African Journal of Biotechnology

Volume 14 Number 42, 21 October, 2015
ISSN 1684-5315

Academic Journals

ABOUT AJB

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

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

Submission of Manuscript

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

Click here to Submit manuscripts online

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

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

Editor-In-Chief

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

Editor

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

Associate Editors

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

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

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

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

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

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

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

Editorial Board

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Dr. Essam A. Zaki
Genetic Engineering and Biotechnology Research
Institute, GEBRI,
Research Area,
Borg El Arab, Post Code 21934, Alexandria
Egypt

Dr. Alfred Dixon
International Institute of Tropical Agriculture (IITA)
PMB 5320, Ibadan
Oyo State, Nigeria

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

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

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

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

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

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

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

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

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

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

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

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

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

Dr. Deborah Rayfield
Physiology and Anatomy
Bowie State University
Department of Natural Sciences
Crawford Building, Room 003C
Bowie MD 20715, USA

Dr. Marlene Shehata
University of Ottawa Heart Institute
Genetics of Cardiovascular Diseases
40 Ruskin Street
K1Y-4W7, Ottawa, ON, CANADA

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Prof. Felix Dapare Dakora
Research Development and Technology Promotion
Cape Peninsula University of Technology,
Room 2.8 Admin. Bldg. Keizersgracht, P.O. 652,
Cape Town 8000,
South Africa

Dr. Geremew Bultosa
Department of Food Science and Post harvest Technology
Haramaya University
Personal Box 22, Haramaya University Campus
Dire Dawa,
Ethiopia

Dr. José Eduardo Garcia
Londrina State University
Brazil

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Dr. Evans C. Egwim
Federal Polytechnic,
Bida Science Laboratory Technology Department,
PMB 55, Bida, Niger State,
Nigeria

Prof. George N. Goulielmos
Medical School,
University of Crete
Voutes, 715 00 Heraklion, Crete,
Greece

Dr. Uttam Krishna
Cadila Pharmaceuticals Limited,
India 1389, Tarsad Road,
Dholka, Dist: Ahmedabad, Gujarat,
India

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

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

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

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

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

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

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

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

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

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

Dr. Thomas Silou
Universit of Brazzaville BP 389
Congo

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

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

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

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

Dr. Linga R. Gutha
Washington State University at Prosser,
24106 N Bunn Road,
Prosser WA 99350-8694.

Dr Helal Ragab Moussa
Bahnay, Al-bagour, Menoufia,
Egypt.

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

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

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

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

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

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

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

Dr Takuji Ohyama
Faculty of Agriculture, Niigata University

Dr Mehdi Vasfi Marandi
University of Tehran

Dr FÜgen DURLU-ÖZKAYA
Gazi Üniversity, Tourism Faculty, Dept. of
Gastronomy and Culinary Art

Dr. Reza Yari
Islamic Azad University, Boroujerd Branch

Dr Zahra Tahmasebi Fard
Roudehen branche, Islamic Azad University

Dr Albert Magrí
Giro Technological Centre

Dr Ping ZHENG
Zhejiang University, Hangzhou, China

Dr. Kgomotso P. Sibeko
University of Pretoria

Dr Greg Spear
Rush University Medical Center

Prof. Pilar Morata
University of Malaga

Dr Jian Wu
Harbin Medical University, China

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

Prof. Pavel Kalac
University of South Bohemia, Czech Republic

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

Dr. Shuyang Yu
Department of Microbiology, University of Iowa
Address: 51 newton road, 3-730B BSB bldg. Iowa
City, IA, 52246, USA

Dr. Binxing Li

Dr. Mousavi Khaneghah
College of Applied Science and Technology-
Applied Food Science, Tehran, Iran.

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

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

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

Instructions for Author

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

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

Article Types
Three types of manuscripts may be submitted:

Regular articles: These should describe new and carefully confirmed findings, and experimental procedures should be given in sufficient detail for others to verify the work. The length of a full paper should be the minimum required to describe and interpret the work clearly.
Short Communications: A Short Communication is suitable for recording the results of complete small investigations or giving details of new models or hypotheses, innovative methods, techniques or apparatus. The style of main sections need not conform to that of full-length papers. Short communications are 2 to 4 printed pages (about 6 to 12 manuscript pages) in length.

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

Review Process

All manuscripts are reviewed by an editor and members of the Editorial Board or qualified outside reviewers. Authors cannot nominate reviewers. Only reviewers randomly selected from our database with specialization in the subject area will be contacted to evaluate the manuscripts. The process will be blind review.
Decisions will be made as rapidly as possible, and the journal strives to return reviewers' comments to authors as fast as possible. The editorial board will re-review manuscripts that are accepted pending revision. It is the goal of the AJFS to publish manuscripts within weeks after submission.

Regular articles

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

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

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

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

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

Materials and methods should be complete enough to allow experiments to be reproduced. However, only truly new procedures should be described in detail; previously published procedures should be cited, and important modifications of published procedures should be mentioned briefly. Capitalize trade names and include the manufacturer's name and address. Subheadings should be used. Methods in general use need not be described in detail.

Results should be presented with clarity and precision. The results should be written in the past tense when describing findings in the authors' experiments. Previously published findings should be written in the present tense. Results should be explained, but largely without referring to the literature. Discussion, speculation and detailed interpretation of data should not be included in the Results but should be put into the Discussion section.

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

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

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

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

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

Examples:

Abayomi (2000), Agindotan et al. (2003), (Kelebeni, 1983), (Usman and Smith, 1992), (Chege, 1998; 1987a,b; Tijani, 1993,1995), (Kumasi et al., 2001)
References should be listed at the end of the paper in alphabetical order. Articles in preparation or articles submitted for publication, unpublished observations, personal communications, etc. should not be included in the reference list but should only be mentioned in the article text (e.g., A. Kingori, University of Nairobi, Kenya, personal communication). Journal names are abbreviated according to Chemical Abstracts. Authors are fully responsible for the accuracy of the references.

