(ch.3)</td>
<td>62.9</td>
<td>73</td>
<td>NAU3654-350</td>
<td>A7(ch.7)</td>
<td>23.067</td>
</tr>
<tr>
<td>26</td>
<td>NAU1200</td>
<td>A5(ch.5)</td>
<td>16.6</td>
<td>74</td>
<td>NAU3695_280</td>
<td>A11(ch.11)</td>
<td>125.6</td>
</tr>
<tr>
<td>27</td>
<td>NAU1218-150</td>
<td>A6(ch.6)</td>
<td>57.568</td>
<td>75</td>
<td>NAU3703_660</td>
<td>A11(ch.11)</td>
<td>67.3</td>
</tr>
<tr>
<td>28</td>
<td>NAU1230</td>
<td>D5(ch.19)</td>
<td>0.2</td>
<td>76</td>
<td>NAU3860_250</td>
<td>A12(ch.12)</td>
<td>85</td>
</tr>
<tr>
<td>29</td>
<td>NAU1233</td>
<td>A10(ch.10)</td>
<td>40.2</td>
<td>77</td>
<td>NAU3901_280</td>
<td>D1(ch.15)</td>
<td>117.3</td>
</tr>
<tr>
<td>30</td>
<td>NAU1254-305</td>
<td>A8(ch.8)</td>
<td>21.557</td>
<td>78</td>
<td>NAU3903_300</td>
<td>D2(ch.14)</td>
<td>110.1</td>
</tr>
<tr>
<td>31</td>
<td>NAU1266</td>
<td>-</td>
<td>-</td>
<td>79</td>
<td>NAU3916-320</td>
<td>D10(ch.20)</td>
<td>30.947</td>
</tr>
<tr>
<td>32</td>
<td>NAU1350-305</td>
<td>D8(ch.24)</td>
<td>107.342</td>
<td>80</td>
<td>NAU3961-210</td>
<td>D12(ch.26)</td>
<td>48.569</td>
</tr>
<tr>
<td>33</td>
<td>NAU1366-700</td>
<td>D11(ch.21)</td>
<td>142.709</td>
<td>81</td>
<td>NAU4073-160</td>
<td>A1(ch.1)</td>
<td>53.3</td>
</tr>
<tr>
<td>34</td>
<td>NAU1369-410</td>
<td>A8(ch.8)</td>
<td>43.809</td>
<td>82</td>
<td>NAU418</td>
<td>D9(ch.23)</td>
<td>99.6</td>
</tr>
<tr>
<td>35</td>
<td>NAU2016-250</td>
<td>A1(ch.11)</td>
<td>18.128</td>
<td>83</td>
<td>NAU437-290</td>
<td>A2(ch.2)</td>
<td>52.833</td>
</tr>
<tr>
<td>36</td>
<td>NAU2169-700</td>
<td>D8(ch.24)</td>
<td>77.052</td>
<td>84</td>
<td>NAU4516</td>
<td>D13</td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>NAU2190-360</td>
<td>D2(ch.14)</td>
<td>24.1</td>
<td>85</td>
<td>NAU453-180</td>
<td>D10(ch.20)</td>
<td>-</td>
</tr>
<tr>
<td>38</td>
<td>NAU2265-380</td>
<td>A2(ch.2)</td>
<td>5.902</td>
<td>86</td>
<td>NAU4565</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>39</td>
<td>NAU2306-380</td>
<td>D8(ch.24)</td>
<td>50.968</td>
<td>87</td>
<td>NAU458-190</td>
<td>D1(ch.15)</td>
<td>12.422</td>
</tr>
<tr>
<td>40</td>
<td>NAU2317-220</td>
<td>A10(ch.10)</td>
<td>2.554</td>
<td>88</td>
<td>NAU462-650</td>
<td>A9(ch.9)</td>
<td>117.544</td>
</tr>
<tr>
<td>41</td>
<td>NAU2336-215</td>
<td>D2(ch.14)</td>
<td>54.308</td>
<td>89</td>
<td>NAU483-250</td>
<td>A3(ch.3)</td>
<td>27.986</td>
</tr>
<tr>
<td>42</td>
<td>NAU2355b</td>
<td>-</td>
<td>-</td>
<td>90</td>
<td>NAU4956-240</td>
<td>D7(ch.16)</td>
<td>50.973</td>
</tr>
<tr>
<td>43</td>
<td>NAU2439-150</td>
<td>D8(ch.24)</td>
<td>70.516</td>
<td>91</td>
<td>NAU5091-160</td>
<td>D11(ch.21)</td>
<td>66.492</td>
</tr>
<tr>
<td>44</td>
<td>NAU2443-150</td>
<td>D13(ch.18)</td>
<td>61.754</td>
<td>92</td>
<td>NAU5189-280</td>
<td>D9(ch.23)</td>
<td>140.07</td>
</tr>
<tr>
<td>45</td>
<td>NAU2477-200</td>
<td>A4(ch.4)</td>
<td>82.172</td>
<td>93</td>
<td>NAU6106</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>46</td>
<td>NAU2503-250</td>
<td>D5(ch.19)</td>
<td>175.169</td>
<td>94</td>
<td>NAU6191</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>47</td>
<td>NAU2527</td>
<td>A10(ch.10)</td>
<td>31.7</td>
<td>95</td>
<td>TMP20-205</td>
<td>A11(ch.11)</td>
<td>72.758</td>
</tr>
<tr>
<td>48</td>
<td>NAU2540_160</td>
<td>D10(ch.20)</td>
<td>25.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3. Mean squares of the ANOVA of morpho-physiological traits.

<table>
<thead>
<tr>
<th>Trait/Source of variation</th>
<th>Block</th>
<th>Water Levels (WL)</th>
<th>Error</th>
<th>Genotypes (G)</th>
<th>Water Levels × Genotypes</th>
<th>Coeff Var. %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shoot Length (cm)</td>
<td>445.7589</td>
<td>21182.3**</td>
<td>12.91</td>
<td>67.8**</td>
<td>18.7*</td>
<td>14.1</td>
</tr>
<tr>
<td>Root Length (cm)</td>
<td>56.1126</td>
<td>3463.6**</td>
<td>18</td>
<td>44.47**</td>
<td>17.73n.s.</td>
<td>22.2</td>
</tr>
<tr>
<td>Fresh Shoot Weight (g)</td>
<td>13.8</td>
<td>2409.6**</td>
<td>1.46</td>
<td>3.93**</td>
<td>2.25**</td>
<td>29.7</td>
</tr>
<tr>
<td>Fresh Root Weight (g)</td>
<td>0.29</td>
<td>34.0**</td>
<td>0.04</td>
<td>0.07**</td>
<td>0.048n.s.</td>
<td>39.4</td>
</tr>
<tr>
<td>Dry shoot weight (g)</td>
<td>2.1758</td>
<td>60.04**</td>
<td>0.089</td>
<td>0.25**</td>
<td>0.14**</td>
<td>34</td>
</tr>
<tr>
<td>Dry root weight (g)</td>
<td>0.0136</td>
<td>0.0072**</td>
<td>0.0044</td>
<td>0.0101**</td>
<td>0.0064.n.s.</td>
<td>51</td>
</tr>
<tr>
<td>Relative Water Contents %</td>
<td>151</td>
<td>14307**</td>
<td>29.11</td>
<td>96.25**</td>
<td>71.13**</td>
<td>12</td>
</tr>
</tbody>
</table>

* = P ≤ 0.05; ** = P ≤ 0.01; *** = P ≤ 0.001

The PCR amplifications were performed in a 10 μl reaction mix containing 1 μl 10× PCR buffer, 0.2 μl dNTPs (5 mM each), 0.1 μl 25 mM MgCl₂, 0.1 μl Taq DNA polymerase, and 1 μl (50 ng) genomic DNA. The microsatellites were amplified using the standard polymerase chain reaction (PCR) procedures described by Zhang et al. (2000). Two millilitre of PCR products was separated vertically on denaturing 16% polyacrylamide gels with 5× TBE buffer at 180 V for 45 min and stained with silver (Bassam et al., 1991). A 50 bp DNA ladder was used to estimate allele sizes in

Silver staining and development of bands

After a specific migration of the band on the gel, the gels were placed in fixing solution (40% ethanol and 10% glacial acetic acid) for 20 min. They were washed three times with distilled water and stained with silver staining solution (0.2% AgNO₃) for 20 min. After staining, the gels were again washed three times with distilled water for 20 s, and the developing solution (3% NaOH and 0.05% formaldehyde) was applied for 3 to 5 min.

Inference of population structure for association mapping

The STRUCTURE software is a DOS, Windows, UNIX (Solaris) and Linux based database that performs a model-based clustering method for gathering the occurrence of population structure, finding diverse genetic populations, allocating individuals to populations, and classifying migrants and admixed individuals. Complementary studies on genotypic data for evaluating the population structure before continuing with LD analysis were performed by a model-based approach; they were implemented in the software package STRUCTURE (Pritchard et al., 2000) to identify subgroups in cotton cultivars under study. Admixture model under independent allele frequencies using the burn-in time of 50,000, and a number of MCMC repeats at 100,000 were used (Pritchard et al., 2000), with the K ranging from 2 to 15.

Extent of linkage disequilibrium and marker-trait association analysis

The genome-wide LD between pairs of SSR marker loci was studied using the software package TASSEL ver. 2.1. Linkage disequilibrium was estimated by a weighted average of squared allele-frequency correlations (R²) between SSR loci. The significance of pairwise LD (P-values≤0.005) among all possible SSR loci was evaluated using TASSEL. The LD values between all pairs of SSR loci were plotted as LD plots using TASSEL to estimate the general view of genome-wide LD patterns and to evaluate LD structures. The marker-trait associations were calculated by GLM association test incorporating Q matrices from

STRUCTURE2.2 into TASSEL software package (Bradbury et al., 2007). To assess significant marker-trait associations P-marker ≤ 0.05 was used.

RESULTS

Phenotypic variation

The cotton varieties under study revealed a wide range of phenotypic variation in morpho-physiological traits under both control and drought treatments (Table 3). Mean squares from analysis of variance for all morpho-physiological traits revealed highly significant variation (P≤0.05) with respect to water levels and genotypes. However, the interaction between water levels and genotypes was significant for shoot length, fresh shoot weight and dry shoot weight (Table 1). Mean value of all 90 genotypes indicated significant reduction in all seedling traits. The considerable amount of genotypic variance apparent in all traits shows that variance under water stress conditions is genetically determined and selection of varieties/lines for drought tolerance on the basis of seedling traits is possible. Correlation coefficients between means in the stressed and non-stressed conditions were positive and highly significant (P ≤ 0.01) for most of the traits (Table 4). The frequency distribution of morpho-physiological traits under control (W1) and water stressed (W2) treatments indicated considerable genotypic variance (Figure 1).

