ABOUT AJB

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

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

Contact Us

Editorial Office: ajb@academicjournals.org
Help Desk: helpdesk@academicjournals.org
Website: http://www.academicjournals.org/journal/AJB
Submit manuscript online http://ms.academicjournals.me/
Editor-in-Chief

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

Associate Editors

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

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

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

Dr. Ismail TURKOGLU
Department of Biology Education, Education Faculty, Fırat University,
Elazığ, Turkey

Prof T.K.Raja, PhD FRSC (UK)
Department of Biotechnology
PSG COLLEGE OF TECHNOLOGY (Autonomous)
(Affiliated to Anna University)
Coimbatore-641004, Tamilnadu, INDIA.

Dr. George Edward Mamati
Horticulture Department,
Jomo Kenyatta University of Agriculture and Technology,
P. O. Box 62000-00200,
Nairobi, Kenya.

Dr. Gitonga
Kenya Agricultural Research Institute,
National Horticultural Research Center,
P.O Box 220,
Thika, Kenya.
Editorial Board

Prof. Sagadevan G. Mundree
Department of Molecular and Cell Biology
University of Cape Town
Private Bag Rondebosch 7701
South Africa

Dr. Martin Fregene
Centro Internacional de Agricultura Tropical (CIAT)
Km 17 Cali-Palmira Recta
AA6713, Cali, Colombia

Prof. O. A. Ogunseitan
Laboratory for Molecular Ecology
Department of Environmental Analysis and Design
University of California, Irvine, CA 92697-7070. USA

Dr. Ibrahima Ndoye
UCAD, Faculte des Sciences et Techniques
Departement de Biologie Vegetale
BP 5005, Dakar, Senegal.
Laboratoire Commun de Microbiologie
IRD/ISRA/UCAD
BP 1386, Dakar

Dr. Bamidele A. Iwalokun
Biochemistry Department
Lagos State University
P.M.B. 1087. Apapa – Lagos, Nigeria

Dr. Jacob Hodeba Mignouna
Associate Professor, Biotechnology
Virginia State University
Agricultural Research Station Box 9061
Petersburg, VA 23806, USA

Dr. Bright Ogheneovo Agindotan
Plant, Soil and Entomological Sciences Dept
University of Idaho, Moscow
ID 83843, USA

Dr. A.P. Njukeng
Département de Biologie Végétale
Faculté des Sciences
B.P. 67 Dschang
Université de Dschang
Rep. du CAMEROUN

Dr. E. Olatunde Farombi
Drug Metabolism and Toxicology Unit
Department of Biochemistry
University of Ibadan, Ibadan, Nigeria

Dr. Stephen Bakiamoh
Michigan Biotechnology Institute International
3900 Collins Road
Lansing, MI 48909, USA

Dr. N. A. Amusa
Institute of Agricultural Research and Training
Obafemi Awolowo University
Moor Plantation, P.M.B 5029, Ibadan, Nigeria

Dr. Desouky Abd-El-Haleem
Environmental Biotechnology Department &
Bioprocess Development Department,
Genetic Engineering and Biotechnology Research
Institute (GEBRI),
Mubarak City for Scientific Research and Technology
Applications,
New Burg-Elarab City, Alexandria, Egypt.

Dr. Simeon Oloni Kotchoni
Department of Plant Molecular Biology
Institute of Botany, Kirschallee 1,
University of Bonn, D-53115 Germany.

Dr. Eriola Betiku
German Research Centre for Biotechnology,
Biochemical Engineering Division,
Mascheroder Weg 1, D-38124,
Braunschweig, Germany

Dr. Daniel Masiga
International Centre of Insect Physiology and Ecology,
Nairobi,
Kenya

Dr. Essam A. Zaki
Genetic Engineering and Biotechnology Research
Institute, GEBRI,
Research Area,
Borg El Arab, Post Code 21934, Alexandria
Egypt
Dr. Alfred Dixon  
International Institute of Tropical Agriculture (IITA)  
PMB 5320, Ibadan  
Oyo State, Nigeria

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

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

Dr. Solomon Olawale Odemuyiwa  
Pulmonary Research Group  
Department of Medicine  
550 Heritage Medical Research Centre  
University of Alberta  
Edmonton  
Canada T6G 2S2

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

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

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

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

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

Dr. Jones Lemchi  
International Institute of Tropical Agriculture (IITA)  
Onne, Nigeria

Prof. Greg Blatch  
Head of Biochemistry & Senior Wellcome Trust Fellow  
Department of Biochemistry, Microbiology & Biotechnology  
Rhodes University  
Grahamstown 6140  
South Africa

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

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

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

Dr. Deborah Rayfield  
Physiology and Anatomy  
Bowie State University  
Department of Natural Sciences  
Crawford Building, Room 003C  
Bowie MD 20715, USA
Dr. Marlene Shehata
University of Ottawa Heart Institute
Genetics of Cardiovascular Diseases
40 Ruskin Street
K1Y-4W7, Ottawa, ON, CANADA

Dr. Hany Sayed Hafez
The American University in Cairo,
Egypt

Dr. Clement O. Adebooye
Department of Plant Science
Obafemi Awolowo University, Ile-Ife
Nigeria

Dr. Ali Demir Sezer
Marmara Üniversitesi Eczacilik Fakültesi,
Tibbiye cad. No: 49, 34668, Haydarpasa, Istanbul,
Turkey

Dr. Ali Gazanchian
P.O. Box: 91735-1148, Mashhad,
Iran.

Dr. Anant B. Patel
Centre for Cellular and Molecular Biology
Uppal Road, Hyderabad 500007
India

Prof. Arne Elofsson
Department of Biophysics and Biochemistry
Bioinformatics at Stockholm University,
Sweden

Prof. Bahram Goliaei
Departments of Biophysics and Bioinformatics
Laboratory of Biophysics and Molecular Biology
University of Tehran, Institute of Biochemistry and
Biophysics
Iran

Dr. Nora Babudri
Dipartimento di Biologia cellulare e ambientale
Università di Perugia
Via Pascoli
Italy

Dr. S. Adesola Ajayi
Seed Science Laboratory
Department of Plant Science
Faculty of Agriculture
Obafemi Awolowo University
Ile-Ife 220005, Nigeria

Dr. Yee-Joo TAN
Department of Microbiology
Yong Loo Lin School of Medicine,
National University Health System (NUHS),
National University of Singapore
MD4, 5 Science Drive 2,
Singapore 117597
Singapore

Prof. Hidetaka Hori
Laboratories of Food and Life Science,
Graduate School of Science and Technology,
Niigata University.
Niigata 950-2181,
Japan

Prof. Thomas R. DeGregori
University of Houston,
Texas 77204 5019,
USA

Dr. Wolfgang Ernst Bernhard Jelkmann
Medical Faculty, University of Lübeck,
Germany

Dr. Moktar Hamdi
Department of Biochemical Engineering,
Laboratory of Ecology and Microbial Technology
National Institute of Applied Sciences and Technology.
BP: 676. 1080,
Tunisia

Dr. Salvador Ventura
Department de Bioquímica i Biologia Molecular
Institut de Biotecnologia i de Biomedicina
Universitat Autònoma de Barcelona
Bellaterra-08193
Spain

Dr. Claudio A. Hetz
Faculty of Medicine, University of Chile
Independencia 1027
Santiago, Chile

Prof. Felix Dapare Dakora
Research Development and Technology Promotion
Cape Peninsula University of Technology,
Room 2.8 Admin. Bldg. Keizersgracht, P.O. 652, Cape
Town 8000,
South Africa
Prof. George N. Goulielmos  
Medical School,  
University of Crete  
Voutes, 715 00 Heraklion, Crete, Greece

Dr. Uttam Krishna  
Cadila Pharmaceuticals limited,  
India 1389, Tarsad Road, Dhalka, Dist: Ahmedabad, Gujarat, India

Prof. Mohamed Attia El-Tayeb Ibrahim  
Botany Department, Faculty of Science at Qena,  
South Valley University, Qena 83523, Egypt

Dr. Nelson K. Ojjio Olang’o  
Department of Food Science & Technology,  
JKUAT P. O. Box 62000, 00200, Nairobi, Kenya

Dr. Pablo Marco Veras Peixoto  
University of New York NYU College of Dentistry  
345 E. 24th Street, New York, NY 10010 USA

Prof. T E Cloete  
University of Pretoria Department of Microbiology and Plant Pathology,  
University of Pretoria, Pretoria, South Africa

Prof. Djamel Saidi  
Laboratoire de Physiologie de la Nutrition et de Sécurité  
Alimentaire Département de Biologie, Faculté des Sciences, Université d’Oran, 31000 - Algérie  
Algeria

Dr. Tomohide Uno  
Department of Biofunctional chemistry,  
Faculty of Agriculture Nada-ku, Kobe., Hyogo, 657-8501, Japan

Dr. Ulises Urzúa  
Faculty of Medicine,  
University of Chile Independencia 1027, Santiago, Chile

Dr. Aritua Valentine  
National Agricultural Biotechnology Center, Kawanda  
Agricultural Research Institute (KARI)  
P.O. Box, 7065, Kampala, Uganda

Prof. Yee-Joo Tan  
Institute of Molecular and Cell Biology 61 Biopolis Drive,  
Proteos, Singapore 138673  
Singapore

Prof. Viroj Wiwanitkit  
Department of Laboratory Medicine,  
Faculty of Medicine, Chulalongkorn University, Bangkok  
Thailand

Dr. Thomas Silou  
Universitt of Brazzaville BP 389  
Congo

Prof. Burtram Clinton Fielding  
University of the Western Cape  
Western Cape, South Africa

Dr. Brnčić (Brncic) Mladen  
Faculty of Food Technology and Biotechnology, Pierottijevo 6,  
10000 Zagreb, Croatia.

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

Dr. Idress Hamad Attitalla  
Omar El-Mukhtar University,  
Faculty of Science, Botany Department, El-Beida, Libya.

Dr. Linga R. Gutha  
Washington State University at Prosser,  
24106 N Bunn Road, Prosser WA 99350-8694
Dr Helal Ragab Moussa  
Bahny, Al-bagour, Menoufia, Egypt.

Dr VIPUL GOHEL  
DuPont Industrial Biosciences  
Danisco (India) Pvt Ltd  
5th Floor, Block 4B, DLF Corporate Park DLF Phase III Gurgaon 122 002 Haryana (INDIA)

Dr. Sang-Han Lee  
Department of Food Science & Biotechnology, Kyungpook National University Daegu 702-701, Korea.

Dr. Bhaskar Dutta  
DoD Biotechnology High Performance Computing Software Applications Institute (BHSAI) U.S. Army Medical Research and Materiel Command 2405 Whittier Drive Frederick, MD 21702

Dr. Muhammad Akram  
Faculty of Eastern Medicine and Surgery, Hamdard Al-Majeed College of Eastern Medicine, Hamdard University, Karachi.

Dr. M. Muruganandam  
Department of Biotechnology St. Michael College of Engineering & Technology, Kalayarkoil, India.

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

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

Dr. Takuji Ohyama  
Faculty of Agriculture, Niigata University

Dr Mehdi Vasfi Marandi  
University of Tehran

Dr Fügen DURLU-ÖZKAYA  
Gazi University, Tourism Faculty, Dept. of Gastronomy and Culinary Art

Dr. Reza Yari  
Islamic Azad University, Boroujerd Branch

Dr Zahra Tahrnasebi Fard  
Roudehen branche, Islamic Azad University

Dr Albert Magri  
Giro Technological Centre

Dr Ping ZHENG  
Zhejiang University, Hangzhou, China

Dr. Kgomoeto P. Sibeko  
University of Pretoria

Dr Jian Wu  
Harbin medical university, China

Dr Hsiu-Chi Cheng  
National Cheng Kung University and Hospital.

Prof. Pavel Kalac  
University of South Bohemia, Czech Republic

Dr Kürsat Korkmaz  
Ordu University, Faculty of Agriculture, Department of Soil Science and Plant Nutrition

Dr. Shuyang Yu  
Department of Microbiology, University of Iowa Address: 51 newton road, 3-730B BSB bldg. Iowa City, IA, 52246, USA
Dr. Mousavi Khaneghah  
*College of Applied Science and Technology-Applied Food Science, Tehran, Iran.*

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

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

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

<table>
<thead>
<tr>
<th>Title</th>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Effect of salt stress on growth and contents of organic and inorganic compounds in noni (<em>Morinda citrifolia</em> L.)</td>
<td>2401</td>
</tr>
<tr>
<td>Ailde Mitsue Watanabe Cova, André Dias de Azevedo Neto, Rogério Ferreira Ribas, Hans Raj Gheyi and Renata Velasques Menezes</td>
<td></td>
</tr>
<tr>
<td>Effect of nitrogen and potassium fertilization on morpho-agronomic traits of three elephant grass (<em>Pennisetum purpureum</em> Schum.) genotypes for biomass production</td>
<td>2411</td>
</tr>
<tr>
<td>Antonio Alonso Cecon Novo, Rogério Figueiredo Daher, Geraldo de Amaral Gravina, Ernany Santos Costa, Juares Ogliari, Kleberson Cordeiro Araújo, Bruna Rafaela da Silva Menezes, Niraldo José Ponciano, Érik da Silva Oliveira and Verônica Britos Silva</td>
<td></td>
</tr>
<tr>
<td>Study of associative effects of date palm leaves mixed with <em>Aristida pungens</em> and <em>Astragalus gombiformis</em> on the aptitudes of ruminal microbiota in small ruminants</td>
<td>2424</td>
</tr>
<tr>
<td>Deffairi Djamila and Arhab Rabah</td>
<td></td>
</tr>
<tr>
<td>Isolation and characterization of cellulolytic <em>Bacillus licheniformis</em> from compost</td>
<td>2434</td>
</tr>
<tr>
<td>Nallusamy Sivakumar, Amira Al Zadjali, Saif Al Bahry, Abdulkhadir Elshafie and Elsadig Abdulla Eltayeb</td>
<td></td>
</tr>
<tr>
<td>Drought tolerant tropical maize (<em>Zea mays</em> L.) developed through genetic transformation with isopentenyltransferase gene</td>
<td>2447</td>
</tr>
<tr>
<td>Leta Tulu Bedada, Miccah Songelael Seth, Steven Maina Runo, Wondyifraw Tefera, Charless Mugoya, Clet Wandui Masiga, Richard Okoth Oduor, Eduardo Blumewald and Francis Wachira</td>
<td></td>
</tr>
<tr>
<td>Transcriptional modulation of genes encoding nitrate reductase in maize (<em>Zea mays</em>) grown under aluminum toxicity</td>
<td>2465</td>
</tr>
<tr>
<td>Talita Cantú, Cris laine Emidio Vieira, Rafaélia David Piffer, Giovanna Carneiro Luiz and Silvia Graciele Hülse de Souza</td>
<td></td>
</tr>
</tbody>
</table>
Full Length Research Paper

Effect of salt stress on growth and contents of organic and inorganic compounds in noni (Morinda citrifolia L.)

Alide Mitsue Watanabe Cova¹, André Dias de Azevedo Neto², Rogério Ferreira Ribas¹, Hans Raj Gheyi¹ and Renata Velasques Menezes¹

¹Centro de Ciências Agrárias, Ambientais e Biológicas, Universidade Federal do Recôncavo da Bahia, Cruz das Almas, 44380, BA, Brasil.
²Centro de Ciências Exatas e Tecnológicas, Universidade Federal do Recôncavo da Bahia, Cruz das Almas, 44380, BA, Brasil.

Received 1 August 2016, Accepted 14 October, 2016

Salinity is one of the abiotic stresses that most affect agricultural production, especially in arid and semi-arid regions; however, among species there are large differences in salt tolerance. In this study, the effects of salinity on the growth and accumulation of organic and inorganic solutes were evaluated in ‘noni’ seedlings at 1, 10, 20, 30 and 40 days of salt stress in a 5 x 2 completely randomized experimental design. Seedlings of ‘noni’ were grown in the nutrient solution at two salinity levels (0 and 100 mM sodium chloride). Plant height, number of leaves, stem diameter and dry matter of leaves, stems and roots, the allocation of biomass and the contents of organic and inorganic solutes were determined in the different plant organs. Salinity reduced all growth variables, being less expressive in stem diameter. The biomass allocation in leaves was higher than in roots, regardless of treatment or time considered. The contents of organic and inorganic solutes varied according to the plant part and the time of exposure to salinity. In general, salinity increased the contents of sodium ion (Na⁺), chloride ion (Cl⁻) and reduced potassium ion (K⁺), nitrogen (N) and phosphorus (P). Soluble carbohydrates and free amino acids were the main organic solutes contributing to osmotic potential of noni to salt stress. The salinity increased proline content in roots more than in leaves, but the proline content in the leaves was, on average 17 and 6 times higher than that of roots of plants under control and stressed conditions, respectively. Quantitatively, proline does not contribute substantially to the osmotic potential of noni, however its increase suggests that it plays a role in the salt stress acclimation or is an indicator of salt-induced metabolic disorders.

Key words: Amino acids, carbohydrates, toxic ions, proline, proteins.

INTRODUCTION

Medicinal plants are used since the antiquity by the population for combating various diseases. Among the different species, noni (Morinda citrifolia L.), which belongs to the Rubiaceae family, has stood out for its...
phytotherapeutic properties. It is a fruit crop with medicinal and nutritional value that has been used for more than 2000 years by the Polynesians (Chan-Blanco et al., 2006). In noni seeds, peel and pulp, significant amounts of carbohydrates, proteins, vitamin C, total carotenoids and total phenolic compounds have been found (Costa et al., 2013). In addition, the plant acclimates to the most diverse climatic situations; soils and environmental stresses including high levels of salts in the soil (Mian-Ying et al., 2002). Salinity is one of the abiotic stresses that most affect agricultural production especially in arid and semi-arid regions. In these regions, the edaphoclimatic conditions and the inadequate management of soil and water favor the process of salinization. However, these areas can be explored with the cultivation of plants tolerant to salts, and noni can be an alternative of extra income for farmers. Sodium chloride is one of the main salts in the areas affected by salinity. The ions Na+ and Cl- are toxic to most glycophytes, but the plants may have mechanisms to survive to certain concentrations of salts in the soil (Munns and Tester, 2008). However, among the species there are large differences in the tolerance to salt stress (Severiano et al., 2014). Plants when cultivated in saline environment may compartmentalize ions like; potassium ion (K+), chloride ion (Cl-) and sodium ion (Na+) in the vacuole and accumulate compatible organic solutes in the cytoplasm to maintain the osmotic homeostasis of the cell (Quéro et al., 2014). The most studied compatible solutes contributing to the osmotic potential in plants under salt stress are soluble carbohydrates, free amino acids, soluble proteins and free proline (Azevedo Neto et al., 2004, Silva et al., 2008, Sacramento et al., 2014). Among the compatible solutes, the accumulation of proline has been considered as one of the adaptive mechanisms to minimize the adverse effects of salinity (Iqbal et al., 2014). In noni, proline accumulation has proved to be an indicator of damages caused by salt stress (Souza et al., 2014).

The accumulation of organic solutes is extremely important to guarantee the survival of plant, under saline conditions. Hence, in the selection of plants tolerant to salinity, the accumulation of these compounds has been proposed as a physiological marker (Azevedo Neto et al., 2004, 2009). The knowledge on the accumulation and compartmentalization of organic and inorganic solutes, in the different organs in noni plants under salt stress may help to understand physio-biochemical mechanisms of tolerance to salinity. In this context, this study aimed to evaluate the growth and accumulation of organic and inorganic solutes in noni seedlings under salt stress.

MATERIALS AND METHODS

Experimental conditions and treatments

The experiment was carried out in a greenhouse, in completely randomized design using a factorial scheme with five time intervals (1, 10, 20, 30 and 40 days after transplanting) x two salinity levels in the nutrient solution (0 and 100 mM sodium chloride), with four replicates. Noni seedlings at recommended age for transplanting that is, three months with four pairs of leaves (de Sousa et al., 2010) were transferred to containers with 12 L of nutrient solution of Furlani (1998), in a floating hydroponic system. Noni seedlings remained in nutrient solution for four days for acclimation. After this period, the seedlings received the respective treatments [nutrient solution without NaCl (control) or nutrient solution containing 100 mM of NaCl (salt stress)]. NaCl was gradually added (25 mM day−1), in order to avoid the osmotic shock. The volume of the nutrient solutions was completed daily with water and the renewal was performed weekly. The pH was evaluated every two days and adjusted to 6.0 by using sodium hydroxide (NaOH) or hydrochloric acid (HCl). The system was maintained under intermittent aeration of 15 min every hour, using an air compressor coupled to a timer.

Growth

At the end of each time period, the plants of all treatments were carefully removed from the nutrient solution. The roots were washed with distilled water and plants were divided into different organs. After drying at 65°C in an oven for 72 h, the dry masses of stem (SDM), leaf (LDM) and root (RDM) were determined. Based on these data, the total dry mass of the plant (TDM) was calculated. These data were used to calculate the biomass allocation of leaves (ABL), stem (ABS) and roots (ABR) according to the equations proposed by Benincasa (2003):

\[
\text{AB organ (%) = (DM organ/DM total) x 100}
\]

Extract preparation and inorganic solute analysis

Plants were separated into leaves, stems and roots, which after drying were ground for the determination of the inorganic solute contents. For the determination of the Na+, K+ and Cl− contents, the extracts were prepared as described by Jones (2001), with minor modifications. 100 mg of the ground material of leaves, stems or roots were mixed with 10 mL of deionized water in test tubes. The tubes were maintained for 1 h at 80°C in water bath, shaking every 15 min. After this period, the tubes were centrifuged at 5,000 x g for 15 min, at room temperature. The Na+ and K+ contents were determined by flame photometry (Faithfull, 2002) and Cl− contents by spectrophotometry (Jones, 2001).

The N and P contents were determined by acid digestion of 0.5 g of plant material in a mixture of concentrated H2SO4 and H2O2 (30%), as described by Jones (2001). The N and P contents were determined by phenol-hypochlorite and molybdovanadate spectrophotometric methods, respectively (Faithfull, 2002).

Extract preparation and organic solute analysis

The organic solutes contents were analyzed in the first pair of fully expanded leaves and in the samples of the lower third of the roots. The leaves were collected, immediately frozen, lyophilized, ground and stored in ultra-freezer (-80°C). For organic solute determinations, the extracts were prepared by maceration of lyophilized leaf and root tissues (200 mg) in a mortar and pestle with 6 mL of 0.1 M potassium phosphate buffer, pH 7.0, containing 0.1 mM ethylenediaminetetraacetic acid (EDTA). The extract was filtered through muslin cloth, centrifuged at 12,000 x g for 15 min, and the supernatant was stored in ultra-freezer and used for soluble carbohydrates, free proline, free amino acids and soluble proteins determinations (Azevedo Neto, 2009).

The soluble carbohydrates were determined at 490 nm by
Plant height (A), stem diameter (B) and number of leaves (C) of noni plants cultivated for 40 days in hydroponic system under conditions of control (●) or presence of 100 mM of NaCl (●) in the nutrient solution. The values indicate the mean of four replicates and respective standard deviations.

Statistical analysis

The obtained data were subjected to analysis of variance (F test) and the means were compared through their respective standard deviations, according to Snedecor (1956).

RESULTS

Plant height (Figure 1A), stem diameter (Figure 1B) and number of leaves (Figure 1C) increased in the plants of both treatments, during the experimental period. However, it can be observed that, in all variables, this increment was more evident in control plants (without NaCl) than in salt stressed ones. In the comparison between treatments, salinity reduced plant height and stem diameter from 10 days onwards and the number of leaves from 30 days onwards. It can also be observed in this figure that these reductions were more pronounced at 40 days of stress, of the order of 31% for stem diameter and 38% for plant height and number of leaves. Thus, stem diameter was less affected by salinity than plant height and number of leaves.

The dry mass of leaves (Figure 2A), stem (Figure 2B) and roots (Figure 2C) of noni plants increased during the experimental period in both treatments. However, comparing the dry mass production at the end of the experimental period, it is observed that the salinity decreased LDM, SDM and RDM by 26, 62 and 71%, respectively. It can also be observed that the salt stress decreased RDM and SDM after 10 days while LDM was reduced only at 40 days.

The data of biomass allocation in the different plant organs are also shown in Figure 2. In stressed plants, ABL increased from 10 days onwards in relation to the respective controls (Figure 2D). In the stem, biomass allocation decreased only at 40 days (Figure 2E). In contrast to ABL, the ABR decreased from 10 days onwards, compared with the control plants (Figure 2F). It can also be observed in this figure that ABL was higher than ABS and ABR, regardless of treatment or time considered.

The Na⁺ and Cl⁻ contents in all parts of the stressed plants abruptly increased at 10 days, remaining relatively stable up to the end of the experimental period (Figures 3A, 3B, 3C, 3D, 3E and 3F). The contents of Na⁺ in the leaves of stressed plants were about 1.4 and 1.7 times, respectively, higher than in stem and roots. However, there were no substantial differences between Cl⁻ contents in the different plant organs. Unlike the result observed for Na⁺ and Cl⁻ contents, from 10 days onwards, salinity decreased K⁺ contents by approximately 31% in leaves, 45% in stem and 25% in roots (Figures 3G, 3H and 3I). In the roots of stressed plants, the K⁺ contents were about 1.5 and 2.0 times higher than those in leaves and stems, respectively. The K⁺/Na⁺ ratios were reduced by salinity in all organs (Figures 3J, 3K and 3L). It is important to point out that, from 10 days of salt stress, the values of this ratio were lower than 0.5 in leaves and stems. In contrast, in the roots, the values of K⁺/Na⁺ in all times varied between 1.0 and 2.5.
In the leaves of stressed plants, N contents decreased during the experimental period and, in the stem, until 20 days (Figures 4A and 4B). The leaf P contents decreased until 20 days and, in the stem, until 10 days (Figures 4D and 4E). In the roots, the salinity did not affect the N and P contents during the studied period (Figures 4C and 4F).

The variations in the soluble carbohydrates, free amino acids, soluble proteins and free proline contents in leaves and roots are presented in Figure 5. By comparing the treatments, the NaCl in the nutrient solution decreased by 14% the contents of soluble carbohydrates in the leaves after 40 days of stress (Figure 5A). In the roots, there were reductions of 30 and 36% in the contents of these solutes at 30 and 40 days of stress, respectively (Figure 5B).

Salinity decreased the leaf free amino acids contents in day one (50%) and day ten (28%) (Figure 5C). In the roots, the amino acids decreased by about 30% after 20 days of stress (Figure 5D). The soluble proteins in leaves and roots increased along the experimental period in both treatments. However, there were no differences between the protein contents in control and stressed plants (Figure 5E and 5F). Salinity increased the content of free proline in leaves (Figure 5H) and in roots (Figure 5G), from day 10 of stress onwards. However, by comparing the proline contents in both plant parts, it is observed that they were, on average, 17 and 6 times higher in the leaves than in the roots of control and stressed plants, respectively.

DISCUSSION

The accumulation of salts in the rhizosphere can limit water absorption, cause ionic imbalance and affect plant growth (Iqbal et al., 2014). One of the most harmful effects of salt stress is the metabolic disorders caused by
Figure 3. Contents of sodium - Na\(^+\) (A, B, C), chloride - Cl\(^-\) (D, E, F), potassium - K\(^+\) (G, H, I) and K\(^+\)/Na\(^+\) ratio (J, K, L) in the different organs of noni plants cultivated for 40 days in hydroponic system under conditions of control (○) or presence of 100 mM of NaCl (●).

Na\(^+\) and Cl\(^-\) accumulation in plant cells (Geilfus et al., 2015). Consequently, the reduction in height, stem diameter, number of leaves and dry mass production are the main effects of the salts observed in the whole plant level (Silva et al., 2008). The salinity reduced all the analyzed growth variables, but the stem diameter and LDM were the least affected. Souto et al. (2013), working with the noni crop, also reported reductions in plant height, stem diameter, number of leaves, leaf area and shoot dry mass with the increase of salinity in irrigation water. The plant stem diameter shows a positive correlation with the survival rate after transplanting (Tatagiba et al., 2010), the vigor and strength (Marçal, 2011), and the initial yield (Carvalho et al., 2010). It is also important to point out that noni plants can produce up to 70,000 kg ha\(^{-1}\) year\(^{-1}\) of fruits (Chan-Blanco et al., 2006), requiring stems resistant to breaking and damping-off. Considering that stem diameter was sensitive variable

Na\(^+\) and Cl\(^-\) accumulation in plant cells (Geilfus et al., 2015). Consequently, the reduction in height, stem diameter, number of leaves and dry mass production are the main effects of the salts observed in the whole plant level (Silva et al., 2008). The salinity reduced all the analyzed growth variables, but the stem diameter and LDM were the least affected. Souto et al. (2013), working with the noni crop, also reported reductions in plant height, stem diameter, number of leaves, leaf area and shoot dry mass with the increase of salinity in irrigation water. The plant stem diameter shows a positive correlation with the survival rate after transplanting (Tatagiba et al., 2010), the vigor and strength (Marçal, 2011), and the initial yield (Carvalho et al., 2010). It is also important to point out that noni plants can produce up to 70,000 kg ha\(^{-1}\) year\(^{-1}\) of fruits (Chan-Blanco et al., 2006), requiring stems resistant to breaking and damping-off. Considering that stem diameter was sensitive variable
to salt stress, these data suggest that noni plants are less subjected to the damages resulting from the breaking of stems due to the weight of the fruits.