Examples:

Chikere CB, Omoni VT and Chikere BO (2008). Distribution of potential nosocomial pathogens in a hospital environment. Afr. J. Biotechnol. 7: 3535-3539.

Moran GJ, Amii RN, Abrahamian FM, Talan DA (2005). Methicillinresistant Staphylococcus aureus in community-acquired skin infections. Emerg. Infect. Dis. 11: 928-930.

Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

Pelczar JR, Harley JP, Klein DA (1993). Microbiology: Concepts and Applications. McGraw-Hill Inc., New York, pp. 591-603.

Short Communications

Short Communications are limited to a maximum of two figures and one table. They should present a complete study that is more limited in scope than is found in full-length papers. The items of manuscript preparation listed above apply to Short Communications with the following differences: (1) Abstracts are limited to 100 words; (2) instead of a separate Materials and Methods section, experimental procedures may be incorporated into Figure Legends and Table footnotes; (3) Results and Discussion should be combined into a single section.
Proofs and Reprints: Electronic proofs will be sent (e-mail attachment) to the corresponding author as a PDF file. Page proofs are considered to be the final version of the manuscript. With the exception of typographical or minor clerical errors, no changes will be made in the manuscript at the proof stage.

Fees and Charges: Authors are required to pay a $650 handling fee. Publication of an article in the African Journal of Biotechnology is not contingent upon the author's ability to pay the charges. Neither is acceptance to pay the handling fee a guarantee that the paper will be accepted for publication. Authors may still request (in advance) that the editorial office waive some of the handling fee under special circumstances

Copyright: © 2015, Academic Journals.
All rights Reserved. In accessing this journal, you agree that you will access the contents for your own personal use but not for any commercial use. Any use and or copies of this Journal in whole or in part must include the customary bibliographic citation, including author attribution, date and article title.

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

Disclaimer of Warranties

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

African Journal of Biotechnology

Table of Contents: Volume 14 Number 42, 21 October, 2015

ARTICLES

Comparison of anther and microspore culture in androgenic embryogenesis and regeneration of broccoli (*Brassica oleracea* L. var. *italica* P.) 2910
Ying Qin, Yunyu Huang, Seppo Pulli and Yang-Dong Guo

Suppressive effects of a polymer sodium silicate solution on powdery mildew and root rot diseases of miniature rose 2917
Mohsen Mohamed Elsharkawy, Tatsuya Hase, Masafumi Shimizu and Mitsuro Hyakumachi

Toxic effects of *Ricinus communis* non-protein trypsin inhibitor on *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera:Noctuidae) 2928
Gislaine A. Carvalho, Custódio D. dos Santos, Dejane S. Alves, Geraldo A. Carvalho, Maria das G. Cardoso and Marcelo M. de Haro

The efficacy of palm oil sludge in reducing ruminal methanogenesis using rumen simulation technique 2937
L. C. Ugwuowo, A. G. Ezekwe, A. O. Ani, S. I. Eze, C. N. Anyanwu and A. Ofomatah

academicJournals

Vol. 14(42), pp. 2910-2916, 21 October, 2015
DOI:10.5897/AJB12.333
Article Number: 6AF925F55880
ISSN 1684-5315
Copyright © 2015
Author(s) retain the copyright of this article
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Comparison of anther and microspore culture in androgenic embryogenesis and regeneration of broccoli (*Brassica oleracea* L. var. *italica* P.)

Ying Qin[1], Yunyu Huang[1], Seppo Pulli[2] and Yang-Dong Guo[1]*

[1]College of Agriculture and Biotechnology, China Agricultural University, Beijing 100193, China.
[2]Department of Biology, University of Turku, FI-20014 Turku, Finland.

Received 20 January 2012; Accepted 4 April, 2012

The aim of this study was to compare the efficiency of broccoli anther and microspore culture methods for doubled haploid (DH) lines production. We evaluated the main influencing factors and optimized the culture methods to improve embryo induction and plant regeneration for efficient doubled haploid production in broccoli breeding. Six broccoli hybrids were used in this study. Our results show that generally, the efficiency of androgenic embryogenesis and regeneration in microspore culture is higher than that in the anther culture. Moreover, the microspore culture eliminated the possibility of plantlets coming from diploid tissue. In this study, the four-day cold pre-treatment yielded the highest number of embryos in both anther and microspore culture methods; the embryo yield at 32.5°C for 24 h was the highest in anther and microspore culture. Optimal plating densities were 30 anthers per dish in anther culture and 4×10^5 microspores per ml in microspore culture. In androgenic embryo production, the PG-96 medium proved to be more effective than NLN medium. Sucrose concentration at 10% for anther culture and 13% (w/v) for microspore culture was recommended. A total of 70 regenerants were obtained from three genotypes including doubled haploids, haploids and aneuploids.

Key words: Anther, broccoli, doubled haploid, microspore, plant regeneration.

INTRODUCTION

Broccoli (*Brassica oleracea* L. var. *italica* P.) is an important crop. Cultivars in use today are almost exclusively F_1 hybrids (Wang et al., 1999). Conventional inbreeding is laborious and time consuming, hence double haploid (DH) production and cross-pollinated DH-progeny as an alternative can be the greatest source in variation for plant breeding or selection. The two major advantages of using DH in plant breeding are the increase of selection efficiency and shortening the time to release new cultivars (Castillo et al., 2000). Furthermore, DH lines are very valuable for quantitative genetics studies and for genome mapping and quantitative trait locus (QTL) analysis (Monforte et al., 2004; Behn et al., 2005). Anther cultures and isolated microspore cultures are two different

*Corresponding author. E-mail: yaguo@cau.edu.cn. Tel: 86 10 62734845.