SSR genotyping, population structure, pairwise linkage disequilibrium and LD decay

The selected SSR primer pairs generated a total of 241 SSR alleles, of which 147 (60%) were polymorphic, resulting in 57.5% polymorphism. The average number of polymorphic alleles per primer was 2.10. To determine the population structure and number of subgroups in cotton cultivars under study, a model-based approach, implemented in the software package STRUCTURE (Pritchard et al., 2000), the distribution of log probability of data, lnP(D), did not show a clear peak against any value of K, but by the use of parameter ΔK, rate of
change in the log probability of the data, graph peaked against a value of $K = 5$. This confirmed 5 subpopulations in the germplasm at significant threshold values ($R^2 \geq 0.05$). 7.1% of the SSR marker pairs showed significant pairwise LD in 90 accessions of *G. hirsutum* in our study. Plots for pairwise LD between SSR markers demonstrated significant LD blocks in the genome-wide LD analysis. We observed a significant (R2) LD between 13 pairs of SSR loci within the same chromosome in range of 180 cM between NAU1230 and NAU3095 loci in chromosome D5 and 1.612 cM and between NAU462 and NAU3414 in chromosome A9. Triangle plots for pairwise LD between SSR markers demonstrated significant LD blocks in the genome-wide LD analysis. Genome-wide LD decay was assessed by plotting r2 LD values as a function of genetic distance in cM. Two long stretches of LD blocks were observed on chromosomes A3 and D9, extending to a distance of 180 and 77 cM respectively (Table 5). Genome-wide LD at $r^2 > 0.1$ rapidly decayed within ~1.61 to 11 cM, indicating a strong potential for association mapping (Saeed et al., 2014). The percentage of SSR loci pairs in LD observed in 90 *G. hirsutum* (7.1%) was comparable with reports in cotton (11 and 12%) (Abdurakhmonov and Addukarimov, 2008) maize (10%) (Remington et al., 2001) and sorghum (8.7%) (Hamblin et al., 2004). A high recombination rate in allopolyploid cottons was reported (Brubaker et al., 1999) and it might be one of the factors explaining the observed low level of pairwise LD in cotton, along with mutation, selection, and genetic drift that occurred in the domestication of *G. hirsutum* germplasm.

**Marker-trait association for morpho-physiological traits**

A total of 21 marker loci identified by GLM analysis were significantly associated (P≤0.001) with phenotyped traits under both control and drought treatment (Table 6). Out of these 21 markers, NAU3414, NAU2691, NAU1141 and NAU1190 were associated with more than one morpho-physiological trait under drought treatments (Table 4). Phenotypic variance explained ($R^2$) value ranging from 9.91 to 19%. Highest phenotypic variance explaining ($R^2$) was ascribed to NAU3011 chromosome D13 significantly (P<0.001) associated with root length under drought treatment. This locus appeared to be a major locus as it is associated with highest phenotypic variance. NAU3414 located on chromosome A9 was associated with

---

**Table 4. Correlation coefficients of morpho-physiological traits under well-watered (W) and drought-stress (W2) treatment.**

<table>
<thead>
<tr>
<th>Traits</th>
<th>PDW</th>
<th>PFW</th>
<th>PH</th>
<th>RDW</th>
<th>RFW</th>
<th>RFW</th>
<th>RSR</th>
<th>SDW</th>
<th>SFW</th>
</tr>
</thead>
<tbody>
<tr>
<td>PFW</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>W1</td>
<td>0.89958**</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>W2</td>
<td>0.6296**</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PH</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>W1</td>
<td>0.7083**</td>
<td>0.7527**</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>W2</td>
<td>0.4677**</td>
<td>0.5698**</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RDW</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>W1</td>
<td>0.7779**</td>
<td>0.7261**</td>
<td>0.5532**</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>W2</td>
<td>0.3963**</td>
<td>0.1202NS</td>
<td>0.1075NS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RFW</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>W1</td>
<td>0.6880**</td>
<td>0.6861**</td>
<td>0.4361**</td>
<td>0.7831**</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>W2</td>
<td>0.2841*</td>
<td>0.5756**</td>
<td>0.2715*</td>
<td>0.1734NS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>W1</td>
<td>0.4815**</td>
<td>0.5279**</td>
<td>0.7717**</td>
<td>0.4702**</td>
<td>0.3889**</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>W2</td>
<td>0.1851NS</td>
<td>0.1836NS</td>
<td>0.7498**</td>
<td>0.0888NS</td>
<td>0.1043NS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RSR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>W1</td>
<td>-0.3833**</td>
<td>-0.3175*</td>
<td>-0.3001*</td>
<td>0.2084NS</td>
<td>0.0163NS</td>
<td>-0.0793NS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>W2</td>
<td>-0.1579NS</td>
<td>-0.2843*</td>
<td>-0.2024NS</td>
<td>0.8251**</td>
<td>-0.0110NS</td>
<td>-0.0482NS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SDW</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>W1</td>
<td>0.9963**</td>
<td>0.8927**</td>
<td>0.7066**</td>
<td>0.7221**</td>
<td>0.6512**</td>
<td>0.4689**</td>
<td>0.4522**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>W2</td>
<td>0.9692**</td>
<td>0.6499**</td>
<td>0.4753**</td>
<td>0.1597NS</td>
<td>0.2585**</td>
<td>0.1740NS</td>
<td>-0.390**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SFW</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>W1</td>
<td>0.8862**</td>
<td>0.9954**</td>
<td>0.7596**</td>
<td>0.6854**</td>
<td>0.6135**</td>
<td>0.5216**</td>
<td>-0.3470*</td>
<td>0.8837**</td>
<td></td>
</tr>
<tr>
<td>W2</td>
<td>0.6367**</td>
<td>0.9897**</td>
<td>0.5733**</td>
<td>0.1001NS</td>
<td>0.4530*</td>
<td>0.1818NS</td>
<td>-0.3085*</td>
<td>0.6636**</td>
<td></td>
</tr>
<tr>
<td>SL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>W1</td>
<td>0.6569**</td>
<td>0.6852**</td>
<td>0.8517**</td>
<td>0.4349**</td>
<td>0.3295*</td>
<td>0.3241*</td>
<td>-0.381**</td>
<td>0.6648**</td>
<td>0.7007**</td>
</tr>
<tr>
<td>W2</td>
<td>0.5268**</td>
<td>0.6762**</td>
<td>0.8041**</td>
<td>0.0795NS</td>
<td>0.3097**</td>
<td>0.2097NS</td>
<td>-0.2562*</td>
<td>0.5481**</td>
<td>0.6825**</td>
</tr>
</tbody>
</table>

PH, Plant length; PFW, Plant fresh weight; SFW, Shoot fresh weight; SDW, Root dry weight; RFW, Root fresh weight; RSR, Root shoot ratio; RWC, Relative water contents.
Figure 1. Frequency distribution of morpho-physiological traits under control (W1) and water stressed (W2) treatments.
Table 5. The pairwise genome linkage disequilibrium (LD) between pairs of SSR markers in same chromosomes and their distances.

<table>
<thead>
<tr>
<th>Locus Name1</th>
<th>Locus Name2</th>
<th>R²</th>
<th>DPrime</th>
<th>pDiseq</th>
<th>Chr</th>
<th>cM</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAU3095</td>
<td>NAU1230</td>
<td>0.181971</td>
<td>0.827381</td>
<td>0.000386</td>
<td>D5</td>
<td>180.772</td>
</tr>
<tr>
<td>NAU2691</td>
<td>BNL1606</td>
<td>0.107744</td>
<td>0.444444</td>
<td>0.047173</td>
<td>D3</td>
<td>30.336</td>
</tr>
<tr>
<td>NAU3011</td>
<td>NAU2697</td>
<td>0.098475</td>
<td>0.373626</td>
<td>0.02466</td>
<td>D13</td>
<td>11.188</td>
</tr>
<tr>
<td>NAU2336</td>
<td>NAU2190</td>
<td>0.083965</td>
<td>0.576803</td>
<td>0.014891</td>
<td>D2</td>
<td>30.208</td>
</tr>
<tr>
<td>NAU462</td>
<td>NAU3414</td>
<td>0.083056</td>
<td>0.288194</td>
<td>0.020138</td>
<td>A9</td>
<td>1.612</td>
</tr>
<tr>
<td>NAU4105</td>
<td>NAU2697</td>
<td>0.080887</td>
<td>0.355656</td>
<td>0.013277</td>
<td>D13</td>
<td>29.299</td>
</tr>
<tr>
<td>NAU483</td>
<td>NAU1167</td>
<td>0.076643</td>
<td>0.835045</td>
<td>0.003996</td>
<td>A3</td>
<td>77.339</td>
</tr>
<tr>
<td>NAU1230</td>
<td>NAU1102</td>
<td>0.065643</td>
<td>0.367246</td>
<td>0.03619</td>
<td>D5</td>
<td>-</td>
</tr>
<tr>
<td>NAU462</td>
<td>BNL1672</td>
<td>0.064808</td>
<td>0.280702</td>
<td>0.036295</td>
<td>A9</td>
<td>57.529</td>
</tr>
<tr>
<td>NAU3703</td>
<td>NAU3695</td>
<td>0.0625</td>
<td>0.4375</td>
<td>0.022808</td>
<td>A11</td>
<td>58.3</td>
</tr>
<tr>
<td>NAU3254</td>
<td>NAU2083</td>
<td>0.061728</td>
<td>0.333333</td>
<td>0.03903</td>
<td>A1</td>
<td>75.02</td>
</tr>
<tr>
<td>NAU3011</td>
<td>NAU2980</td>
<td>0.060445</td>
<td>0.340909</td>
<td>0.078648</td>
<td>D13</td>
<td>-</td>
</tr>
<tr>
<td>NAU2336</td>
<td>NAU1070</td>
<td>0.048902</td>
<td>0.511364</td>
<td>0.003996</td>
<td>A3</td>
<td>40.796</td>
</tr>
</tbody>
</table>

Table 6. Marker-trait associations assessed by GLM analysis with their phenotypic variance explained (R²) values (P ≤ 0.001).