Silva et al. (2008) and Souza et al. (2014) also reported that, plant height and number of leaves of noni also decreased in the presence of salts. According to Mazher et al. (2007), the reduction in plant height may be due to the negative effects of salts on photosynthetic rate, enzyme activity and on the contents of carbohydrates and growth hormones. Additionally, the salt accumulation in cell walls and cytoplasm limit the production of leaves. In contrast, the reduction in number of leaves and leaf area has a negative effect on photosynthesis, water and nutrient uptake and, consequently, on growth and production (Souza et al., 2014).

Regarding the dry mass production, roots and stems were the most sensitive organs to salt effect when compared with the leaves. Abreu et al. (2008) reported that roots have higher capacity of osmotic adjustment and better protection against oxidative stress under salt stress conditions. However, the higher reduction in RDM observed in noni may be a consequence of the roots which are directly exposed to salt stress. Souza et al. (2014) also observed that RDM of noni was more reduced by salinity than shoot dry mass. In plants of *Ricinus communis*, the roots were more affected by salinity than stems and leaves when subjected to 150 mM NaCl (Rodrigues et al., 2014).

Analyzing the effect of duration of stress on the growth variables, it can be inferred that plant height, stem diameter, SDM and RDM were precaucious indicators of salinity effect on the noni crop. The increase in ABL occurred simultaneously to the reduction in ABR. These results corroborate with those of dry mass production with respect to the higher sensitivity of noni roots to salt stress and are similar to those observed in *Ricinus communis* seedlings (Rodrigues et al., 2014). This response suggests a sensitivity of noni to salt stress, due to the higher growth of the parts with greater transpiration capacity in relation to the roots (Rodrigues et al., 2014). This hypothesis is supported by the significant reduction in growth, as well as the presence of toxicity symptoms, such as curving of older leaves, coriaceous texture and greenish-blue color, observed at 40 days (data not shown), which are considered symptoms of sensitivity to salinity (Munns and Tester, 2008).

In the present study, the increase of salinity in the nutrient solution caused a sharp increase in the Na⁺ and Cl⁻ contents in all plant organs. However, these

Figure 4. Contents of nitrogen - N (A, B, C) and phosphorus - P (D, E, F) in different organs of noni plants cultivated for 40 days in hydroponic system under conditions of control (○) or presence of 100 mM of NaCl (●) in the nutrient solution. The values indicate the mean of four replicates and respective standard deviations.
Contents of soluble carbohydrates (A and B), free amino acids (C and D), soluble proteins (E and F) and free proline (G and H) in leaves and roots of noni plants cultivated for 40 days in hydroponic system under conditions of control (○) or presence of 100 mM of NaCl (●) in the nutrient solution. The values indicate the mean of four replicates and respective standard deviations.

Figure 5. Contents of soluble carbohydrates (A and B), free amino acids (C and D), soluble proteins (E and F) and free proline (G and H) in leaves and roots of noni plants cultivated for 40 days in hydroponic system under conditions of control (○) or presence of 100 mM of NaCl (●) in the nutrient solution. The values indicate the mean of four replicates and respective standard deviations.

Increments were more pronounced in the leaves than in the roots. Similar results were also observed in Spondias tuberosa (Silva et al., 2008). The Na⁺ accumulation in the leaves may be due to the transpiration flow rate and/or the reduction in cell volume induced by the lower water absorption caused by salinity (Munns and Tester, 2008). Na⁺ and Cl⁻ are the most abundant ions in saline soils and the excessive absorption of these salts may affect
the electrochemical stability and the plant growth. Na$^+$ is a 
cytotoxic ion that destabilizes membranes and proteins, 
disturbs fundamental physiological processes such as 
cell division and expansion, and alters the primary and 
secondary metabolisms and nutrient homeostasis (Munns 
and Tester, 2008). Cl$^-$ at high concentrations induces 
chlorophyll degradation, which may result in structural 
impact in photosystem II, reducing the photosynthetic 
capacity and yield (Marschner, 2012) thus, salt-induced 
growth inhibition in noni plants can at least partially be 
related to the toxic effects of Na$^+$ and Cl$^-$ ion 
accumulation.

In contrast with Na$^+$, salinity reduced the K$^+$ contents in 
the different plant organs. It is well established that K$^+$ 
uptake can be affected by the antagonism between this 
ion and the Na$^+$ in the absorption sites resulting from 
physico-chemical similarities between them (Abreu et al., 
2008; Rodrigues et al., 2013). This antagonism frequently 
results in decrease of K$^+$ contents thereby leading to 
metabolic disorders in the plants (Mekawy et al., 2015).

Salinity decreased the contents of K$^+$ and increased 
those of Na$^+$ in all plant organs thereby altering the 
K$^+$/Na$^+$ ratios. Similar results were reported by Azevedo 
et al. (2004), Rodrigues et al. (2014) and Sacramento et 
al. (2014). According to Greenway and Munns (1980), the 
K$^+$/Na$^+$ ratio in glycophytes must be higher than 1.0 for 
the maintenance of ion homeostasis and an optimal 
metabolic efficiency. Recently, Rodrigues et al. (2013) 
reported that K$^+$/Na$^+$ ratios between 1.0 and 2.0 were the 
ones that promoted maximum photosynthesis and growth in 
Jatropha curcas plants under salt stress. In the present 
study, from day 10 onwards, the values of K$^+$/Na$^+$ ratio in 
stressed plants were below 1.0 which suggests that the 
plant metabolism was affected by salinity and can 
partially explain the salt-induced growth reduction.

The contents of N and P also decreased with salinity 
especially in the leaves. This behavior corroborates the 
growth reduction observed in the present study, because 
biomass production is directly related to the nutritional 
balance of the plants (Lucena et al., 2012; Marschner, 
2012). A few physiological and biochemical reasons have 
been proposed for the reduction of N and P uptake in 
salt-stressed plants. NO$_3^-$ and H$_2$PO$_4^-$ are the main 
sources of N and P in agricultural soils, and high salt 
concentrations may affect their absorption, resulting from 
the competitive mechanism established with Cl$^-$ (Feijão et 
al., 2013; Lucena et al., 2012).

In the present study, the reductions in N and P contents 
in conjunction with the salt-induced growth decrease 
indicate an antagonism (Imo, 2012) between these 
nutrients and Cl$^-$ ions of the nutrient solution. In addition, 
the observation that salinity decreased N and P contents 
in shoot organs, but did not affect those in the roots, 
suggests the occurrence of disorders in the translocation 
of these nutrients from roots to shoots (Marschner, 2012). 
In the present study, the salinity reduced the soluble 
carbohydrate contents in leaves and roots. This reduction 
was probably due to the decrease in net photosynthesis 
observed after 30 and 40 days of stress (data not 
shown). Consequently, the transport of carbohydrates 
from leaves to roots was also reduced (Silva et al., 2008). 
The reduction in the leaf carbohydrates is frequently 
associated with disorders in their biosynthesis or in the 
translocation to the other plant parts (Azevedo Neto et 
al., 2004, 2009). Various studies have reported changes 
in the soluble carbohydrates in different organs and plant 
species under salt stress conditions. In Zea mays and 
Anacardium occidentale, the carbohydrates were not 
affected in the different organs (Abreu et al., 2008, 
Azevedo Neto et al., 2004). In Spondias tuberosa there 
was an increment in the leaves and reduction in the roots 
(Silva et al., 2008) and in Spartina alterniflora an 
increment in the shoots (Li et al., 2010). Thus, the 
content of carbohydrates may vary with the species, plant 
organ and time of exposure to salts.

The NaCl addition in the nutrient solution reduced the 
free amino acid contents, in both leaves and roots 
(Figures 5C and D), but did not affect the soluble protein 
contents (Figures 5E and F). The decrease in the amino 
acids is often associated with increased degradation or 
inhibition of their biosynthesis, along with the reduction in 
protein degradation or increase in protein synthesis (Silva 
et al., 2008). N availability is another factor that can limit 
the amino acids synthesis (Feijão et al., 2013). Thus, the 
data of this study suggest that the reduction in the amino 
acid contents in stressed plants is resulted from the 
biosynthesis inhibition. The observation that salinity did 
not affect the soluble proteins (Figures 5E and F) 
and decreased the N content (Figures 4A and B) supports 
this hypothesis.

Unlike soluble carbohydrates, free amino acids and 
soluble proteins, the contents of proline significantly 
increased in salt-stressed noni plants, along the entire 
experimental period. Similar results were obtained by 
Souza et al. (2014), which indicate that the proline 
accumulation was primarily due to “de novo” synthesis 
coupled with the reduction of proline degradation and 
utilization (Carillo et al., 2011).

Proline is an osmolyte that accumulates in various plant 
species in response to biotic and abiotic stresses, 
however its role in the osmotolerance still remains 
controversial. Thus, while some authors consider proline 
as an important amino acid for the osmotic adjustment in 
plants under salt stress (Iqbal et al., 2014, Li et al., 2010), 
others consider that the proline contents are not sufficient 
for a significant contribution to the osmotic adjustment 
(Oliveira et al., 2013). In this study, salinity increased 
proline contents in noni leaves and roots however, the 
contents of soluble carbohydrates and free amino acids 
were quantitatively much higher than those of proline. 
This indicates that in noni, soluble carbohydrates and 
free amino acids were the main organic solutes involved 
in the osmotic potential.

Furthermore, since proline was the only organic solute
whose concentration increased with the salt stress despite its importance in osmoregulation, this result suggests that proline accumulation plays an important role in the acclimation of noni to salinity. In this scenario, other important functions have been attributed to the proline accumulation, such as stabilization of proteins, protein complexes and membranes, removal of free radicals, maintenance of cell redox homeostasis, increase in the enzyme activities, reserve of carbon and nitrogen, cytosolic pH control, and detoxification of NH₄⁺ excess (Azevedo Neto and Silva, 2015). Alternatively, the increase in proline concentration in conjunction with the growth reduction suggests that the sensitivity to stress is conditioned to the salt-induced metabolic disorders, as proposed by Oliveira et al. (2013).

Conclusions

The increase in the contents of sodium and chloride ions (Na⁺ and Cl⁻) leads to nutritional imbalance of N, P and K. This accumulation of toxic ions associated with the nutritional imbalance can at least explain the growth reduction and the change in the pattern of biomass allocation in noni seedlings under salt stress. Among the evaluated growth variables, plant height, stem diameter and root dry mass are the earliest indicators of the salt effects on noni seedlings. The results also show that soluble carbohydrates and free amino acids are the main organic solutes contributing to the osmotic potential in leaves and roots of noni and that proline, although not contributing substantially to the osmotic potential, it either play a role in noni acclimation to salt stress or is an indicator of the salt-induced metabolic disorders.

Conflict of Interests

The authors have not declared any conflict of interests.

REFERENCES


Carvalho AM, Mendes ANG, Carvalho GR, Botelho CE, Gonçalves FMA, Ferreira AD (2010). Correlation between growth and yield of coffee cultivars in different regions of the state of Minas Gerais, Brazil. Pesq. Agropec. Bras. 45(3):269-275.


Quéro A, Molinié R, Elboutatchfalt R, Petit E, Pau-Roblot C, Guilot X,


Effect of nitrogen and potassium fertilization on morpho-agronomic traits of three elephant grass (*Pennisetum purpureum* Schum.) genotypes for biomass production

Antonio Alonso Cecon Novo¹*, Rogério Figueiredo Daher², Geraldo de Amaral Gravina², Ernany Santos Costa¹, Jáures Ogliari¹, Kleberson Cordeiro Araújo¹, Bruna Rafaela da Silva Menezes², Niraldo José Ponciano², Érik da Silva Oliveira² and Verônica Britos Silva²

¹Instituto Federal Fluminense, Campus Bom Jesus do Itabapoana RJ. Setor de Agropecuária. Postal Code: 28360-000, RJ, Brazil.
²Universidade Estadual do Norte Fluminense Darcy Ribeiro (UENF), Laboratório de Engenharia Agrícola. Postal Code: 28013-600, Campos dos Goytacazes, RJ, Brazil.

Received 16 August, 2016; Accepted 26 September, 2016

Elephant grass has been proposed for the energy sector as a possible source of renewable energy, because of its high biomass production. The aim of this study was to evaluate the effect of the mineral nutrients nitrogen and potassium on the morpho-agronomic traits (dry mater yield (DMY), percentage of DM (%DM), number of tillers per linear meter (NT), plant height (PH), stem diameter (SD) and leaf blade width (LW)) in different elephant grass genotypes in a randomized-block experimental treatment in a split-plot arrangement with three replications, in which the genotype factor ('Cuban Pinda' - G1; 'IAC Campinas' - G2; and 'Cameroon' - G3) was randomized in the plot, and the N and K factor was randomized in the sub-plot. The increase in nitrogen and potassium doses utilized influenced very little or almost did not influence the response of the three genotypes for the different morpho-agronomic traits assessed. The three genotypes had high number of tillers, height, and stem diameter at the lowest N and K doses, demonstrating a possible trend of high doses not providing a highly significant increase in these traits. The study of DMY showed that under a low nitrogen dose and with increase in potassium concentrations, dry matter yield increased; however, as the nitrogen dose increased in associated with potassium doses, dry matter yield did not augment, but was rather suppressed. The three elephant grass genotypes: ‘Cuban Pinda’, ‘IAC Campinas’, and ‘Cameroon’, had average dry yields of 52.66, 50.60, and 48.57 t ha⁻¹, respectively. Results are highly promising and prove the possibility of using elephant grass as an alternative source for biomass production.

Key words: Renewable energy, mineral nutrients, production capacity.

INTRODUCTION

The tropical-climate conditions of Brazil have characteristics that facilitate production of biomass such as sugarcane bagasse, charcoal, and wood (Rocha et al., 2015). The tropical climate is very favorable, especially to
C4 plants, which have a high plant mass accumulation in a short period, making the use of solar energy efficient. In this regard, the Poaceae family stands out for their greater plant mass production in relation to other plants. Elephant grass is species with great photosynthetic (C4 metabolism) and dry matter accumulation capacities, which make it comparable to sugarcane (Daher et al., 2014). This grass has shown tremendous advantages in relation to the other energy sources in research conducted so far (Oliveira et al., 2013). Elephant grass is highly productive in smaller areas, has a lower production, allows total mechanization, and provides renewable energy, greater carbon assimilation, and increased productivity by increasing the applications of nitrogen and potassium (Santos et al., 2014; Woodward and Sollenberger, 2015).

Nitrogen is one of the nutrients that most limits the development and biomass production of most Brazilian crops. The adequate management of fertilizers, especially those containing nitrogen, given the deficient availability of this nutrient in the soil, is fundamental for obtaining gains in productivity (Flores et al., 2013). Thus, it is important to adequate the use of nutrients in the elephant-grass production system so as to optimize the gains in renewable energy. Today, because of the elevated biomass production potential of elephant grass, it is being used in the form of direct combustion, in heat supply, replacing wood or charcoal. This energy source will be able to replace the man-made coal extracted in a predatory manner from native forests, which has caused environmental damage, like siltation and consequent death of several rivers.

Potassium is the second most widely required mineral in quantity by plant species, after nitrogen. It is highly mobile in the plant at any concentration, be it in the 57 cell, in the plant tissue, in the xylem, or in the phloem. Potassium is not metabolized in the 58 plants and binds to easily-reversible organic molecules. Furthermore, it is the most 59 abundant ion in plant cells (Marschner, 1995).

In plant breeding, associations among traits are important, as they allow for several traits to be improved simultaneously. To meet the demands of producers of charcoal from elephant grass, varieties adapted to the different ecosystems, to generate biomass production, are greatly sought. Characteristics such as faster growth, high yield, high energy efficiency, efficient mineral nutrient use, more uniform distribution of the dry matter production throughout the year, and less or more resistance to pests and diseases are used to discriminate between promising genotypes (Menezes et al., 2015; Santos et al., 2014).

Given the earlier-stated facts, this study was conducted to evaluate the effect of different nitrogen and potassium fertilization levels on the main morpho-agronomic traits of three elephant grass genotypes.

MATERIALS AND METHODS

Cultivation conditions and genetic materials

The experiment was conducted in the Cattle Unit at the Federal Institute of Science and Technology of Rio de Janeiro State, on Bom Jesus do Itabapoana Campus (UTM 223877 m E and 7660277 m N, 24K zone, 84 m altitude Climate Northwest Fluminense is, according to the classification Köppen, Aw (with hot and rainy summer and dry winters).

The soil was sampled from the 0 to 20 cm layer for particle-size and chemical analyses, and showed the following results: sand, 55.8%; silt, 13.6%; clay, 30.6%; pH in H2O, 5.1; P, 3.0 mg dm-3; K, 36.0 mg dm-3; Na, 0.06 mg dm-3; Ca, 84.1 mg dm-3; Mg, 31.6 mg dm-3; Al, 2.6 mg dm-3; H+Al, 47.6 mg dm-3; effective CEC, 154.3 mg dm-3; CEC at pH 7.0, 199.3 mg dm-3; SB, 151.7 mg dm-3; base saturation, 68%; and aluminum saturation, 3.0%. The analysis was performed in the Soil Physics Laboratory at the Center for Agricultural Sciences at the Federal Rural University of Rio de Janeiro. The soil was classified a Red-Yellow Ultisol (Embrapa, 2013).

The experiment was implemented as a randomized-block design, in a split-plot arrangement, with three replications, in which the genotype factor (‘Cuban Pinda’ - G1; ‘IAC Campinas’ - G2; and ‘Cameroon’ - G3), which originated from the breeding program at UENF, was randomized in the plot, and the N and K factor, in the sub-plot. Four nitrogen fertilization doses (100, 400, 1500, and 2200 kg N ha-1 year-1 of urea) and four potassium fertilization doses (50, 400, 750, and 1100 kg K2O ha-1 year-1 of potassium chloride) were applied on the subplot, with three replications.

The experimental area was formed by nine 54-m rows spaced 1.50 m apart, and each block was formed by 16 experimental units (plots) with 3.0 m linear extension. The grass was harvested twice, at 365-day intervals. The usable area of 2.25 m2 was obtained.
by removing 1.50 m from each plot. Stems were planted aligned inside the furrows, with the base of a plant touching the apex of another plant, and subsequently cut and covered with 3 cm of soil.

Planting took place on October 02, 2012. A plot-leveling cut was made on February 12, 2013 (130 days after planting). The 1st harvest for evaluation occurred on February 12, 2014 (365 days after the plot-leveling cut) and the second on February 12, 2016 (365 days after the 1st harvest). The mean of both harvests were used to evaluate the morpho-agronomic traits, to have more consistent numbers for the discussion of results.

**Evaluated traits**

The dry matter yield (DMY) of the whole plant was estimated as the product between the fresh matter of whole plants (kg), obtained on a digital scale, originating from 2.25 m², by the percentage of dry matter of the whole plant (%DM), obtained from the sampling of these plants; this variable was estimated from samples of whole plants extracted at random among the plants cut from the usable area, and the obtained value (kg/m²) was converted to t ha⁻¹. The percentage of dry matter of the whole plant (%DM) was estimated in whole-plant samples extracted at random among the plants from the usable area which were weighed and pre-dried in a forced-air oven at 65°C for 72 h (air-dried sample, ADS) and weighed again to obtain the percentage of dry matter of the whole plant (%DM), following the method described by Silva and Queiroz (2002). After drying, samples were ground (1 mm) through a Wiley mill and packed in bottles. The dry matter contents were obtained by drying the material in a forced-air oven at 105°C for 24 h (oven-dried sample, ODS), and this parameter served as basis to express the dry matter yield in t ha⁻¹ (DMY). The following variables were also determined: average plant height (PH), expressed in meters, measured with a ruler graduated in centimeters from the base of the plant to the apex of erect leaves during the harvest for evaluation, based on the height of five plants from the usable area; number of tillers per linear meter (NT), obtained by counting the number of tillers higher than 70 cm from the usable area of the plot, moments before the harvest for evaluation; average stem diameter at the plant base (SD), expressed in centimeters, taken from five plants from the usable area of the plot, measured 10 cm above the soil level with a digital caliper during the harvest for evaluation; and average leaf width (LW), described in the middle third of the leaf blade during the harvest for evaluation.

**Statistical analysis**

Analyses were performed using the Genes (Cruz, 2013) and SAEG (version 9.0) computer programs developed at the Federal University of Viçosa.

\[ Y_{ijkl} = \mu + B_i + G_j + \varepsilon_{(a)} + N_{i,j} + K_{k} + G_i K_j + N_{i,j} K_k + G_i N_{i,j} K_k + \varepsilon_{(b)} \]

where \( Y_{ijkl} \) is the observed value referring to genotype \( i \), at nitrogen dose \( j \), at potassium dose \( k \), on block \( l \); \( \mu \) is the overall mean of the experiment; \( G_j \) is the effect of genotype \( j \); \( B_i \) is the effect of block \( l \); \( N_{i,j} \) is the effect of nitrogen dose \( j \); \( K_k \) is the effect of potassium dose \( k \); \( G_i K_j \) is the effect of the interaction between genotype \( i \), and potassium dose \( k \); \( N_{i,j} K_k \) is the effect of the interaction between nitrogen dose \( j \) and potassium dose \( k \); and \( \varepsilon_{(a)} \) is the effect of error \( a \) associated with genotype \( i \) with nitrogen dose \( j \) and potassium dose \( k \), on block \( l \). \( \varepsilon_{(a)} \) and \( \varepsilon_{(b)} \) ~ NID (0, \( \sigma^2 \epsilon_{a,b} \)).

**RESULTS AND DISCUSSION**

**Variance analysis**

The results of the analyses of variance for the morpho-agronomic traits evaluated involving three genotypes and four nitrogen and four potassium levels showed a significant effect (P<0.05) for dry matter yield (DMY) from all factors and interactions. For percentage of dry matter (%DM) and average leaf width (LW), however, no significant effect was detected (P>0.05) from any factor or interactions, indicating independence among the factors. For the genotype factor, there was no effect on any of the studied traits.

In the analysis of the sources of variation nitrogen and potassium × nitrogen and potassium × genotype interactions, there was a significant effect (P<0.05) on the traits DMY, number of tillers per meter (NT), and plant height (PH). However, there was a highly significant effect (P<0.01) of the sources of variation nitrogen, potassium, and potassium × genotype and potassium × nitrogen × genotype interactions on the morpho-agronomic traits DMY, NT, and stem diameter (SD). Hence, it was observed that the effect of nitrogen fertilization did not influence percentage of dry matter (%DM), NT, SD, or average LW; potassium fertilization did not influence %DM, PH, or average LW of the elephant grass; and the variation in these traits may be due to the genetic factor.

**Comparisons between mean of genotypes within the different doses of N and K for each of the evaluated morpho-agronomic traits**

The evaluated morpho-agronomic traits DMY, %DM, PH, and LW, described in Table 1, differed statistically according to Tukey’s test at the 5% probability level at specific N and K doses. The NT and SD traits, however, did not differ statistically.

For the DMY trait, a significant effect was detected by Tukey’s test at the 5% probability level at the N2K1 doses for genotype G1 and also at the N4K2 doses for genotype G2. For the %DM trait, there was an effect for genotype G2 at the N3K4 doses and also for PH and LW at the K4N4 doses, respectively.

The evaluated genotypes (G1, G2, and G3) showed high estimates for DMY at the lowest N and K doses: 48.77, 46.06, and 52.08 t ha⁻¹, respectively. These estimates differed from those obtained by Rossi (2010), who found DMY in genotypes ‘Cuban Pinda’, ‘IAC Campinas’, and ‘Cameroon’, of 37.34, 25.6, and 48.77, 46.06, and 52.08 t ha⁻¹, respectively. These estimates differed from those obtained by Rossi (2010), who found DMY in genotypes ‘Cuban Pinda’, ‘IAC Campinas’, and ‘Cameroon’, of 37.34, 25.6, and 48.77, 46.06, and 52.08 t ha⁻¹, respectively, in a period of 10 months under fertilization with 25 kg ha⁻¹ ammonium sulfate and potassium chloride.

With the lower nitrogen fertilization level of 100 kg ha⁻¹ and increasing potassium doses, all genotypes expressed elevated production, though no statistical differences
Table 1. Mean values for the morpho-agronomic traits (dry mater yield (DMY), percentage of DM (%DM), number of tillers per linear meter (NT), plant height (PH), stem diameter (SD)) evaluated in three elephant grass genotypes ('Cuban Pinda' - G1; 'IAC Campinas' - G2; and 'Cameroon' - G3) under different nitrogen (N1 = 100, N2 = 800, N3 = 1500, and N4 = 2200 kg ha\(^{-1}\) N) and potassium (K1 = 50, K2 = 400, K3 = 750, and K4 = 1100 kg ha\(^{-1}\) K\(_2\)O) levels, in a time interval of two years, for energy purposes.

<table>
<thead>
<tr>
<th>Doses of K Kg ha(^{-1})</th>
<th>GEN</th>
<th>Doses of N Kg ha(^{-1})</th>
<th>Morpho-agronomic traits (DMY, t ha(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>N1</td>
<td>N2</td>
</tr>
<tr>
<td>K1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td></td>
<td>48.77(^{a})</td>
<td>54.95(^{a})</td>
</tr>
<tr>
<td>G2</td>
<td></td>
<td>46.06(^{a})</td>
<td>44.00(^{ab})</td>
</tr>
<tr>
<td>G3</td>
<td></td>
<td>52.08(^{a})</td>
<td>41.71(^{b})</td>
</tr>
<tr>
<td>G1</td>
<td></td>
<td>58.58(^{a})</td>
<td>50.16(^{a})</td>
</tr>
<tr>
<td>K2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G2</td>
<td></td>
<td>47.05(^{a})</td>
<td>40.32(^{a})</td>
</tr>
<tr>
<td>G3</td>
<td></td>
<td>57.80(^{a})</td>
<td>53.60(^{a})</td>
</tr>
<tr>
<td>G1</td>
<td></td>
<td>58.50(^{a})</td>
<td>40.81(^{a})</td>
</tr>
<tr>
<td>K3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G2</td>
<td></td>
<td>50.71(^{b})</td>
<td>48.26(^{a})</td>
</tr>
<tr>
<td>G3</td>
<td></td>
<td>48.62(^{a})</td>
<td>42.55(^{a})</td>
</tr>
<tr>
<td>G1</td>
<td></td>
<td>72.95(^{a})</td>
<td>53.75(^{a})</td>
</tr>
<tr>
<td>K4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G2</td>
<td></td>
<td>70.62(^{a})</td>
<td>63.77(^{a})</td>
</tr>
<tr>
<td>G3</td>
<td></td>
<td>57.15(^{a})</td>
<td>46.19(^{a})</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Morpho-agronomic traits (%DM, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
</tr>
<tr>
<td>K1</td>
</tr>
<tr>
<td>G2</td>
</tr>
<tr>
<td>G3</td>
</tr>
<tr>
<td>G1</td>
</tr>
<tr>
<td>K2</td>
</tr>
<tr>
<td>G3</td>
</tr>
<tr>
<td>G1</td>
</tr>
<tr>
<td>K3</td>
</tr>
<tr>
<td>G2</td>
</tr>
<tr>
<td>G3</td>
</tr>
<tr>
<td>G1</td>
</tr>
<tr>
<td>K4</td>
</tr>
<tr>
<td>G2</td>
</tr>
<tr>
<td>G3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Morpho-agronomic traits (NT/m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
</tr>
<tr>
<td>K1</td>
</tr>
<tr>
<td>G2</td>
</tr>
<tr>
<td>G3</td>
</tr>
<tr>
<td>G1</td>
</tr>
<tr>
<td>K2</td>
</tr>
<tr>
<td>G3</td>
</tr>
<tr>
<td>G1</td>
</tr>
<tr>
<td>K3</td>
</tr>
<tr>
<td>G2</td>
</tr>
<tr>
<td>G3</td>
</tr>
<tr>
<td>G1</td>
</tr>
<tr>
<td>K4</td>
</tr>
<tr>
<td>G2</td>
</tr>
<tr>
<td>G3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Morpho-agronomic traits (PH, m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
</tr>
<tr>
<td>K1</td>
</tr>
<tr>
<td>G2</td>
</tr>
<tr>
<td>G3</td>
</tr>
<tr>
<td>G1</td>
</tr>
</tbody>
</table>

---
were found between doses. A different outcome was noted with the increasing interactions of N and K doses, in which yield did not increase, but a statistical difference was found with the N2K1 and N4K2 interaction. Thus, the increased nitrogen and potassium fertilization did not provide an increase in dry matter yield (Table 1).