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

methods widely used for DH production in broccoli. DH plants of broccoli were obtained from anther culture (Keller and Armstrong, 1983; Cogan et al., 2001) and isolated microspore culture (Takahata and Keller, 1991; Duijs et al., 1992). DH parental lines were already obtained using anther culture and introduced into breeding schemes (Farnham et al., 1998). Despite these improvements, anther and isolated microspore culture methods have some limitations in broccoli. One big problem is that the yield of embryo in some broccoli genotypes is still very low, and certain genotypes do not appear amenable to the process at all (Dias, 2001; Yuan et al., 2011). Meanwhile, a general problem encountered in the use of anther culture with *B. oleracea* is that resulting populations contain a mixture of variable ploidy individuals (Chauvin et al., 1993).

Isolated microspore culture is initiated by extracting microspores from anthers, and in turn the microspores are cultured free of anther tissue, which offer the opportunity of producing large amounts of plants with less effort and reduced cost (Duijs et al., 1992). Further advantage of microspore embryogenesis would be the capability of plant regeneration from a single cell, as the isolated microspore culture eliminates the possibility of plants from diploid tissue (such as septum, anther wall and tapetum), thus resulting in an efficient androgenic embryogenesis. Androgenesis and subsequent DH production are influenced by genetic, physiological, physical and chemical factors, which affect the pollen grain while entering into a new developmental pathway. In androgenic embryogenesis, the donor genotype is one of the most important influences. In broccoli, genotype is considered to be the key factor for obtaining microspore-derived embryos (Keller and Armstrong, 1983). In androgenic cell cultures, the density of anthers or isolated microspores can influence the embryogenesis and plant regeneration. Cultured anthers release endogenous hormones and certain chemicals which could regulate and affect embryogenesis (George, 1993).

Microspores have a remarkable capacity to develop into haploid plants *via* embryogenesis *in vitro*; stress treatment triggers the induction of this sporophytic pathway. These include nitrogen starvation, short days and low temperature treatment of donor plants, which have a strong enhancing effect on microspore embryo formation in anther cultures, as well as cold or heat shock and chemical treatment of excised inflorescences, flower buds or anthers (Touraev et al., 1997). Today, heat shock is the method of choice for androgenic cell culture (Arnison et al., 1990; Duijs et al., 1992). Cold pretreatment has been used in a variety of plant species for induction of androgenesis. Such treatments given to donor materials (spike, inflorescence, flower bud etc.) before inoculation promote embryo induction, produce more embryos and enhance the green plant yield in anther and microspore cultures (Sato et al., 2002; Osolnik et al., 1993).

According to Nitsch (1974), cold treatment increases the frequency of embryoid formation by increasing the number of pollen with similar nuclei and maintaining pollen in viable condition.

In plant tissue culture, the culture medium is always an important factor, providing both nutrient and osmotic environments. Many factors have been made in order to optimize medium for plant anther and microspore culture. The induction medium has not only the task to nourish the microspores but also to redirect their developmental pathway to the formation of embryos (Jähne and Lörz, 1995). All tissue culture media require the presence of a sugar(s) as a source of carbon and energy. Sugars also act as osmotic regulators in the medium. Sucrose has been the most widely used carbohydrate source in the culture medium, also commonly employed in androgenic cell culture (Pescitelli et al., 1990). The main objective of this study was to compare the efficiency of anther and isolated microspore culture for the production of DH lines from F_1 hybrids of broccoli. The study evaluated the main influencing factors and optimized the culture methods to improve embryo induction and plant regeneration for efficient practical breeding use. To our knowledge, the present comparative study of androgenic culture methods is the first one in broccoli.

MATERIALS AND METHODS

Six F_1 hybrids that came from Xiaotangshan company were investigated: namely "Mantuolu", "Bishan", "Luling", "Beilu", "Zhulu" and "Meilu118". Seeds were germinated in a greenhouse under controlled conditions (25/20°C, day/night temperature, 16 h photoperiod at 70 µmol m^{-2} s^{-1} light intensity) in July. Seedlings were vernalised in a cold room (6°C, 12 h photoperiod at 30 µmol m^{-2} s^{-1} light intensity) for 5 to 6 weeks, after which the plants were transferred to a greenhouse under the same conditions as before. Anther and microspore cultures were performed after harvest of flower buds. For the experiments (1 to 4), only genotype "Bishan" was used as test materials.

Experimental procedure

Microspore developmental stage was checked by acetocarmine staining. Buds were surface-sterilized with 1.6% (final concentration) sodium hypochlorite solution and 0.5% Tween 20 for 15 min, then rinsed five times in sterile water before excised. For anther culture, anthers from the same plant were cultured in a 90-mm Petri dish containing 25 ml the PG-96 solid medium (Guo et al., 1999). For microspore isolation, anthers were put into the NLN liquid induction medium (Takahata and Keller, 1991) and microspores were released using a glass rod homogenizer and a microspore suspension was obtained by filtration through 45 µm nylon mesh screen. This suspension was centrifuged three times at 100 g for 3 min and resuspended in the same medium. Microspores were resuspended in the required amount of liquid induction medium, final density of microspore was determined with a haemocytometer and adjusted to required densities. The microspore suspension was incubated in a 60 mm Petri dish. Each test has five replicates.

After five to seven weeks of culture at 25°C, well-developed embryos from anther and microspore culture were transferred to MS

Table 1. Requirements of cold treatment on androgenic embryo induction of broccoli on PG-96 media (variety "Bishan" was used).