<table>
<thead>
<tr>
<th>Traits</th>
<th>Marker</th>
<th>Chr.</th>
<th>W1</th>
<th>W2</th>
<th>Relative value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SL</td>
<td>NAU3414</td>
<td>A9</td>
<td>0.113</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>BNL1606</td>
<td>D3</td>
<td>-</td>
<td>-</td>
<td>0.118</td>
</tr>
<tr>
<td></td>
<td>BNL1421</td>
<td>A1</td>
<td>-</td>
<td>-</td>
<td>0.1232</td>
</tr>
<tr>
<td></td>
<td>NAU2691</td>
<td>D3</td>
<td>0.0793</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>NAU2697</td>
<td>D13</td>
<td>0.1021</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>RL</td>
<td>NAU3011</td>
<td>D13</td>
<td>-</td>
<td>0.1904</td>
<td>-</td>
</tr>
<tr>
<td>PL</td>
<td>NAU5091</td>
<td>D11</td>
<td>-</td>
<td>0.1529</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>NAU1190</td>
<td>A3</td>
<td>0.1357</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FSW</td>
<td>NAU3414</td>
<td>A9</td>
<td>-</td>
<td>0.1791</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>NAU3903</td>
<td>D2</td>
<td>0.0996</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>JESP220</td>
<td>D4</td>
<td>0.1687</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FRW</td>
<td>NAU6191</td>
<td>A6</td>
<td>-</td>
<td>0.102</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>NAU1141</td>
<td>A13</td>
<td>0.0942</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>NAU3100</td>
<td>D9</td>
<td>-</td>
<td>-</td>
<td>0.1133</td>
</tr>
<tr>
<td>FPW</td>
<td>NAU1190</td>
<td>A3</td>
<td>0.1263</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>NAU2974</td>
<td>D7</td>
<td>-</td>
<td>-</td>
<td>0.1096</td>
</tr>
<tr>
<td></td>
<td>NAU1190</td>
<td>A3</td>
<td>0.1226</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>NAU1070</td>
<td>D2</td>
<td>-</td>
<td>-</td>
<td>0.1219</td>
</tr>
<tr>
<td>DSW</td>
<td>NAU3414</td>
<td>A9</td>
<td>-</td>
<td>0.1129</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>NAU2974</td>
<td>D7</td>
<td>-</td>
<td>-</td>
<td>0.0991</td>
</tr>
<tr>
<td>DRW</td>
<td>NAU2265</td>
<td>A2</td>
<td>0.1068</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>RSR</td>
<td>NAU1167</td>
<td>A3</td>
<td>0.1193</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>NAU1369</td>
<td>A8</td>
<td>0.1193</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>RWC</td>
<td>NAU1141</td>
<td>A13</td>
<td>0.1096</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>NAU2016</td>
<td>A11</td>
<td>-</td>
<td>0.1068</td>
<td>-</td>
</tr>
</tbody>
</table>

maximum number of traits (shoot length, shoot fresh weight and dry shoot weight whose value ranged from 11.3, 17.9 and 11.29% respectively). Six markers were exclusively associated with drought treatment (W₂).

DISCUSSION
Currently, association mapping methods has been used in diverse plant species such as bread wheat (Yu et al.,
2012; Phumichai et al., 2012), barley (Cockram et al., 2008), triticale (Niedziela et al., 2012) and bean (Shi et al., 2011). For molecular studies, there should be a reasonable degree of variability present among the organism of interest; only then the molecular approaches can identify the genetic case underlying this variability. As there was a significant variability shown in our experimental material under greenhouse conditions, thus our molecular study findings are of future significance. In our cotton germplasm, the number of polymorphic alleles detected per primer pair ranged from one to eight, with 2.10 alleles per primer pair. The SSR markers revealed a considerable amount of variation in the sampled genome, even though the overall polymorphism detected for these cotton cultivars was relatively low. The narrow genetic base of cotton has been mentioned in many studies using such molecular markers as SSRs (Bertini et al., 2006; Zhang et al., 2011; Kalivas et al., 2011), within Upland cultivars, which generally reveal a low level of genetic variety. There was little variation in the estimation of the molecular diversity among the upland cultivars (G. hirsutum). However, Abdurakhmonov et al. (2007) reported that the genetic distance for the Upland cultivars was in 0.01 to 0.28 range. The simple sequence repeats (SSR) allelic diversity found in our population for association analysis is approximately the same as the total diversity presented in more extended studies. The same mean number of alleles per locus as in our study was found in a collection of 106 accessions, with 2.13 SSR alleles (Guo et al., 2006). In this study at significant threshold values (R^2≥0.05), 7.1% of the SSR marker pairs showed significant pairwise LD in a total of 90 accessions of G. hirsutum. This is comparable with previous reports on cotton: 11 to 12% of SSR loci pairs in the exotic G. hirsutum accessions (Abdurakhmonov and Abdurakimov, 2008), 4% SSR markers in G. hirsutum variety accessions (Abdurakhmonov et al., 2009) and 3% cotton germplasm from China and USA (Saeed et al., 2014). In our study, cotton germplasms used are from Pakistan; whereas in previous reports, the cotton germplasm used were of African, Australian, and Latin American, Mexican, Uzbek, China and USA ecotypes. The identification of QTLs’ morpho-physiological traits related to drought tolerance has been reported in many studies: they include drought-related QTLs for morphological traits (Liang et al., 2014), physiological traits (Saeed et al., 2011), earliness (Li et al., 2013), yield (LiFang et al., 2010) and fibre traits (Islam et al., 2014). In our study, significant marker-trait associations were found in a total of 21 marker loci which were significantly associated (P ≤0.001) with phenotyped data under both control and drought treatment. Markers, NAU3414, NAU2691, NAU1141 and NAU1190 were associated with more than one traits under drought treatments. Highest phenotypic variance explaining (R^2) was ascribed to NAU3011 loci significantly (P≤0.001) associated with root length under drought treatment. This locus appeared to be a major locus as it is associated with highest phenotypic variance. NAU3414 located on chromosome A9 was associated with maximum number of traits (shoot length, shoot fresh weight and dry shoot weight whose value ranged from 11.3, 17.9 and 11.29% respectively). Six markers were exclusively associated with drought treatment (W_s). This study also proved that association mapping approach has strong potential to assess significant marker-trait associations, save much time and cost compared to linkage mapping approach.

Conflict of interests

The authors have not declared any conflict of interests.

Abbreviations

QTL, Quantitative trait loci; SSR, simple sequence repeat; TASSEL, trait analysis by association, evolution and linkage; GLM, general linear model; PL, plant length, PFW, plant fresh weight, SL, shoot length, RL, root length, SFW, shoot fresh weight, SDW, shoot dry weight, RFW, root dry weight, RSR, root shoot ratio; RWC, relative water contents; LD, linkage disequilibrium.

REFERENCES


Full Length Research Paper

Inbreeding depression in crosses of coerulea clones of Walker’s Cattleya (Cattleya walkeriana Gardner)

Miléia Ricci Picolo, Ceci Castilho Custódio, Nelson Barbosa Machado-Neto*

Department of Agronomy, Universidade do Oeste Paulista (UNOESTE), Presidente Prudente, São Paulo, Brazil.

Received 25 July, 2016; Accepted 21 September, 2016

Orchids are among the most beautiful flowers and endangered due to habitat destruction and over-collection. Cattleya walkeriana is one of the most beautiful flowers joining the small sized plant with medium large and heavily scented flowers. It is widely known and appreciated by its beautiful clones and it has much to offer to breeders because their plants have besides other attributes as small habit and big flowers, many colour variations, form and precocity, becoming flower only four years in ex vitro culture. However, in some of the original places it is becoming a red listed species. Notwithstanding, very little is known about the genetics of these flowers and the variability in the species that is widespread in the Brazilian territory. The aim of this work was to estimate the variability among cultivated materials using the F statistics and to verify if there was inbreeding in plant crosses with similar characteristics, employing as a tool the RAPD simple methodology. The results obtained showed that RAPD was good enough to estimate the variability in C. walkeriana. The selected primers were able to define colour group, especially the coerulea. Inbreeding will occur in crosses of clones with the same colour.

Key words: Orchidaceae, deoxyribonucleic acid (DNA), random amplified polymorphic DNA (RAPD), variability, domestication.

INTRODUCTION

Currently, molecular markers have been used in plant breeding for several objectives, which makes possible more detailed and consistent analysis of their genetics. The establishment of deoxyribonucleic acid (DNA) molecular patterns serves as a parameter for identification of clones and varieties and as a tool for a better taxonomical classification and variability determination in orchids (Chung et al., 2006; Minoo et al., 2006, 2008; Parab et al., 2008; Niknejad et al., 2009; Verma et al., 2009; Oliveira et al., 2010; Xue et a l., 2010; Machado Neto and Vieira, 2011; Sharma et al., 2011; Manners et al., 2013).

The variability estimated by these markers can be used, as any other markers, to estimate the population genetics (F statistics) (Wright, 1978). However, these tools (F statistics) are barely used to follow populations in plant breeding (Sallam et al., 2015). Marker Assisted Selection (MAS) has been a useful tool for plant breeders,
but has had limited success in improving complex traits (Heffner et al., 2010).

According Minoo et al. (2008), random amplified polymorphic DNA (RAPD) technique has some advantages over other techniques as the easy, rapid achievement of polymorphic markers, low cost, absence of hybridization, detection of polymorphism in highly repetitive genome, and high level of polymorphism compared to other molecular markers. However, either RAPD or any other single primer amplification method (SPAR) (Gupta et al., 1994), present a few disadvantages, such as ambiguity in the interpretation of the bands co-migrating fragments of equal size or close and dominant character in most of the markers obtained, what cannot be a penalty for this (Simmons, 2007). In the orchids study, this method can be used to indicate genetic similarity between these plants, their hybrids and wild ancestors, to off-spring prediction of a cross, based on the information from the genotype of early and efficiently, as well as helping in the species classification, or just to measure the raw variability (Costa et al., 2006; Minoo et al., 2008; Niknejad et al., 2009; Oliveira et al., 2010; Xue et al., 2010; Machado-Neto and Vieira, 2011; Manners et al., 2013).

The analysis at molecular level is an advantage for studies on perennial plants, enabling the evaluation of genetic similarity between genotypes (Ambiel et al., 2008, 2010; Machado-Neto and Vieira, 2011) and to collect information on the level of genetic diversity of wild orchids to enable a better conservation of the species (Manners et al., 2013).

In the world flora, orchids are the second largest family, with almost 736 genera and over 26,000 species (Chase et al., 2015), ranging from 7 to 10% of the flowering plant species (Cowan et al., 2006) distributed in five subfamilies: Apostasioidae, Cypripedioideae, Epidendroideae, Orchidoideae, and Vanilloideae (Cameron, 2006). Cattleya together with Cymbidium, Oncidium, Phalaenopsis and Dendrobium are important commercial ornamental species due to its large spectrum of colours and relatively high cross ability with other genera. Orchid commercialization, both pot plants and cut flowers, is highly significant worldwide, about US$504 million (De and Medhi, 2015) and is increasing year after year.

The number of described species in Cattleya is still a matter of debate, ranging from 49 to 114 species (excluding Guarianthe and Cattleyella and including Sophronitis (Van den Berg, 2014) and Brazil has 98 endemics (Forzza et al., 2013).

Cattleya walkeriana belongs to the unifoliate group of the genus and within which it can be considered a small plant. It has a stout rhizome, with three internode pseudobulbs, close to each other. It has long roots, thick and often branched (Menezes, 2011). It is widely known and appreciated by its beautiful clones. It is found in different regions of Brazil, growing over rocks or trees in the states of Goiás, São Paulo, Mato Grosso do Sul, and Minas Gerais, nearby lakes, rivers or swamps and it could be easily cultivated (Menezes, 2011). It has much to offer to breeders because their plants have small habit, large flowers, many colour variations (type: pink, alba, semi-alba and coerulea, Figure 1), form and precocity (Menezes, 2011), becoming flower only four years in ex vitro culture. However, in some of the original places, it is becoming a red listed species (Brasil, 2008).