The dry matter yield of genotype ‘Cuban Pinda’ of 42.25 t ha⁻¹ year⁻¹, at the N dose of 2200 kg ha⁻¹, corroborates Santos et al. (2014), who obtained 38.7 t ha⁻¹ from cv. ‘Cameroon-Piracicaba’ in harvest 2 (300 days) at the N dose of 1000 kg ha⁻¹, in Alegre - ES, Brazil. It is also in line with Oliveira et al. (2015), who obtained total yields in two harvests of 27.25 t ha⁻¹ year⁻¹ at the N dose of 1000 kg ha⁻¹ in Campos dos Goytacazes - RJ, Brazil.

The study of dry matter yield showed that under low nitrogen supply and with increased potassium doses, DMY increased, but as the nitrogen dose was increased in associated with potassium doses, this yield did not increase but was rather suppressed. Morais et al. (2009)

### Table 1. Contd.

<table>
<thead>
<tr>
<th></th>
<th>G1</th>
<th>G2</th>
<th>G3</th>
<th>G4</th>
</tr>
</thead>
<tbody>
<tr>
<td>K1</td>
<td>3.08</td>
<td>3.08</td>
<td>2.99</td>
<td>3.22</td>
</tr>
<tr>
<td>K2</td>
<td>3.08</td>
<td>3.04</td>
<td>3.01</td>
<td>3.06</td>
</tr>
<tr>
<td>K3</td>
<td>2.97</td>
<td>3.01</td>
<td>3.01</td>
<td>3.20</td>
</tr>
<tr>
<td>K4</td>
<td>3.01</td>
<td>2.82</td>
<td>2.82</td>
<td>3.18</td>
</tr>
<tr>
<td></td>
<td>3.05</td>
<td>2.96</td>
<td>2.98</td>
<td>3.22</td>
</tr>
<tr>
<td></td>
<td>3.00</td>
<td>2.98</td>
<td>3.08</td>
<td>3.08</td>
</tr>
<tr>
<td></td>
<td>3.25</td>
<td>3.18</td>
<td>2.94</td>
<td>3.5²b</td>
</tr>
<tr>
<td></td>
<td>3.05</td>
<td>3.05</td>
<td>3.15</td>
<td>3.19</td>
</tr>
<tr>
<td></td>
<td>3.03</td>
<td>2.93</td>
<td>3.03</td>
<td>2.86</td>
</tr>
</tbody>
</table>

**Morpho-agronomic traits (SD, cm)**

<table>
<thead>
<tr>
<th></th>
<th>G1</th>
<th>G2</th>
<th>G3</th>
<th>G4</th>
</tr>
</thead>
<tbody>
<tr>
<td>K1</td>
<td>1.37</td>
<td>1.33</td>
<td>1.34</td>
<td>1.38</td>
</tr>
<tr>
<td>K2</td>
<td>1.41</td>
<td>1.45</td>
<td>1.37</td>
<td>1.45</td>
</tr>
<tr>
<td>K3</td>
<td>1.50</td>
<td>1.45</td>
<td>1.48</td>
<td>1.47</td>
</tr>
<tr>
<td>K4</td>
<td>1.48</td>
<td>1.43</td>
<td>1.42</td>
<td>1.43</td>
</tr>
<tr>
<td></td>
<td>1.51</td>
<td>1.47</td>
<td>1.50</td>
<td>1.48</td>
</tr>
<tr>
<td></td>
<td>1.47</td>
<td>1.45</td>
<td>1.49</td>
<td>1.42</td>
</tr>
<tr>
<td></td>
<td>1.44</td>
<td>1.42</td>
<td>1.49</td>
<td>1.46</td>
</tr>
<tr>
<td></td>
<td>1.40</td>
<td>1.49</td>
<td>1.53</td>
<td>1.38</td>
</tr>
<tr>
<td></td>
<td>1.52</td>
<td>1.46</td>
<td>1.44</td>
<td>1.45</td>
</tr>
<tr>
<td></td>
<td>1.42</td>
<td>1.48</td>
<td>1.45</td>
<td>1.43</td>
</tr>
<tr>
<td></td>
<td>1.50</td>
<td>1.47</td>
<td>1.54</td>
<td>1.62</td>
</tr>
<tr>
<td></td>
<td>1.48</td>
<td>1.41</td>
<td>1.46</td>
<td>1.44</td>
</tr>
</tbody>
</table>

**Morpho-agronomic traits (LW, cm)**

<table>
<thead>
<tr>
<th></th>
<th>G1</th>
<th>G2</th>
<th>G3</th>
<th>G4</th>
</tr>
</thead>
<tbody>
<tr>
<td>K1</td>
<td>4.35</td>
<td>4.28</td>
<td>4.27</td>
<td>4.32</td>
</tr>
<tr>
<td>K2</td>
<td>4.71</td>
<td>4.12</td>
<td>4.30</td>
<td>4.53</td>
</tr>
<tr>
<td>K3</td>
<td>4.14</td>
<td>4.17</td>
<td>3.96</td>
<td>4.28</td>
</tr>
<tr>
<td>K4</td>
<td>4.43</td>
<td>4.28</td>
<td>4.12</td>
<td>4.15</td>
</tr>
<tr>
<td></td>
<td>4.52</td>
<td>4.32</td>
<td>4.46</td>
<td>4.58</td>
</tr>
<tr>
<td></td>
<td>4.26</td>
<td>4.40</td>
<td>4.58</td>
<td>4.30</td>
</tr>
<tr>
<td></td>
<td>4.59</td>
<td>4.25</td>
<td>4.36</td>
<td>4.39</td>
</tr>
<tr>
<td></td>
<td>4.37</td>
<td>4.24</td>
<td>4.42</td>
<td>4.19</td>
</tr>
<tr>
<td></td>
<td>4.05</td>
<td>4.22</td>
<td>4.20</td>
<td>4.23</td>
</tr>
<tr>
<td></td>
<td>4.40</td>
<td>4.25</td>
<td>4.41</td>
<td>4.12</td>
</tr>
<tr>
<td></td>
<td>4.44</td>
<td>4.42</td>
<td>4.61</td>
<td>4.75</td>
</tr>
<tr>
<td></td>
<td>4.18</td>
<td>4.11</td>
<td>4.39</td>
<td>4.09</td>
</tr>
</tbody>
</table>

Means followed by the same lowercase letter in the columns do not differ statistically, according to Tukey’s test, at the 5% probability level. Whole-plant dry matter yield, in t ha⁻¹ = DMY; Percentage of whole dry matter = %DM; Number of tillers per meter = NT; Plant height, in m = PH; Average stem diameter, in cm = SD; Average leaf width, in cm = LW.
worked with six elephant grass genotypes and a nitrogen dose of 50 kg ha$^{-1}$ in three crop cycles, each with six months, totaling 18 months, and did not find a significant effect, corroborating the present study, in which no significant statistical difference was observed.

Overall, the genotypes did not differ from each other as to their dry matter production potential in the harvests made with the different nitrogen and potassium doses utilized, demonstrating that they are highly productive.

The average DMY of genotype ‘Cameroon’, of 48.57 t ha$^{-1}$ was higher than the 38.7 t ha$^{-1}$ obtained by Santos et al. (2014) with a harvest interval of 300 days, using a N dose of 1000 kg ha$^{-1}$. It was also higher than the 24.71 t ha$^{-1}$ found by Rossi (2010) in genotype ‘Cameroon’ in a period of 10 months and with fertilization with 25 kg ha$^{-1}$ ammonium sulfate and potassium chloride. Morais et al. (2009) obtained, in Ponta Ubú, Anchieta - ES, Brazil, for the same genotype, 8.17 t ha$^{-1}$ in the 3rd cycle in 18 months of growth, using less nitrogen fertilization, which is also a lower value than that obtained in our study.

The overall mean for the dry matter percentage (%DM) of the genotypes was 33.21% (Table 1) and the lowest value, 31.05%, was obtained in genotype ‘Cuban Pinda’, under N and K doses of 800 and 50 kg ha$^{-1}$, respectively. The highest result, however, was found in ‘IAC Campinas’, at the N and K doses of 1500 and 1100 kg ha$^{-1}$, respectively. This percentage of dry matter corroborates Rossi (2010), who used less nitrogen fertilization in a period of 10 months and obtained %DM for genotypes ‘Cuban Pinda’, ‘IAC Campinas’ and ‘Cameroon’ of 35.85, 34.73, and 31.90%, respectively. These values published by Rossi et al. (2014) differ from those obtained by Santos (2013), who reported an overall mean of 24.72%; the lowest value, 23.22%, found in ‘Cameroon’ at 1500 kg ha$^{-1}$ N; and the highest, 25.77%, in ‘Guacu/I2.2’, at 500 kg ha$^{-1}$ N, with a harvest interval of 180 days. These data also differ from those reported by Souza-Sobrinho et al. (2005), who obtained an average %DM of 24.47% with harvest intervals shorter than 100 days.

Santos (2013) evaluated the chemical composition of elephant grass cv. ‘Roxo’, ‘Guacu/I2.2’, ‘Cameroon’, and ‘Canad’Af’rica’ and found an average %DM of 19.7 and 24.72%, respectively, whereas in this study an average of 33.21% was found for the three genotypes (Table 1). The observed dry matter contents, as compared with the results, confirm that the dry matter increases when the interval between harvests is increased, due to the increased stem diameter, and plant height.

The dry matter content of observed compared with the results confirm that there is an increase of dry matter when the cutting interval increases, due to increased stem diameter, and plant height.

For NT, the genotypes did not differ statistically with the different N and K doses. The overall mean of the trait NT of the genotypes was 24.59 (Table 1) and the lowest obtained value was 20.52, referring to genotype ‘Cuban Pinda’, with N and K doses of 1500 and 400 kg ha$^{-1}$, respectively, while the highest value, 33.94, was obtained with genotype ‘IAC Campinas’ at the N dose of 800 and 1100 kg ha$^{-1}$ K. Genotypes had higher tillering with the lowest N and K doses, demonstrating a possible trend for high doses to not increase the number of tillers.

Mean values for NT were similar to those found by Santos et al. (2014), who observed 28.43, 23.00, and 30.58 in genotypes ‘Guacu/I2.2’, ‘Cameroon-Piracicaba’, and ‘Canad’Af’rica’, respectively, in the second harvest, with a harvest interval of 10 months, using a lower N dose of 1000 kg ha$^{-1}$. Our values were also close to those obtained by Oliveira et al. (2013), whose cv. ‘Guacu/I2.2’, ‘Cameroon-Piracicaba’, ‘Canad’Af’rica’, and ‘Cuban’ showed 28, 28, 23, and 24 tillers per meter, respectively, in a six-month interval, also using a lower nitrogen fertilization dose. According to Silva et al. (2010) and Daher et al. (2014), the traits number of basal and aerial tillers per meter have high heritability, indicating little or no influence of the environment on the variability among the studied genotypes. The higher yield coincides with the higher number of tillers per area and plant height.

Concerning PH, only genotype ‘IAC Campinas’ differed statistically from the others, standing out in relation to ‘Cuban Pinda’ and ‘Cameroon’ at the N4K4 dose utilized, with the respective value of 3.19 (Table 1). The height of genotype ‘Cameroon’ varied negatively with the increasing nitrogen and potassium levels; therefore, fertilization might have suppressed the height of this genotype, though this trait is known to possibly be due to the genetic factor.

Elephant grass varieties can reach great heights depending on climate and management conditions. Kannika et al. (2011) evaluated the height of elephant grass with different harvest intervals and found that at 12 months of age the grass reached 5 m. Oliveira et al. (2013), found an average genotype height of 1.88 m with a cycle of six months. In this study, the average height was 3.02, with a 12-month cycle. According to Xia et al. (2010), this variable is positively correlated with yield.

Analyzing the SD values, the overall mean of the genotypes was 1.45 cm (Table 1); the lowest value of 1.33 was found for genotype ‘Cuban Pinda’ at the N dose of 800 kg ha$^{-1}$ and K dose of 50 kg ha$^{-1}$; and the highest, 1.62 cm, for genotype ‘IAC Campinas’, with the N and K doses of 2200 and 1100 kg ha$^{-1}$. Santos (2013) obtained, for genotype ‘Cameroon’, higher values: 1.80, 1.86, 1.82, 1.92, and 1.79 cm at the N doses of 0, 500, 1000, 1500, and 2000 kg ha$^{-1}$, respectively, while the lowest value, 1.52 cm, was found in genotype ‘Canad’Af’rica’, at the N level of 500 kg ha$^{-1}$.

For the leaf width (LW) trait, there was a significant effect on genotype ‘IAC Campinas’, whose mean value was 4.75 cm at the N dose of 2200 and 1100 kg ha$^{-1}$ K and on ‘Cuban Pinda’ and ‘Cameroon’, which showed the lowest LW: 4.12 and 4.09 cm, respectively (Table 1).
Genotype 'IAC Campinas' responded positively to the K dose of 1100 kg ha\(^{-1}\) and the N increase. Santos (2013), adopting a harvest interval of 180 days, found a LW of 6.16 cm at the N dose of 1500 kg ha\(^{-1}\), which shows that the harvest interval can influence the width of elephant grass leaves.

Regression analysis for the evaluated morpho-agronomic traits for the three elephant grass genotypes

The aspects of the most representative biometric models (1st degree, 2nd degree, and lack of regression) are represented in Table 2 and Figures 1 to 3, which show the mean square estimates for the sources of variation due to regression and deviations and the regression graphs, respectively, for the three elephant grass genotypes.

For the DMY involving the three elephant grass genotypes, a significant 1st-degree linear effect was observed as a result of K doses within N1 for genotype ‘Cuban Pinda’ and a 2nd-degree linear effect within N4 and N2 for genotypes ‘IAC Campinas’ and ‘Cameroon’, with the respective coefficients of determination of 36.54, 91.81, and 93.34%, respectively (Table 2).

Santos (2013) found an average yield of 26.74 t ha\(^{-1}\) in three elephant grass cultivars (‘Guaçu/IZ.2’, ‘Cameroon-Piracicaba’, and ‘Cana D’África’) and three harvest cycles under two nitrogen fertilization doses (500 and 1000 kg ha\(^{-1}\) N). Quesada (2005) obtained, in eight months of growth, DM values of up to 30 t ha\(^{-1}\) in ‘Cameroon’ genotypes without application of N fertilizer.

Morais et al. (2009) found, in 18 months of growth, 44.7 t ha\(^{-1}\) DM from genotype ‘Cameroon’ with application of 50 kg ha\(^{-1}\) N. These results confirm the good selection of elephant grass varieties that has been performed aiming at high biomass production and at its use as an alternative energy source (Quesada, 2005), thereby providing positive results that potentiate the use of elephant grass as an alternative energy source by the direct biomass combustion.

In evaluating the DMY, as shown in Table 2, a significant effect of nitrogen doses (N1, N4, and N2) was observed on genotypes ‘Cuban Pinda’, ‘IAC Campinas’, and ‘Cameroon’, respectively. The highest N and K doses provided the highest estimated DMY for all genotypes. There was also an increase in DMY as the K doses were elevated, regardless of N (Figure 1).

In their study in the experimental area of IFES, Alegre - ES Campus, in the 2012 to 2013 period, Santos (2013) demonstrated a trend towards increased dry matter yield of elephant grass as the nitrogen doses were increased. The ratio of kilograms of N per ton of dry matter produced confirms the behavior of this trend, up to the limit at which N depresses this productivity, since the three cultivars (‘Guaçu/IZ.2’, ‘Cameroon-Piracicaba’, and ‘Cana D’África’) responded positively to the increased nitrogen fertilization levels used.

Carvalho et al. (1995) studied the application of nitrogen (0, 100, 200, and 400 kg ha\(^{-1}\) year\(^{-1}\)) and potassium (0, 75, and 150 kg ha\(^{-1}\) year\(^{-1}\)) in Brachiaria grass grown on a Red-Yellow Latosol (Hapludox) and found that under low potassium supply, the response to nitrogen fertilization was limited. However, the effect of nitrogen fertilization on dry matter yield was not significant, and this effect was not deeply influenced by the application of potassium, suggesting that with low potassium and nitrogen supply, the plant response was higher, but with the increase in potassium fertilization there was no marked response to nitrogen fertilization.

Oliveira et al. (2015), found an average dry matter yield in six genotypes of 35.03 t ha\(^{-1}\) year\(^{-1}\), with a 10-month cycle, showing that the results found in this study are higher, which indicates that fertilization provided an increase in dry matter yield.

The overall mean for percentage of dry matter (%DM) trait of the genotypes as a function of the N and K doses was 32.88% (Table 2). This dry matter percentage corroborates the results obtained by Souza Sobrinho et al. (2005), who found an average of 24.47%, with intervals shorter than 100 days.

The estimates for the 1st- and 2nd-degree linear models applied to the mean values of NT involving the three elephant grass genotypes referring to the two-year crop cycle are shown in Table 2 and Figure 2. In the regression analysis for the trait NT, it was found that the genotype which showed regression was ‘IAC Campinas’ at the N4 dose, whose coefficient of determination was R\(^2\) = 83.13% at the 5% significance level by the “F” test. The best-fitting model was the 1st-degree type.

Genotype ‘IAC Campinas’ showed a higher NT, estimated at 23.53, with the highest N dose, differing from genotypes ‘Cuban Pinda’ and ‘Cameroon’, whose NT were 28.82 and 22.75, respectively, under the same dose. Among the N doses, genotype ‘Cameroon’ manifested a reduced number of tillers, 22.75, with the highest N dose, 2200 kg ha\(^{-1}\).

The obtained values for NT did not differ from those found by Santos et al. (2014), who observed for the cv. ‘Guaçu/IZ.2’, ‘Cameroon-Piracicaba’, and ‘Cana D’África’, in the 2nd harvest, the respective values of 28.43, 23.00, and 30.58 in a 10-month interval under a lower N dose; they also did not differ from those obtained by Oliveira et al. (2013), whose cultivars ‘Guaçu/IZ.2’, ‘Cameroon-Piracicaba’, ‘Cana D’África’, and ‘Cuban Pinda’ showed 28, 28, 23, and 24 tillers per meter, respectively, in a six-month interval, also using lower doses of nitrogen fertilization. According to Silva et al. (2010), the traits number of basal and aerial tillers per meter showed high heritability, demonstrating little influence of the environment on the variability among clones. The higher productivity coincides with the higher number of tillers per
Table 2. 1st- and 2nd-degree linear regression models for the morpho-agronomic traits (dry mater yield (DMY), percentage of DM (%DM), number of tillers per linear meter (NT), plant height (PH), stem diameter (SD)) of three genotypes ('Cuban Pinda' - G1; 'IAC Campinas' - G2; and 'Cameroon' - G3) under four nitrogen levels (100, 800, 1500, and 2200 kg ha\(^{-1}\) N) and four potassium levels (50, 400, 750, and 1100 kg ha\(^{-1}\) K\(_2\)O) and two years of growth.

<table>
<thead>
<tr>
<th>GEN</th>
<th>Doses of N</th>
<th>Model</th>
<th>Regression equation</th>
<th>R(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Morpho-agronomic traits (DMY, t ha(^{-1}))</td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>N1</td>
<td>1</td>
<td>(\hat{y} = 5.79 - 5.5 \times 10^{-3}K^*)</td>
<td>36.54</td>
</tr>
<tr>
<td></td>
<td>N2</td>
<td>Absence</td>
<td>(\hat{y} = 56.34)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>N3</td>
<td>Absence</td>
<td>(\hat{y} = 52.28)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>N4</td>
<td>Absence</td>
<td>(\hat{y} = 67.73)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Independent of N</td>
<td>-</td>
<td>(\hat{y} = 43.42 + 0.0161K^{**})</td>
<td>81.87</td>
</tr>
<tr>
<td>G2</td>
<td>N1</td>
<td>Absence</td>
<td>(\hat{y} = 46.33)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>N2</td>
<td>Absence</td>
<td>(\hat{y} = 41.23)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>N3</td>
<td>Absence</td>
<td>(\hat{y} = 49.04)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>N4</td>
<td>2</td>
<td>(\hat{y} = 73.60 - 0.1973K^* + 4.34 \times 10^{-5}K^{2*})</td>
<td>91.81</td>
</tr>
<tr>
<td></td>
<td>Independent of N</td>
<td>-</td>
<td>(\hat{y} = 42.07 + 0.0148K^{**})</td>
<td>97.54</td>
</tr>
<tr>
<td>G3</td>
<td>N1</td>
<td>Absence</td>
<td>(\hat{y} = 49.04)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>N2</td>
<td>2</td>
<td>(\hat{y} = 56.97 + 1.5 \times 10^{-3}K^{ns} - 3.8 \times 10^{-5}K^{2ns})</td>
<td>93.34</td>
</tr>
<tr>
<td></td>
<td>N3</td>
<td>Absence</td>
<td>(\hat{y} = 47.48)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>N4</td>
<td>Absence</td>
<td>(\hat{y} = 54.18)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Independent of N</td>
<td>-</td>
<td>(\hat{y} = 46.91 + 0.0029K^{ns})</td>
<td>20.32</td>
</tr>
</tbody>
</table>

Morpho-agronomic traits (%DM, %)

<table>
<thead>
<tr>
<th>GEN</th>
<th>Doses of N</th>
<th>Model</th>
<th>Regression equation</th>
<th>R(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>N1</td>
<td>Absence</td>
<td>(\hat{y} = 31.48)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>N2</td>
<td>Absence</td>
<td>(\hat{y} = 31.54)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>N3</td>
<td>Absence</td>
<td>(\hat{y} = 31.78)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>N4</td>
<td>Absence</td>
<td>(\hat{y} = 32.51)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Independent of N</td>
<td>-</td>
<td>(\hat{y} = 32.39 + 3 \times 10^{-5}K^{ns})</td>
<td>0.14</td>
</tr>
<tr>
<td>G2</td>
<td>N1</td>
<td>Absence</td>
<td>(\hat{y} = 34.06)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>N2</td>
<td>Absence</td>
<td>(\hat{y} = 33.37)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>N3</td>
<td>Absence</td>
<td>(\hat{y} = 33.68)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>N4</td>
<td>Absence</td>
<td>(\hat{y} = 33.68)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Independent of N</td>
<td>-</td>
<td>(\hat{y} = 33.20 + 0.001K^{ns})</td>
<td>75.97</td>
</tr>
<tr>
<td>G3</td>
<td>N1</td>
<td>Absence</td>
<td>(\hat{y} = 32.89)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>N2</td>
<td>Absence</td>
<td>(\hat{y} = 33.24)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>N3</td>
<td>Absence</td>
<td>(\hat{y} = 33.46)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>N4</td>
<td>Absence</td>
<td>(\hat{y} = 32.87)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Independent of N</td>
<td>-</td>
<td>(\hat{y} = 33.62 - 0.0003K^{ns})</td>
<td>14.75</td>
</tr>
</tbody>
</table>

Morpho-agronomic traits (NT/m)

<table>
<thead>
<tr>
<th>GEN</th>
<th>Doses of N</th>
<th>Model</th>
<th>Regression equation</th>
<th>R(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>N1</td>
<td>Absence</td>
<td>(\hat{y} = 23.50)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>N2</td>
<td>Absence</td>
<td>(\hat{y} = 22.26)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>N3</td>
<td>Absence</td>
<td>(\hat{y} = 27.40)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>N4</td>
<td>Absence</td>
<td>(\hat{y} = 28.82)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Independent of N</td>
<td>-</td>
<td>(\hat{y} = 20.79 + 0.0059K^{**})</td>
<td>86.81</td>
</tr>
<tr>
<td>G2</td>
<td>N1</td>
<td>Absence</td>
<td>(\hat{y} = 24.09)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>N2</td>
<td>Absence</td>
<td>(\hat{y} = 21.02)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>N3</td>
<td>Absence</td>
<td>(\hat{y} = 26.32)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>N4</td>
<td>1</td>
<td>(\hat{y} = 35.19 - 5.3 \times 10^{-3}K^*)</td>
<td>83.13</td>
</tr>
</tbody>
</table>
Table 2. Contd.

Independent of N  

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th>( \hat{y} = 22.35 + 0.0061K^{**} )</th>
<th>90.52</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1</td>
<td>Absence</td>
<td>( \hat{y} = 25.33 )</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>N2</td>
<td>Absence</td>
<td>( \hat{y} = 23.63 )</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>N3</td>
<td>Absence</td>
<td>( \hat{y} = 24.80 )</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>N4</td>
<td>Absence</td>
<td>( \hat{y} = 22.75 )</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

**Morpho-agronomic traits (PH, m)**

<table>
<thead>
<tr>
<th></th>
<th></th>
<th>( \hat{y} = 2.84 + 1.5 \times 10^{-4}K^* )</th>
<th>56.33</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1</td>
<td>Absence</td>
<td>( \hat{y} = 3.06 )</td>
<td>-</td>
</tr>
<tr>
<td>N2</td>
<td>Absence</td>
<td>( \hat{y} = 2.94 )</td>
<td>-</td>
</tr>
<tr>
<td>N3</td>
<td>Absence</td>
<td>( \hat{y} = 3.21 )</td>
<td>-</td>
</tr>
</tbody>
</table>

Independent of N  

|     |     | \( \hat{y} = 3.03 + 7 \times 10^{-5}K^{ns} \) | 40.00 |

**Morpho-agronomic traits (SD, cm)**

<table>
<thead>
<tr>
<th></th>
<th></th>
<th>( \hat{y} = 1.35 )</th>
<th>-</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1</td>
<td>Absence</td>
<td>( \hat{y} = 1.46 )</td>
<td>-</td>
</tr>
<tr>
<td>N2</td>
<td>Absence</td>
<td>( \hat{y} = 1.44 )</td>
<td>-</td>
</tr>
<tr>
<td>N3</td>
<td>Absence</td>
<td>( \hat{y} = 1.45 )</td>
<td>-</td>
</tr>
</tbody>
</table>

Independent of N  

|     |     | \( \hat{y} = 1.37 + 8 \times 10^{-5}K^{ns} \) | 68.77 |

**Morpho-agronomic traits (LW, cm)**

<table>
<thead>
<tr>
<th></th>
<th></th>
<th>( \hat{y} = 4.32 )</th>
<th>-</th>
</tr>
</thead>
<tbody>
<tr>
<td>N1</td>
<td>Absence</td>
<td>( \hat{y} = 4.40 )</td>
<td>-</td>
</tr>
<tr>
<td>N2</td>
<td>Absence</td>
<td>( \hat{y} = 4.47 )</td>
<td>-</td>
</tr>
<tr>
<td>N3</td>
<td>Absence</td>
<td>( \hat{y} = 4.41 )</td>
<td>-</td>
</tr>
</tbody>
</table>

Independent of N  

|     |     | \( \hat{y} = 4.29 + 3 \times 10^{-5}K^{ns} \) | 6.15 |
Table 2. Contd.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Treatment</th>
<th>Model</th>
<th>Coefficients</th>
</tr>
</thead>
<tbody>
<tr>
<td>G2</td>
<td>N1 Absence</td>
<td>$\hat{y} = 4.47$</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>N2 Absence</td>
<td>$\hat{y} = 4.41$</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>N3 Absence</td>
<td>$\hat{y} = 4.37$</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>N4 Absence</td>
<td>$\hat{y} = 4.37$</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Independent of N</td>
<td>$\hat{y} = 4.39 + 7 \times 10^{-5}K^{ns}$</td>
<td>10.37</td>
</tr>
<tr>
<td>G3</td>
<td>N1 Absence</td>
<td>$\hat{y} = 4.11$</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>N2 Absence</td>
<td>$\hat{y} = 4.34$</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>N3 Absence</td>
<td>$\hat{y} = 4.08$</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>N4 Absence</td>
<td>$\hat{y} = 4.18$</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Independent of N</td>
<td>$\hat{y} = 4.23 - 9 \times 10^{-6}K^{ns}$</td>
<td>0.12</td>
</tr>
</tbody>
</table>

**, *, and "ns"Significant at the 1 and 5% probability levels and not significant according to the F test, respectively. Whole-plant dry matter yield, in t ha$^{-1}$ = DMY; Percentage of whole dry matter = %DM; Number of tillers per meter = NT; Plant height, in m = PH; Average stem diameter, in cm = SD; Average leaf width, in cm = LW.