Duration of cold treatment (day)	Yield of embryo (embryos per 100 anthers) ± S. D[a]	
	Anther culture	Microspore culture
0	20.3 ± 3.2[a]	30.3 ± 4.1[b]
2	41.1 ± 9.9[b,c]	73.2 ± 12.6[c]
4	57.0 ± 13.4[c]	89.1 ± 15.0[c]
6	39.7 ± 6.3[b,c]	67.3 ± 11.5[c]
8	32.9 ± 5.6[b]	19.4 ± 2.7[a]

[a] Means followed by the same letters within columns are not significantly different according to the LSD test at the 5% level of significance. Means ± standard deviation are from five replicates.

regeneration medium (Murashige and Skoog, 1962) supplemented with 1 μM indole-3-butyric acid (IBA) and 4.4 μM 6-benzylaminopurine (BA) for four weeks and then transferred to a half-strength mineral salts MS medium for rooting. Cultures were incubated in a growth chamber with 70 μmol m^{-2} s^{-1}, and 16 h light at 25°C. Prior to transferring plantlets to soil, the roots of the plantlets were immersed in a 0.2% colchicine solution for 2 h followed by several water washes to double the chromosome. Root tip chromosomes of regenerants were counted using the Feulgen staining method (Sinkovic and Bohanec, 1988) after chromosome doubling. Every treatment was replicated five times in this study. The number of embryos and the yield of regenerants were scored and the data were analyzed with SAS 6.0 software. Statistical analyses were performed using ANOVA. Mean values were compared according to LSD test at P = 0.05.

Experiment 1: Effect of cold pre-treatment of donor materials on anther and microspore culture

Flower buds with length 3.0 to 4.0 mm were used as sources for anther and microspore culture were harvested when microspore were mostly at late uninucleate development stage, with 10 to 30% binucleate microspore and stored at 4°C in darkness with stalks in water to test the cold pre-treatment effects of different durations (0, 2, 4, 6 and 8 days). The plating densities were 30 anthers per Petri dish for anther culture and 4 × 10^5 microspores per ml for isolated microspore culture.

Experiment 2: Optimization of incubation temperature regime

In the incubation temperature regime experiment, microspores were incubated in the dark conditions at 27.5, 30 and 32.5°C for 48 h and then maintained at 25°C in the dark. The plating densities were 30 anthers per Petri dish for anther culture and 4 × 10^5 microspores per ml for isolated microspore culture. Anthers were subcultured every four weeks. For the microspore cultures, the cultures were transferred to a shaker at 60 rpm in the dark at 25°C after four weeks incubation.

Experiment 3: Optimization of plating density of anther and microspore culture

In our tests, plating density ranged from 10 to 50 anthers per dish (90 mm) for anther culture. Three microspore densities were tested for isolated microspores in liquid induction medium, including 2×10^5, 4×10^5 and 8×10^5 microspores per ml.

Experiment 4: Effects of basal medium and sugar concentrations

The PG-96 and the NLN were employed as induction media. Liquid media which were used in microspore culture were sterilized by filtration; solid media which were used in anther culture were sterilized by autoclaving at 120°C for 25 min. Additionally, α-naphthalene acetic acid (NAA) at 0.54 μM, 2,4-dichlorophenoxyacetic acid (2,4-D) at 0.45 μM and $AgNO_3$ at 11.8 μM were filter-sterilized and added after autoclaving. For anther culture, sucrose concentration (w/v) at 6, 10 and 13% were tested on embryo induction across PG-96 and NLN media and six genotypes. For microspore culture, sucrose concentration (w/v) at 10, 13 and 17% were tested for embryo induction across both media and six genotypes. The levels of sucrose were set according to former similar study (Guo and Pulli, 1996; Guo et al., 1999). Activated charcoal was added to the culture media at 150 mg L^{-1} in anther and microspore cultures.

Experiment 5: Effect of genotypes on embryo induction

Six hybrids were tested for androgenic embryo induction under the following conditions; the stalks of donor plants were treated in sterile distilled water held at a cold temperature of 4°C in the dark for four days, incubated initially at 32.5°C for 24 h, and then transferred to 25°C in the darkness. For anther culture, plating density was 30 anthers per dish; for isolated microspore culture, plating density was 4×10^5 microspores per ml. The PG-96 with sucrose at 10% for anther culture and 13% for microspore culture was used as culture medium. The embryos bigger than 1.5 mm were counted under microscope and then transferred to MS regeneration medium.

RESULTS

Influence of cold per-treatment on anther and microspore culture

Our results reveal that androgenic embryogenesis was generally enhanced by the cold pre-treatment in broccoli. Table 1 shows the yield of embryo in genotype "Bishan" significantly improved by 2 to 8 days cold pre-treatment to anther culture and 2 to 6 days to isolated microspore culture.

Effect of cold pre-treatment on embryo induction in

Table 2. Effects of heat shock on embryo induction of anther and microspore culture of broccoli on PG-96 media (variety “Bishan” was used).

Heat shock treatment	Number of embryos per 100 anthers ± S. D.[a]	
	Anther culture	Microspore culture
25°C (control)	15.4 ± 7.2[a]	20.2 ± 7.0[a]
27.5°C (48 h)	20.4 ± 11.2[a]	24.9 ± 12.3[a]
30°C (48 h)	31.6 ± 13.5[a,b]	67.2 ± 14.5[b]
32.5°C (24 h)	54.9 ± 19.9[b]	83.5 ± 20.4[b]
32.5°C (48 h)	45.1 ± 12.9[b]	59.7 ± 12.4[b]

[a]Means followed by the same letters within columns are not significantly different according to the LSD test at the 5% level of significance. Means ± standard deviation are from five replicates.