Very few is known about the genetics of Cattleya species; Cattleya intermedia (Machado-Neto and Vieira, 2011) using RAPD; Cattleya elongata (Cruz et al., 2011) with isozymes and ISSR; Cattleya coccinea (Novello et al., 2013); with ISSR in Cattleya bicolor, Cattleya labiata and Cattleya schofieldiana (Fajardo et al., 2014) with SSR and one in C. labiata using RAPD and ISSR (Pinheiro et al., 2012). And lesser is known about the inheritance of some characters especially because these plants are perennials and the time between one generation and the following is almost 5 years.

Plant breeding and evolution are related for two reasons; the first is that plant breeding might be defined as evolution guided by man and the second is that both processes have their basis in, and a major effect on, biodiversity (Ceccarelli, 2009).

In populations submitted to constant selection, where just the superior individuals were promoted for reproduction, the alleles controlling characters of interest had their frequency increased, leading to diversity loss in crop plants. In orchids, flower shape and colours have been improved by breeding (Machado Neto and Vieira, 2011). While in wild specimens of C. walkeriana, another colour than the type are often not found; it is common to find alba, coerulea and different colours in bred C. walkeriana with exceptionally well-shaped flowers. This species has much to offer for breeders, but it counts just with 109 direct hybrid offspring and it is not much used as parent; for example two related species, Cattleya loddigesii and C. intermedia, counts with 230 and 217 primary hybrids respectively (RHS, 2016).

This study aimed at the measurement of the variability and inbreeding in a population submitted to selection and directional crosses of coerulea clones of C. walkeriana, by means of F statistics (Wright, 1978; Sallam et al., 2015), and to evaluate the ability of RAPD markers in grouping phenotypes of these plants and measure the fixation of the coerulea characteristic in the species and among clones. To our knowledge, this work is the first of this kind with this species.

MATERIALS AND METHODS

Plant

In this work, young and adult plants of C. walkeriana, coerulea (blue) colour, type (pink), semi-alba and alba (white flowers), were used. Plants have diverse origin, except the offspring. They are listed in Table 1.

DNA extraction

DNA extraction and amplifications were done as in Machado-Neto
and Vieira (2011). To identify markers, 120 primers from Operon (Alameda, USA) were initially tested in four plants, one representative of each generation (Parental, F1, F2, BC1) and 33 were primarily chosen (A1, A2, A5, A10, A11, A14, A18, A19, A20, C1, C2, C4, C5, C6, C7, C8, C11, C12, C14, C16, D1, D2, D13, G1, G3, G5, G6, G7, G8, G11, G12, G14, G16) as they were polymorphic. PCR was carried out in a reaction volume of 25 μl containing Tris buffer (20 mM Tris-HCl, pH 8.4), 50 mM KCl, 1.5 mM MgCl₂, 0.4 μM of primer, 0.2 μM of each dNTP, 1U Taq polymerase and two concentrations of template DNA 25 and 50 ng. RAPD amplifications were performed in a thermo cycler under the following conditions: 94°C for 3 min for initial denaturation and then 40 cycles of 1 min at 94°C, 1 min at 37°C for primer annealing and 90 s at 72°C for chain elongation in a PTC-100 Thermocycler. An extra step of 5 min at 72°C for final elongation was included. Amplification products were separated by electrophoresis in 1.5% agarose gel. Gels were stained with ethidium bromide and visualized using Electrophoresis Analysis System (Biosystems). Each amplification reaction was repeated at least twice and only clearly distinct and reproducible bands were scored. Weak or low intensity bands were not considered to avoid ambiguous interpretations. The analysis of the bands was performed with the Quantum program - Capt (Vilber -Lourmat) to determine the electrophoretic pattern. The primers selected for the final analysis were
A2, A5, A10, G5, G11 and G13, because they successfully amplified a total of 54 fragments. Polymerase chain reaction (PCR) was performed as described earlier.

Bands were used to construct a similarity matrix based on the Jaccard coefficient, coding 1 as presence and 0 as absence. The grouping analysis was done using the Unweighted Pair-Group Method Using an Arithmetic Average (UPGMA) algorithm. This analysis was performed with the software NTSYS 2.1 (Rohlf, 2004). Molecular variance analysis (AMOVA) was calculated by total decomposition of its components among and between accessions using the square distances with the Arlequin software (Excoffier et al., 2015).

The fixation index or F statistics of Wright (F<sub>ST</sub>) was generated by the Arlequin software (v. 3.5). The inbreeding coefficient (F<sub>IS</sub>) was calculated by the formula:

\[
F_{IS} = 1 + \frac{(1 - F_{IT})}{(1 - F_{ST})}
\]

and the general fixation index (F<sub>IT</sub>) was calculated by the formula:

\[
F_{IT} = 1 - \frac{Ho}{He}
\]

where Ho and He were the observed and await heterozygosity respectively, obtained in Arlequin software.

RESULTS AND DISCUSSION

Table 2 shows the nucleotide sequences used. The fragments generated ranged from 150 to 2500 bp, lying within the boundaries according to Xue et al. (2010), in which the RAPD technique has good reproducibility.

After primers selection, the construction of a dendrogram was made with all RAPD markers selected. According to the band analysis, it was possible to estimate the ability of these markers to group plants by their colour, that efficiency is demonstrated by the dendrogram (Figure 2), which exhibit a group of coerulea plants, where P<sub>1</sub> and RSP<sub>1</sub> are in the same branch, but in a different location of P<sub>2</sub> and RSP (P). That both P1 and P2 plants could be regarded as coerulea but to different subclades, P1 for group IA and P2 for group IB.

It was expected that all plants from a cross between “Rancho Sereno” and “Patricia” were in the same branch, because both plants are coerulea. All coerulea plants showed up together in a larger branch. In another clade, F<sub>1</sub> plants, although the same phenotype, were separated by branches indicating genetic proximity, but no similarity (group II – Figure 2). The CWV plant showed next to semi-alba (group III – Figure 2), these last sharing the same clade, and the plant CA in other branch bellow, but close to the semi-alba (group IV – Figure 2). This figure shows the clustering of plants that have flourished (relatives, control and F<sub>1</sub>) and plants that have not flowered (F<sub>2</sub> and BC<sub>1</sub> generations). Some F<sub>2</sub> plants were close to F<sub>1</sub> ones which indicates a probable phenotypic similarity. The proximity of these plants with those of already known phenotype may indicate that their flower will have the same colour.

As shown in Table 3, for the population studied, there was a high variability, indicated by the overall F<sub>ST</sub> (0.017), considering Wright (1978) in which F<sub>ST</sub> > 0.25 was considered low variability. However, even using coerulea of different origins an increase in the F<sub>ST</sub> values was showed (0.337 for the parents, 0.539 for F<sub>1</sub> and 0.567 for F<sub>2</sub>), 0.465 for all the coerulea, and 0.492 for BC<sub>1</sub> explained by the fact that plants with similar characteristics were crossed and there was a decreasing in the variability meaning a strong differentiation between those plants and the population (F<sub>ST</sub> 0.017). The selection of plants with the same colour for the initial cross led to endogamy showed in this study by the cross between Patricia x Blue City (Table 1), but it could happen in nature, as a pollinator would choose flower with the same colour, by chance, creating an inbreed population.

In Cattleya intermedia (Machado-Neto and Vieira, 2011) and Cattleya elongata (Cruz et al., 2011) F<sub>ST</sub> values were low indicating a high gene flux among plants (0.016 and 0.100 respectively), but for Fajardo et al. (2014) these values are much higher (from 0.177 in Cattleya labiata to 0.322 in Cattleya granulosa) indicating loss of variability in the last case.

The F<sub>IS</sub> values shown (Table 3) are also very informative, as the values are closer to -1, in the overall sample, meaning that there is more heterozygosity in

<table>
<thead>
<tr>
<th>Primers</th>
<th>Nucleotide sequence (5’ 3’)</th>
<th>Polymorphic bands</th>
<th>Fragment size (pb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A2</td>
<td>TGC CGA GCT G</td>
<td>10</td>
<td>280 - 1380</td>
</tr>
<tr>
<td>A5</td>
<td>AGG GGT CTT G</td>
<td>9</td>
<td>300 - 1190</td>
</tr>
<tr>
<td>A10</td>
<td>GTC ATC GCA G</td>
<td>10</td>
<td>250 - 1370</td>
</tr>
<tr>
<td>G5</td>
<td>CTG AGA CCG A</td>
<td>10</td>
<td>280 - 1380</td>
</tr>
<tr>
<td>G11</td>
<td>TGC CCG TCG T</td>
<td>8</td>
<td>400 - 1460</td>
</tr>
<tr>
<td>G13</td>
<td>CTC TCC GCC A</td>
<td>7</td>
<td>350 - 1060</td>
</tr>
<tr>
<td>Total</td>
<td>-</td>
<td>54</td>
<td>-</td>
</tr>
</tbody>
</table>
this. On the other hand, there was more homozygosis as the values approaches to zero, exemplified by -0.022 in the F₁ population. Intermediate values as the parents (−0.403), F₂ (−0.320), Coerulea (−0.243) and BC₁ (0.447) were more heterozygous than F₁. These kind of data are very useful for perennials (Guries and Ledig, 1981). The values found in this work for Ho and He were not statistically different for the populations. In both, F_{ST} and F_{IS}, there were indications that general population has a good gene flux and driven crosses led to gene diversity loss.

Li and Ge (2006) using RAPD markers found low genetic diversity within populations and high among the studied populations of Changnienia amoena (an orchid species). These results were due to small population size, the local extinction because of habitat destruction

**Figure 2.** Grouping of plants of *Cattleya walkeriana* progeny F₁, F₂, BC₁ and other colour forms.

**Table 3.** Wright's measure of population differentiation (F_{ST}) and inbreeding (F_{IS}), observed (H₀) and estimated Heterosigosity (Hₑ) using RAPD markers for *Cattleya walkeriana* offsprings.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>F_{ST}</th>
<th>F_{IS}</th>
<th>H₀ (±SD)</th>
<th>Hₑ (±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parents</td>
<td>0.377**</td>
<td>-0.403**</td>
<td>0.496 (0.273)</td>
<td>0.353 (0.140)</td>
</tr>
<tr>
<td>F₁</td>
<td>0.539**</td>
<td>-0.022</td>
<td>0.456 (0.197)</td>
<td>0.446 (0.126)</td>
</tr>
<tr>
<td>F₂</td>
<td>0.567**</td>
<td>-0.320**</td>
<td>0.454 (0.198)</td>
<td>0.344 (0.145)</td>
</tr>
<tr>
<td>Coerulea</td>
<td>0.465**</td>
<td>-0.243**</td>
<td>0.496 (0.213)</td>
<td>0.399 (0.135)</td>
</tr>
<tr>
<td>BC₁</td>
<td>0.492**</td>
<td>-0.447**</td>
<td>0.450 (0.211)</td>
<td>0.311 (0.149)</td>
</tr>
<tr>
<td>Overall</td>
<td>0.017</td>
<td>-0.734**</td>
<td>0.463 (0.211)</td>
<td>0.267 (0.153)</td>
</tr>
</tbody>
</table>

**P<0.01.**
and restricted gene flow.

In *Platanthera leucophaea*, another species of orchid, rare and endangered species, the values of $F_{ST}$ for RAPD and isoenzymes (0.889 and 0.754, respectively) showed a large amount of inbreeding consistent with each other (Holsinger et al., 2002). Moreover, in a study by Pressoir and Berthaud (2004), the allelic fixation index in corn landraces showed little variation between populations ($F_{ST}$ 0.003 to 0.011).