Figure 1. Characteristic straight for dry matter yield of genotypes ‘Cuban Pinda’ (G1), ‘IAC Campinas’ (G2), and ‘Cameroon’ (G3) with increasing potassium fertilization doses (50, 400, 750, and 1100 kg ha$^{-1}$ K$_2$O) irrespective of nitrogen fertilization.

The estimates for the 1st- and 2nd-degree linear models applied to the mean values for PH involving three elephant grass genotypes referring to the two-year crop cycle are described in Table 2 and Figure 3. For this variable, there was no model fit for genotypes ‘IAC Campinas’ and ‘Cameroon’, which showed no regression, while ‘Cuban Pinda’ showed a 1st-degree regression, with $R^2 = 56.33\%$. As reported by Oliveira et al. (2015), in regression analysis of PH, ‘Guaçu/I.Z.2’ did not obtain regression and for ‘Cameroon-Piracicaba’, the best-fitting regression model was the 2nd-degree type, with a coefficient of determination of $R^2 = 41.93\%$ at the 1% significance level by the “F” test.

In the analysis of the average height (PH) trait, genotypes ‘IAC Campinas’ and ‘Cameroon’ did not differ statistically across the nitrogen doses. The growth of genotype ‘Cameroon’ with increasing potassium and nitrogen doses did not influence PH, demonstrating that neither element provided a highly significant increase in area and plant height.
the height of genotypes ‘Cuban Pinda’ and ‘IAC Campinas’ and led to a decrease in the PH of genotype ‘Cameroon’ (Figure 3).
grasses with different harvest intervals and found that at 12 months of age the grass had reached 5 m. Oliveira et al. (2013) found that the average of the studied genotypes was 1.88 m, at 24 weeks. In our study, the average height was 3.02 cm, with a 12-month cycle. According to Xia et al. (2010) and Menezes et al. (2015), this variable is positively correlated with dry matter yield.

The estimates for the 1st- and 2nd-degree linear models applied to the mean values for SD involving three elephant grass genotypes referring to the two-year crop cycle are shown in Table 2. Analyzing the average SD, genotypes ‘Cuban Pinda’ and ‘Cameroon’ showed no regression for the N and K doses. For the N3 dose, genotype ‘IAC Campinas’ displayed a 2nd-degree regression, with $R^2 = 90.0\%$, at the 5% significance level by the “F” test.

Genotype ‘IAC Campinas’ differed from genotypes ‘Cuban Pinda’ and ‘Cameroon’ at the N3 dose. With the lower N and K doses, the genotypes already showed an increase in stem diameter. According to Oliveira et al. (2015), for the genotype ‘Cuban Pinda’, the model that best fit was the 2nd-degree model, with a coefficient of determination of $R^2 = 18.62\%$, at the 5% significance level by the “F” test. For genotype ‘Cameroon-Piracicaba’, the 1st-degree model had the best fit, with 24.03% at the 5% significance level by the “F” test.

Studying the stem diameter and number of basal tillers per meter, Silva et al. (2010) observed the existence of high heritability. However, heritability estimates are part of the set of genotypes evaluated and of a certain environmental condition. In a small-sized Pennisetum clone, researchers (Silva and Rocha, 2010; Silva et al., 2010) observed 98% heritability for stem diameter and 83% for number of tillers, because the lower genetic variation found for these clones indicates that a large part of phenotypic variability has genetic causes.

Estimates for the 1st- and 2nd-degree linear models applied to leaf width involving the three elephant grass genotypes are shown in Table 2. There was no model fit for the genotypes that showed no regression.

Conclusions

The three genotypes higher number of tillers, height, and stem diameter with the lowest N and K doses, showing a possible trend of elevated doses not providing a highly significant increase in these traits.

The study of dry matter yield showed that under low nitrogen doses and with increased potassium doses, dry matter yield increased; however, as the nitrogen level was increased in association with potassium doses; this production did not increase but was rather suppressed.

The three elephant grass genotypes, ‘Cuban Pinda’, ‘IAC Campinas’, and ‘Cameroon’, obtained average yields of 52.66, 50.60, and 48.57 t.ha$^{-1}$, respectively. These results are quite promising and prove the possibility of using elephant grass as an alternative source for biomass production.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors thank the National Council for Scientific and Technological Development (Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPQ) for the financial support to develop this research and the Rio de Janeiro Federal Institute (Instituto Federal Fluminense), Bom Jesus do Itabapoana campus, for providing the area to conduct the experiment.

Abbreviations

GEN, Genotype; G1, Cuban Pinda; G2, IAC Campinas; G3, Cameroon; DMY, dry matter yield; %DM, percentage of whole dry matter; NT, number of tillers per meter; PH, plant height in m; SD, average stem diameter in cm; LW, average leaf width in cm.

REFERENCES


Full Length Research Paper

Study of associative effects of date palm leaves mixed with *Aristida pungens* and *Astragalus gombiformis* on the aptitudes of ruminal microbiota in small ruminants

Deffairi Djamila* and Arhab Rabah

Department of Food Sciences, Blida University, Algeria.

Received 22 September 2015, Accepted 27 June, 2016

The objectives of this trial were to evaluate interactions between microbial degradation of three substrates: date palm leaves as tannin-rich substrate, *Astragalus gombiformis* (as nitrogen source) and *Aristida pungens* (as carbon source). The model included the principal effects of single substrate and mixtures of multiple interactions. The forages were fermented alone or mixed with date palm leaves in various combinations (0, 10, 20, 30, 40 and 50%), in buffered rumen fluid using *in vitro* gas techniques. Gas production (mmol/g of dry matter) at 3, 6, 9, 24, 48 and 72 h of incubation was measured from all feed combinations. Treatments were carried out in triplicate and data were submitted to analysis of variance using a mixed procedure with repetition considered as the random effect. The interactions between feeds were evaluated on the basis of differences between single and mixed substrates on the different variables measured. The results showed that the included palm leaves reduced gas production when associated with *A. pungens* (p<0.0001), whereas this effect was less significant with *A. gombiformis*. This effect was more pronounced at earlier times of incubations. The *in vitro* organic matter digestibility decreased linearly with the increasing inclusion of date palm leaves in the mixtures. It is concluded that date palm leaves may be a suitable feed supplement for small ruminants browsing *A. pungens* and *A. gombiformis* in arid regions. The present results also showed that the inclusion level should be lower than 20% and for a short period in order to minimise tannins effect. The description of the fermentation profile of *in vitro* gas production showed that Sandoval model was poor and not appropriate for the characteristic varieties of arid and semi-arid areas in Algeria and the multiple regression models revealed a good linear regression for both mixtures.

Key words: Date palm leaves, forage, semi-arid zone, associative effects, rumen, *in vitro* fermentation.

INTRODUCTION

The problem of forage availability and quality of animal feed are aggravated in arid, semi-arid and tropical regions and because of the scarcity and irregular rainfall that limit the growth of herbaceous species and biomass

*Corresponding author. Email: deffairi.reu@yahoo.fr.

Author(s) agree that this article remains permanently open access under the terms of the Creative Commons Attribution License 4.0 International License.
yield in rangelands. Thus, breeding in these regions have to survive the shortage of insufficient resources for most parts of the year (Robles et al., 2008; Bouffennara et al., 2012). In semi-arid regions, and in the dry season, crude protein content of the herbaceous rangeland vegetation decreases significantly which induces a prolonged period of under-nutrition of livestock (Yayneshet et al., 2009). In developing countries where food resources for food and feed are deficient, only low quality forages, crop residues and agro-industrial by-products available are used for feeding ruminants. Under these conditions, the use of supplementation is inevitable. This pathway provides to the rumen microorganisms the nutritive elements necessary for their growth, thus ensuring the favorable conditions for cellulolysis in the rumen (Moujehed et al., 2000).

In Algeria, the rangelands represent two-thirds of the total land widespread, mainly in the arid regions. Some species may be crucial for grazing ruminants where these plants contain anti-nutritional secondary compounds (phenolics and tannins) with potential side effects such as inhibition of rumen microbial fermentation, as well as decreased feed digestibility and animal performance (Min et al., 2003; Waghorn and McNabb, 2003; Mueller-Harvey, 2006).

In oasis areas, local farmers often offer date-palm leaves as a supplement for ruminants in spite of their high tannin content (Arhab et al., 2006). The anti-nutritive effect of dry leaves of the date palm has been studied in animal models of ruminants and mono-gastric and it seems that both reduced digestibility and toxicity may limit the potential of this plant as a feed supplement. The nutritional value of date palm crop residues has been quite extensively studied due to their high availability in the countries where date production is important (Alananbeh et al., 2015). Both the energy and protein values of these by-products are low as compared to that of cereal straw (Arbouche et al., 2008). However, no study has been conducted on the associative effect of palm leaves with other forage on in vitro fermentation.

The influence of different feed ingredients on rumen microbial activity can be variable and contradictory. For instance, increasing the level of cereal grain supplementation reduced ruminal fermentation of fiber (Mould et al., 1983a), whereas supplementation of alfalfa hay with corn stalks resulted in positive associative effects on N utilization that caused reduced intake of corn stalk (Wang et al., 2008). Rations formulated for ruminants are generally a mixture of individual feeds, and its net (NE) or metabolizable (ME) energy value is generally calculated by adding up the energy values of the individual feeds in it, on the hypothesis that the NE or ME value of individual feeds will be unchanged when they are fed in combination with other feeds. This assumption may not be true, as some published reports indicated that a level of association exists among feeds in rations (Dixon and Stockdale, 1999; Franci et al., 1997; Haddad, 2000; Hong et al., 2001, 2002; Mould et al., 1983a, b; Wang et al., 2008). However, while associative effects are often discussed in ruminant nutrition, at least theoretically, they are seldom taken into account in feed formulation. While the meaning of an associative effect is clear (the sum of the parts being less, or more, than the combination of the parts), reasons for an associative effect are not clear. On the other hand, a true associative effect does not relate to correction of a known nutrient deficiency, such as fermentable N, but to unknown (or obscure) nutrient interactions.

In 1979, Menke et al. proposed an estimation of the energy value of feedstuffs from their in vitro gas production associated with chemical parameters. The gas test (HFT or Hohenheimer Futterwert Test) method is based on the assumption that the accumulated 24 h gas production by a substrate, incubated in a syringe with rumen liquor and a nutritive solution, is proportional to the amount of digestible carbohydrates, and thus highly correlated to the energy value of feedstuffs or to the in vivo organic matter digestibility (OMD).

The objectives of this study were to (i) investigate the associative effects on fermentation abilities of ruminal microbiota of sheep and gas production of plant leaves of the date palm and characteristics of two perennial plants in arid zones: Astragalus gombiformis and Aristida pungens (Stipagrostis pungens) mixed with different combinations and (ii) establish an interaction between the main components of different substrates.

MATERIALS AND METHODS

Forage material

Forages were collected from El Oued, located in South-East area of Algeria. The climate of this region is arid with a mean annual rainfall of 75 mm, and average temperature of 1°C in January and 43°C in July.

The studied forages consisted of two native species of North Africa: A. gombiformis (Foulet el Ibel), rich in protein (Arhab, 2006), A. pungens (Stipagrostis pungens) (drinn) rich in fibers (Arhab, 2006) and a by-product of the date palm: the leaves, rich in tannins (Arhab, 2006). These plants were harvested at a mature stage while date palm leaves were harvested at senescence. About six to ten specimens of each plant species were sampled to obtain a representative sample of the plant biomass.

The aerial part of the plants, leaves, thin branches (young stems) and some flowers (when existing) were clipped with scissors and taken immediately to the laboratory where the samples from the different specimens were pooled and dried in an circulating air oven at 60°C for 48 h. The samples were then coarsely ground in a laboratory using a chopper and were then ground again and passed through a 1 mm screen; the latter are mixed with the leaves of date palm at different percentages (0, 10, 20, 30, 40, 50 and 100%).

Animal material

The experiment was conducted on three Texel (origin of these...
sheep Texel island) (breed of sheep), aged 12 months and an average live weight of 82 kg, deemed healthy by veterinary control, castrated and fitted with ruminal fistula. The animals were housed in individual pens. These subjects received a daily ration of 1200 g of oat vetch hay in two equal meals (8:00 am and 4:00 pm) with free access to water. Two weeks adaptation period were planned to allow a good adaptation of ruminal microbiota.

Analysis methods

Chemical analysis

The determination of the dry matter (DM) and organic matter of samples was conducted following the methods of AOAC (1999, 942.05 method ID). The analysis of the compounds of the plant cell wall was performed as described by Van Soest et al. (1991). The crude protein content of plants on one hand and content of crude protein associated with the fraction of neutral detergent fiber (NDF neutral detergent fiber) on the other were determined by the Kjeldahl method.

All determinations were conducted in triplicate and expressed as a percentage of DM. Phenolics were determined by addition of two or three drops of ferric chloride (FeCl₃) in 1 ml of the methanol extract that was diluted in 50% of distilled water. The change in color indicated the presence of phenolic compounds as follows: no change indicates absence of phenolic compounds, dark blue indicates presence of phenols or hydrolysable tannins, and dark green indicates presence of condensed tannins. The method followed was Folin-Ciocalteau as described in Julkunen-Titto (1985).

Quantitative analysis of phenolic compounds

Total phenols (TP) and total condensed tannins (TCT) was dosed separately.

Total Phenols (TP): The phenolic compounds analysis was carried out in three replicates. The dried plant material (200 mg) was extracted with acetone (10 ml, 70% v/v), then the solution was subjected, at ambient temperature for 20 min, to ultrasonic treatment. The content was centrifuged (4°C, 10 min and 3000 g) and stored in ice for analysis, then the centrifugate was treated as described above. The total phenols were estimated using Folin-Ciocâlteau reaction (Makkar et al., 1993). Tannic acid was used to perform calibration curve. TP was quantified as tannic acid equivalents and expressed as tannic acid eqg/kg DM.

Tanins: For the total condensed tannins (TCT), 0.5 ml of extract was treated with n-Butanol HCl (3 ml, 95%) in the presence of ferric ammonium sulfate (0.1 ml). The reactants were heated for 1 h in a boiling water bath. Absorbance was read at 550 nm. TCT were expressed as leucocyanidin using the equation:

\[
TCT = Ax78.26x(\text{dilution factor})/(\text{weight of sample on DM})
\]

where: A is the absorbance at 550 nm assuming that E1% efficiency, 1 cm, 550 nm is 460 leucocyanidin (Porter et al., 1986). Total tannins (TT) were determined as the difference in total phenolic compounds (measured by Folin-Ciocalteau reagent) before and after treatment with insoluble polyvinylpyrrolidone (Makkar et al., 1993).

In vitro study

The in vitro fermentation inoculum was obtained from the filtered rumen juice taken from three sheep. This juice was mixed to obtain a homogeneous inoculum. Rumen fluid was collected 1 h before the morning meal, placed in a container preheated to 39°C and saturated with CO₂. This container was sealed immediately and transported to the laboratory for further analysis within 2 h of collection (Menke et al., 1979).

Sheep rumen fluid was mixed well, then filtered through four layers of muslin and bubbled with CO₂ at 39°C. All the handling was carried out under a constant stream of CO₂. The activity of ruminal microbiota requires an anaerobic and the solution was bubbled with a continuous flow of CO₂, resulting in the reduction of artificial saliva indicated by the color change from pink to transparent.

In vitro fermentation of forages by rumen microbiota

The technique followed was that of Theodorou et al. (1994). In vitro gas production technique is a simulation of food degradation by the rumen microflora. At the end of fermentation, the gas is measured using a pressure transducer (Delta Ohm DTP704-2BGI, Herter Instruments SL). It is a simple and inexpensive technique, based on the measurement of gas production.

In vitro gas production

The method of Theodorou et al. (1994) was used for gas production. Four hundred milligrams of each feed were weighed into bottles of 125 ml serum and incubated in a water bath at 39°C with 15 ml of ruminal fluid and 25 ml of artificial saliva (Van Soest, 1994). Monitoring fermentation kinetics was performed by measuring the gas pressure produced at different incubation hours: 3, 6, 9, 24, 48 and 72 h.

Statistical analysis

The evolution of gas production was followed according to the incubation time and the percentage of A. pungens or A. gomphiformis in nutrient mixtures. A factorial ANOVA (the factorial ANOVA is based on design S <A*B> treating the effects of two crossed factors. The groups are assumed to be independent in each of the conditions defined by the intersection of two factors. The sources of variation to take into account are the factors A (time) and B (percentage) and optionally AB (time*percentage) interaction) was performed to find out the effects of the incubation time, the percentage of added plants and their mutual interaction on gas production. A fermentation profile model from the literature, based on Levenberg-Marquardt algorithm (Levenberg, 1944; Marquardt, 1963) which is an improvement of the Gauss-Newton method for the resolution of nonlinear least-squares regression problems, was tested. Then, a multiple regression was performed to determine the equation giving the best estimate of gas production based on the independent variables and their interaction. Statistical analyses were conducted with Statistica 10, Statsoft Inc, Tulsa, USA.

RESULTS AND DISCUSSION

The chemical composition of forages

The chemical composition of the substrates is shown in Table 1. There was a change in all nutrient elements of forages. The highest fiber content was recorded in A. pungens (Stipagrostis pungens) (509.4 g/kg DM),
followed by *A. gombiformis* (445.2 g/kg DM) and the leaves of dry palms (422.1 g/kg DM). The highest crude protein content was noted in *A. gombiformis* (125 g/kg DM) followed by palm leaves (45.9 g/kg DM). Regarding phenolic compounds (total phenols, TT and TCT) were 61.8, 49.1 and 36.2 g/kg DM for dry palm leaves, by *Astragalus* and *A. pungens*, respectively. The change in the mineral and organic elements concentration for the studied plants was strongly associated with the type of soil, climate, stage of maturity and harvest season genotypic characteristics, and the factors affecting the nutritional properties of forages (Arhab, 2007; Bahman et al., 1997; Pascual et al., 2000; Genin et al., 2004; Ramirez et al., 2004; Ammar et al., 2004).

According to the level of crude protein, *A. gombiformis* had high nitrogen level; in fact it was richer than the green forage such as *Setaria sphacelata* in which nitrogen content was between 47.4 and 69.2 g/kg of DM (Rakotozandriny, 1993). However, *A. gombiformis* is considered as the most digestible plant with a high content of crude protein (125 g/kg DM), which shows that the forage resource has an interesting nutritional potential for ruminants (Boufennara, 2012). On the other hand, Pascual et al. (2000) and Genin et al. (2004) showed that the high level of protein in *A. gombiformis* indicates its possible use as a protein supplement for ruminants.

The high content of the cell wall could be preserved because of the climate in the arid zone. In general, high temperatures and low rainfall tend to increase cell wall polysaccharides and decrease the soluble carbohydrates (Pascual et al., 2000).

The concentration of phenolic compounds varies considerably among plant species, the highest levels were observed in the dry leaves of the date palm. This could be due to the fact that the radial diffusion method, based on the measurement of the potential biological activity of tannins in food, will depend on the bonding force of the tannins and their mode of binding to the protein (Frazier et al., 2003), while chemical methods, based on the chemical properties of tannins, indicate the chemical nature of tannins (Silanikove et al., 1996).

**Chemical composition of mixtures**

For mixtures, it was noticed that the addition of palm leaves increased the DM level especially the level of tannins (TT and TCT) of mixtures and reduced the levels of fiber and crude protein in proportion to percentages (Tables 2 and 3). This can be explained by the ability of tannins to form complexes with proteins and therefore the fall of the levels of proteins (Dalzell and Kerven, 1998). Furthermore, it was found that for less percentage of mixtures (10-20%), TCT and TT levels were low, 7.22-10.44 and 24.08-26 86 g/kg DM, respectively, while the level of total nitrogen contents was higher (118.5 and 111.9 g/kg DM) in the palm leaves mixture and *A. gombiformis* as compared to those obtained in the leaves of date palm only.

For the mixture of date palm leaves and *A. pungens*, it was noted that at the same percentage of incorporation, the level of the nitrogen material was low as compared to the first mixture (54.2-54.8 g/kg DM) while the tannins levels were similar for both mixtures. According to a research carried out by Paterson et al. (1996), forages which have MAT contents below 70 mg/g DM require nitrogen supplementation to improve their ingestion by ruminants.

**Gas production**

The gas production of mixture of the date palm leaves with *A. pungens* (*S. pungens*) or *A. gombiformis* at different percentages of fermentation for individual substrates and mixtures are presented in Figures 1 and 2. The results of factorial ANOVA showed that adding palm leaves decreased gas production when they were associated with *A. pungens*, with significant effects (P < 0.0001), whereas, this effect was significant but less pronounced with *A. gombiformis*. For the mixture of the leaves of date palms with palm *A. pungens*, a highly significant effect of time (F=1341.6, p<0.0001), percentage (F=23.64, p<0.0001) and interaction of time with percentage (F=7.28, p<0.0001) was found. For the mixture of the leaves of date palms with *A. gombiformis*, there was a highly significant effect of time (F=323.5, p<0.0001), percentage (F=10.84, p<0.0001) while no effect of interaction of time with percentage (F=0.78, p=0.785>0.05) was found.

Indeed, there is an increase in the amount of gas generated as a function of time. The cumulative volume

---

**Table 1.** Chemical composition of simple fodder (in g/kg DM).

<table>
<thead>
<tr>
<th>Forages</th>
<th>DM</th>
<th>CP</th>
<th>NDF</th>
<th>ADF</th>
<th>ADL</th>
<th>TP</th>
<th>TT</th>
<th>TCT</th>
<th>PPC</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. gombiformis</em></td>
<td>551.7</td>
<td>125.0</td>
<td>614.9</td>
<td>445.2</td>
<td>78.1</td>
<td>34.0</td>
<td>21.3</td>
<td>4.0</td>
<td>ND</td>
</tr>
<tr>
<td><em>A. pungens</em></td>
<td>797.3</td>
<td>53.6</td>
<td>794.7</td>
<td>509.4</td>
<td>84.4</td>
<td>2.4</td>
<td>1.6</td>
<td>3</td>
<td>ND</td>
</tr>
<tr>
<td>Date palm leaves</td>
<td>896.3</td>
<td>59.5</td>
<td>586.1</td>
<td>422.1</td>
<td>97.1</td>
<td>61.8</td>
<td>49.1</td>
<td>36.2</td>
<td>55.5</td>
</tr>
</tbody>
</table>

DM, Dry matter; CP, crude protein; NDF, neutral detergent fibre; ADF, acid detergent fibre; ADL, lignin determined by solubilisation of cellulose with sulphuric acid; TP, total phenols; TT, total tannins; TCT, total condensed tannins; PPC, protein precipitating capacity.
of gas increased with the increase of incubation time (Figures 1 and 2). Significant differences in gas production were found between the substrates for all the incubation times. Gas production decreased in the presence of dry palms; this could be explained by the fact that they contained substances (tannins) that affect the hydrolytic way (enzymes) of A. pungens (mainly cellulose enzymes) (Sweeney et al., 2001). The best gas production can be obtained at high time but for low palm percentages (10 and 20%); according to Robinson et al. (2009). Gas production was much more pronounced in the early hours of incubation with mixture levels of 15 to 25%; this effectively proves that the incorporation of dry palms inhibited A. pungens degradation by ruminal microbiota. This can be explained by the chemical composition of dry palms in tannins. Indeed, effects of this are well established on fermentation activity. According to McSweeney et al. (2001), tannins can directly influence the ruminal microbiota and enzyme activity; in fact, condensed tannins exert an inhibitory action on growth of rumen microorganisms. Furthermore, Makkar and Becker (2009) suggested that TT have an influence on the reduction of methane production.

Unlike the association of palms-drinn, palms did not

Table 2. Chemical composition of the mixture (Aristida and palm leaves).

<table>
<thead>
<tr>
<th>A. pungens</th>
<th>Feuilles</th>
<th>0% Palm</th>
<th>10%</th>
<th>20%</th>
<th>30%</th>
<th>40%</th>
<th>50%</th>
<th>100%</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS g/kg</td>
<td>7973</td>
<td>8963</td>
<td>797,3</td>
<td>807,2</td>
<td>817,1</td>
<td>827</td>
<td>836,9</td>
<td>846,8</td>
</tr>
<tr>
<td>MM g/kg</td>
<td>119,6</td>
<td>109,5</td>
<td>119,6</td>
<td>118,6</td>
<td>117,6</td>
<td>116,6</td>
<td>115,6</td>
<td>114,6</td>
</tr>
<tr>
<td>Azote</td>
<td>8,56</td>
<td>9,5</td>
<td>8,56</td>
<td>8,65</td>
<td>8,75</td>
<td>8,84</td>
<td>8,94</td>
<td>9,03</td>
</tr>
<tr>
<td>MAT (Nx6,25)</td>
<td>53,6</td>
<td>59,5</td>
<td>53,6</td>
<td>54,2</td>
<td>54,8</td>
<td>55,4</td>
<td>56</td>
<td>56,6</td>
</tr>
<tr>
<td>N-NDF</td>
<td>3,75</td>
<td>5,3</td>
<td>3,75</td>
<td>3,91</td>
<td>4,06</td>
<td>4,22</td>
<td>4,37</td>
<td>4,53</td>
</tr>
<tr>
<td>Azote libre</td>
<td>4,81</td>
<td>4,2</td>
<td>4,81</td>
<td>4,75</td>
<td>4,69</td>
<td>4,63</td>
<td>4,57</td>
<td>4,51</td>
</tr>
<tr>
<td>NDF</td>
<td>794,7</td>
<td>568,1</td>
<td>794,7</td>
<td>772</td>
<td>749,4</td>
<td>726,7</td>
<td>704,1</td>
<td>681,4</td>
</tr>
<tr>
<td>ADF</td>
<td>509,4</td>
<td>422,1</td>
<td>509,4</td>
<td>500,7</td>
<td>491,9</td>
<td>483,2</td>
<td>474,5</td>
<td>465,8</td>
</tr>
<tr>
<td>Hémicelluloses</td>
<td>285,3</td>
<td>164,9</td>
<td>285,3</td>
<td>273,3</td>
<td>261,2</td>
<td>249,2</td>
<td>237,1</td>
<td>225,1</td>
</tr>
<tr>
<td>ADL</td>
<td>84,4</td>
<td>97,1</td>
<td>84,4</td>
<td>85,7</td>
<td>86,9</td>
<td>88,2</td>
<td>89,5</td>
<td>90,8</td>
</tr>
<tr>
<td>Cellulose</td>
<td>425</td>
<td>324</td>
<td>425</td>
<td>414,9</td>
<td>404,8</td>
<td>394,7</td>
<td>384,6</td>
<td>374,5</td>
</tr>
<tr>
<td>TP</td>
<td>2,4</td>
<td>61,8</td>
<td>2,4</td>
<td>8,34</td>
<td>14,28</td>
<td>20,22</td>
<td>26,16</td>
<td>32,1</td>
</tr>
<tr>
<td>TT</td>
<td>1,6</td>
<td>49,1</td>
<td>1,6</td>
<td>6,35</td>
<td>11,1</td>
<td>15,85</td>
<td>20,6</td>
<td>25,35</td>
</tr>
<tr>
<td>TCT</td>
<td>3</td>
<td>36,2</td>
<td>3</td>
<td>6,32</td>
<td>9,64</td>
<td>12,96</td>
<td>16,28</td>
<td>19,6</td>
</tr>
<tr>
<td>PPC</td>
<td>ND</td>
<td>55,45</td>
<td>ND</td>
<td>ND</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3. The chemical composition of mixture of A. gombiformis and palm leaves.