Table 3. Effects of plating density on embryo yield on PG-96 media in anther and microspore culture of broccoli (Variety “Bishan” was used).

Plating density	Yield of embryo (embryos per 100 anthers) ± S. D[a]	
	Anther culture	Microspore culture
10 anther/dish	12.1 ± 6.2[ab]	
20 anther/dish	26.4 ± 8.2[b]	
30 anther/dish	49.9 ± 14.2[c]	
40 anther/dish	29.6 ± 11.5[b,c]	
50 anther/dish	6.8 ± 2.8[a]	
2×10^5 microspores/ml		57.5 ± 13.7[b]
4×10^5 microspores/ml		77.8 ± 20.8[b]
8×10^5 microspores/ml		23.1 ± 9.8[a]

[a]Means followed by the same letters within columns are not significantly different according to the LSD test at the 5% level of significance. Means ± standard deviation are from five replicates.

microspore cultures was greater than that in anther cultures. In our test, a 4-day cold treatment gave the best results for both anther and microspore cultures; the embryo yields of anther and microspore cultures were 57.0 per 100 anthers and 89.1 per 100 anthers for genotype “Bishan”, respectively (Table 1).

The effect of heat shock regime on anther and microspore culture

In this study, a heat shock pre-culture at 32.5°C given at the beginning of culture initiation, instead of normal dark culture at 25°C, resulted in an increase of micro embryo formation.

The best result was achieved from a heat shock at 32.5°C for 24 h; the number of embryo per 100 anthers were 54.9 and 83.5 in anther and microspore culture, respectively (Table 2). The influence of heat shock regime on microspore culture was more important than that of anther culture (Table 2).

Effects of the plating density on anther and microspore culture

In our current test, culture density significantly influenced embryo induction efficiency in anther and microspore cultures. For anther culture, the density of 30 anthers per dish gave the maximum yield, while for microspore culture, the density of 4×10^5 gave the best result (Table 3).

Influences of basal media, sugar concentration on anther and microspore culture

In the current experiments, the PG-96 and the NLN used as embryo and shoot induction media were employed for both anther and microspore cultures. In the present tests, the highest embryo yields of genotype “Bishan” on the PG-96 media were 53.9 per 100 anthers and 87.6 per 100 anthers in the anther and microspore culture, and those in the NLN medium were 46.1 per 100 anthers and 64.5 per 100 anthers, respectively (Table 4). Results of the present

Table 4. Effects of sugar concentration and culture media on embryo induction of anther and microspore culture in broccoli (variety “Bishan” was used).

Method	Sucrose (%)	Number of embryo per 100 anthers ± S. D[a]	
		NLN	PG-96
Anther culture	6	34.2 ± 10.6[a,b]	46.7 ± 18.6[a]
	10	46.1 ± 17.3[b]	53.9 ± 16.9[a]
	13	20.4 ± 8.1[a]	43.5 ± 14.8[a]
Microspore culture	10	44.3 ± 6.9[a]	51.8 ± 9.0[b]
	13	64.5 ± 24.9[a]	87.6 ± 11.2[c]
	17	49.3 ± 13.9[a]	24.9 ± 6.1[a]

[a]Means followed by the same letters within columns are not significantly different according to the LSD test at the 5% level of significance. Means ± standard deviation are from five replicates.

Table 5. Effects of genotype on androgenic embryo production using PG-96 media in anther and microspore culture of broccoli.

Genotype	Number of embryo per 100 anthers ± S. D[a]	
	Anther culture	Microspore culture
Bishan	50.1 ± 18.4[a]	78.3 ± 20.1[a]
Mantuolu	29.9 ± 7.6[a]	56.9 ± 19.9[a,b]
Luling	32.1 ± 12.0[a]	41.5 ± 14.3[b,c]
Meilu 118	45.3 ± 16.1[a]	38.3 ± 6.9[b,c]
Beilu	12.6 ± 6.0[b]	23.5 ± 11[c]
Zhulu	3.9 ± 0.9[c]	5.6 ± 2.4[d]

[a]Means followed by the same letters within columns are not significantly different according to the LSD test at the 5% level of significance. Means ± standard deviation are from five replicates.

study demonstrate the advantage of the PG-96 to the NLN medium for embryo induction. In the present experiments, anther cultures produced the highest embryo yields in media with 10% of sucrose, which produced the lowest ones in 6% sucrose concentration; microspore cultures produced the highest embryo yields in media with 13% of sucrose, which produced the lowest ones in 10% sucrose concentration (Table 4).

Effects of genotype on anther and microspore culture

In the case of broccoli, anther and microspore culture ability is genetically controlled and that culture efficiency is thus genotype dependent (Takahata and Keller, 1991). This study shows that genetic order, from the highest to the lowest embryo producer in microspore culture, was “Bishan” (78.3 embryos per 100 anthers), “Mantuolu” (56.9 embryos per 100 anthers), “Luling” (41.5 embryos per 100 anthers), “Meilu118” (38.3 embryos per 100 anthers), “Beilu” (23.5 embryos per 100 anthers) and “Zhulu” (5.6 embryos per 100 anthers), respectively (Table 5).

Plant regeneration and ploidy levels of regenerant

Regeneration was relatively difficult in the present study. Many androgenic micro embryos stopped growth or developed into plantlet-like structures with vitrification. Approximately 3 to 5% of androgenic embryos developed into regenerated plants. A total of 70 regenerants had been obtained so far including doubled haploids (2n = 18), haploids (n = 9) and aneuploids (Table 6). Ploidy levels of regenerant were estimated by chromosome counting. Frequencies of chromosome doubling are shown in Table 6.