Ambiel et al. (2008, 2010) estimated in *Brachiaria brizantha*, an apomictic species, lower values of $F_{ST}$ (0.216 and 0.276) indicate a high gene flow. According to Wright (1978) populations with low levels of selection showed lower values of $F_{ST}$. Sallam et al. (2015) used the F statistics to follow selection during a barley breeding process, so this could be a very useful tool to follow improvement during breeding program, especially in perennial orchids as orchids.

Wild and cultivated populations differ statistically in various characteristics likely to be human selected, although some cultivated plants are morphologically indistinguishable from their relative wild plants (Pickersgill, 2007). So, the increase in the $F_{ST}$ measured during the breeding generations followed in this work was the same as an increase in the inbreeding and in the loss of variability. The targeted character (flower colour) was not being fixed in the F generations after the initial cross, but the primers were wide enough to group the plants according flower colours.

**Conclusion**

RAPD was good enough to estimate the variability in *C. walkeriana*. The selected primers were able to define colour group, especially the *coerulea*. $F_{ST}$ was a good of inbreeding, occurring in crosses of clones with the same colour and in the loss of variability driven by the selection.

**Conflict of Interests**

The authors have not declared any conflict of interest.

**REFERENCES**


Full Length Research Paper

The protective role of honey against cytotoxicity of cadmium chloride in mice

Asmaa Shahrour, Mohamed Zowail and Khaled Sharafeldin*

Department of Zoology, Faculty of Science, Benha University, Benha, Egypt.

Received 21 April, 2016; Accepted 16 August, 2016

The present study aimed to investigate the honey (HY) protective role in opposition to cadmium (Cd) induced chromosomal aberrations of bone marrow and sperm abnormalities. Forty five (45) adult male albino mice were caged into six groups. Mice were injected, i.p, 300 mg HY/kg and/or 0.67 mg CdCl₂/kg b.w for 96 h, separately and alternated. The alternated trials were continued for consecutive eight days. Results show that mice injected with cadmium had significant increase in the frequency of aberrant chromosomes as fragment, centric fusion, gap, stickiness and aneuploidy and in sperm abnormality. The administration of HY improved the frequency of the chromosomal aberrations and sperm abnormality induced by Cd.

Key words: Cadmium, Honey, sperm, chromosome aberrations.

INTRODUCTION

The toxic adverse of cadmium (Cd) is known as environmental and industrial pollutant. Its physical and chemical properties constitute the industrial individuality for applications (Krichah et al., 2003). As a heavy metal, Cd causes severe injuries (Suzuki et al., 1989). People often develop health disorders starting from vomiting, stomach pain and diarrhea to bone fracture, lung and reproductive failure, particularly those who live in vicinity of factories that release cadmium or work in metal refinery industry (Nordberg, 2009). Amara et al. (2011) and Singh et al. (2007) mentioned that the damaged central nervous system and DNA or cancer progression appeared as consequences of Cd exposure. Cadmium also causes severe soft tissues and bone damages (Cucu et al., 2011; Ercal et al., 2001; Ersan et al., 2008). Cadmium is known to enhance the production of reactive oxygen species (ROS) (Liu et al., 2001). The toxic effect of Cd is controlled by the oxidation of cellular organelles by generating ROS reactions which lead to lipid peroxidation, apoptosis, damage of DNA and altered gene expression (Stohs et al., 1999; Wu et al., 2002; Thévenod, 2003). Therefore, to relieve Cd adverse effect the antioxidants induction is considered as an important therapeutic approach (Renugadevi and Prabu, 2010; Sinha et al., 2009).

At least 181 substances are included in the composition of...
of honey (Wang and Li, 2011). This composition varies primarily depending on the floral source rather than seasonal and environmental factors. Glucose (31%) and fructose (38%) represents the main contributors of honey solution. Minor constituents of honey have antioxidant properties as phenolic acids, enzymes, ascorbic acid, carotenoid-like substances and flavonoids (Andrade et al., 1997; Ferreres et al., 1992; Wang and Li, 2011; Cherchi et al., 1994). The present study aimed to investigate the protective role of honey (HY) opposing the chromosomal and sperm abnormalities induced by cadmium.

MATERIALS AND METHODS

Cadmium was used as cadmium chloride (CdCl₂) (Oxford Laboratory, Mumbai, India). The concentrations used was 0.67 mg/kg or 1/10 LD₅₀ according to Bench et al. (1999). Honey (HY) clover flower was purchased from Isis Company, Cairo, Egypt. 300 mg HY/kg b.w. was used as an optimal dose according to El-Raby (2010).

Animals

In this study, 30 adult Swiss albino mice (MUS musculus) were used, varying from 20 to 25 g in weight and aged three month old. These mice were obtained from the National Research Center (N.R.C.), Dukki, Cairo, Egypt, were caged individually under standard conditions of light, temperature, humidity and fed with standard pellet diet and water ad libitum.

Experiment

The experiment was categorized in six groups of five animals each. Group I was the control group, group II: mice were i.p injected with 0.5 ml saline solution daily for 96 h, as positive control, group III: mice were i.p injected, as a single dose, with 0.67 mg CdCl₂/kg b.w. dissolved in 0.5 ml saline solution, for 96 h, group IV: mice were injected i.p with 300 mg honey (HY)/kg b.w. dissolved in 0.5 ml saline solution daily for 96 h, group V (HYCd): mice were injected i.p with HY daily for 96 h, then single dose of CdCl₂ for 96 h (as a protective trial), and group VI (CdHY): mice were i.p injected, single dose, CdCl₂ for 96 h then followed by four consecutive honey doses, for 96 h (as a treatment trial).

Cytogenetical study

Colchicine was injected intraperitoneally 2 to 3 h before sacrificing. Bone marrow was extracted from femur bone and metaphases according to the method of Yosida and Amano (1965). The preparations of mitotic chromosome were made according to Ford and Hamerton (1956). Giemsa stain (7%) in phosphate buffer (pH 6.8) was used for slides. Hundred spreads metaphases per animal were investigated for chromosomal aberration analysis.

Sperm head morphology assay

Cauda epididymides were excised and both epididymides were minced together in isotonic medium then filtered to exclude large fragments. The cells' smears were prepared and stained with 5% Eosin Y. Light microscope (100x) green filter was used to examine smears. Thousand sperms were assessed for each animal to investigate the morphology of sperm abnormality according to the criteria of Wyrobek and Bruce (1975). Any overlay or contact sperms or heads without tails were ignored.

Statistical analysis

Mean ± SE was expressed to all values where five animals were evaluated, n=5, in each group. Statistical analysis of cytogenetic was performed on SPSS software (version 18) using one-way analysis of variance (ANOVA) test. Significance was considered when P values less than 0.05.

RESULTS

Various chromosomal aberrations were observed in the bone marrow cells of albino mice injected with cadmium. These were of structural and numerical type, were identified and quantitated relative to non-treated control (Figure 1A).

Structural aberration included chromatid deletions (Figure 1B); fragments (Figure 1C); centric fusion (Figure 1D); centromeric attenuation (Figure 1E); end to end (Figure 1F); rings; gap (Figure 1G) (Figure 1H); polyploidy (Figure 1I); stickiness (Figure 1J). A chromatid was considered to have a gap when it had an unstained area shorter than its diameter or equal to it (Figure 1G). End to end association was scored when two chromatids of different chromosomes appeared attached this could result from reciprocal translocation or stickiness (Figure 1F). The stickiness is considered as assort of chromosomal agglutination of unknown nature which resulted in a pycnotic or sticky appearance of chromosome (Figure 1J).

As shown in Table 1, the mean number nuclei with chromosomal aberration in mice treated with cadmium appear high significant of chromosomal aberrations in all animals compared with control. The more types of aberration appeared with chromosomal fragments, centric fusion, gaps, stickiness and aneuploidy. Also the mitotic activity shows decrease in the treated groups with cadmium chloride when compared to control. Honey administration has the same results as control of chromosomes abnormalities and the rate of cell division. Honey as a protective or treatment dose lead to decrease of chromosome aberration with increase in cell division that shows Cd HY and HY Cd groups could protect the body from cytotoxicity arising from cadmium.

Figure 2 shows normal sperm (a), hummer sperm (b), without hook (c) and banana shape (d). Honey recorded increased in number normal sperm head (Figure 3). Honey as protective or treatment group (Cd HY or HY Cd) could protect the body from cytotoxicity arising from...
Figure 1. Metaphase spread from mouse bone marrow cells showing: (a) normal chromosomes spread, (b) deletion, (c) fragment, (d) centric fusion, (e) centromeric attenuation, (f) centric attenuation, (g) end to end, (h) ring, (i) polyploidy, (j) stickiness. All aberrations are indicated by arrows.
Table 1. Average of chromosomal aberration observed in bone marrow cells of male mice treated with Cd and HY.

<table>
<thead>
<tr>
<th>Group</th>
<th>Fragment</th>
<th>Centric fusion</th>
<th>Gap</th>
<th>Stickiness</th>
<th>Trisomy</th>
<th>Total</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.0±0.5</td>
<td>8.2±0.7</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>0.2±0.8</td>
<td>12.4±2</td>
<td>1.24</td>
</tr>
<tr>
<td>+Control</td>
<td>5.8±0.7</td>
<td>8.6±1.0</td>
<td>0.0±0.0</td>
<td>0.0±0.0</td>
<td>0.2±0.6</td>
<td>14.6±2</td>
<td>1.46</td>
</tr>
<tr>
<td>HY</td>
<td>4.8±0.6</td>
<td>5.4±0.7</td>
<td>0.2±0.2</td>
<td>0.0±0.0</td>
<td>0.2±0.7</td>
<td>10.6±2</td>
<td>1.1</td>
</tr>
<tr>
<td>Cd</td>
<td>6.8±0.4</td>
<td>11.8±1.5</td>
<td>0.4±0.2</td>
<td>1.0±0.3</td>
<td>1.4±0.3</td>
<td>21.4±2</td>
<td>2.1</td>
</tr>
<tr>
<td>CdHY</td>
<td>5.0±1.0</td>
<td>8.0±1.1</td>
<td>0.0±0.0</td>
<td>0.8±0.3</td>
<td>7.4±2.8</td>
<td>0.74</td>
<td></td>
</tr>
<tr>
<td>HYCd</td>
<td>5.0±0.7</td>
<td>9.0±1.2</td>
<td>0.0±0.0</td>
<td>0.6±0.2</td>
<td>15.2±2.4</td>
<td>1.52</td>
<td></td>
</tr>
</tbody>
</table>

Average was expressed as mean±standard error. The significances were indicated as follow: ‘a’significant with control, ‘b’significant with +control, ‘c’significant with HY, ‘d’significant with Cd, ‘e’significant with Cd Hy, ‘f’significant with HY Cd. Significant means P<0.05.