<table>
<thead>
<tr>
<th>A. gombiformis</th>
<th>Feuilles</th>
<th>0% Palm</th>
<th>10%</th>
<th>20%</th>
<th>30%</th>
<th>40%</th>
<th>50%</th>
<th>100%</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS g/kg</td>
<td>551,7</td>
<td>896,3</td>
<td>551,7</td>
<td>586,2</td>
<td>620,6</td>
<td>655,1</td>
<td>689,5</td>
<td>724</td>
</tr>
<tr>
<td>MM g/kg DM</td>
<td>231,7</td>
<td>109,5</td>
<td>231,7</td>
<td>219,5</td>
<td>207,3</td>
<td>195</td>
<td>182,8</td>
<td>170,6</td>
</tr>
<tr>
<td>Azote</td>
<td>20</td>
<td>9,5</td>
<td>20</td>
<td>18,95</td>
<td>17,9</td>
<td>16,85</td>
<td>15,8</td>
<td>14,75</td>
</tr>
<tr>
<td>MAT (Nx6,25)</td>
<td>125</td>
<td>59,5</td>
<td>125</td>
<td>118,5</td>
<td>111,9</td>
<td>105,4</td>
<td>98,8</td>
<td>92,3</td>
</tr>
<tr>
<td>N-NDF</td>
<td>6,4</td>
<td>5,3</td>
<td>6,4</td>
<td>6,29</td>
<td>6,18</td>
<td>6,07</td>
<td>5,96</td>
<td>5,85</td>
</tr>
<tr>
<td>Azote libre</td>
<td>13,6</td>
<td>4,2</td>
<td>13,6</td>
<td>12,66</td>
<td>11,72</td>
<td>10,78</td>
<td>9,84</td>
<td>8,9</td>
</tr>
<tr>
<td>NDF</td>
<td>614,9</td>
<td>568,1</td>
<td>614,9</td>
<td>610,2</td>
<td>605,5</td>
<td>600,9</td>
<td>596,2</td>
<td>591,5</td>
</tr>
<tr>
<td>ADF</td>
<td>445,2</td>
<td>422,1</td>
<td>445,2</td>
<td>442,9</td>
<td>440,6</td>
<td>438,3</td>
<td>436</td>
<td>433,7</td>
</tr>
<tr>
<td>Hémicelluloses</td>
<td>122,7</td>
<td>164,9</td>
<td>122,7</td>
<td>126,9</td>
<td>131,1</td>
<td>135,4</td>
<td>139,6</td>
<td>143,8</td>
</tr>
<tr>
<td>ADL</td>
<td>78,1</td>
<td>97,1</td>
<td>78,1</td>
<td>80</td>
<td>81,9</td>
<td>83,8</td>
<td>85,7</td>
<td>87,6</td>
</tr>
<tr>
<td>Cellulose</td>
<td>356,4</td>
<td>324</td>
<td>356,4</td>
<td>353,2</td>
<td>349,9</td>
<td>346,7</td>
<td>343,4</td>
<td>340,2</td>
</tr>
<tr>
<td>TP</td>
<td>34</td>
<td>61,8</td>
<td>34</td>
<td>36,78</td>
<td>39,56</td>
<td>42,34</td>
<td>45,12</td>
<td>47,9</td>
</tr>
<tr>
<td>TT</td>
<td>21,3</td>
<td>49,1</td>
<td>21,3</td>
<td>24,08</td>
<td>26,86</td>
<td>29,64</td>
<td>32,42</td>
<td>35,2</td>
</tr>
<tr>
<td>TCT</td>
<td>4</td>
<td>36,2</td>
<td>4</td>
<td>7,22</td>
<td>10,44</td>
<td>13,66</td>
<td>16,88</td>
<td>20,1</td>
</tr>
<tr>
<td>PPC</td>
<td>ND</td>
<td>55,45</td>
<td>ND</td>
<td>ND</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 1. Gas production based on percentage of date palm leaves and *A. pungens* (*S. pungens*) at different times.

Figure 2. Gas production based on percentage of date palm and *A. gombiformis* at different times.

affect the fermentation of *A. gombiformis*. This may be explained by the fact that dry palms, rich in tannins, do not affect the proteolytic enzymes (specificity of action and structure), or that the chemical composition of *A. gombiformis* limits the effects of palm leaves on the fermentation parameters. *A. gombiformis* is rich in
nitrogen matter, it generates the production of biomass. Indeed, the degradation of crude protein of *A. gombiformis* generates biomass production. Liu et al. (2002) demonstrated in an *in vitro* system, that an amount of nitrogen is sufficient to sustain microbial growth. In addition, it has been shown that the nitrogen can make only a small contribution to gas production. According to McCweeney (2001), the nitrogen digestibility in ruminants reported favorable responses when providing as supplement. Wang et al. (2008) had shown that supplementation with nitrogen increase digestibility of forage.

Our results are consistent with those reported by Aregheore et al. (2000), Long et al. (1999) and Khazaal et al. (1993). These authors report that the contribution of the total nitrogenous matter (MTA) on gas production is not a significant influencing factor. This suggests an intensification and stimulation of rumen fermentation activity of the microbiota when the latter is in the presence of a food rich in nitrogen and energy (Tendonkeng, 2004; Getachew et al., 2000 and Florence et al., 1999).

Otherwise, supplementation with greater amounts of energy-rich feeds with a source of protein could reduce the time taken to finish cattle for market and increase profitability. Numerous reports in the literature indicate substantial increases in live weight (LW) gain by supplementing cattle consuming low digestibility forages with energy and protein supplements (Hennessy and Morrison, 1982; Lee et al., 1987; Hennessy et al., 1995).

A positive effect on associative gas production has been reported when the leaves of forage trees were mixed with concentrate diets (Sandoval-Castro et al., 2002) and when the straw was mixed with tree leaves (Liu et al., 2002).

**Modeling of gas production**

The model of Sandoval-Castro (2000) was used for the description of the fermentation profile of the *in vitro* gas production. Therefore, the authors tried to stimulate the development of gas production according to the equation: (Gas Prod) = a + b (1-e^-kt) based on the percentage of *A. pungens* or *A. gombiformis*. The estimation method is that of Levenberg-Marquardt where a and b were two coefficients to be determined, k the hourly rate of gas production (%.t) and t the time. The results are summarized in Table 4. The coefficients determination R^2 were always >0.90. Negative values of “a” (Orskov and Ryle, 1990) were noted. According to several authors, a negative value of “a” is the result of the existence of a latent phase during which microorganisms attach and colonize food particles before any damage is detectable (Ahmed and El-Hag, 2004). This could also indicate the difficulty of ruminal microbiota to degrade these substrates and the need for an adjustment period so that it begins this degradation.

Despite the values of negative “a” and high coefficients of determination R^2>0.90, the convergence remained mediocre. Accordingly, this theoretical model remains poor, inflexible and not suitable for varieties of arid and semi arid areas. This mathematical model does not apply to plants of Saharan zones. That is why the authors proposed the model of multiple regression (Figures 3 and 4) with a relationship type Gas Production = a + bt + cP + dP t for the two mixtures where a, b, c and d are constant coefficients to be determined, t the time (h) and P the percentage of *A. pungens* or *A. gombiformis*. The analysis showed a good regression with multiple r = 0.896, with highly significant effects of t and interaction Pt (p<0.0001), but with non sensible effect of the percentage (p=0.365). Gas Production = 2.332 + 0.045t - 0.395P + 0.0453Pt for mixtures of leaves palm/A. pungens. For the palm mixture with *A. gombiformis*, a good regression with multiple r = 0.799, with significance of t (p=0.001), but with no sensible effect of P or interaction Pt (p=0.41 and p=0.10 respectively) was found. The equation was Gas Production = 2.723 + 0.0548t + 0.518P + 0.0233Pt. So, the regression model can be considered as available

<table>
<thead>
<tr>
<th>Mixture (% Drinn)</th>
<th>a</th>
<th>b</th>
<th>R^2</th>
<th>Mixture (% A. gombiformis)</th>
<th>a</th>
<th>b</th>
<th>R^2</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-3.765</td>
<td>8.551</td>
<td>0.961</td>
<td>0</td>
<td>-4.154</td>
<td>9.234</td>
<td>0.961</td>
</tr>
<tr>
<td>50</td>
<td>-2.896</td>
<td>7.958</td>
<td>0.937</td>
<td>50</td>
<td>-7.490</td>
<td>13.924</td>
<td>0.973</td>
</tr>
<tr>
<td>60</td>
<td>-3.702</td>
<td>9.162</td>
<td>0.945</td>
<td>60</td>
<td>-7.066</td>
<td>13.402</td>
<td>0.973</td>
</tr>
<tr>
<td>70</td>
<td>-3.276</td>
<td>8.764</td>
<td>0.936</td>
<td>70</td>
<td>-7.054</td>
<td>13.864</td>
<td>0.970</td>
</tr>
<tr>
<td>80</td>
<td>-3.512</td>
<td>9.351</td>
<td>0.936</td>
<td>80</td>
<td>-8.031</td>
<td>14.941</td>
<td>0.973</td>
</tr>
<tr>
<td>90</td>
<td>-2.432</td>
<td>7.935</td>
<td>0.905</td>
<td>90</td>
<td>-4.953</td>
<td>10.984</td>
<td>0.948</td>
</tr>
<tr>
<td>100</td>
<td>-4.499</td>
<td>10.932</td>
<td>0.945</td>
<td>100</td>
<td>-7.418</td>
<td>14.236</td>
<td>0.965</td>
</tr>
</tbody>
</table>

a and b are the two coefficients of (Gas prod)=a+b(1-e^-kt), where k is the hourly rate of the gas production (%.t), t the time (h), R^2 is the coefficient of determination.
Gas production with A. pungens (S. pungens). In addition, palm leaves can be a food supplement suitable for small ruminants browsing A. gombiformis or A. pungens (S. pungens) in arid regions. The present study results also demonstrated that the percentages of the incorporated palm leaves had to be less than 20% and for short periods in order to minimize the effect of tannins. The description of the fermentation profile of in vitro gas production revealed that Sandoval (2000) model is poor and therefore not appropriate for the characteristics of varieties of arid and semi-arid areas in Algeria, whereas the model of multiple regression has revealed a good linear regression for the two types of mixtures.

Conflict of Interests

The authors have not declared any conflict of interests.

REFERENCES


**Figure 3.** Rate of gas production of dry palm leaves and A. pungens mixtures.

**Figure 4.** Rate of gas production of dry palm leaves and A. gombiformis mixtures.


Isolation and characterization of cellulolytic *Bacillus licheniformis* from compost

Nallusamy Sivakumar*, Amira Al Zadjali, Saif Al Bahry, Abdulkhadir Elshafie and Elsadig Abdulla Eltayeb

1Department of Biology, College of Science, Sultan Qaboos University, P. O. Box:36, PC 123, Muscat, Sultanate of Oman.  
2Higher College of Technology, Muscat, Sultanate of Oman.

Received 1 September, 2016; Accepted 18 October, 2016

Eight cellulose degrading bacteria were isolated from compost and were identified as *Bacillus licheniformis* by 16S rRNA sequencing. Among the eight isolates, *Bacillus licheniformis* B4, B7 and B8 showed the highest cellulase activity. *B. licheniformis* B4 and B8 showed the maximum cellulase activity during the stationary phase of growth; but for B7, the maximum activity of cellulase was observed during the log phase. Reducing sugar released in the media, increased with increasing cellulase activity for all the three isolates. Significant correlation was observed between cellulase activity and protein content. The crude cellulase from B7 strain showed activity towards carboxymethyl cellulose and filter paper, but there was no detectable activity towards p-nitrophenyl-β-D-glucopyranoside (PNPG). The crude cellulase of *B. licheniformis* B7 exhibited maximum activity at 50°C and at pH 6 to 7.

**Key words:** *Bacillus licheniformis*, 16S rRNA, cellulase, reducing sugar, compost, viscosity.

INTRODUCTION

Accumulation of municipal solid waste is becoming a serious problem in all developing countries (Al-Khatib et al., 2015). In most developing countries, the inadequate treatment of municipal solid waste causes a serious threat to the environment (Pin-Jing, 2012). Cellulosic wastes such as paper, wood, agricultural residues and cardboard constitute a major component of municipal solid waste. Improper management of these wastes contaminates air, soil and water. Disposal of solid wastes in landfills contaminates the ground water and cause the emission of greenhouse gases such as carbon dioxide and methane. Most of the carbon dioxide and methane are produced from biodegradable cellulosic wastes such as wood, leaves, other agricultural residues and waste papers. Therefore, recycling of such cellulosic wastes can decrease the greenhouse effect (Kazaragis, 2005) and these can be used as one of the main renewable sources of energy (Zhou et al., 2015).

Cellulose a linear polysaccharide composed of β-1,4 linked D-glucopyranosyl units is synthesized by all
plants and other organisms such as bacteria, fungi, protists and invertebrates. It is considered as the most abundant renewable natural biological resource on the earth (Zhang et al., 2006). There is an increasing interest in the production of biofuel using cellulosic biomass as a renewable source of energy by breaking them into sugars using cellulase enzymes (Demain et al., 2005). The production of fuels and chemicals from cellulosic substrates using cellulas would reduce the use of fossil fuels and decrease the air pollution (Zhou et al., 2001). In biorefining, renewable resources such as agricultural crops or wood are utilized either for extraction of intermediates or for bioconversion into chemicals, commodities and fuels (Kamm and Kamm, 2004; Fernando et al., 2006). Thermostable enzymes has an advantage as catalysts in these processes, as high temperatures often promote better enzyme diffusion and cell-wall disorganization of the raw materials (Paes and O’Donohue, 2006). Furthermore, the conversion of plant-derived cellulosic biomass into useful commodities as biofuels always depends on the cellulase producing efficiency of bacteria.

Cellulases have extensive applications in various industries. Traditionally, they are useful in food and brewery production, animal feed processing, detergent production and laundry, textile processing and paper pulp manufacturing (Kuhad et al., 2011; Karmakar and Ray, 2011). The applications of cellulase enzymes in cellulose biorefinery for producing fermentable sugars are expected to rapidly increase in the foreseeable future due to the problems in sustainable supply of fossil fuel and the increased demand for production of biofuels and chemicals from renewable resources (Juturu and Wu, 2014). Because of the booming biotechnology industries, the demand for thermostable enzymes has increased immensely due to its high thermostability and feasibility (Haki and Rakshit, 2003).

Reactions at higher temperatures has decreased viscosity and hence increased diffusion coefficient of substrates leading to the favourable equilibrium displacement in endothermic reactions (Kumar and Swati, 2001). Conducting biotechnological processes at high temperatures reduce the risk of contamination by common mesophiles. In addition, process at high temperatures has major impact on the bioavailability and solubility of organic compounds leading to the effective bioremediation (Becker, 1997). Thus the demand for cellulase producing bacteria is steadily increasing, and the search of cellulase degrading bacteria from different sources is continuously needed.

This study was conducted with an objective to isolate cellulase producing bacteria from compost, an important component in the organic farming. The organic matter in compost is mainly cellulose (Jurak et al., 2014) and hence compost can be a good source to isolate cellulase producing bacteria.

MATERIALS AND METHODS

Screening of cellulolytic bacteria from compost samples

Twenty five compost samples were collected from the Gulf mushroom company Muscat, Oman for isolation of cellulase producing bacteria. The temperature at the time of collection was 50°C, and the samples were brought to the laboratory immediately after collection and well assay was used to screen the cellulose degrading capacity of bacteria from different compost samples. Five wells were made in a sterile carboxymethyl cellulose (CMC) medium (1 g of CMC, 0.1 g of NaCl, 0.1 g of NaNO₃, 0.1g of K₂HPO₄, 0.1g of KCl, 0.05 g of MgSO₄, 0.05 g of yeast extract, 1.7 g of agar and 100 ml of distilled water).

Compost samples (0.5 g) were dissolved in 4.5 ml Ringer solution (1/4 strength – Sigma). After homogeneous mixing, 100 µl of samples was added to each well. The plates were incubated at 50°C for 24 h. After incubation the CMC plates were flooded with Congo red, incubated for 30 min and then destained with 1 M NaCl. The compost samples with large clearance zones were selected for the isolation of cellulase producing bacteria. To isolate cellulolytic bacteria, 1 g of the selected compost samples were serially diluted using an automatic spiral platter (Autoplate 4000, Spiral Biotech, and UK), 100 µl of the sample was plated on the CMC agar medium and incubated at 50°C for 24 h. Colonies of cellulolytic bacteria were counted and the clearing zone diameters of the colonies were measured in mm (Apun et al., 2000).

Molecular identification of the isolates

Among the cellulase producing bacteria, eight isolates with the largest diameter of clearing zones were selected, isolated and identified by 16S rRNA sequence. Bacterial DNA was extracted and purified (MoBio kit, USA). The purified DNA was amplified by 30 cycles of PCR in a thermal cycler (Applied Biosystem, USA). Two external primers annealing at 5’ and 3’ end of the 16S rRNA were used: B27f 5’-AGAGTTTGATCCTGGCTCAG-3’ and U1492r 5’-GGTTACCTTGTTACGACTT-3’. The amplified products were analyzed in 1% agarose gel and purified (Aquick PCR purification kit, UK). The purified products were subjected to a second PCR and the products were sequenced using 3130X1 Genetic Analyzer (Applied Biosystem, UK). In the second PCR, two internal primers A341f 5’-CTAXGGGGXGXGACXACG-3’ and A1041r 5’-GGCCATGCACXWCTCTC-3’were used at 5’ and 3’ ends of 16S rRNA. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013).

Utilization of substrates

A preliminary study was conducted to demonstrate the ability of the eight isolates in utilizing glucose, cellulose, mannose, D-xylose, L-xylose, D-arabinose, L-arabinose and methyl-β-D-xylopyranose in phenol broth. Bacteria were inoculated into the broth aseptically and incubated at 50°C for 24 h. Change of the phenol broth from red to yellow is an indication of utilization of the above sugars.

Inoculum preparation and cellulase fermentation

For inoculum preparation, the eight isolates were cultured in...
nutrient broth at 50°C. Once OD reached 0.5 (at 600 nm), it will be use as inoculum for the production media. Inoculum of each bacteria (3%) was inoculated into 50 ml in two production media containing 1 and 2% CMC in 250 ml Erlenmeyer flasks and incubated at 50°C in a rotary shaker at 100 rpm. The supernatants which was obtained after centrifugation at 10,000 rpm (Beckman Coulter) at 24, 48 and 72 h were used for cellulase activity.

Cellulase activity

The cellulase activity was estimated using 2% CMC in 0.05 M citrate buffer (pH 4.8) as a substrate. Substrate (0.5 ml) was added to culture filtrate (0.5 ml) and incubated for 30 min at 50°C. Reducing sugar released was estimated by dinitrosalicylic acid (DNS) method (Ghose, 1987). The cellulase activity was determined by using a calibration curve of glucose. One unit of CMCase activity was expressed as 1 µmol of glucose which was liberated per ml enzyme per minute.

Cellulase production

To find out the cellulase producing ability, the selected isolates were inoculated into the CMC broth at 50°C. During fermentation, samples was withdrawn for every 2 h time interval and analysed for cellulase activity, reducing sugar, cell growth, viscosity, pH and protein content (Lowry et al., 1951).

Effect of temperature and pH on the activity of cellulase

The optimum temperature for CMCase activity of the crude cellulase was determined by measuring the enzyme activity at different temperatures ranging from 30 to 90°C. The optimum pH was also determined by measuring the enzyme activity at different pH ranging from 3 to 11 at optimum temperature. Different buffers such as 50 mM sodium acetate buffer (pH 3, 4 and 5), 50 mM sodium phosphate buffer (pH 6, 7, and 8) and 50 mM glycine-NaOH buffer (pH 9, 10 and 11) were used to adjust the pH. Further, crude cellulase was also tested for its ability to degrade CMC, filter paper, cellobiose, p-Nitrophenyl β-D-glucopyranoside (PNPG) and methyl-β-D-xylopyranoside.

Statistical analysis

The mean values and standard deviations were calculated form the data obtained from three different experiments. Analysis of variance was performed by the one way ANOVA procedures followed by Tukey HSD Post Hoc tests using PASW statistics 8. Statistical difference at p<0.05 was considered significant.

RESULTS AND DISCUSSION

In this study, compost was selected as a source for the isolation of cellulase producing bacteria, because the organic content in compost is mainly made up of cellulose and the temperature during composting was 50°C and above. Cellulase producing microbes have been isolated from different sources such as soil, water, compost etc. (Al-Kharousi et al., 2015). All the compost samples were positive for cellulolytic bacteria by forming clear zones in different diameters ranging from eight to nineteen mm. However, only 11 out of 25 samples showed that clearing zones is equal or greater than 10 mm. These samples were selected for isolation of cellulase producing bacteria. The colony forming units (CFU) of these samples ranged from 20×10^3 to 82×10^3 CFU/ml. Totally 25 bacteria were isolated from the 11 samples. Out of which, eight isolates with a large diameter of the clear zone were selected for further studies. The nucleotide sequencing of 16S rRNA confirmed that all the eight isolates were different strains of Bacillus licheniformis and named as B. licheniformis B1 to B8 (NCBI accession no JQ700446 to JQ700453).

Amplified 16S ribosomal ribonucleic acid (rRNA) gene fragments from the isolated B. licheniformis strains were sequenced and blast searched through the The National Center for Biotechnology Information (NCBI) database. Closely related sequences were downloaded and aligned using CLUSTAL W. These sequences were analyzed using the maximum likelihood method. Acetobacter pasteurianus 386B with accession number 102925.1 was taken as an out group (Figure 1). The presence of only B. licheniformis could be due to the high temperature of compost which allows only the endospore formers to exist. Bacillus spp. are the potential producers of cellulases. Other investigators reported that moderately thermophilic B. licheniformis was found to hydrolyze carboxymethylcellulose and p-nitrophenylcelllobioside (Bischoff et al., 2006). B. licheniformis with high cellulase activity at 65°C was isolated from mangroves (Tabao and Monsalud, 2010). B. licheniformis was able to degrade rice straw by secretion of cellulase and hemicellulase (Hong et al., 2007). B. amyloquefaciens hydrolyzing rice hull was isolated from soil (Lee et al., 2008). Most cellulolytic Bacillus spp. secrete endoglucanases which are capable of degrading carboxymethyl cellulose (Robson and Chambilis, 1984). Mostly Bacillus spp. are used in industries because they are not pathogenic, grow and reproduce easily, do not produce foul odors or gases, some species can survive in alkaline condition and at high temperature, secrete proteins extracellularly and are considered relatively safe to use with regard to health and environmental aspects (Beukes and Pletschke, 2006).

Utilization of different substrates

The ability of the eight isolates to utilize different substrates and derivatives of cellulose as carbon sources were studied. All the isolates were able to utilize xylan, cellobiose, glucose and mannose. None of the isolates utilized methyl-β-D-xylopyranose, L-xylose and D-arabinose. However, D-xylose was utilized only by B. licheniformis strains B4, B7 and B8 and L-arabinose by B4 and B7 (Table 1). Both xylose and arabinose are the
building blocks of hemicellulose. This indicates that all the eight bacteria isolated from compost have the ability to secrete xylanase, cellobiase, mannase and CMCase. Hydrolysis of complex plant cellulotic materials require the presence of different enzymes to degrade cellulose and hemicellulose into simple sugars. Enzymes, such as CMCase, xylanase, cellobiohydrolases, β-glucosidases, endo-1,4-β-xylanases, β-xylidosases, α-1-arabinofuranosidases, acetyl xylan esterase, α-glucuronidase, pectate lyase, and endo-β-1,4-d-mannanase are needed in biodegradation of cellulosic biomass (Van Dyk et al., 2009). This suggests that the isolates of this study were able to degrade the cellulose.

**Influence of CMC on cellulase production**

Cellulases are inducible enzymes produced by microorganisms when grown on cellulose and cellulose derivatives. The influence of CMC concentration on cellulase production and microbial density was assessed for the eight isolates using 1 and 2% CMC media. In 1% CMC medium, B3 showed the high cell density while B5 and B7 showed the lowest cell density (Figure 2a). The changes in cell densities during 24 h (p=0.089) and 48 h (p=0.103) were insignificant for all the isolates. However, cell density of B5 decreased significantly at 72 h and B3 increased significantly at 72 h (p=0.03). In 1% CMC medium, B7 showed the maximum cellulase activity at 48 h (Figure 2b). On the other hand, B3, B5 and B6 showed high cellulase activity at 72 h. All the eight isolates didn’t show any significant difference in their cellulase activity at 24 h (p>0.05). However, significant differences were found in the cellulase activity among all the eight isolates during 48 and 72 h (p=0.047 and 0.049 respectively).

In 2% CMC medium, cell densities of the isolates varied significantly from each other (p>0.05). For all the isolates microbial density declined during 72 h (Figure 3a). B2 showed lower cell density but B6 exhibits high cell density among the isolates. High cellulase activity was observed in B7 at 24 h. Among the isolates, cellulase activity was low in B5 and B6 even though they showed a good growth in the medium (Figure 3b). The induction of enzyme activity is significantly higher in 2% CMC medium than 1% medium. CMC is the most effective carbon source for cellulase production by *B. alcalophilus* and *B. amyloliquefaciens* (Abou-Taleb et al., 2009). *B. pumilus* EB3 was induced to produce CMCase when grown in the CMC medium (Ariffin et al., 2006). In this study, it is interesting to note that some isolates showed high cell density in the medium but their cellulase activity is still low. Some other strains with moderate cell density showed high cellulase activity in both 1% and 2% CMC media suggesting that the bacteria with high cell density may not be a high cellulose producer (Tong and
Table 1. Utilization of different carbon sources by *B. licheniformis* strains isolated from compost samples.

<table>
<thead>
<tr>
<th>Strains with NCBI accession number</th>
<th>xylan</th>
<th>Celllobiose</th>
<th>methyl-βD-xylopyranose</th>
<th>D-xylose</th>
<th>L-xylose</th>
<th>D-arabinose</th>
<th>L-arabinose</th>
<th>Glucose</th>
<th>Mannose</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. licheniformis</em> B1 (JQ700446)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>B. licheniformis</em> B2 (JQ700447)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>B. licheniformis</em> B3 (JQ700448)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>B. licheniformis</em> B4 (JQ700449)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>B. licheniformis</em> B5 (JQ700450)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>B. licheniformis</em> B6 (JQ700451)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>B. licheniformis</em> B7 (JQ700452)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>B. licheniformis</em> B8 (JQ700453)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

+ able to utilize carbon source; - unable to utilize carbon source.

Rajendra 1992; Emtiaz et al., 2007). Components in the media, other than CMC, might influence the growth to a certain extent. Among the eight isolates, the best three (B4, B7 and B8) were selected for further studies after enrichment in CMC medium.

**Relationship between cell growth and cellulase activity**

Both B4 and B8 had a high positive significant correlation between cellulase activity and pattern of cell growth (Figure 4a and c). For B4 and B8 strains, the cellulase activity reached a maximum between 14 and 18 h when the growth is at the stationary phase. This could be due to the decreasing concentration of CMC in the medium which results in the slowing down of growth rate and the cell density maintained at the same level. Hence, the cellulase production does not increase after this level (Robson and Chambliss 1984) and the enzyme activity follows the cell growth pattern. A positive significant correlation between cellulase activity and cell growth was observed (*p*<0.05). For B7, maximum cellulase activity occurred after 4 h, in the log phase of growth. This clearly indicates that these bacteria utilize CMC as a source of carbon and energy by producing cellulase as a primary metabolite. Further, enrichment in CMC medium increased the cellulase activity of B7. Cellulase activity and growth of B7 strain were not significantly correlated (Figure 4b). The time to reach the maximum cellulase activity for B4, B7 and B8 could be due to the difference in the triggering of cellulase pathways by cellulose. This also indicates that the correlation between cell growth and cellulase activity depends on the bacterial strain.

Among the isolates B4, B7 and B8, the B7 has a novel feature of producing maximum cellulase activity at 4 h of incubation. This variation could be due to the differences in genetic make-up for different strains, and the cultural conditions employed during the production process (Bajaj et al., 2009). Further, the arid environment in Oman from which the organic matters is collected for composting would influence the metabolic activities significantly. A further complete optimization study is required to get increased cellulase production from these strains.

**Changes in reducing sugar, protein content, viscosity and pH**

Changes in reducing sugar content, protein content, viscosity and pH of the CMC medium were studied. Reducing sugar released into the media indicates the biodegradation of CMC by cellulase enzyme into simple sugar (Alam et al., 2005). The reducing sugar released by all the three isolates followed the trend of cellulase activity (Figure 5a, b and c). A significant positive correlation was observed between cellulase
activity and reducing sugar released ($p<0.05$). Cellulase activity is related to soluble enzymes secreted into the medium and therefore the concentration of soluble proteins was analyzed. The increase in cellulase activity was significantly correlated with the increase in soluble protein in the media ($p=0.000$ for B8 and $p<0.05$ for B4 and B7).