DISCUSSION

Some important factors, such as stress treatments, optimal plating densities, the basal media, sugar concentrations as well as the genotypes of donor plants strongly influence the induction of androgenic embryogenesis and the regeneration of broccoli. Cold pre-treatment of anthers is commonly employed to enhance the frequency of androgenic embryogenesis. It has been used extensively

Table 6. Ploidy levels of regenerant in anther and microspore culture of broccoli.

Genotype	Anther culture			Isolated microspore culture		
	DH (%) 2n = 18	Haploid (%) n = 9	Others (%)	DH (%) 2n = 18	Haploid (%) n = 9	Others (%)
Bishan	11 (68.8)	3 (18.8)	2 (12.5)	15 (62.5)	4 (16.7)	5 (20.8)
Mantuolu	4 (50.0)	3 (37.5)	1 (12.5)	7 (63.6)	2 (18.2)	2 (18.2)
Meilu118	3 (37.5)	3 (37.5)	2 (25.0)	1 (33.3)	2 (66.7)	0 (0.0)

in many crops such as *Brassica campestris* (Guo and Pulli, 1996) and *Triticum aestivum* (Li et al., 1988). Instead of asymmetry of normal first pollen mitosis, more symmetric divisions have been reported following cold pretreatment (Kiviharju and Pehu, 1998) likely due to blocking the normal gametophytic development. Stress treatments including low temperature treatment and heat shock act as triggers for promoting the sporophytic pathway (Touraev et al., 1996, 1997). Synthesis of proteins has been reported as a result of stress treatments. Moreover,

the involvement of cytoskeletal elements or the delay of pollen or anther wall senescence has been suggested (Cordewener et al., 1995; Kiviharju and Pehu, 1998). For isolated microspore culture, the swollen microspores have specific cytological characters, comprising a large central vacuole, thin tonoplast, parietal cytoplasm and peripheral nucleus. Many microspores between very late uninucleate and early binucleate stages will “swell” after the heat shock, whereas younger and older microspores will not. Only such “swollen” microspores have the potential to continue to develop, divide and finally form microcalluses. Microspores have a remarkable capacity to develop into haploid plants *via* embryogenesis *in vitro*; stress treatment can further trigger the induction of this sporophytic pathway. Deviation from a gametophytic to a sporophytic developmental pathway has been induced in microspores by applying various pretreatments either *in vivo* or *in vitro* (Touraev et al., 1997). Currently, the method of heat shock is commonly used for androgenic cell culture in *Brassica oleracea* (Takahata and Keller, 1991; Duijs et al., 1992; Fabijanski et al., 1991). Furthermore, the combination of cold pretreatment and heat shock significantly enhances microspore embryogenesis efficiency and results in higher diploid frequency of the regenerated population compared to traditional microspore culture protocol which typically uses heat shock in broccoli (Yuan et al., 2011).

Plating density is an important factor influencing androgenesis since cultured anthers and microspores release endogenous hormones and other metabolic by-products which not only affect embryogenesis, but also competitively exploit nutrients and oxygen supply as well. Meanwhile, dead microspores are likely to release toxic substances into the culture medium. Those toxic substances tamper with the development of viable microspores (George, 1993). Constituents of the basal medium are important factors in eliciting successful androgenesis. By now, the NLN medium is recognized as a suitable medium for broccoli androgenesis (Takahata and Keller, 1991; Duijs et al., 1992). The PG-96 medium composed of relatively complex organic acids and vitamin compounds. In previous studies, the modified PG-96 induction medium promoted androgenic embryogenesis and regeneration in timothy (Guo and Pulli, 1999). Sugar as the source of carbon and energy also functions as an osmotic regulator in culture media. Carbon source in induction medium has a profound effect on anther culture response. In the case of *Brassica*, sucrose has proved effective for increasing embryo induction or improving embryo development and plant regeneration (Takahata and Keller, 1991; Duijs et al., 1992; Na et al., 2011).

Auxin and cytokinin as a whole are essential in embryo induction and shoot formation. Low level or free of growth regulator has been proven suitable for *Brassica* androgenesis (Arnison et al., 1990; Takahata and Keller, 1991; Duijs et al., 1992). In broccoli androgenic cell cultures, spontaneous diploidization has occurred with great variation. A general problem encountered in the use of anther and microspore culture with *B. oleracea* results in the mixture of regenerants with variable ploidy (Chauvin et al., 1993). The anther and microspore culture methods for the induction of androgenic embryogenesis and requirements for successful regeneration of broccoli were compared in this paper. All the important factors mentioned above had been optimized to improve embryo induction and green plant regeneration for efficient practical breeding use. The efficiency of androgenic embryogenesis and regeneration in isolated microspore culture was higher than that in the anther culture. Both anther culture and isolated microspore culture methods could be used for DH breeding in broccoli.

Conflict of interests

The author(s) did not declare any conflict of interest.

ACKNOWLEDGEMENTS

This work was partly supported by grants to Guo (BLVT-03) and Beijing Key Laboratory of Growth and Developmental Regulation for Protected Vegetable Crops.

REFERENCES

Arnison PG, Donaldson P, Jackson A, Semple C, Keller W (1990). Genotype-specific response of cultured broccoli (*Brassica oleracea* var. *italica*) anthers to cytokinins. Plant Cell Tiss. Org. 20:217-222.

Behn A, Hartl L, Schweizer G, Baumer M (2005). Molecular mapping of QTLs for non-parasitic leaf spot resistance and comparison of half-sib DH populations in spring barley. Euphytica, 141:291-299.