Figure 2. (a) Normal sperm, (b) hummer sperm, (c) without hook, (d) Banana shape.
cadmium. The same values of normal sperm were recorded (Figure 3).

**DISCUSSION**

Currently, chromosomal fragment, centric fusion, gap, stickiness and aneuploidy were produced by Cd injection. Other study revealed that Cd induced chromosomal break, centric fusion, terminal association and C-mitosis in *Oreochromis mossambica* (Chandra and Khuda-Bukhszh, 2004). Cd burden has been correlated with chromosomal aberrations (IARC 1993). Singh et al. (2007) showed that low dose of Cd (1 mg/kg/day) for 30 days resulted in chromosomal aneuploidy, breaks, gaps and centromeric fusion. However, dose of 25 mg Cd/kg/day for 20 days and 200 mg/kg/day for five days resulted in severe damage of chromosome. Singh and Sankhla (2010) have assured that Cd increased the number of chromosomal aberrations and declined the mitotic index. On the other hand, the Cd exposure has been shown to stimulate free radical production, resulting in various pathological conditions in humans and animals (El-Demerdash et al., 2004; Shaikh et al., 1999). Acute or chronic exposure of Cd has been associated with increased lipid peroxidation in testes, erythrocytes, and other soft tissues (Manca et al., 1991; Sarkar et al., 1997). Free-radicals can originate from exposure to cadmium, resulting in elevated chromosomal aberrations and declined mitotic index. Abnormal sperm population was observed in the present study. Due to the effect of Cd-induced ROS on specific gene loci of germ cell chromosomes, it may dysfunction the maintenance of normal sperm structure. Most of the germ cells have been destroyed in Cd-treated mice due to either membranous or macromolecular damage incurred by formation of ROS leading to declined sperm count and ultimately testicular weight loss (Acharya et al., 2003; Oldereid et al., 1994); as well as the decreased sperm count and alterations in motility have been associated with cigarette smoking (Kulikaukas et al., 1985).

Honey administration has the same results as control of chromosomes abnormalities and the rate of cell division. Honey as protective or treatment dose lead to decrease in chromosome aberration with increase in cell division that shows Cd HY and HY Cd groups could protect the body from cytotoxicity arising from cadmium. Honey could be used to prevent and eliminate mutation caused by aflatoxins (Sharmanov et al., 1986). This was in accordance with reducing the chromosomal aberrations induced by aflatoxins (Sharmanov et al., 1986). The chromosomal aberrations induced by mycotoxins could be minimized by honey administration (Ezz El-Arab et al., 2006). Using 300 mg HY/kg against the brodifacoum pesticide, mitomycin-c anticancer drug and zinc phosphide pesticides lead to chromosome aberration, micronucleus formation and sperm head abnormalities (El-Raby, 2006, 2007, 2010). The association of honey
with cyclophosphamide (CPM) ameliorates the evaluated reduced glutathione (GSH), malondialdehyde (MDA) and chromosomal aberrations. Zoheir et al. (2015) also stated that honey inhibited the cytotoxic and genotoxic risks associated with the treatment by CPM in mice. Royal jelly has shown a protective effect in opposition to chromosomal abnormalities in bone marrow and histological alterations in kidney produced by valproic acid in male mice (Galaly et al., 2014). The treatment with flavonoids significantly reduced the chromosome abnormalities and delayed tumorigenesis in adult mice exposed to fetal irradiation (Uma Devi and Satyamitra, 2004). Honey sugar displayed complex behavior toward the enhanced or inhibited mutagenic activity in a model by cooked food systems (Skog, 1993). Royal jelly (RJ) had shown highly efficient antioxidant where it scavenges the produced free radicals (Cemek et al., 2010; El-Nekeety et al., 2007; Türkmen, 2009). El-Monem (2011) also revealed that RJ caused a significant recovery in antioxidant status. However RJ reduced glutathione (GSH) and inhibited malondialdehyde production which ameliorated DNA damage and genotoxicity induced by malathion in rat cells. Inoue et al. (2003) and Narita et al. (2006) previously reported the protection effect of RJ to DNA and the stimulation of bone marrow formation (Narita et al., 2006).

Honey recorded increased in the number of normal sperm head. Honey as protective or treatment group (Cd HY or HY Cd) could protect the body from cytotoxicity arising from cadmium. The same values of normal sperm were recorded. Syazana et al. (2011) observed the increased sperm count, percentage of normal sperm and reduced percentage of sperm abnormalities as a result of the Malaysian Gelam honey administration in male rats. Interperitoneal injection of nicotine could adversely affect sperm qualities, and Gelam honey was useful to increase the sperm motility and number that might increase the fertility of juvenile male rats (Asiyah et al., 2011). On the other hand, flavonoids and phenolic acids were found to improve semen quality and quantity, as well as decrease the sperm-shape abnormalities and histological damage in reproductive tissues of different animals exposed to several toxicants (Hala et al., 2010; Purdy et al., 2004; Türk et al., 2008). Moreover, the sexual behavior and fertility have been increased in male rats by honey administration against the toxic effect of cigarette smoke (Mohamed et al., 2012). Haron and Mohamed (2015) observed increased tests and epididymis weights as well as the sperm motility and percentages of normal spermatzoa in male rat offspring after honey supplementation during prenatal restraint. The honey supplementation seemed to reduce the adverse effects of restraint stress on reproductive organs weight and sperm parameters in male rat offspring (Haron and Mohamed, 2015). Also Mohamed et al. (2012) added that honey at dose of 1.2 g/kg may enhance spermatogenesis in adult rats.

Conclusion

From the current study, we can conclude that both the treatment and protection with honey ameliorated the adverse effects of Cd on chromosome and sperm. The cover honey as monofloral honey was the type of honey collected by most beekeepers and consumed by Egyptians.

Conflict of Interests

The authors have not declared any conflict of interests.

REFERENCES


Shelf life improvement of sorghum beer (pito) through the addition of *Moringa oleifera* and pasteurization

Florence Adwoa Ayirezang¹*, Courage Kosi Setsoafia Saba¹, Francis Kweku Amagloh² and Hellie Gonu¹

¹Department of Biotechnology, Faculty of Agriculture, University for Development Studies, Tamale, Ghana.
²Food Processing Technology Unit, Faculty of Agriculture, University for Development Studies, Tamale, Ghana.

Received 22 July, 2016; Accepted 7 September, 2016

Pito is a traditional alcoholic beverage that is mostly brewed in the three northern regions of Ghana. Although widely consumed and used in many festivities, poor storability limits its economic potential as an income-generating venture for most women. This study was carried out to improve the shelf-life of pito through the addition of *Moringa oleifera* leaf extract and pasteurization (75 to 80°C). Microbial enumeration, physico-chemical parameters (pH, extract (%) and alcohol) and consumer preference scores were used as quality indices of each pito treatment carried out. There was a general decline in coliform and fungi growth and in the physico-chemical (pH, extract (%) and alcohol) properties during the 56 days of storage. Microbial load, extract (%), alcohol content and pH were significantly different (P<0.05) among treatments. Pasteurized moringa pito had the least microbial load. The treated pito samples had higher values in pH, extract (%) and alcohol content than the untreated pito during storage. There was high consumer acceptability of pasteurized pito from the 0 day to the 28 days of storage, with a mean score of 4.27 ± 0.75 to 3.61 ± 1.36. However, the moringa treated pito (pasteurized moringa pito and moringa pito) was less preferred (with a mean score of 2.86 ± 1.19 to 1.87 ± 0.92) from the 0 day to the 28 days of storage. The untreated pito was also acceptable for a period of seven days.

Based on the findings of this research, it can be concluded that pasteurization and/or the addition of *M. oleifera* leaf extract can improve the shelf-life of pito for four weeks, but addition of moringa extract in pito reduced consumer preference for it. Further research using other antimicrobial plants is recommended as consumers did not like pito with the *M. oleifera* leaf extract.

**Key words:** *Moringa*, pasteurization, pito, shelf-life, storage.

**INTRODUCTION**

The brewing and drinking of traditional beverages are intrinsic part of the culture of the African people. Traditional brewed beverages are characterized by good mineral composition such as calcium, magnesiu,
sodium, zinc, potassium and iron, which are necessary for regulating and building the living cells (Kolawole et al., 2007; Duodu et al., 2012) and also contain probiotic properties (Moslehi-Jenabian et al., 2010). One of such traditionally brewed beverage in Ghana is pito (sorghum beer). Pito is brewed and consumed by people of the West African sub regions (Demuyakor and Ohta, 1993). Pito brewing is usually processed at households by women and linked with the people of northern (Upper West, Upper East and Northern Region) Ghana. It is an income generating business which serves as a source of employment in areas in which they are produced. However, pito production is limited by short shelf-life; as a result, the product needs to be consumed within a day (Demuyakor, 1994).

The causes of short shelf-life of pito have been intensively studied. Microbial contamination and activity from poor pito brewing practices is mainly the cause of spoilage (Novellie, 1962; Ekundayo, 1969; Paparusi et al., 1973; Dhir, 1978; Tisekwa, 1989). These microbes include diverse array of bacteria and wild yeasts with lactic acid bacteria being the dominant spoilers (Sakamoto and Konings, 2003). Spoilage of pito is attributed to undesirable changes in sensory characteristics in terms of texture, smell, taste or appearance (Doyle, 2001) which lead to discarding the whole product. Microbial spoilage of alcoholic beverages is of critical importance hence, different methods have been adapted to reduce spoilage. Among these methods are thermal treatment (pasteurization), filtration and chemical treatment (addition of artificial preservatives) (Ellis et al., 2005; Onagbe and Izuagbe, 1989; Osseyi et al., 2011). Instances when pasteurized at 75°C for 30 min and addition of sorbic acid concentration of 5% improved the shelf-life of pito for a period of four weeks (Onagbe and Izuagbe, 1989). Some of these methods can be sophisticated and expensive for the small scale operator to adopt. Ellis et al. (2005) also improved the shelf-life of filtered pito for eight weeks by pasteurization (60 to 70°C for 15 min) and adding sodium metabisulphite. Increasing the shelf life of pito via chemical means such as sodium metabisulphite can have adverse effects such as respiratory tract irritation and anaphylactic symptoms which is life threatening (Pavord et al., 1991; Vally et al., 2009).

Although synthetic antimicrobial and antioxidant agents are approved in many countries, its usage has created environmental and health concerns, which has called for natural, safe and effective preservatives by consumers and producers (Ortega-Ramirez et al., 2014; Regnier et al., 2012). Ortega-Ramirez et al. (2014) proposed that medicinal plants traditionally used to treat health disorders and prevent diseases can serve as a source of bioactive compounds for food additives. This is because these medicinal plants are rich in antimicrobial phytochemicals. M. oleifera leaf, in that regard is also found to possess antimicrobial properties (Eilert et al., 1981).