During cellulase fermentation, the viscosity of B4 inoculated CMC medium decreased up to 8 h and then increased slightly. The viscosity of the B7 inoculated medium dropped sharply at 2 h of incubation and remained constant at later stages of incubation. In B8 inoculated into CMC medium, the viscosity decreased continuously up to 10 h, then increased slightly and remained at the same level during the later stages of incubation (Figure 6 a, b and c). The drop in the viscosity of the CMC medium during the initial hours of fermentation was due to the degradation of CMC by cellulase secreted by the bacteria. The slight increase in viscosity during the later stages of fermentation could be
due to the production of exopolysaccharides by the three isolates (Dupont et al., 2000). For all the three bacteria, pH changed from 8.4 to around 9 during cellulase fermentation.

**Activity of cellulase at different temperature and pH**

During cellulase fermentation B7 showed more activity in a short time than B4 and B8. Hence, the crude cellulase of B7 was tested at different pH and temperature to determine the optimum conditions of activity. The crude cellulase activity was higher at pH 6 and 7 and it retains 80% activity from pH 3 to 10 (Figure 7a). The activity of cellulase in a wide range of pH indicates that this enzyme could be used in different industrial processes with different pH ranges. Cellulases produced by *Bacillus* spp. from different sources have been found to have a different optimum pH.

![Graph showing cell density and cellulase activity of B. licheniformis B1 – B8 in 2% CMC medium at different incubation time.](image)
Figure 4. Changes in cell density and cellulase activity of *B. licheniformis*: (a) B4, (b) B7 and (c) B8 during cellulase fermentation in CMC broth.
Figure 5. Reducing sugar released and protein content of CMC medium during fermentation by *B. licheniformis*. (a) B4, (b) B7 and (c) B8.
Figure 6. Changes in medium viscosity and cellulase pH of *B. licheniformis* (a) B4, (b) B7 and (c) B8 during cellulase fermentation.
The optimum pH for *Bacillus* spp. CH43 and HR68 ranges from 5 to 6.5 (Mawadza et al., 2000), and pH 7.5 for *B. licheniformis* NLRI-X33 (Tae-IK et al., 2000). However, other studies have reported that the optimum pH for purified cellulase from *B. circulans* was 4.5 (Kim, 1995) while cellulase produced by *Bacillus* sp. C14 was 11 (Aygan and Arikan 2008). The crude cellulase activity was tested at different temperatures. The optimum temperature for cellulase activity was 50°C (Figure 7b). At 30°C it retains 73% activity and 75% activity at 70°C. The cellulase activity decreased significantly above 70°C and retains only 32% activity at 90°C. It has been reported that the optimum temperature for purified cellulase activity of *Bacillus* strains was 50°C (Kazaragis 2005; Lee et al., 2008; Kim 1995) and for *Anoxybacillus flavithermus* was 75°C (Shabeb et al., 2010). Further, crude cellulase from B7 showed the ability to degrade CMC, filter paper (FPase activity of 0.035 U/mg) and cellobiose (cellobiase activity of 0.04 U/mg). However, there was no detectable activity towards PNPG and methyl-βD-xylopyranose.

**Conclusions**

Eight cellulolytic *B. licheniformis* strains have been isolated from compost samples which were able to utilize
xylan, cellobiose, manno and CMC. Among the eight isolates, B4, B7 and B8 were found to produce the highest cellulase activity. B7 strain was found to produce the maximum cellulase activity at 4 h of incubation.

Moreover, the crude cellulase from B7 was active in a wide range of pH with an optimum temperature of 50°C at pH 6 to 7. Because of the ability to retain good activity at higher temperatures and a wide range of pH, cellulase from *B. licheniformis* B7 could be useful in biorefineries. An optimization study would further increase the production of cellulase by these bacteria. Further, the compost would be a good resource to isolate cellulose degrading bacteria.

**Conflict of Interests**

The authors have not declared any conflict of interests.

**ACKNOWLEDGEMENT**

We would like to thank The Research Council of Oman for their financial support (ORG/EBR/14/003).

**REFERENCES**


Tae-IK, Han JD, Jeon BS, Yang GB, Kim KN, Kim MK (2000). Isolation from cattle manure and characterization of *Bacillus licheniformis*

Drought tolerant tropical maize (Zea mays L.) developed through genetic transformation with isopentenyltransferase gene

Leta Tulu Bedada\textsuperscript{3*}, Miccah Songela Seth\textsuperscript{2}, Steven Maina Runo\textsuperscript{1}, Wondyifraw Tefera\textsuperscript{3}, Charless Mugoya\textsuperscript{4}, Clet Wandui Masiga\textsuperscript{4}, Richard Okoth Oduor\textsuperscript{1}, Eduardo Blumewald\textsuperscript{5} and Francis Wachira\textsuperscript{4}

\textsuperscript{1}Plant Transformation Laboratory, Department of Biochemistry and Biotechnology, Kenyatta University, P. O. Box 43844, GPO 00100, Nairobi, Kenya.
\textsuperscript{2}Mikocheni Agricultural Research Institute, Mikocheni B Light Industrial Area, Plot No. 24B, Along Coca Cola Road, P. O. Box 6226, Dar Es Salaam, Tanzania.
\textsuperscript{3}Ethiopian Institute of Agricultural Research, P. O. Box 2003, Addis Ababa, Ethiopia.
\textsuperscript{4}Association for Strengthening Agricultural Research in East and Central Africa (ASARECA), P. O. Box 765, Entebbe, Uganda.
\textsuperscript{5}Department of Plant Sciences, University of California, Davis, CA, USA.

Maize is a staple food crop for millions of Africans. Despite this fact, African farmers have been harvesting average grain yield of not more than 2 t/ha while there is a potential of producing more than 10 t/ha. Drought is one of the major abiotic constraints contributing to this low productivity. Drought diminishes crop productivity mainly by causing premature leaf senescence. The \textit{ipt} gene codes for isopentenyltransferase (IPT) enzyme which catalyzes the rate limiting step in the biosynthesis of cytokinin and has been shown to enhance tolerance to drought in transgenic crops by delaying drought-induced leaf senescence. This created interest to investigate if \textit{ipt} gene can be useful in enhancing drought tolerance in locally adapted African tropical maize genotypes. The tropical maize inbred line CML216 was transformed with \textit{ipt} gene using Agrobacterium-mediated transformation method. Five transgenic lines which were proved to be stably transformed through Southern blot analysis with copy number of 2 to 4 per event were developed. In drought assay carried out in the glass house, transgenic lines expressing the \textit{ipt} gene showed tolerance to drought as revealed by delayed leaf senescence compared to the wild type plants. Transgenic plants maintained higher relative water content and total chlorophyll during the drought period and produced significantly higher mean grain yield of 44.3 g/plant while the wild type plants produced mean grain yield of 1.43 g/plant. It is proposed that the transgenic lines developed in this study can be further tested for tolerance to drought under contained field trials. Furthermore, transgenic lines developed can be used in breeding programs to improve drought tolerance in other commercial tropical maize genotypes through conventional breeding.

Key words: Cytokinin, delayed leaf senescence, drought inducible, CML216, \textit{ipt} gene.

INTRODUCTION

Drought is a major limitation to crop production worldwide. It is now a decade and a half since drought was reported to affect maize production in about 20 to 25% of the global maize area (Heisey and Edmeades, 1999).
Two decades have been counted since yield losses of more than 70% was reported as a result of drought affecting tropical maize in over 60 million hectares in the tropics (Edmeades et al., 1994). With the forthcoming global climate change (Battisti and Naylor, 2009), more yield losses are expected with consequences of affecting 140 million people each year (Jones and Thornton, 2003). By 2050, crop yields are expected to diminish further by 10 to 20% as a result of higher temperature and reduced rainfall (Jones and Thornton, 2003). To reverse this problem, it is mandatory to develop agricultural technologies adapted to such changing environment (Rivero et al., 2007). New maize varieties having improved tolerance to drought stress rank higher in the list of such technologies. The contribution of conventional breeding towards this goal has become insufficient because of limited genetic diversity in the maize gene pool (Hardy, 2010; Shiferaw et al., 2011) and lack of suitable selection criteria for tolerance to drought stress (Nigussie et al., 2002). This brought the need to diversify the genetic basis of locally adapted tropical maize germplasm by introgressing genes responsible for improving tolerance to drought stress.

The ipt gene from Agrobacterium tumefaciens codes for isopentenyltransferase (IPT) enzyme which catalyzes the rate limiting step in the cytokinin biosynthesis pathway (Akiyoshi et al., 1984). Crops genetically engineered with this gene showed increased level of cytokinin and enhanced tolerance to drought stress as a result of delayed leaf senescence (Rivero et al., 2007; Rivero et al., 2009; Peleg et al., 2011). In maize, the upper eight to nine leaves form major sources contributing 75 to 90% of the assimilate to the grain (Allison and Watson, 1996). Maize responds to drought by launching leaf senescence as a strategy to avoid drought by reducing canopy size and to mobilize nutrients to support the growth of the upper younger leaves and grains (Grabau, 1995). This regulation of leaf senescence has an obvious adaptive value in wild plants allowing them to complete their life cycle even under stressful conditions. In crop plants, drought induced leaf senescence is often associated with reduced grain yield (Gan and Amasino, 1996; Gungula et al., 2005) causing premature death of photosynthetically active leaves. Drought induced production of cytokinin has been shown to play a major role in changing this source/sink relationships and was applied as an important component for the development of drought-tolerant rice (Peleg et al., 2011). It is hypothesized that the same strategy can be applied to tropical maize, which is also a monocot, ending with improved drought tolerance.

Transgenic crops developed with ipt gene manifest different morphological and physiological abnormalities depending on the promoter driving the gene. Such abnormalities occur in condition where constitutive promoters like the cauliflower mosaic virus promoter CaMV35S or the ubiquitin maize promoter is used to drive the gene to the extent of preventing studies on cytokinin overproduction in normal plant tissues (McKenzie et al., 1998). In maize, the ipt gene has been expressed using senescence-activated promoters (Young et al., 2004; Robson et al., 2004). In PSAG12::ipt maize, senescence was not delayed despite the observed expression of ipt in senescing leaves (Young et al., 2004). Extended greenness accompanied by a delay in senescence induced by nitrogen stress has been reported in temperate maize transformed with ipt gene driven by native promoter of the senescence enhanced (SEE) gene (Robson et al., 2004). Similar to previous report by Jordi et al. (2000), transgenic lines failed to recycle internal nitrogen from senescing lower leaves which has accounted for significant chlorosis in emerging younger leaves when plants were grown in low nitrogen stress condition (Robson et al., 2004). Hence, these drawbacks limited the use of the senescence enhanced promoter to drive ipt gene to delay leaf senescence in maize.

The other option was, therefore, to resort to drought inducible expression of the ipt gene as a strategy for maize transformation with the objective of delaying drought induced leaf senescence. This strategy could also delay drought induced leaf senescence by synchronizing cytokinin production with the onset of drought stress in the plant (Rivero et al., 2007). A drought inducible senescence-associated receptor kinase (SARK) promoter was identified from a gene of which expression is up-regulated at the earliest stage of leaf senescence before any visible sign like leaf yellowing in haricot bean (Phaseolus vulgaris L.) (Hajouj et al., 2000). This was linked to the ipt gene and used to transform tobacco (Rivero et al., 2007) and rice (Peleg et al., 2011), using A. tumorfaciens. In both crops, transgenic lines expressing PSARK::IPT did not differ in appearance from the wild type. They further exhibited extreme tolerance to drought by delaying senescence compared to wild type plants.

Unlike the previous report of Gan and Amasino (1996) on PSAG::IPT transgenic lines, the expression of PSARK::IPT during drought was not only enhanced in basal leaves but also in the middle and apical leaves, confirming the drought responsiveness of the SARK promoter. Their work shows that nitrogen mobilization was not affected in the PSARK::IPT plants, because the basal leaves displayed chlorophyll degradation during drought.

The performance of the PSARK::IPT rice lines (Peleg et al., 2011) created interest that the ipt gene has the potential to bring improved tolerance to drought in tropical
maize which is also a monocot crop. This study was therefore designed to investigate if ipt gene driven by the drought inducible SARK promoter can be useful in enhancing tolerance to drought stress in maize genotypes adapted to tropical African environment. For this purpose, a construct carrying $P_{\text{SARK}}$::ipt::NOST cassette was obtained from the Department of Plant Sciences, University of California, Davis, CA, USA. We sub-cloned the expression cassette to the binary vector, pNOV2819, to avoid use of antibiotic resistance marker gene and to take advantage of the pmr gene as plant selectable marker and mannose as selective agent (Negrotto et al., 2000) to develop a product, which is safe to the environment and the consumers. The tropical maize inbred line CML216 was transformed using Agrobacterium-mediated transformation technique and successfully regenerated normal and fertile transgenic plants. Though ipt gene has already been used to transform temperate maize under the promoter of SAG12 gene and SEE gene of Arabidopsis and maize, respectively, we are reporting for the first time on successful enhancement of tolerance to drought stress in tropical African maize by delaying drought induced leaf senescence through inducible expression of the ipt gene under the SARK promoter.

MATERIALS AND METHODS

Construct preparation

The AF234301 (pCAMBIA1380::pSARK::IPT) (10.4 kbp) construct (Figure 1A) carrying the $P_{\text{SARK}}$::ipt::NOST expression cassette was received from the Department of Plant Sciences, University of California, Davis, CA, USA having the hygromycin phosphotransferase gene (hptII) as a plant selectable marker. The expression cassette was sub-cloned to the pNOV2819 binary vector provided by Syngenta (Figure 1B), to avoid use of hptII gene as a marker and to take advantage of the pmr gene in pNOV2819 vector as a plant selectable marker and mannose as selective agent (Negrotto et al., 2000).

Forward 5'-GTGCTCCACCATGTTGCGCCGCAGCAGCGGA-3' and reverse 5'-GCGAATTCCTCGGATGTAAGCATGAT-3' primers were designed on the sequences of the PUC multiple cloning site and SARK promoter, and nos terminator (NOST), respectively, using FastPCR software. The forward primer incorporated restriction sites for AscI and EcoRI from the PUC multiple cloning site available in AF234301 (pCAMBIA1380::pSARK::IPT) while HindIII site was engineered to the 5' of the reverse primer. The PCR reaction was carried out in Eppendorf (Eppendorf AG, Hamburg, Germany) PCR machine using Pfu DNA polymerase (5 U/μl) (Fermentas Inc, Maryland, USA). The PCR programme was composed of initial denaturation at 98°C for 5 min, followed by 35 cycles of denaturation at 98°C for 30 s, annealing at 45°C for 30 s, extension at 72°C for 2 min, and final extension at 72°C for 15 min. The PCR product was inserted in the multiple cloning site of pNOV2819 vector as AscI/HindIII fragment. The $P_{\text{SARK}}$::ipt::NOST cassette was then sequenced to check the originality of the sequences. The result indicated complete consensus of the sequences with the original sequences of the SARK promoter, ipt gene and nopaline synthase terminator. The map of this construct drawn using vector NTI software is shown in Figure 1B. The pNOV2819 vector carrying the $P_{\text{SARK}}$::ipt::NOST expression cassette (PNOVIPT1) was then inserted in to the Agrobacterium strain EHA 101 for subsequent use in maize transformation.

Pre-induction of Agrobacterium for infecting immature zygotic embryos

In preparation for infecting immature zygotic embryos, the Agrobacterium strain EHA 101 carrying the gene construct PNOVIPT1 was grown on LBA (Luria Bertani Agar) medium supplemented with 100 mg.l$^{-1}$ spectinomycin and 100 mg.l$^{-1}$ kanamycin at 28°C for two days in dark. This plate was kept at 4°C as a source of inoculum for experiments up to one month after which it was regularly refreshed from long term glycerol stock kept at -80°C. One full loop (3 mm) of bacteria was scooped from this fresh culture and suspended in 10 ml of Linsmaier and Skoog (LS), (1965) infection medium (Table 1) supplemented with 100 μM acetosyringone in a sterile 50 ml falcon tube. The tube was sealed with parafilm and covered with aluminium foil and fixed on a shaker in a horizontal position and the culture was left to grow for 3 to 4 h at 250 rpm and temperature of 28°C until optical density (OD) of 0.4 to 0.6 was attained at A260 nm. This procedure called pre-induction step was routinely carried out before all transformation experiments.

Plant material

Seeds of the tropical maize inbred line CML216 were obtained from the International Maize and Wheat Improvement Centre (CIMMYT-Nairobi), Twenty-five (25) plants were grown in the glasshouse at the Biosafety Level II Plant Transformation Laboratory (PTL) of Kenyatta University to supply immature zygotic embryos for in vitro culture. Glasshouse conditions were temperature of 30/25°C (day/night) and relative humidity of 30% and 16/8 h (light/dark) photoperiod. Soils for growing maize were prepared by mixing sandy loam soil with compost in the ratio of 2:1 and about 15 kg was filled into buckets having diameter of 3 mm and height of 3.1 mm. Each bucket was planted with one seed and then fertilized with 10 g di-ammonium phosphate (DAP) having 18% (w/w) ammonical nitrogen and 46% (w/w) available P$_2$O$_5$ avoiding contact with the seed. The soil was then watered fully up to complete saturation with 2 L of water for the first time. Subsequent watering was done by applying 1 L of water daily. The plants were top dressed with 7 g of urea having 46% (w/w) nitrogen when they had grown to a height of 10 cm and this top dressing was repeated depending on plant demand. All the 25 plants were self-pollinated to produce genetically and true-to-type immature zygotic embryo explants. Production, collection and sterilization of maize cobs and excision of immature zygotic embryos were carried out following protocol described by Seth et al. (2012).

Media for maize transformation

Infection, co-cultivation, resting, selection and maturation media were based on LS salts with specific formulations modified from Negrotto et al. (2000) and contained LS modified vitamins and 1.5 mg.l$^{-1}$ 2,4-dichlorophenoxyacetic acid (2,4-D) (Table 1). Infection medium contained 1 g.l$^{-1}$ casein hydrolysate, 68.5 g.l$^{-1}$ sucrose and 36 g.l$^{-1}$ glucose and 100 μM acetylsyringone. The pH of this medium was adjusted to 5.2 using 1 N NaOH and/or 1 N HCl and then filter sterilized using 0.2 μm pore size filter. It was then aliquoted into volume of 50 ml and kept at -20°C until it was used. Both co-cultivation and resting media contained 0.7 g.l$^{-1}$ L-proline, 0.5 g.l$^{-1}$ 2-(N-morpholino) ethanesulfonic acid (MES), and 30 g.l$^{-1}$ sucrose. Co-cultivation differs from resting medium as it contained 10 g.l$^{-1}$ glucose and 100 μM acetylsyringone while resting medium also
Figure 1. AF234301 construct carrying the $P_{SARK}$::IPT::NOST expression cassette (A) and pNOV2819 binary vector carrying the CMPS (cestrium yellow leaf curling virus promoter short version), the selectable marker $pmi$ gene with NOST and the $P_{SARK}$::IPT::NOST expression cassette (B).

differs as it contained 1.6 mg.l$^{-1}$ silver nitrate and 250 mg.l$^{-1}$ carbenicillin for counter selecting Agrobacterium. Selection medium was similar to resting medium except that it contains 25 g.l$^{-1}$ sucrose and 5 g.l$^{-1}$ D-mannose and it lacks silver nitrate. Maturation medium is similar to selection medium except that the amount of mannose was reduced by half and it was supplemented with 0.5 mg.l$^{-1}$ kinetin instead of 2,4-D. Regeneration medium was based on Murashige and Skoog (MS), (1962) and hormone free. Except infection medium, all media were solidified with addition of 0.8% (w/v) agar and sterilized by autoclaving at 121°C and 15 Psi after adjusting pH to 5.8. Acetosyringone, LS vitamins, silver nitrate and carbenicillin were added to the respective medium after autoclaving.
Table 1. Media used for infection, callus induction, selection and regeneration of transgenic maize plants.

<table>
<thead>
<tr>
<th>Media</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>LS* infection</td>
<td>LS macro- and micro-salts, 1.5 mg l⁻¹ 2,4-D, 1 g l⁻¹ casein hydrolysate, 68.5 g l⁻¹ sucrose, 36 g l⁻¹ glucose, modified LS vitamins, 100 µM l⁻¹ Acetosyringone, pH=5.2, filter sterilized</td>
</tr>
<tr>
<td>LS co-cultivation</td>
<td>LS macro- and micro-salts, 1.5 mg l⁻¹ 2,4-D, 0.7 g l⁻¹ proline, 0.5 g l⁻¹ MES, 30 g l⁻¹ sucrose, 10 g l⁻¹ glucose, 8 g l⁻¹ agar, pH=5.8, autoclave, LS vitamins, 100 µM l⁻¹ Acetosyringone</td>
</tr>
<tr>
<td>LS resting</td>
<td>LS macro- and micro-salts, 1.5 mg l⁻¹ 2,4-D, 0.7 g l⁻¹ proline, 0.5 g l⁻¹ MES, 30 g l⁻¹ sucrose, 8 g l⁻¹ agar, pH=5.8, autoclave, 1.6 g l⁻¹ silver nitrate, LS vitamins, 250 mg l⁻¹ carbenicillin</td>
</tr>
<tr>
<td>LS selection</td>
<td>LS macro- and micro-salts, 1.5 mg l⁻¹ 2,4-D, 0.7 g l⁻¹ proline, 0.5 g l⁻¹ MES, 25 g l⁻¹ sucrose, 5 g l⁻¹ D-mannose, 8 g l⁻¹ Agar, pH=5.8, autoclave, LS vitamins, 250 mg l⁻¹ carbenicillin</td>
</tr>
<tr>
<td>LS maturation</td>
<td>LS macro- and micro-salts, 0.5 g l⁻¹ kinetin, 0.7 g l⁻¹ proline, 0.5 g l⁻¹ MES, 25 g l⁻¹ sucrose, 2.5 g l⁻¹ D-mannose, 8 g l⁻¹ Agar, pH=5.8, autoclave, vitamins, 250 mg l⁻¹ carbenicillin</td>
</tr>
<tr>
<td>MS** regeneration</td>
<td>4.43 g l⁻¹ MS premix, 30 g l⁻¹ sucrose, 2.5 g l⁻¹ D-mannose, 0.7 g l⁻¹ proline, 0.5 g l⁻¹ MES, 8 g l⁻¹ agar pH=5.8 autoclave, 250 mg l⁻¹ carbenicillin</td>
</tr>
</tbody>
</table>

*LS: Linsmaier and Skoog, ** Murashige and Skoog

and cooling to 40 to 50°C.

Infection and co-cultivation

After being aseptically removed from the cob, the immature zygotic embryos were placed in Petri plates containing infection medium. Infection was carried out by removing the medium of the entire Petri plates to which pre-induced Agrobacterium culture was introduced and mixed by slightly swirling the plate. The plates were then covered with aluminium foil and incubated in dark for 5 min to encourage attachment of the Agrobacterium cells to the immature zygotic embryos. The infected zygotic embryos while still in infection medium were transferred to co-cultivation medium and the entire infection medium was carefully drained off by using sterile pipette tips. All the immature zygotic embryos were then rearranged ensuring direct contact with the medium, embryo axis down and scutellum side up. The plates were sealed with parafilm and incubated at 23°C for three days in dark. After three days of co-cultivation, the embryos were transferred to resting medium for callus induction. Each infected embryo was picked carefully and put on resting medium with 20 to 25 embryos in 90 x 15-mm plate still ensuring contact of the embryos with the medium. The culture was incubated at 27±1°C in dark for 10 to 15 days with occasional observation on the process of callus induction.

Selection of putatively transformed events and plant regeneration

The immature zygotic embryos which did and did not produce callus were transferred to selection medium having 5 g l⁻¹ D-mannose, for selection of transformed events and incubated at 27±1°C in dark for four weeks with sub-culturing onto fresh selection medium every 15 days after transfer to selection medium. Healthy embryogenic type I and type II calli were transferred to maturation medium to mature the somatic embryos and incubated at 27±1°C in dark for 15 days. Type I calli were identified by their compact nature and white to creamy color, while type II were friable and light yellow in color growing faster than type I calli. After 15 days of culture on LS maturation medium, embryogenic calli were transferred to MS regeneration medium to regenerate putative transgenic maize plants. The culture was incubated at temperature of 27±1°C and 16/8 h light/dark photoperiod until plantlets have grown fully with well-developed shoots and roots. Transformation frequency was determined as the ratio of the total number of mannose resistant independent callus events that regenerated transgenic plants to the total number of immature zygotic embryos infected and expressed in percentage.

Acclimatization and glasshouse growth of putative transgenic plants

Putative transgenic maize plants with well-developed shoots and roots were transferred to small pots filled with sterile peat moss (Kekkilä Co. Ltd, Tuusula, Finland) for acclimatization and hardening. Acclimatization was carried out following procedure described by Seth et al. (2012). After 7 to 10 days the plantlets were transferred to nursery pots containing sandy loam soil mixed with manure and sand at a ratio of 2:2:1, and kept in containment in the glasshouse till they grew to maturity. Individual transgenic plants were self-pollinated to give T₀ seeds for further analysis.

PCR analyses of transgenic plants

Polymerase chain reaction analyses of putative transgenic plants were carried out using forward 5'-ATAGGCCGCGCCAAATTCTTCTTCCTTA-3' and reverse 5'-GCCAAGCTTCCAGTCTCTTAGATAGAT-3' primers flanking the SARK promoter and the NOST region targeting the whole expression cassette of 2 kbp size. The PCR programme included: initial denaturation for 10 min at 98°C followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 2 min and final extension at 15 min at 72°C. Each of 25 µl PCR reaction contained 0.5 µl of forward and reverse primers (10 pmol µl⁻¹ each), 2.5 µl PCR buffer (×10), 0.5 µl dNTPs.
Southern blot analysis

Total genomic DNA was extracted from 2 to 3 g of young T1 and wild type (WT) plant leaves using the cetyltrimethylammonium bromide (CTAB) method (Allen et al., 2006). Ten micrograms of genomic DNA per sample were digested completely with HindIII restriction enzyme (New England Biolabs, UK) overnight with incubation at 37°C. Genomic DNA obtained from WT plants (CML216) was included as a negative control. For positive control, the pNOV2819 vector carrying the FpSARK::ipt::NOST cassette (PNOVIP1) plasmid DNA was diluted to 20 ng µl⁻¹, and 1 µl was used. Digested products of each sample were loaded per lane and subjected to gel electrophoresis on 1% (w/v) agarose gel at 30 V overnight. The DNA was then transferred overnight to Hybond™-N+ membrane optimized for nucleic acid transfer (Amersham) in 20× standard sodium citrate (3 M NaCl, 0.3 M Na-citrate, pH 7.0) following procedure described in Sambrook et al. (1989). Hybridization probes were prepared by PCR amplification of specific region of the pmi gene using forward 5'-ACAGCACGTCTCCATCCA-3' and reverse 5'-GGTTGGCCATCATCCATTCA-3' primers with the same PCR condition indicated for Negrotto et al. (2000). The probes were labelled with alkaline phosphatase and used to hybridize the blots using Gene Images Alkaphose direct DNA labelling and detection system near the field capacity was determined following the procedure described by Dastane (1967). Plants were watered with 1 l of water, as established through this method, daily (every 24 h) from planting to physiological maturity whenever they are grown under optimal moisture condition. Drought was imposed by withdrawing watering from the transgenic and wild type plants for three weeks when they were at the age of eight weeks (six fully grown leaf stage). Watering was restored in all stressed plants after these three weeks of drought, giving 1 l of water daily (every 24 h) up to physiological maturity (black layer formation).