Castillo AM, Valles MP, Cistue L (2000). Comparison of anther and isolated microspore cultures in barley. Effects of culture density and regeneration medium. Euphytica, 113:1-8.

Chauvin JE, Yang Q, Jeune B, le Herve Y (1993). Androgenic embryos obtained by anther culture of *Brassica oleracea* (*ssp italica* and *ssp botrytis*) and estimation of the value of regenerated material in plant breeding programs. Agronomie 13:579-590.

Cogan N, Harvey E, Robinson H, Lynn J, Pink D, Newbury HJ, Puddephat I (2001). The effects of anther culture and plant genetic background on *Agrobacterium rhizogenes*-mediated transformation of commercial cultivars and derived doubled-haploid *Brassica oleracea*. Plant Cell Rep. 20:755-762.

Cordewener JHG, Hause G, Görgen E, Busink R, Hause B, Dons HJM, Lammeren AAM, van Lookeren Champagne MM, van Pechan P (1995). Changes in synthesis and localization of members of the 70-kDa class of heat-shock proteins accompany the induction of embryogenesis in *Brassica napus* L. microspores. Planta 196: 747-755.

Dias JCD (2001). Effect of incubation temperature regimes and culture medium on broccoli microspore culture embryogenesis. Euphytica 119:389-394.

Duijs JC, Voorrips RE, Visser DL, Custers JBM (1992). Microspore culture is successful in most crop types of *Brassica oleracea* L. Euphytica, 60:45-55.

Fabijanski SF, Altosaar I, Arnison PG (1991). Heat shock response during anther culture of broccoli (*Brassica oleracea* var. *italica*). Plant Cell Tiss. Org. 26:203-212.

Farnham MW, Caniglia EJ, Thomas CE (1998). Efficient ploidy determination of anther-derived broccoli. Hort. Sci. 33:323-327.

George EF (1993). Plant propagation by tissue culture. Part 1, the technology. London: Exegetics Ltd. 1993:327.

Guo Y-D, Pulli S (1996). High-frequency embryogenesis in *Brassica campestris* microspore culture. Plant Cell Tiss. Org., 46: 219-225.

Guo Y-D, Sewón P, Pulli S (1999). Improved embryogenesis from anther culture and plant regeneration in timothy. Plant Cell Tiss. Org. 57:85-93.

Jähne A, Lörz H (1995). Cereal microspore culture. Plant Sci., 109: 1-12.

Keller WA, Armstrong KC (1983). Production of haploids via anther culture in *Brassica oleracea* var. *italica*. Euphytica, 32: 151-159.

Kiviharju E, Pehu E (1998). The effect of cold and heat pretreatments on anther culture response of *Avena sativa* and *A. sterilis*. Plant Cell Tiss. Org., 54: 97-104.

Li HC, Qureshi JA, Kartha KK (1988). The influence of different temperature treatments on anther culture response of spring wheat (*Triticum aestivum* L.). Plant Sci. 57:55-61.

Monforte AJ, Oliver M, Gonzalo MJ, Alvarez JM, Dolcet-Sanjuan, R., Arus, P. (2004). Identification of quantitative trait loci involved in fruit quality traits in melon (*Cucumis melo* L.). Theor. Appl. Genet., 108: 50-758.

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

Na H, Hwang G, Kwak JH, Yoon MK, Chun C (2011). Microspore derived embryo formation and doubled haploid plant production in broccoli (*Brassica oleracea* L. var *italica*) according to nutritional and environmental conditions. Afr. J. Biotechnol. 10:12535-12541.

Nitsch C (1974). Pollen culture - a new technique for mass production of haploid and homozygous plants. In: Kasha, K.J. (ed.). Haploids in Higher Plants-Advances and Potential. Guelph University Press, pp. 123-135.

Osolnik B, Bohanec B, Jelaska S (1993). Stimulation of androgenesis in white cabbage (*Brassica oleracea* var. *capitata*) anthers by low temperature and anther dissection. Plant Cell Tiss. Org. 32:241-246.

Pescitelli SM, Johnson CD, Petolino JF (1990). Isolated microspore culture of maize: effects of isolation technique reduced temperature and sucrose level. Plant Cell Rep. 8:628-631.

Sato S, Katoh N, Iwai S, Hagimori M (2002). Effect of low temperature pretreatment of buds or inflorescence on isolated microspore culture in *Brassica rapa* (syn. *B. campestris*). Breeding Sci. 52:23-26.

Sinkovic T, Bohanec B (1988). Chromosome count and karyotype analysis in buckwheat (*Fagopyrum esculentum Moench*). Fagopyrum, 8:20-22.

Takahata Y, Keller WA (1991). High frequency embryogenesis and plant regeneration in isolated microspore culture of *Brassica oleracea* L. Plant Sci. 74:235-242.

Touraev A, Pfosser M, Vicente O, Heberle-Bors E (1996). Stress as the major signal controlling the developmental fate of tobacco microspores: towards a unified model of induction of microspore/ pollen embryogenesis. Planta 200:144-152.

Touraev A, Vicente O, Heberle-Bors E (1997). Initiation of microspore embryogenesis by stress. Trends Plant Sci. 2:297-302.

Wang M, Farnham MW, Nannes JSP (1999). Ploidy of broccoli regenerated from microspore culture versus anther culture. Plant Breed. 118:249-252.