Information on the use of M. oleifera in the brewing of sorghum beer has not received any attention. Therefore, using pasteurization and a natural preservative (M. oleifera), which is easily accessible in Ghana and in the tropics (Fahey, 2005; Quarcoo, 2008) in pito comparatively can be less expensive.

In light of the highlighted aforementioned problem associated with the pito industry, ways are needed to supply it to the citizens in a more quality and presentable manner. Hence the relevance of this study is to improve the shelf-life of pito by the addition of M. oleifera leaves under producers’ condition, combined with pasteurization at 75 to 80°C for 15 min.

MATERIALS AND METHODS

Sample collection

Moringa oleifera processing

Fresh young green moringa (M. oleifera) leaves were collected from the Nyankpala community into a clean polyethylene bag. The leaves were washed thoroughly under tap running water, and dried at a room temperature (29 ± 2°C) and relative humidity of 46.5 to 61.7% (Ghana Meteorological Agency, 2014) for 72 h. The dried leaves were milled into fine powder using Philips blender HR2000/16. The moringa powder was collected into a clean airtight bowl, and then boiled in 1 litre conical flask, stoppered and kept for 1 week in a refrigerator at 5°C.

Obtaining Moringa oleifera extracts

Dried moringa powder of 50 g was added to 500 ml distilled water in 1 litre conical flask, stoppered and kept for 1 week in a refrigerator (5°C) with periodical manual shaking. The extract was filtered using a clean, sterilized muslin cloth, and then boiled for 30 min with continuous stirring.

Sourcing of pito

Dagarti pito samples were obtained from a commercial brewer in Nyankpala Township, in the Tolon District of Northern Region. Dagarti pito was prepared as shown in the flow chart.

Treatments

In this study, four treatments were applied thus: untreated pito (control), pasteurised pito, moringa pito (moringa leaf extract + pito) and pasteurized moringa pito. Nine (9) litres of each treatment was dispensed from kegs into sterilised 300 ml glass bottles and crowned immediately using a manual hand crowner, then packaged into its respective box and labelled. Moringa pito treatments had M. oleifera extracts and pito in the ratio 1:3 (v/v). The ratio of moringa to pito was achieved through preliminary study of moringa pito, which showed that 25% moringa extract composition of the beverage was preferred by consumers. All pasteurised treatment samples were carried at 75 to 80°C for 15 min using a water bath. Pasteurised samples were allowed to cool before storage. The samples were stored for two months at an ambient temperature. Analysis were carried during 0, 7, 14, 28 and 56 days of storage.

Microbial analysis of samples

Microbial analysis was carried out for each treatment thus untreated
pito, pasteurised pito, moringa pito, and pasteurized moringa pito during 0, 7, 14, 28 and 56 days of storage. MacConkey agar and potato dextrose agar were used to determine coliforms and fungi (moulds and yeast), respectively. MacConkey agar and potato dextrose agar media were prepared using the manufacturer’s protocol from Sigma-Aldrich. The pour plate method as described by Hoben and Somasegaran (1982) was used in the microbial count.

Analysis of physico-chemical properties

Four replicates of each treatment (untreated pito, pasteurised pito, moringa pito and pasteurized moringa pito) were sampled and examined for pH, sugar and alcohol levels at 0, 7, 14, 28 and 56 days of storage. The pH was determined using basic 20 pH meter and the hand held refractometer was used to measure extract % in mass saccharose. The alcohol content was carried out using the specific gravity method described by Mathapati et al. (2010).

Sensory evaluation of bottled pito

Sensory evaluation of each treatment (untreated pito, pasteurised pito, moringa pito, and pasteurized moringa pito) was assessed by 150 subjects (taste panel). The panel consists of students and lecturers of UDS Nyankpala Campus, as well as community members from Nyankpala, selected based on their familiarity with pito. Consumer preferences of the samples listed were compared at 0, 7, 14, 28 and 56 days of storage. Sensory ballot sheet was provided for each subject and the sensory scale adapted was the 5-point hedonic scale, just-about right scale and the forced-choice (yes/no) scale (Stone and Sidel, 2004). Samples were coded and presented to the assessors to indicate their preferences. Samples were served in clean, transparent plastic containers with tight lids.

Statistical analysis

Data obtained from the paired preference test were analysed using the Microsoft Excel Programme. All other data were subjected to analysis of variance (ANOVA) for variation of means of treatments with their respective period of storage, using the Genstat Discovery edition 4. Multiple mean comparisons were also carried out with the Minitab. Statistical significance was set at P < 0.05.

RESULTS

Microbial quality of samples during storage

Microbial quality of pito beverage in this study reveals the presence of enterobacteriaceae (coliforms) when samples were analysed on MacConkey. Coliform count of samples of untreated pito, pasteurized pito, moringa pito and pasteurized moringa pito were between $1.5 \times 10^3$ to $40 \times 10^3$, $2.6 \times 10^3$ to $3.1 \times 10^5$, $1.3 \times 10^5$ to $7.1 \times 10^5$ and $0.8 \times 10^5$ to $1.5 \times 10^5$, respectively during 56 days of storage (Figure 5). Coliform growth/population was significantly different (P < 0.05) among treatment during storage. Coliform growth and population reduced in each pito treatment during the period of storage (Figure 2). Varied levels of coliforms were, however, observed with respect to treatments as well as the duration of storage.

There was a general decline of coliform growth during storage. Pasteurized pito, moringa pito and pasteurized moringa pito had similar (P > 0.05) coliform count. The treated pito (pasteurized, moringa and pasteurized moringa pito) showed a relative reduction of coliform growth as compared to the untreated pito 0 day of storage. The reduction of pasteurized moringa pito was two times that of the pasteurized pito and four times that of the moringa pito. The untreated pito recorded the highest coliform growth than the treated pito samples.

As in the coliform growth, there was also a general decline of fungi growth during storage (Figure 3). The study reveals the presence of fungi in each treatment. Mycological count of samples of untreated pito, pasteurized pito, moringa pito and pasteurized moringa pito ranged between $4.6 \times 10^5$ to $37 \times 10^5$, $2.1 \times 10^5$ to $0.9 \times 10^5$, $21 \times 10^5$ to $1.4 \times 10^5$ and $0.7 \times 10^5$ to $0.2 \times 10^5$, respectively within 56 days of storage as shown in Figure 5. Pasteurized pito and pasteurized moringa pito had the low fungi load compared to untreated pito and moringa pito prior to storage and after storage. There was significance difference (P < 0.05) in fungi population among treatments. Generally, pasteurized moringa pito had relatively low fungi load.

Physico-chemical properties

pH levels

The pH value of pito treatments during storage is presented in Figure 1. Untreated pito, pasteurized pito, moringa pito and pasteurized pito had pH between 3.2 to 3.4, 3.2 to 3.5, 3.5 to 3.2 and 3.2 to 3.6, respectively during the entire period of storage (Figure 4). pH values were decreased during storage. The pasteurized pito and pasteurized moringa pito showed significantly (P < 0.05) higher pH values than the untreated pito from the 0 day to the 56 days of storage and with moringa pito from 0 day to the 28 days of storage. The level of pH reduction in the untreated pito, moringa pito and pasteurized pito was 0.2, 0.1 and 0.04 times that of pasteurized moringa pito, respectively.

Levels of extract in % mass saccharose

Levels of extract (%) in the various treatment of pito during storage are shown in Figure 5. There was a general decline in the levels of extract (%) in each treatment. Levels of extract (%) in untreated pito, pasteurized pito, moringa pito, and pasteurized moringa pito during 56 days of storage ranged from 5.00 to 7.00, 6.00 to 7.00, 4.20 to 6.52 and 5.00 to 6.28%, respectively. Pasteurized moringa pito were significantly (P < 0.05) higher in the extract (%) than in the untreated pito from the 7 days to the 56 days of storage, and for the
**Figure 1.** A flow chart of the brewing process of Dagarti pito (Demuyakor, 1994).

**Figure 2.** Viable number of coliforms when conventional and treated pito were stored for two months. Values are means and standard deviations of colony forming unit per millilitres; values that do not share the same letter are significantly different (P < 0.05). All treatments were stored at an ambient room temperature.
Figure 3. Viable number of fungi for conventional and modified pito stored for two months. Values are means and standard deviations of fungi forming units per millilitres; values that do not share the same letter are significantly different (P < 0.05). All treatments were stored at an ambient room temperature.

Figure 4. Levels of pH during 56 days. Values are the means ± standard deviation of quadruple determinations. Values that do not share the same letter are significantly different (P < 0.05). All treatments were stored at an ambient room temperature.

moringa pito from the 14 days to the 56 days of storage. The extract (%) in all the treatments was reducing with increasing duration of storage. Untreated pito, moringa pito and pasteurized pito, recorded a percentage difference of 19, 11 and 8%, in that order with respect to pasteurized moringa pito during storage. From 0 day to 14 days of storage, there was no significant (P > 0.05) reduction of extract (%) of the pasteurized pito and
Alcohol levels were between 1.96 and 3.93, 2.62 and 3.93, 2.10 and 3.44 and 2.62 and 3.28% in untreated pito, pasteurized pito, moringa pito and pasteurized moringa pito, respectively. Pasteurized pito, moringa pito and pasteurized moringa pito had higher alcohol content than the untreated pito from 7 days to 56 days of storage.

### Alcohol levels

The alcohol content decreased during storage (Figure 6).
There was significant difference ($P < 0.05$) in alcohol value in the samples investigated. The percentage difference of the untreated pito during the 56 days of storage with respect to pasteurized moringa pito was 33%; and moringa pito, pasteurized pito, recorded 24 and 20%, respectively. Statistically, no significant ($P > 0.05$) reduction in the alcohol levels was observed when the treated pito samples: pasteurized pito, moringa pito and pasteurized moringa pito, were stored for seven days.

**Sensory evaluation of bottled pito**

**Consumer preference for the degree of sourness of pito samples**

The degree of sourness of pito samples (Figure 7) showed that untreated pito samples were very sour after 0 day of storage; followed by moringa pito, then the pasteurized pito samples (pasteurized pito and pasteurized moringa pito) which became very sour after 14 and 28 days of storage, respectively. The preference of pito samples in the Ghanaian market (Figure 8) showed that respondents favoured pito samples without moringa extract in the Ghanaian market than those with the moringa extract.