Reverse transcription-polymerase chain reaction (RT-PCR)

For total RNA extraction, leaf samples of about 100 mg were collected from drought stressed transgenic and WT plants and immediately frozen in liquid nitrogen. The leaf samples were then crushed into powder using mortar and pestle under liquid nitrogen. Subsequent RNA extraction steps were carried out using Qiagen RNeasy® Plant Mini Kit (Qiagen N. V. Valencia, USA) following the manufacturer’s instruction. Extracted RNA was re-suspended in 50 µl of RNase free water and kept at -70°C. Complementary DNA (cDNA) was synthesized following SuperScript III™ first-strand synthesis system for RT-PCR (Invitrogen Corp. Carlsbad CA, USA). Forward 5'-CCAACTTGCAAGGAAAGACGACG-3' and reverse 5'-TCCTAGTGAAGACGGTGCG-3' primers were used to amplify 0.69 kbp of the ipt gene transcripts. The PCR programme was based on initial denaturation for 5 min at 95°C, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min and final extension of 15 min at 72°C. Each PCR reaction contained 0.5 µl each of forward and reverse primers (10 pmol µl⁻¹ each), 2.5 µl PCR buffer (x10), 0.5 µl dNTPs (10 mM), 1.25 µl MgCl₂ (25 mM) and 0.5 µl Taq polymerase (5 U/µl) and 1 µl template (20 ng). The final volume was brought up to 25 µl by adding 17.5 µl RNase free water. The ZmAct forward 5'-ACCACAGGCTAACCGTGAG-3' and ZmAct reverse 5'-TTGTTGGCCATCATCCATTCA-3' primers were used to amplify 0.426 kbp of the ACTIN gene transcripts as internal control. The PCR reaction of 25 µl was set up with the same PCR condition indicated for the ipt gene specific primers. The PCR programme was based on initial denaturation at 94°C for 3 min followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 30 s and final extension of 72°C for 2 min. The PCR products were loaded in a 1% (w/v) agarose gel and electrophoresis was carried out at 70 V for 1 h.

Glasshouse drought experiment

Glasshouse drought experiment

Seeds obtained from selfed T1 generation plants of stable transgenic events of FpSARK::ipt::CML216 and non-transformed (wild type, CML216) plants were planted in the glasshouse at Kenyatta University Plant Transformation Laboratory. Glasshouse conditions were temperature of 40/35°C (day/night) and relative humidity of 35% and 16/8 h (light/dark) photoperiod. Both the transgenic and wild type plants were treated alike in terms fertilizer application and watering frequency. The amount of water needed to keep soil moisture near the field capacity was determined following the procedure described by Dastane (1967). Plants were watered with 1 l of water, as established through this method, daily (every 24 h) from planting to physiological maturity whenever they are grown under optimal moisture condition. Drought was imposed by withdrawing watering from the transgenic and wild type plants for three weeks when they were at the age of eight weeks (six fully grown leaf stage). Watering was restored in all stressed plants after these three weeks of drought, giving 1 l of water daily (every 24 h) up to physiological maturity (black layer formation).

Evaluation of physiological parameters

Plants’ physiological responses to drought stress was monitored by measuring relative water content, chlorophyll a, b and total carotenoid levels before dehydration, at weekly intervals during the drought period, and 24 h after one time re-watering with 1 l of water and a week after re-watering daily with the same volume of water.

Relative water content

Relative water content (RWC) was measured in leaf samples collected at 2 to 3 pm, when plants were expected to experience the most drought stress as result of high temperature and soil moisture deficit, and fresh weight was recorded immediately using sensitive balance. The samples were then immersed in distilled water and kept in darkness at 4°C overnight to minimize respiration losses and turgid weight was recorded after blotting the adhering water and kept in an oven (Combi-H12, FINEPCR, Korea) set at 70°C for 24 h and dry weight was recorded. RWC was calculated according to Rivero et al. (2007) as:

\[
RWC = \frac{(FW-DW) \times 100}{DW} \tag{1}
\]

Where, FW is fresh weight, DW is dry weight and TW is turgid weight of leaf samples.

Chlorophyll extraction and quantification

For chlorophyll pigment analyses, leaf samples were collected at the same time point as for relative water content and punctured into uniform circular sizes using paper puncture. Ten punctured pieces were then transferred to a mortar and were thoroughly homogenized under 4 ml of 100% acetone using a mortar and pestle in the dark. The homogenates were centrifuged at 12,000 rpm for 10 min. The supernatants were then collected and the absorbencies were measured at 662, 645 and 470 nm using an ultraviolet (UV)-visible spectrophotometer (722 Visible
Spectrophotometer, China). Then chlorophyll a, chlorophyll b, total chlorophylls, and total carotenoids (xanthophyll + β-carotene) content were calculated applying the following equations (Lichtenthaler and Wellburn, 1983) as:

1. Chlorophyll a (µg/gfw) = \(11.75A_{662} - 2.35A_{645}\)  
2. Chlorophyll b (µg/gfw) = \(18.61A_{645} - 3.96A_{662}\)
3. Total carotenoid (µg/gfw) = \(1000(A_{470} - 2.27)\) chlorophyll a (µg/gfw) - \([81.4 \text{ chlorophyll b (µg/gfw)}]/227\)

### Induction of leaf senescence in dark

To investigate on the occurrence of leaf senescence in transgenic and WT plants in the dark, senescence was induced in young leaf tissues collected from the drought stressed WT and transgenic plants at intervals of four days over 12 days time. The leaves from the WT plants were kept in Petri plates filled with sterile distilled water and kinetin and 6-Benzylaminopurine (BAP) each at concentration of 5 mg/l while leaves from transgenic plants were exclusively kept in sterile distilled water (Peleg et al., 2011). All samples were kept in dark at 30°C with frequent supervision for development of senescence. On the 12th day, all the leaves were taken from the dark and picture was taken. Chlorophyll a and b were extracted from the leaf tissues before dark assay and on the 12th day of dark treatment and quantified using the method described in Lichtenthaler and Wellburn (1983).

### Evaluation of important agronomic parameters

Important agronomic parameters were evaluated both in the transgenic and wild type plants. Phenological traits were evaluated in terms of days to anthesis and silking. Days to anthesis were determined as the number of days a plant has taken from germination to the day half of the tassel has started pollen shading. Days to silking was also determined as the number of days a plant has taken from germination to the day silks have emerged at least 2 cm above the ear sheath. Anthesis-silking interval (ASI) was recorded as the difference between days to silking and days to anthesis. Plant and ear heights were, respectively, measured as the height from the soil level to the base of the tassel and to the node bearing the uppermost ear. The whole plant was cut at the soil level and the weight was recorded as biological yield in grams per plant. After recording the biological yield the whole plant was divided in to leaf, stalk, and ear which were dried separately in the oven at 70°C for 24 h after removing the cob bearing the kernels. The cob was dried separately after removing the kernels, which were air dried to uniform moisture of 13% before recording dry weight. Dry weight of the different separate parts was then added to give total plant dry weight.

The weight of the seed obtained after drying in the sun to uniform moisture content of 13% was recorded as seed yield per plant. Thousand seeds were counted and weighed on sensitive balance and the value was recorded as thousand seed weight in grams.

### Statistical analysis of physiological and agronomic data

Analysis of variance (ANOVA) was carried out using genstat discovery edition 4 (VSN international software for biosciences, www.vsn.co.uk/software/genestat) to test the statistical significance of genotypes and drought on RWC and chlorophyll concentration at different time points and growth, seed yield and agronomic characters. Whenever the ANOVA revealed significant differences, the means were separated using the least significant difference (LSD) test at 5% probability level.

### RESULTS

Nine independent putative transgenic events were generated through Agrobacterium-mediated transformation of the tropical maize inbred line CML216 with the SARK promoter linked to the ipt gene. All plants were normal and fertile despite phenotypic aberrations that appeared as a result of tissue culture induced variation (Larkin and Scowcroft, 1981). Polymerase chain reaction analyses indicated the presence of the transgene in all events (Figure 2) indicating transformation efficiency of 100% in transforming tropical maize following the pmi/manose based selection system. Southern blot analysis revealed stable integration of the transgene in T1 generation of five independent events with copy number of 2 to 4 per event (Figure 3) indicating stable transformation efficiency of 55% as opposed to transformation efficiency of 100% obtained with PCR analysis in the T0 generation. Individual plants of the stably transformed events were then advanced to T2 generation through self-pollination. Reverse transcription PCR also indicated presence of the ipt mRNA transcript in transgenic plants. The result is shown only for four
Figure 3. Southern blot analysis of nine independent events of \(P_{SARK}'::IPTCML216\). Ten micrograms of genomic DNA extracted from young leaf tissues of T\(_1\) plants (E\(_1\)-E\(_9\)) was digested with \(HindIII\) restriction enzyme and hybridized with the \(pmi\) probe. WT, Genomic DNA from non-transformed maize inbred line CML216 plants taken as a negative control; +Cr, 20 ng of plasmid DNA digested with \(HindIII\) used as a positive control.

Figure 4. \(P_{SARK}'::IPTCML216\) transgenic and wild type plants growing in the glasshouse. Delayed leaf senescence contributed to strong source/sink relationship, which led to better ear development and enhanced grain productivity under drought in transgenic plants expressing \(ipt\) gene (middle and right). Wild type plants (left) suffered loss of green leaf area, which affected ear development and grain yield under drought.

Development of stress and leaf senescence

Drought stress symptoms were observed in the WT plants faster than in the transgenic plants as revealed by leaf wilting on the fifth day after withdrawing watering. The transgenic plants did not show any sign of wilting during this time. One week of drought could induce senescence in the lower leaves in the WT plants and this progressed further to the second leaves within the second week of drought. Senescence began on leaf tips and margins and progressed to the centre of the leaves as drought intensified further. By the end of the second week of drought, two to three leaves died from senescence in the WT plants. The transgenic plants did not show any sign of senescence during this time though symptom of drought stress was observed by wilting and rolling of few leaves temporarily in the hottest part of the day. These plants could show temporary recovery from drought stress in the morning hours as indicated by leaf unrolling and achieving fully turgid condition. On the contrary, the wild type plants could not show any sign of recovery from drought stress. Rather leaf rolling, wilting and senescence continued at much magnified level up to the third week of drought. No recovery was observed even 24 h after one time rewatering with 1 l of water.

The strongest drought stress developed in the transgenic plants at the end of the third week of drought when leaf wilting and rolling combined with development of dark green color was observed. At this time, point only the lowest leaves could show complete death from senescence. These plants could recover completely from this drought stress within 24 h after 1 time re-watering with 1 l of water. The relative water content measured after 24 h of one time re-watering with 1 l of water was almost equivalent to the amount measured at pre-dehydration indicating faster recovery of the transgenic plants from drought stress.
The wild type plants showed deficit of 20% tissue water content compared to their pre-dehydration relative water content even after a week of re-watering with 1 L of water daily.

Despite restoration of optimal soil moisture, senescence was more accelerated in the wild type plants as consequence of previous drought experienced at the age of 8 to 11 weeks. Figure 6 shows both transgenic and wild type plants 20 days after pollination (one month after drought). By this time, more than 50% of the photosynthetically active green leaf area was lost in the wild type plants. On the contrary, leaf senescence was delayed significantly and was confined to the margins and tips of leaves below the ears in the transgenic plants. Only the lower most two leaves died per plant as result of senescence, which should be expected in maize even under normal production circumstances. Within one month from this time (at the age of four months and two months after drought) 80% of the WT leaves died as a result of the previous stress (Figure 6D).

**Effect of drought stress on leaf relative water content**

Both the wild type and transgenic plants had RWC of 95% before drought (Figure 7). The wild type plants showed drastic reduction of tissue water content down to 70% (25% loss) within the first 1 week of drought stress. The transgenic plants showed loss of only 10% and maintained that level until the end of the third week when it further lost 10% of the tissue water content. By this time, the wild type plants lost 40% and RWC stood at 57.9%. One time re-watering with 1 l of water did not improve the RWC in these plants while it replenished to 88.7% in the transgenic plants, which maintained the same level after one week of re-watering with the same volume of water daily. At this time point, the RWC of the

---

**Figure 5.** RT-PCR analysis of ipt gene expression in drought stressed $P_{SARK}$::IPTCML216 transgenic plants ($P_1$-$P_4$). A, ipt gene specific primers amplified 0.69 kbp of the gene transcripts; M, 1 kbp Molecular marker; +Cr, Plasmid DNA used as positive control; RT-, cDNA synthesis mix with DNAs I treated RNA but without Superscript III; WT, cDNA from WT plant exposed to drought stress at the age of 8 weeks; B, Actin primers amplified 0.426 kbp of the ACTIN gene transcripts as internal control.

**Figure 6.** Drought induced leaf senescence was delayed in $P_{SARK}$::IPTCML216 transgenic plants (A & C) compared to wild type plants (B and D). The $P_{SARK}$::IPTCML216 transgenic plants at the age of four months (A) did not show sign of senescence compared to the wild type plants of equivalent age (B) which had lost 50% of the green leaf area to drought stress imposed for three weeks at the age of 8 weeks. At the age of five months the $P_{SARK}$::IPTCML216 transgenic plants (C) still maintained green leaf area compared to the equivalent age wild type plants (D) which lost 80% of the green leaf area as a result of drought.
Figure 7. Relative water content measured in 8 weeks old transgenic and wild type plants at different time points during three weeks of drought stress assay in the glass house. Each data point represents mean of four replications ±SE. Mean values followed by different letters at specific time points are significantly different from each other according to LSD test at P<0.05.

Wild type plants refilled to 75% only still showing deficit of 20% compared to the values before drought.

Effect of drought stress on total chlorophyll

Total chlorophyll declined with increasing time of drought stress in both the wild type and transgenic plants (Figure 8A). However, the transgenic plants had higher total chlorophyll compared to the wild types at all-time points. The wild type plants experienced 35.6% loss in total leaf chlorophyll content during the third week of drought stress compared to the level recorded at pre-drought period. During this time the transgenic plants lost only 4.3% of the total leaf chlorophyll content. In both cases total chlorophyll continued to decline despite restoration of moisture in the soil.

Effect of drought stress on chlorophyll a and b

Chlorophyll a was almost stable in both the wild type and transgenic plants during drought except where it declined in the wild type after the third week of drought (Figure 8B). In general, changes in leaf chlorophyll b content followed the same trend as the total chlorophyll with time of drought stress in both the wild type and transgenic plants (Figure 8C). Both had almost the same level of chlorophyll b content before drought. However, as drought progressed, the wild type plants manifested substantial reduction as a result of which the transgenic plants had relatively higher level throughout the drought period. The wild type plants experienced loss of 54 and 70% during the third week of drought and after one week of rewatering, respectively, while the transgenic plants manifested loss of 11.9 and 33.3% during the same time points compared to their respective values recorded before drought. This drastic reduction was equally observed in all the wild type plants while there was variation among the transgenic ones.

Effect of drought stress on total carotenoid content

Contrary to total chlorophyll content, total carotenoid content increased progressively with increase in drought intensity in both the wild type and transgenic plants. However, the wild type plants showed substantial increment compared to the transgenic counterparts over the three weeks of drought period. This increment was mild during the first week of drought both in the wild type and transgenic plants though it was still higher in the wild type plants (Figure 8D). It then peaked up exponentially in the wild type plants during the third week of drought stress recording 75% increment compared to the level at the end of the second week. It followed the same trend in the transgenic plants but at lower magnitude compared to the wild type plants. The exponential phase lagged behind by one week in the transgenic plants. This started with re-watering at the end of the third week and continued one week after re-watering when transgenic plants recorded 97% increment compared to the level before re-watering. During this time, the increment in the wild type plants has already stabilized.
Dark assay of leaf senescence

Dark assay of leaf senescence was carried out to compare the effect of cytokinin externally applied and endogenously produced by transgenic maize in delaying leaf senescence. Leaf segments taken from wild type drought stressed plants and kept in distilled water manifested senescence on the 8th day of time lapsed in darkness (Figure 9). When leaf segment from the same plant was kept in 5 mg l⁻¹ cytokinin (BAP or kinetin) senescence was delayed up to the 12th day. Leaf segments detached from drought stressed transgenic maize plants and kept in distilled water in dark did not show sign of senescence at the end of the 12th day. This result showed that endogenously produced cytokinin is better off in delaying leaf senescence than externally applied ones even in detached leaves in maize. Under the dark assay leaf chlorophyll a and b, and hence total chlorophyll contents were reduced significantly in the wild type leaf tissues 12 days after the dark assay (Figure 10). Leaf tissues obtained from the transgenic plants maintained higher level of chlorophyll content even better than externally applied cytokinin supporting the result obtained with physical senescence of the leaf tissues after dark assay. In general, the dark assays were very much supportive of the senescence data obtained from in vivo evaluation made in the glass house drought assay.

Effect of drought stress on growth and agronomic performance of transgenic and wild type plants

The effect of drought on growth was monitored in terms of fresh and dry weight of plants at harvest. Transgenic plants produced significantly (P<0.05) higher root fresh and dry weight than the WT plants. They also produced higher above ground plant fresh and dry matter, and higher total plant fresh and total plant dry matter, both including the root fresh and dry matter, respectively, than the WT plants though the differences were not statistically significant. Higher root growth was due to massive fibrous roots produced in all the transgenic plants as opposed to the WT plants, which produced few taproots with a small number of secondary roots (Figure 11). Better cob growth and stay green character of the transgenic plants have also contributed to their higher
Figure 9. Dark assay of leaf senescence in leaves detached from drought stressed transgenic and wild type plants. W+H₂O: leaf segments detached from wild type plant and kept in water, T+H₂O: leaf segments detached from transgenic plants and kept in water, WT+BAP: leaf segments detached from wild type plants and kept in 5mg.l⁻¹ 6-Benzylaminopurine, WT+K: leaf segments detached from wild type plants and kept in 5mg.l⁻¹ kinetin.

Figure 10. Concentration of chlorophyll a and b, and their total measured in wild type and transgenic leaves before senescence (BS) and 12 days after senescence (DAS) of dark assay. WT+H₂O: wild type leaves incubated in sterile distilled water, T+H₂O(1) and H₂O(2) leaves from transgenic event 1 and 2, respectively, incubated in sterile distilled water; WT+BAP and WT+K: leaves from wild type plants incubated in 5mg.l⁻¹ Benzyleaminopurine and kinetin, respectively. Values are mean ±SE (n=3). BS: Before senescence; DAS: Days after senescence.

total shoot dry matter. Data recorded on important phenological and agronomic characters, and seed yield and major yield components in WT and transgenic plants are shown in Tables 2 and 3, respectively. Transgenic
Figure 11. Root architecture of \( P_{\text{SARK}}::\text{IPTCML216} \) transgenic (A) and wild type (B) plants after watering/drought/rewatering experiment.

Table 2. Important phenological and agronomic characters of wild type and transgenic plants recorded after watering/drought/re-watering in the glasshouse.

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Days to anthesis (No)</th>
<th>Days to silking (No)</th>
<th>ASI(^a) (No)</th>
<th>Plant height (cm)</th>
<th>Ear Height (cm)</th>
<th>Leaf width (cm)</th>
<th>Leaf length (cm)</th>
<th>Total number of leaves/plant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>96±5.0</td>
<td>110±1.85(^a)</td>
<td>14±4.0</td>
<td>145.8±3.9</td>
<td>78.3±3.7</td>
<td>6.5±1.0</td>
<td>54±5.6</td>
<td>15.5±0.53</td>
</tr>
<tr>
<td>Transgenic</td>
<td>103.3±6.4</td>
<td>113.8±1.7(^b)</td>
<td>10±3.4</td>
<td>143±10.6</td>
<td>72.8±10.0</td>
<td>7.5±0.4</td>
<td>59±6.9</td>
<td>15.3±0.9</td>
</tr>
</tbody>
</table>

Mean values followed by different letter for a particular parameter are significantly different from each other according to LSD test at 5% probability level. Values are mean±SE (n=4), \(^a\) ASI: Anthesis-silking interval.

Table 3. Seed yield and major yield components of wild type and transgenic plants recorded after watering/drought/re-watering in the glasshouse.

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Ear length (cm)</th>
<th>Cob dry weight (g)</th>
<th>Seeds/plant (No.)</th>
<th>1000 seeds weight (g)</th>
<th>Seed yield (g/plant)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>10.5±2.2(^a)</td>
<td>7.8±1.5(^a)</td>
<td>8.0±6.9(^a)</td>
<td>1.43±2.6(^a)</td>
<td>1.43±2.6(^a)</td>
</tr>
<tr>
<td>Transgenic</td>
<td>12.13±2.2(^b)</td>
<td>19.6±3.7(^b)</td>
<td>145±4.7(^b)</td>
<td>36.2±4.8(^b)</td>
<td>44.3±2.9(^b)</td>
</tr>
</tbody>
</table>

\(^a\) Weight of 32 seeds produced per a single plant. Mean values followed by different letters for a parameter are significantly different from each other according to LSD test at 5% probability level. Values are mean±SE (n=4).

Plants did not differ significantly from the WT plants in days to anthesis, ASI, plant and ear height, leaf size and number. They, however, had significantly (p<0.05) higher major yield components such as ear length, cob dry weight, seed number/plant, hundred seeds weight and seed yield/plant than the WT plants.

The WT plants came to anthesis within 96 days after germination while the transgenic plants extended this to 103 days. The WT plants also produced silks within 110 days that took the transgenic plants 113 days. Hence, in the WT and transgenic plants, there was ASI of 14 and 10 days, respectively. Wider ASI combined with poor receptivity of the late coming silks might have contributed to poor seed setting in the WT plants that produced seed yield of only 1.43 g/plant (Figure 12). On the other hand, the transgenic plants produced significantly (p<0.05)
higher seed yield of 44.3 g/plant. Significant differences were also observed among the transgenic and WT plants in ear development that led to significantly (p<0.05) higher cob dry matter accumulation and ear size (length) in transgenic plants (Table 3).

**DISCUSSION**

Drought has more damaging effect on maize when it occurs at the most critical growth stages, which coincide with two weeks’ time before and after anthesis (Bänziger et al., 2000). The three weeks drought applied in this study extended well to the pre-anthesis stage that marks the beginning of this susceptible growth stage in maize. This could, therefore, yield an insight into the role of ipt gene in saving plants and in securing reasonable crop productivity in tropical maize under drought. Glasshouse growth condition that combined sunny days with average day/night temperature of >40/25°C simulated the actual combination of stresses maize plants encounter under the real field condition in tropical environment.

The fact that drought stress inhibited grain formation in most of the WT plants confirms susceptibility of maize to the level of stress treatment applied at pre-anthesis growth stage. This might have happened because of previous morphological and physiological changes that had taken place in response to drought. Initially WT plants launched dehydration avoidance strategies such as leaf rolling and hence reduced leaf area, senescence of bottom leaves and possibly stomatal closure as well. Leaf senescence was much accelerated with crop developmental stages where at post-anthesis growth stage, out of the 16 leaves produced/plant, 14 were either fully or partially affected by senescence in the WT plants while the transgenic plants still had more than 14 leaves/plant fully functional.

Oneto et al. (2016) also transformed the temperate maize inbred line H-II with ipt and reported that transgenic plants treated with water deficit for two weeks around anthesis had prolonged total green leaf area, and maintained normal photosynthetic rate and stomatal conductance. With extended drought stress of three weeks imposed before anthesis, the current study yields added information that tropical maize transformed with IPT gene can still withstand drought for extended period of time. Prior to the onset of the senescence syndrome, the WT plants encountered massive chlorophyll degradation as a result of intensive dehydration at the end of the three-weeks drought period.

Gummuluru et al. (1989) reported higher total chlorophyll (chlorophyll a + chlorophyll b) as an indicator of drought tolerance in cereals. Chlorophyll b forms important component of the chlorophyll complex supporting more efficient energy conversion into ATP and NADPH which are then used as sources of energy to build carbohydrates from CO₂ (Bänziger et al., 2000). Hence, chlorophyll degradation in the WT plants can be an indication that photosynthesis might have been inhibited in these plants before the massive leaf senescence was observed. Consequently, the WT plants failed to recover from drought and continued to lose more green leaf area within a period of one month after anthesis/silking.

The $P_{sark::iptcml216}$ transgenic plants demonstrated rapid recovery from dehydration may be as a result of total and chlorophyll b content that were maintained at higher levels during the drought period indicating chloroplast integrity even under dehydration. Peleg et al. (2011) reported similar results in rice. Faster replenishment of tissue water content after re-watering once with 1 L of water substantiated that carbon...
assimilation was taking place in these plants in a manner similar to pre-dehydration. As a result, more grain yields were obtained in the \( P_{\text{SARK}}::\text{IPTCML216} \) plants.

Bolaños and Edmeades (1993) and Edmeades et al. (1999) reported narrow ASI as an indicator of tolerance to drought stress in maize. The relatively narrower ASI obtained with the \( P_{\text{SARK}}::\text{IPTCML216} \) transgenic maize plants could then indicate the role of \( ipt \) gene in enhancing tolerance to drought. Drought delays silk growth as opposed to the tassel, interfering with allocation of assimilates to ears, ovules and silks (Edmeades et al., 1993) and hence removes the synchrony of the male and female organs to the extent of inhibiting seed setting. To establish the damaging effect of drought on grain yield, all plants were self-pollinated except two WT plants that were sib-pollinated by fresh pollen from WT plants grown under optimal watering. Even then, seed setting did not improve showing that the late coming silks were not receptive enough for fertilization to take place. Otegui et al. (1995) reported that applying fresh pollen from unstressed plants did not improve seed setting in late coming silks in maize supporting lack of seed setting in the WT plants is related to poor receptivity of the late appearing silks instead of poor pollen viability.

Pospíšilová et al. (2005) reported that abscisic acid (ABA) and cytokinin (CK) contents have inverse relationship in plants under drought condition. Drought treatment reportedly encouraged increased ABA content in both WT and \( P_{\text{SARK}}::\text{IPT} \) tobacco plants without any effect on CK content indicating that changes in ABA was not related with drought tolerance displayed by transgenic tobacco plants (Rivero et al., 2007). In the context of maize, Liu et al. (2005) reported an increase in ABA content in the reproductive organs to play a role in yield reduction in response to drought stress. In former studies, the ABA concentration of the ovary was reported to increase substantially as the result of pre-flowering stress compared with irrigated maize plants (Ash et al., 2001) revealing the possibility that ABA may play a role in the abortion of female flowers.

In the current study, high ABA concentration might have weakened, the receptivity of the female flowers in the WT plants as silk growth was not affected. In the \( P_{\text{SARK}}::\text{IPTCML216} \) plants increased level of CK might have antagonized the negative effect of ABA. Pospíšilová et al. (2005) reported application of benzyladine inhibits water stress induced accumulation of ABA in maize. Substantial seed setting achieved in the transgenic plants despite delayed anthesis could therefore be attributed to the protective role of CK under drought stress (Rivero et al., 2009).

In maize, the upper 8 to 9 leaves contribute the lion’s share of the assimilate fed to the sink (Allison and Watson, 1996). Loss of these leaves had negative effect on productivity of WT plants. Drought inducible expression of \( ipt \) gene has sufficiently shown itself to improve drought tolerance by delaying loss of these leaves through senescence in tropical maize. \textit{In vitro} dark treatment of leaf segments has yielded results supportive to this effect. Peleg et al. (2011) report delayed senescence in \( P_{\text{SARK}}::\text{IPT} \) transgenic rice plants under pre-anthesis drought for 15 days and in flag leaf sections treated in dark from the same plants. Contrary to this report, however, 12 days of incubation in externally applied CK did not keep WT leaves greener than leaves from the transgenic plants. This could be due to concentration effect of the externally applied CK or uptake of externally applied CK by maize leaf tissues that may not be as efficient as in rice leaf tissues (Gan and Amasino, 1996).

Cytokinin delays senescence through its diverse influences on many plant metabolic processes (Smart et al., 1991). During senescence, genes encoding enzymes such as RNAses, proteasines and lipases that degrade RNA, proteins and lipids, respectively, are expressed (Gan and Amasino, 1997). Cytokinin delays senescence by inhibiting transcription of these genes (Buchanan-Wollaston, 1997). Rivero et al. (2007) reported that the protective role of CK under drought comes through enhancement of stress-related gene expressions encoding antioxidant enzymes playing role in ROS scavenging. They further elaborate that suppression of drought-induced leaf senescence in the transgenic plants is also accompanied by enhanced expression of stress-response transcripts, such as dehydrins and heat-shock proteins, possibly also contributing to the enhanced tolerance and suppressed ROS concentrations in the transgenic plants during drought.

In this study, total carotenoids content increased significantly in the WT plants following maximum dehydration (57% RWC) at the end of the three-weeks drought period. Munné-Bosch et al. (1999) reported an increase of 26% in leaf carotenoid content in rosemary plants grown under drought during hot summer season at midday when plants were dehydrated to less than 50% RWC. Carotenoids are non-enzymatic antioxidant quenching activated oxygen species. Its substantial increment in the WT plants under drought may indicate that the level of this antioxidant was insufficient before drought treatment to guarantee protection against oxidative stress by quenching reactive oxygen species.