The findings of this study (Table 1) clearly showed significant difference ($P < 0.05$) in the overall degree of liking. Pito samples without moringa extract were highly acceptable by the assessors than those with moringa extract. The untreated pito was disliked extremely by assessors after a week of storage. Pasteurized pito was extremely liked throughout a storage period of 4 weeks.
Table 1. Presentation of the acceptability of treatments during storage.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Storage (days)</th>
<th>Untreated pito</th>
<th>Pasteurized pito</th>
<th>Moringa Pito</th>
<th>Pasteurized moringa pito</th>
<th>Fpr &lt; 0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall degree of liking</td>
<td>0</td>
<td>4.41±0.70a</td>
<td>4.27±0.75ab</td>
<td>2.86±1.19abfg</td>
<td>2.83±1.27efg</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>3.87±0.99bc</td>
<td>4.29±0.69ab</td>
<td>2.60±1.22g</td>
<td>2.78±1.24g</td>
<td></td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>3.26±1.23de</td>
<td>4.23±0.73ab</td>
<td>2.69±1.08fg</td>
<td>2.65±1.11fg</td>
<td></td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>3.30±1.38de</td>
<td>3.61±1.36cd</td>
<td>2.55±1.37fg</td>
<td>2.65±1.31fg</td>
<td></td>
</tr>
<tr>
<td></td>
<td>56</td>
<td>2.51±1.51g</td>
<td>3.08±1.36ef</td>
<td>1.87±0.92h</td>
<td>1.91±1.09h</td>
<td></td>
</tr>
</tbody>
</table>

Values are means and standard deviations of triplicate determinations; values within interactions of individual sensory attributes that do not share the same letter are significantly different (P< 0.05). A 5-hedonic scale was used (1= dislike extremely/least acceptable; 3= neutral; 5= like extremely/highly acceptable).

DISCUSSION

Moringa pito exhibited antimicrobial activity which is a characteristic of moringa extract hence contributed to the reduction of coliforms in pito at zero day compared to the high coliform load in untreated pito. The heat shock from pasteurized pito and pasteurized moringa pito reduced coliform load in samples prior to storage. The lowest reduction of microbial load in pasteurized moringa pito can be attributed to the cocktail of treatment hence can enhanced microbial stability in pito during 56 days of storage. However the reduction of coliform load in untreated pito during storage would be as a result of spontaneous microbial activity; this include accumulation of lactic acid and acetic acids during storage produced by bacteria, which are detrimental to some sensitive bacteria (Ekundayo, 1969; Fadahunsi et al., 2013) as well as the lowering of pH values in pito during the storage which some coliforms cannot survive (Ray and Bhunia, 2013) hence explaining the drastic reduction of coliform population in untreated pito samples. Similar inference could be made for the reduction of coliform in various treatments applied to the sorghum beer (pito).

Unlike coliform, the mould population did not reduce very significantly in moringa pito. This implied that, antimicrobial activity of moringa varied with the type of microbe which confirms the findings made by some researchers, on the inhibitory effect of *M. oleifera* leaf extract on some selected fungal strains (Bukar et al., 2010; Devendra et al., 2011) but a combination of pasteurization and moringa extract treatment can reduce mould population to abiterally acceptable levels. This corroborates with previous reports that pasteurization is capable of inactivating microbial activity in traditionally brewed sorghum beers (Ellis et al., 2005; Osseyi et al., 2011). The significant reduction of the number of fungi growth in the untreated pito during storage might have resulted from the exhaustion of nutrients in the products, thus reducing the overall food availability for the microorganisms as reported by other researchers (Fadahunsi et al., 2013). Also, the growth of fungi might have been impeded by unfavourable conditions as stated in the case of coliforms. Generally, the addition of moringa extract and/or the heat treatment might have been the major contributing factor, influencing the overall reduction of microbial growth in moringa pito, pasteurized pito and pasteurized moringa pito.

In order to improve the shelf life of sorghum beer (pito), the addition of *M. oleifera* to and pasteurization of sorghum beer were employed in comparison with traditionally brewed sorghum beer (pito). The pH values for all pito samples (untreated pito, pasteurized pito, moringa pito, and pasteurized moringa pito) were within the stipulated pH range for sorghum beer of 3 to 4 as indicated in previous study of increasing shelf of pito and microbial assessment of pito during storage (Fadahunsi et al., 2013; Ellis et al., 2005; Kalawole et al., 2007). However, each treatment lacked stability with respect to pH (3.2 to 3.5) value, which can be attributed to activities of microbes with the pito samples during the period of storage. Microbial activities were much more in untreated pito which led to a lower pH value compared to moringa pito, pasteurized pito and pasteurized moringa pito which had higher pH values. The lower pH can also be attributed to organic acid produced by some microorganisms (bacteria, moulds and yeast) that were isolated in the pito treated samples and also suggested by Fadahunsi et al. (2013). The pH values recorded did not corroborate with Ellis et al. (2005) and Osseyi et al. (2011) reports in which sorghum beer pasteurized at 60 to 70°C had a stabilized pH value of 3.4 and 3.45 for 8 weeks and 6 months, respectively during storage. The high pH in moringa pito, pasteurized pito and pasteurized moringa pito signifies low microbial activity in the treated pito samples. Comparatively, pH variation in moringa pito, pasteurized pito, and pasteurized moringa was due to the difference in each treatment carried out. Moringa pito suggested that *Moringa oleifera* inclusion only could minize microbial activity to some extent when compared to untreated pito. Both pasteurized pito, and pasteurized moringa pito were better off in reducing microbial activity hence having a higher pH value than moringa pito and untreated pito. This implies that pasteurized pito, and pasteurized moringa pito would be less acidic compared
to moringa pito and untreated pito, since it has been reported that the souring in sorghum beer is owned to the presence of lactic acid bacteria or acetic acid bacteria (Ekundayo, 1969; Demuyakor, 1994; Steinkraus, 2004; Lyumugabe et al., 2012).

Levels of extract (% mass saccharose) in sorghum beer (pito) brewing were determined by fermentation period and yeast cells activities in pito brewing. The decline of the extract (%) was expected because sugar was converted into alcohol. The extract (%) showed a decline in all the treatments. This implied that fermentation was still on-going, hence the presence of some microbes and yeast cell as revealed in the microbiology of the pito sampled. The decline of the extract (%) which showed a reduction in sugar content in pito during 56 days storage corroborates with earlier report of Demuyakor and Ohta, (1993), thus the sugar content in pito reduced during storage since it served as carbon source for energy by the microorganisms present. The low extract (%) levels in moringa pito and pasteurized moringa prior to storage might be due to the 25% reduction in the volume of pito to allow for the addition of the moringa extract. Despite Moringa oleifera leaves known to contain carbohydrate (Mustapha and Babura, 2009), conversion rate into fermentable sugars may be low because of Saccharomyces cerevisiae inability to convert starch/simple carbohydrate to simple sugars and later to alcohol as well as the large proportion of carbohydrate in malted sorghum prior to fermentation (Lyumugabe et al., 2010). In addition, M. oleifera extract might have influenced S. cerevisiae activity hence translating into the low percentages of sugar in the pito at the initial stage of storage when treated with moringa extract (moringa and pasteurized moringa pito). Untreated pito encountered high microbial activity hence resulting in the rapid decline of the amount of sugar for 14 days of storage. Pasteurized pito had minimum about of microbial load hence resulting in sugar stability for 14 days of storage. This period could be described as lag periods/phase as sugar usage was almost negligible. The decline in extract (%) in pasteurised pito from day 14 to 28 might be due to microbial build up hence sugar utilization increased.

It was expected that, the amount of the sugar utilized should be equivalent to alcohol produced but this was not observed. Stability of alcohol level observed for a week (seven days) in pasteurized pito, pasteurized moringa pito and moringa pito could be attributed to treatment shock on microbes and change in the physico-chemical properties of the medium (pito), and hence sugar utilization as a carbon source by microbes and yeast cell was not efficient for the first seven days. Unlike untreated pito, it was characterized by alcohol reduction from day 1 to 14. Pasteurized pito, pasteurized moringa pito and moringa pito had a decrease in alcohol level after seven days. This implied that alcohol reducing microbes were associated with each sample. The reduction of alcohol produced more acids contributing to the sourness of pito during storage hence the low pH recorded for each sample during storage. The reduction of alcohol in all samples during storage would be linked to alcohol degrading microbes. Similar inference could be made for untreated pito. The significant reduction of alcohol in the untreated pito led to early spoilage. This observation indicated that for the efficient conversion of sugar to alcohol involves other factors (Demuyakor and Ohta, 1993; Lin, et al., 2012). The stability of alcohol level in untreated pito might have resulted from inadequate carbon source for the microorganisms present and also the low pH of the medium might not have been appropriate for some microbial activity. This study suggests that moringa and/or pasteurization treatments were capable of maintaining the alcohol content in the pito during storage for seven days of storage.

The undesirable sensory characteristics of sorghum pito are as a result of microbial presence and activity (metabolic processes) that leads to the sorghum beer spoilage. The presence of coliforms and moulds in the pito samples may have resulted from unhygienic practices during and after the brewing process of the pito, causing the relatively high increase of coliform and mould growth at the initial stage of storage in the untreated pito. The high coliform and fungi count in the fresh pito might have led to the present coliform in the pito samples after pasteurization and/or the addition of moringa extract. The intensity of the sourness is a reflection of the product acidity. The right degree of sourness is considered as part of the general characteristics of a good pito (Demuyakor, 1994); if it becomes very sour it is an indication of deterioration. Also, the untreated pito was very sour after seven days of storage indicating the impact of the decrease of pH level during storage. The panelists reported that the sourness was just about right for pasteurized pito, moringa pito and pasteurized moringa pito, until after 28 days of storage.

The overall degree of liking and potential of products being accepted on the Ghanaian market mirrored the choices made by panels in each of the sensory attributes earlier discussed. On the whole, pasteurized pito was mostly liked up to 28 days of storage. The untreated pito was further not preferred after seven days of storage. The reason may be attributed to off-flavours which alter the quality of the beer causing it to deteriorate (Harayama et al., 1991; Rodrigues et al., 2011). Also the pito treated with moringa extract which was less liked throughout the storage period shows that consumers are not familiar with the product, and this confirms report by Barcellos et al. (2009) that, some consumers find it very difficult to change.

**Conclusion**

The addition of moringa extract and/or pasteurization had
reduced the fungi and coliform growth in the treated pito samples than the untreated pito. The physical composition, that is, pH, sugar and alcohol levels were significantly influenced by pasteurization and the addition of *Moringa oleifera* leaf extract: The untreated pito had lower pH than the treated pito samples during storage. Comparatively causing the untreated pito to be very sour than the treated pito samples. Also the pasteurized pito, moringa pito and pasteurized moringa pito had higher sugar and alcohol content than the untreated pito during storage. Although pito with the moringa did improve the shelf life, organoleptically, it was less liked by the assessors. The sensory results showed that pasteurized pito was more preferred by the consumers. Pasteurized pito was liked for the four weeks of storage. Based on the findings of this study, the research concludes that the shelf life of pito can be improved through pasteurization and/or the addition of *Moringa oleifera* leaf extract for 28 days, however pito samples that contained the moringa extract was less favoured by consumers.

**Conflict of Interests**

The authors have not declared any conflict of interests.

**REFERENCES**


African Journal of Biotechnology

Related Journals Published by Academic Journals

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