Mannose based selection system increases transformation frequencies compared to selection system based on antibiotics like kanamycin (Joersbo et al., 1998) which may adversely affect the growth of transformed plant cells through release of toxic compounds from necrotic non-transformed tissues (Lindsey and Gallois, 1990). Higher transformation frequency observed with \textit{pmi} selection could be attributed to the metabolic advantage with mannose selection and absence of any toxic metabolites released from the dying non-transformed cells. In wheat transformation, \textit{pmi} is preferred to \textit{bar} gene that confers resistance to the herbicide bialaphos (Gadaleta et al., 2006). Gadaleta et al. (2008) reported \textit{pmi} as a useful marker even when used as minimal gene cassette in a linear DNA fragment.
In the current study, mannose based *Agrobacterium*-mediated transformation of the tropical maize inbred line CML216 yielded transgenic plants at transformation frequency of 1.7 per cent. This is very low when compared to transformation frequencies of temperate maize. Wright et al. (2001) report average transformation frequency of 45% for recovery of transgenic maize via particle bombardment using the *pmi* gene with mannose as a selective agent.

Negrotto et al. (2000) also reported transformation frequency of 32% through *Agrobacterium*-mediated transformation of the temperate inbred A188 using the same selection system. While genotypic differences could be one of the factors playing the leading role in causing these differences, poor competence of the immature zygotic embryos has also been well known to contribute to low transformation frequency (Negrotto et al., 2000). The desirable aspect of the *pmi*/mannose based selection system in the current study is its stringency in identifying transformed plants as revealed by transformation efficiency of 100%. Such high selection efficiency can be an advantage in saving time and resources that would otherwise be needed to handle plants that are not actually transformed or escapes.

The *pmi* gene is ubiquitous in nature and has been cloned from several bacteria and yeast species. Its use as selectable marker is, therefore, not expected to cause any hazard to environment. Furthermore, safety assessment of PMI reports no toxicity to mammals and no undesirable effects on the agronomic or nutritional composition of transgenic plants (Reed et al., 2001). Studies have proved that this gene causes no allergenicity to consumers and no undesirable agronomic traits in transgenic maize developed using *pmi* gene as plant selectable marker. Moreover, mannose is the cheapest and a ‘user-friendly’ selection agent for use in plant genetic transformation (Wright et al., 2001). Hence, commercial cultivation of the transgenic line developed in this study should not be suspected to bring negative effects to the environment or to the consumers.

The *ipt* gene has been used to develop transgenic temperate maize for delayed leaf senescence under the control of the senescence activated SAG promoter (Young et al., 2004) and the native promoter of the senescence enhanced (*SEE*) maize gene (Robson et al., 2004). Under the control of the SAG promoter, *ipt* gene could not delay leaf senescence in transgenic plants though RT-PCR showed expression of its transcript (Young et al., 2004). With the native, *SEE* maize promoter transgenic plants displayed delayed leaf senescence when grown under nitrogen stress condition. The major problem with this system was nitrogen deficiency in the upper younger leaves as a result of cytokinin accumulation in the lower senescing leaves that caused nitrogen immobilization (Robson et al., 2004). As a result, the transgenic plants could not be proposed for cultivation in soils poor in nitrogen.

The drought inducible SARK promoter was found to be the appropriate promoter driving the *ipt* gene expression resulting in the expected delayed leaf senescence in the transgenic maize under drought condition. Nitrogen deficiency in the upper younger leaves was not a problem with this promoter, as the gene expression is not limited to lower leaves but in all plant tissues facing drought stress (Rivero et al., 2007). In addition, the older lower leaves showed senescence, which caused nitrogen mobilization to the younger leaves. Transgenic plants were normal and the source/sink relationship was not affected. As reported by Rivero et al. (2007) and Peleg et al. (2011) *P_{SARK}::ipt* transgenic tobacco and rice plants differed from their respective WT plants exclusively in delayed leaf senescence. In this study, transgenic and WT plants had distinct root morphological architecture in addition to differences in delayed leaf senescence, where the transgenic plants had multi-branched fibrous roots as opposed to the few long primary roots dominating the WT plants.

In transgenic *Arabidopsis* plants transformed with *ipt* gene under the control of the promoter region from a maize gene encoding a heat shock protein (HSP70), similar results were reported showing root hairs emerging closer to the root tip suggesting a reduction in elongation zone in the transgenic plants (Medford et al., 1989). Such increased root branching and enhanced fibrous root formation was reported under condition of low CK content in CK-deficient transgenic plants which over express the cytokinin oxidase/dehydrogenase (*CKX*) genes that result in an enlarged root meristem and formation of lateral roots closer to the root apical meristem (Schmülling et al., 1989). Hence, the factor that caused increased volume and growth of branched root in transgenic maize plants showing enhanced expression of CK could not be established in this study and can be considered as an area of interest for further investigation. However, whatever the cause may be, this root architecture might have contributed positively to the drought tolerance exhibited by the transgenic plants by enhancing nutrient mining and water absorption from the soil.

The *ipt* gene has shown itself to improve drought tolerance by delaying leaf senescence in tropical maize. This can be considered as a breakthrough in improving drought tolerance in much faster manner compared to conventional breeding that takes long time with unpredictable outcome. Maize produces CK naturally, and increasing the level of this hormone by over expressing the *ipt* gene ectopically is not expected to cause any safety problem to the consumers and the environment. Considering all the positive aspect of the transgenic maize developed in this study, future research should focus on further evaluation of transgenic maize in confined field-trials for tolerance to drought. The line can be used as a source of the transgene for improving drought tolerance in commercial and well-adapted maize genotypes through conventional breeding. The *ipt* gene...
can be used further to improve drought tolerance in important tropical monocot and dicot crops.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

This work is part of the research project on Genetic Engineering of Maize for Drought Stress Tolerance in East and Central Africa funded by the United States Agency for International Development and the Multi-donor Trust Fund of the World Bank to the Association for Strengthening Agricultural Research in East and Central Africa. The tropical maize inbred line used in this study was kindly provided by the International Maize and Wheat Improvement Centre.

REFERENCES


Transcriptional modulation of genes encoding nitrate reductase in maize (Zea mays) grown under aluminum toxicity

Talita Cantú, Crislaine Emidio Vieira, Rafaélla David Piffer, Giovanna Carneiro Luiz and Silvia Graciele Hülse de Souza*

Laboratory of Molecular Biology, University Paranaense, Umuarama, 87502-210, Brazil.

Received 22 August 2016, Accepted 18 October, 2016

The free aluminum (Al) content in soil can reach levels that are toxic to plants, and this has frequently limited increased productivity of cultures. Four genes encoding nitrate reductase (NR) were identified, named ZmNR1–4. With the aim of evaluating NR activity and the transcriptional modulation of the ZmNR1, ZmNR2, ZmNR3, and ZmNR4 genes in leaves, 30-day-old hybrid maize BRAS 3010 plants were irrigated with a solution of Al2(SO4)3·18H2O for 16 days. The transcriptional levels of ZmNR2, ZmNR3, and ZmNR4 and NR activity will exhibit standard changes similar in the leaves, where, from the second week of stress onwards, there was a decrease in enzymatic activity and in the accumulation of transcripts. An increase ZmNR1 mRNA levels were observed, indicating that this gene may be associated with other metabolic pathways. This study resulted in the identification and characterization of different genes that encode NR and are involved in nitrogen metabolism in maize, in which the ZmNR2, ZmNR3, and ZmNR4 genes regulate the activity of NR in response to aluminum stress. The characterization of these genes may help in our understanding of the genetic-molecular and physiological mechanisms of maize subjected to aluminum stress.

Key words: Abiotic stress, Al, gene expression, nitrogen, metal toxicity, mineral nutrition.

INTRODUCTION

Maize (Zea mays) is the main cereal produced in Brazil. In 2015/2016 season, the cultivated area of maize was 15.7 million hectares with a total production of 76.2 million tons of grain (CONAB, 2016). Nowadays, Brazil is the world’s third largest producer of maize, only behind the United States and China (USDA, 2016). The economic importance of maize is due to the different ways it is used, including its wide consumption as food...
and its use in technological industries as a raw material for films, biodegradable packages, and biofuel (Boone et al., 2016).

Toxicity induced by aluminum (Al) is the main factor limiting agricultural productivity in acidic soils (pH ≈ 5.5) (Garzón et al., 2011). Estimates have revealed that approximately 50% of global arable land is potentially usable for food and biomass production is acidic and, thus, subjected to Al^{3+} toxicity (Kochian et al., 2004; Ma, 2007).

The primary manifestation of Al phytotoxicity is the inhibition of root growth and interference in the absorption, transportation, and use of water, and in mineral nutrition and metabolic changes, such as organic acid and nitrogen metabolism (Matsumoto, 2000; Azmat and Hasan, 2008; Azmat et al., 2015). As a consequence, important enzymatic processes are affected, among which the activity of nitrate reductase (NR), and consequently nitrogen absorption, are notable. Nitrogen is an essential nutrient required in large amounts by plants (Zhao et al., 2016). This element acts as an essential nutrient and regulates root development, leaf expansion and expression of genes encoding enzymes involved in carbon and nitrogen metabolism (Wang et al., 2003). Nitrogen (N) is used to form glutamine, which is considered as a precursor for the synthesis of many amino acids, nucleic acids, enzymes, and proteins, as well as secondary metabolites (Bagh et al., 2004; Kusano et al., 2011). Despite the high levels of N in the Earth’s atmosphere, most living organisms do not have the capacity to absorb this element in a gaseous form. This explains why plants use nitrate and ammonia as inorganic sources of nitrogen, which are absorbed by the roots (Wickert et al., 2007).

The varied forms and concentrations of nitrogen present in the soil have led plants to evolve strategies to locate and absorb this mineral in better ways. To regulate the demands of N incorporation in plants, gene families encode products with functions that are directed by enzymes. In this case, nitrate reductase (NR, EC1.6.6.1), which is the first enzyme is involved in nitrogen assimilation (Liseron-Monfils et al., 2013). Nitrate (NO_{3}^{-}) is reduced to nitrite (NO_{2}^{-}) in the cytosol, and is catalyzed by NR. It is then converted to ammonium (NH_{4}^{+}) by the nitrite reductase enzyme (NiR, EC 1.7.7.1) and incorporated into amino acids by glutamine synthetase (GS, EC 6.3.1.2) and glutamate synthetase (GOGAT, EC 1.4.1.14), forming glutamine and glutamate, which are essential to amino acids and are required for protein synthesis (Crawford, 1995; Debouba et al., 2013).

In *Arabidopsis*, two genes, NIA1 (At1g7760) and NIA2 (At1g37130), have been identified and responsible for nitrate reduction. NIA2 is the main gene, responsible for 90% of the enzymatic activity (Wilkinson and Crawford, 1991; Zhouand Kleinhofs, 1996; Debouba et al., 2013). Light is known to induce the expression of the NR genes (Tischner, 2000; Lillo et al., 2001). Liseron-Monfils et al. (2013) identified four expression clusters of the genes that encode the NR enzyme, where each one of these genes has a specific function during the plant’s life. Studies have shown that aluminum can inhibit root growth in maize and cause protein oxidation, which triggers changes in various biochemicals, physiological, and genetic processes (Boscolo et al., 2003; Purcino, et al., 2003; Souza et al., 2016). There is insufficient Information in the literature in respect to the transcriptional regulation of nitrate and nitrogen assimilation in maize subjected to aluminum stress. The aims of the present study were to evaluate the expression of genes encoding NR and to investigate the modulation of NR activity in maize leaves exposed to aluminum toxicity.

**MATERIALS AND METHODS**

**Identification of nitrate reductase (NR) genes in maize**

NR protein sequences have been identified in the model plant *Arabidopsis thaliana* (At1g37130 and At1g77760) and were obtained from the TAIR database (http://www.arabidopsis.org/) and was used for studies on the Phytozome Database of *Z. mays* (https://phytozome.jgi.doe.gov/pz/portal.html), using the program BlastP (Atschul et al., 1997). The sequences were blasted against others deposited in the National Center for Biotechnology Information (NCBI) GenBank Database (http://www.ncbi.nlm.nih.gov), using the programs BlastP and BlastX, to confirm their identity. The sequences deduced from amino acids were obtained using the program Open Reading Frame Finder (ORF Finder; NCBI, http://www.ncbi.nlm.nih.gov/orf/gorf.html). The protein sequences were aligned utilizing Clustal Omega algorithm version 2.0.3 (Sievers et al., 2011).

**Characteristics of predicted NR proteins in maize**

The physical and chemical characteristics of NRs in maize were determined using the tool ProtParam online (http://web.expasy.org/protparam), including the number of amino acids (AA), molecular weight (MW), and theoretical isoelectric point (pI).

**Phylogenetic analysis**

Phylogenetic analysis was performed by aligning NR protein sequences using the algorithm Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). Searches for similar proteins were performed in the program BlastP (http://www.ncbi.nlm.nih.gov). The phylogenetic tree was constructed using the neighbor-joining (NJ) method and also using the pair-wise deletion option with help from MEGA version 6.0 (Tamura et al., 2013). One-thousand bootstrap replicates were utilized to test for confidence.
Vegetal material

Seeds of hybrid maize BRAS 3010 were planted in pots with a 1-kg capacity containing soil and vermiculite at 1:1, and were watered weekly with the nutritive solution as proposed by Hoagland and Arnon (1950). The experiment was conducted in a greenhouse. Thirty-day-old plants were irrigated with a solution of aluminum sulfate (Al₆ [SO₄]₃·18H₂O) on the first and second days at a concentration of 50 and 100 µM, respectively. From the third day until the end of the experiment, the plants were irrigated daily with 150 µM aluminum sulfate (Pimenta et al., 1989). Simultaneously, the same amount of pots with material been irrigated with water (control) was kept. Samples were obtained on days 0, 2, 4, 8, and 16. At each sampling (control and stress), leaves were collected (three pools of leaves from different plants; under the same stress conditions where each pool constituted a biological repetition). The collected materials were immersed immediately in liquid N and stored in a freezer at -80°C.

Activity of NR

In vitro determination of NR activity in maize leaves was performed according to the methods proposed by Jaworski (1974) and Carelli et al. (1990). Incubation solution (buffer phosphate 0.1 M pH 7.5, KNO₃, n-propanol 3%) was added to the materials collected in vivo, and samples were then filtered by vacuum and incubated in a warm bath at 30°C for 40 min. After that, sulfanilamide and o-nitrophenyl-ethylen-diamino solution was added, and the samples were incubated in a warm bath for 10 min. Then, 100 µL of each sample was added to 150µL 60% ethanol, 250 µL sodium citrate (0.2% ascorbic acid pH 5.2%), and 500 µL ninhydrin, placed in a warm bath and cooled on ice before being read in a spectrophotometer at 540 nm.

Amino acids

The concentration of amino acids was determined using the method proposed by Praxedes et al. (2006) with a few modifications. A sample of 20 mg vegetal material was weighed, 250 µL 80% ethanol was added, and the samples were incubated in a warm bath at 80°C for 20 min in a microcentrifuge, following which the supernatant was removed. This procedure was repeated twice. The samples were then placed on ice. To prepare the standard curve, 60% ethanol, sodium citrate, and ninhydrin were mixed and this solution was placed in a warm bath at 90°C for 20 min. Then, 100 µL of each sample was added to 150µL 60% ethanol, 250 µL sodium citrate (0.2% ascorbic acid pH 5.2%), and 500 µL ninhydrin, placed in a warm bath and cooled on ice before being read in a spectrophotometer at 570 nm.

RNA extraction and cDNA synthesis

Total RNA was extracted from maize leaves utilizing the SV Total RNA Isolation System commercial kit (Promega, Madison, USA) following the manufacturer’s instructions. RNA integrity was analyzed by electrophoresis in 1.2% agarose gel. The first cDNA was synthesized utilizing 2 µL of the total purified RNA and the GoScript™ Reverse Transcription System commercial kit (Promega, Madison, USA) and oligo primers (dT)₁₅ following the manufacturer’s instructions.

Semi-quantitative analysis by RT-PCR

The sequences of primers utilized for semi-quantitative RT-PCR analysis of ZmNR and ubiquitin (ZmUBQ) (Christensen and Quail, 2005), which was used as a control, given in Table 1. RT-PCR was performed in a final volume of 15µL, containing: 1.5 µL 10× PCR buffer, 0.8 µL MgCl₂ (50 mM), 0.5 µL dNTPs (10 mM), 0.5 µL each primer (10 mM), 0.4 µL Taq DNA polymerase (5U), and 2 µL cDNA diluted 1:10. The reactions were performed in a thermocycler model AG 22331 (Eppendorf, Hamburg, Germany), with the following conditions: one initial cycle of 94°C for 5 min and 30 cycles of: 94°C for 30 s for denaturing, 60°C for 40 s for annealing, 72°C for 30 s for extension, and one final cycle of 72°C for 10 min (Table 1). The quantification of gene transcripts was standardized using the constitutively expressed gene ZmUBQ as normalizer. The amplified PCR product was subjected to electrophoresis in agarose gel 1.2% stained with ethidium bromide. The gel images were captured by a photo documenting system, L-PIX Molecular Imaging (Locus Biotecnologia, Cotia, Brazil) and analyzed by densitometry (Freschiet al., 2009). IMAGEJ (http://rsbweb.nih.gov/ij/download.html) software was used to quantify band intensities.

Statistical analysis

The results were evaluated and submitted to analysis of variance (ANOVA) and the averages were compared by Tukey’s test (p ≤ 0.05) using the statistical software SISVAR (Ferreira, 1999).

RESULTS AND DISCUSSION

Nitrogen assimilation is crucial for plants, and the enzyme NR plays an important role in this pathway. In higher plants, this enzyme has been structurally, biochemically, and genetically well defined (Ovečka and Takač, 2014). Based on in silico analyses of the Phytozone Database, four NRs were identified and named Zea mays NR (ZmNR1–4). The complete cDNA sequences of ZmNR1 (GRMZM2G568636), ZmNR2 (GRMZM2G428027),

| Table 1. Sequences of primers used for semi-quantitative RT-PCR analysis of nitrate reductase (NR) genes. |
|-----------------|------------------|-----------------|-----------------|
| Genes           | Forward primer 5’-3’ | Reverse primer 5’-3’ | Tm (°C) | N’ of cycles |
| ZmNR1           | GAGTCGACAGCTACTACCA | GATCACCAGTGTATGGAC | 60     | 30           |
| ZmNR2           | CGGAGTCGACAAATTACTACC | CTGGTACGGTGGTGGAC | 60     | 30           |
| ZmNR3           | TACGATCAAAGGTAGCATACTC | CGTTACCCGAGTTATGTCAG | 60     | 30           |
| ZmNR4           | CGACAACACTACCATACAGGA | CGTTACCCGAGTTATGTCAG | 60     | 30           |
| ZmUBQ           | TGGGTGTGGCGGTGTT | GCTGCAGAGGTGGTGGTACA | 60     | 30           |
Sorghum (Sesamum indicum), which shares the same functions and have a common ancestor (Kleinhofs, 1996). Although a comparison between mono- and dicotyledonous species is not always possible, the structure of NR genes in cereals have previously been characterized (Figure 1). The following sequences were included in the analysis: three sequences from rice (Oryza sativa), one each from citrus (Citrus clementina), sorghum (Sorghum bicolor), millet (Setaria italica), sesame (Sesamum indicum), barley (Hordeum vulgare), and vine (Vitis vinifera), and two from Arabidopsis (A. thaliana), in addition to the four NR sequences identified in maize. Figure 1 shows that genes encoding ZmNR1 to 4 are in the same group as the NR genes of other monocotyledon species, while those from dicotyledonous species are in a second group. Sequences of paralogous genes were identified in the genomes of maize, sorghum, rice, barley, and millet. Phylogenetic analysis identified homologous proteins of NR from monocotyledons in dicotyledonous species. Although a comparison between mono- and dicotyledonous species is not always possible, the structure of NR genes in cereals appears to have evolved in a different way, becoming more complex than those found in dicotyledonous species (Plett et al., 2010; Buchner and Hawkesford, 2014). This may be due to the fact that, based on non-synonymous substitution rates, the estimated divergence between monocotyledon and dicotyledonous species occurred approximately 340 million years ago when fungi and plants or algae and plants were used as reference points (Zhou and Kleinhofs, 1996).

In general, functional domains are conserved in genes from the same group, and for this reason, they probably share the same functions and have a common ancestor (Kranz et al., 1998). The sequences that encode NR, ZmNR1 to 4, were found to contain three functional domains (Figure 2). Crawford et al. (1988) identified

**Table 2.** Physicochemical characteristics of ZmNR genes.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Nucleotide CDS bp</th>
<th>Amino acids</th>
<th>Molecular weight</th>
<th>Isoelectric point</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZmNR1</td>
<td>2733</td>
<td>910</td>
<td>101.57</td>
<td>6.37</td>
</tr>
<tr>
<td>ZmNR2</td>
<td>2634</td>
<td>877</td>
<td>97.59</td>
<td>6.36</td>
</tr>
<tr>
<td>ZmNR3</td>
<td>2673</td>
<td>890</td>
<td>98.52</td>
<td>6.39</td>
</tr>
<tr>
<td>ZmNR4</td>
<td>2802</td>
<td>933</td>
<td>103.04</td>
<td>6.32</td>
</tr>
</tbody>
</table>

AA, amino acid; bp, base pair; CDS, coding sequence; MW, molecular weight (kDa); pl, isoelectric point.

**Table 3.** Comparison among the predicted complete sequences of NR Amino acids of Zea mays (ZmNR) cDNAs. The identity values are over the diagonal whereas the similarity values are under the diagonal.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Identity and Similarity of Amino acids Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ZmNR1</td>
</tr>
<tr>
<td>ZmNR1</td>
<td>1.00</td>
</tr>
<tr>
<td>ZmNR2</td>
<td>0.85</td>
</tr>
<tr>
<td>ZmNR3</td>
<td>0.85</td>
</tr>
<tr>
<td>ZmNR4</td>
<td>0.90</td>
</tr>
</tbody>
</table>
Figure 1. Phylogenetic analysis of nitrate reductase (NR) in Zea mays and other plants. Protein names with their respective accession numbers are: A. thaliana (AT1G37130, AT1G77760), S. bicolor SORG_NR (gi|242079443), S. italica SETAR_NR (514796865), O. sativa japonica ORZ_NR1 (Os08g0468100), O. sativa ORZ_NR2 (Os08g0468700), O. sativa ORZ_NR3 (gi|27527625), H. vulgare HORD_NR (326494090), V. vinifera NITIS_NR (526117535), S. indicum SENS_NR (747046092), and C. clementina CITR_NR (567883105). Protein sequences of maize NR are marked with molybdenum binding domain in the N-terminal region of the protein, a heme domain in the central region, and a FAD binding domain in the C-terminal region of the protein. In higher plants, in NR, a molybdoheme-Flavo protein catalyzes the first and rate-limiting step of velocity in the nitrogen assimilation of the plant. NR comprises an enzymatic complex constituted by a mini electron transport chain, in which the FAD binding domain accepts two electrons from NADH or NADPH (Campbell and Smarelli 1986; Campbell and Kinghorn, 1990). The electrons are displaced by the heme domain to the molybdenum complex, which are then transferred to nitrate. The mechanisms of transcription, post-translation, and regulation are extremely complex, and are controlled by various endogenous and environmental factors (Viegas and Silveira, 2002).

NR activity in the leaves of the control plants was kept constant, and did not change significantly among the treatment days throughout the experimental period (Fig 3A). However, following the addition of $\text{Al}_2(\text{SO}_4)_3\cdot18\text{H}_2\text{O}$ to the soil, an increase in NR activity was observed up to the eighth day of treatment; however, this activity was lower by 50% than that of the control by the 16th day of treatment. The effects of aluminum toxicity on NR activity are varied, and studies have shown that plants respond differently to the same kinds of stress. A reduction in the NR activity was observed in grasses such as wheat (Foy and Fleming, 1982) and rice (Sharma and Dubey, 2005) and in the legumes, where high concentrations of Al decreased the efficiency of the nitrogen fixation (Gomes et al., 2002; Fernandes, 2006).

Some studies have shown that high concentrations of aluminum in the soil stimulate NR activity, as observed in the leguminous forage crop Stylosanthes macrocephala (Amaral et al., 2000) and in beans (Wang et al., 2010). Other authors have revealed that there is no change in NR activity in response to aluminum toxicity in the aerial parts of young plants of sugar cane cultivar IAC91-5155 (Carlin et al., 2012). In the present study, exposure to aluminum stress during 16 days of treatment may have negatively affected the nutrition and nitrogen assimilation in leaves. The NR activity (Figure 3A) in leaves was
markedly decreased in response to aluminum stress from day 16 onwards. It is possible that this reduction was the result of aluminum toxicity in the cells of the vegetal tissues. In high concentrations, aluminum in the soil can prevent the assimilation of essential nutrients into the vegetal tissues; therefore, compromising metabolism and limiting the development of the plant (Martins et al., 2011; Souza et al., 2016). The results of the control treatments, which were irrigated with water, were observed, the activity of NR did not differ significantly over time, reinforcing the hypothesis that, the toxicity caused by aluminum negatively affects the assimilation of N and consequently, NR activity in maize.

The amino acid content in response to aluminum stress was similar in the leaves of control plants and in those that were stressed until the fourth day of treatment (Figure 3B). From the eighth day of treatment, a marked increase in the amino acid content occurred in the plants.
subjected to aluminum stress. The amino acid content in the leaves of plants subjected to aluminum stress was around 100 and 400% higher than that in the leaves of the control plants. The increased amino acid content in the leaves is possibly due to the relationship between amino acids and aluminum stress. Plants exposed to stress synthesize and accumulate multiple metabolites as a defense mechanism against the stress (Sharma and Dietz, 2006). Souza et al, (2016) attributed the increase in total amino acids to an increase in protease activity, which broke the reserve proteins in the plants exposed to the aluminum toxicity, increasing the content of total soluble amino acids, aiming for osmotic adjustment. In addition, decreased NR activity also affects the concentrations of amino acids, particularly asparagine and glutamine (Prinsi et al., 2009).

To determine the effect of aluminum stress on NR genes, the corresponding RNA levels were quantified. The NR genes were found to be differentially expressed in response to stress (Figure 4). In maize, leaves subjected to aluminum stress has a decrease in the levels of ZmNR2, ZmNR3, and ZmNR4 (Figure 4B, 4C

**Figure 4.** Expression of the ZmNR1 (4A), ZmNR2 (4B), ZmNR3 (4C), and ZmNR4 (4D) genes in response to aluminum stress in maize leaves. Plants were irrigated with water or Al$_2$(SO$_4$)$_3$·18H$_2$O solution on days 0, 2, 4, 8, and 16. Uppercase letters compare control and aluminum-stressed plants and low case letters compare the treatments days. Different letters represent significant between means at P ≤ 0.05 level determined by Tukey multiple comparison procedure. The values represent the averages ± standard deviation of three biological replications.
and 4D) mRNA relative to that of the control, while ZmNR1 levels increased throughout the experiment (Figure 4A). The levels of ZmNR1 transcript increased quickly following the addition of aluminum to the soil from the second day of treatment (Figure 4A). At the end of the treatment (day 16), the transcript levels of ZmNR2 were decreased by about 50% relative to that of the control leaves (Figure 4B). Generally, the accumulation of ZmNR3 (Figure 4C) and ZmNR4 (Figure 4D) transcripts in leaves was lower in the plants submitted to stress as compared with the control plants; however during the days of stress the answer was kept. Analysis of the accumulation of NR transcripts (ZmNR1 to 4) revealed that the levels of ZmNR2, ZmNR3, and ZmNR4 (Figure 4B, 4C and 4D), as well as NR activity (Figure 3A) were affected in a similar way in the leaves. Therefore, from the second week of aluminum stress, there was a significant decrease in both enzymatic activity and transcript accumulation. A decrease in the levels of ZmNR2, ZmNR3, and ZmNR4 mRNA may be associated with a parallel inhibition of NR activity by aluminum in leaves. Conversely, an increase in the levels of ZmNR1 mRNA (Figure 4A) was observed. It appears that NR activity in response to aluminum stress is predominantly associated with ZmNR2, ZmNR3, and ZmNR4. The expression of ZmNR1 was increased by aluminum stress in maize leaves. It is possible that ZmRN1 is not involved in NR regulation; however, this gene may be involved in other metabolic pathways. Debouba et al. (2013) suggested that the transcript levels of NIA2 (At1g37130) are more important in the regulation of NR by saline stress in leaves and roots than those of NIA1 (At1g77760). Desikan et al. (2002) suggested that NIA1, which encodes NR, assures that NO produced by saline stress in the treatment (day 16), the accumulat

Conflict of interests
The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

TC thanks a fellowship received from CAPES for her Master’s studies. The authors acknowledge financial support from UNIPAR.

REFERENCES


---