Yuan SX, Liu YM, Fang ZY, Yang LM, Zhuang M, Zhang YY, Sun PT (2011). Effect of combined cold pretreatment and heat shock on microspore cultures in broccoli. Plant Breed. 130:80-85.

academicJournals

Vol. 14(42), pp. 2917-2927, 21 October, 2015
DOI:10.5897/AJB2015.14649
Article Number: 5D6BDD155882
ISSN 1684-5315
Copyright © 2015
Author(s) retain the copyright of this article
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Suppressive effects of a polymer sodium silicate solution on powdery mildew and root rot diseases of miniature rose

Mohsen Mohamed Elsharkawy[1], Tatsuya Hase[2], Masafumi Shimizu[2] and Mitsuro Hyakumachi[2]*

[1]Department of Agricultural Botany, Faculty of Agriculture, Kafrelsheikh University, 33516 Kafr El-Sheikh, Egypt.
[2]Laboratory of Plant Pathology, Faculty of Applied Biological Sciences, Gifu University, 1-1 Yanagido, Gifu City 501-1193, Japan.

Received 16 April, 2015; Accepted 8 October, 2015

Sodium silicate was dissolved in water in either a monomer form or polymer form; the effects of both forms of sodium silicate aqueous solution on rose powdery mildew and root rot diseases of miniature rose were examined. Both forms of sodium silicate aqueous solution were applied to the roots of the miniature rose. Potassium silicate aqueous solution was used as a control and was compared to the effect of sodium silicate aqueous solution. The polymer sodium silicate aqueous solution was the most effective treatment against both powdery mildew and root rot diseases. Moreover, no inhibition effects of silicate solutions were observed *in vitro* on *Pythium helicoides,* the causal pathogen of rose root rot disease. The silicon contents in the roots of the miniature rose treated with polymer sodium silicate were significantly greater than that in plants treated with monomer sodium silicate. In conclusion, the suppressive effects of sodium silicate in the polymer form were confirmed against powdery mildew and root rot diseases of the miniature rose.

Key words: *Podosphaera pannosa*, *Pythium helicoides,* miniature rose, polymer and monomer sodium silicate.

INTRODUCTION

Potted miniature roses (*Rosa hybrida* Hort.) have dime- to quarter-size flowers in single, double and semi-double forms. Miniature roses grow well indoors or out and are useful colorful plantings in areas with limited space. The roses are available in almost every color, except blue. Flowers can be pressed or dried for use in arrangements and potpourri. Several hundred cultivars are available, ranging in size from 3 to 18 inches in height and spread. The smallest varieties ("micro-minis") grow to 6 inches or less. Recently, environment-friendly agriculture attempts to minimize the use of fungicides (Elsharkawy et al., 2012a, 2012b, 2013; Hassan et al., 2014b, 2014c; Taguchi et al., 2014; 2015). Disease control by materials with low environmental effects is desired ((El-kazzaz et al., 2015).

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

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

Silicon (Si), as a major soil constituent, is an element that is the second most abundant (after oxygen) in the surface layer of the earth (Exley, 1998). Silicic acid is often abundant in rock samples. Large differences in Si accumulation were observed in different parts of the identical plant (Hodson et al., 2005). The beneficial effects of silicon were discovered first in grasses and extended to only a few number of plant species (Jones and Handreck, 1967). Si is considered as a useful element for specific-species of plants, but it is not an essential element for the plant. Silicic acid application in crops (such as rice) controls diseases and could reduce the load of fungicides released into the environment, even when applied in large quantities in agriculture (Kim et al., 2002; Ma et al., 2004). Several hypotheses have been suggested concerning the role of Si in plant development including the positive effects on reproduction, alleviation of metal toxicity and nutrient imbalance, provision of structural rigidity and increased resistance to fungal diseases such as powdery mildews and root rots (Epstein, 1994; Fawe et al., 1998). Si positively affects rice plants, promotes photosynthesis and increases dry matter production. Root treatment could improve the resistance to insect damage and disease resistance. Rice blast has been suppressed through Si treatment in rice (Maekawa et al., 2001, 2002; Seebold et al., 2001; Hayasaka et al., 2005).

Additionally, disease suppression of powdery mildew using Si has been reported in strawberries, cucumbers, grapes, *Arabidopsis*, wheat and cantaloupe (Bowen et al., 1992; Cherif and Belanger, 1992; Menzies et al., 1992; Belanger et., 2003; Ghanmi et al., 2004; Kanto et al., 2004, 2006). Si can also be found in the form of monosilicic acid, colloidal silicic acid, or organosilicon compounds in plant tissues (Yoshida et al., 1962). The absorption and movement of silicic acid to leaf blades and the end of the transpiration stream, such as rice husks, have been reported as major factors involved in impeding mycelial invasion of blast fungus. Si treatment has been reported to promote the production of phytoalexins and antibacterial substances (Cherif et al., 1994; Fawe et al., 1998). Si is usually present as silicate (SiO_2) in the soil. Some silicic acid molecules are polymerized and high concentrations of this polymerization concentration is known as the polymer state (Takahashi, 1987).

Therefore, the low molecular state is known as the monomer state and the high molecular state is the polymer state. Limited research considers plant disease control with both forms. Therefore, we focus on using both forms of Si in the present study. Sodium silicate was used as the silicate materials. Sodium silicate, when dissolved in water, is divided into silicate ions with a negative charge and sodium ions with a positive charge. When the pH is 7.0 or less, silicate ions become molecular silicic acid, losing charge. The polymerization concentration is high in these silicic acid molecules. Because of the difference in concentration of sodium silicate, low molecular states are characterized in two forms: polymer and monomer. This is the first report using the polymer state to control powdery mildew and root rot diseases. Until now, calcium silicate and potassium silicate were the most used as silicate materials to control plant disease (Moyer et al., 2008). This study investigates the effect of using monomer and polymer sodium silicate on powdery mildew and root rot diseases of miniature rose.

MATERIALS AND METHODS

Powdery mildew experiments

Preparation of test plants

Miniature roses (variety, silk red) were prepared by cuttings. Seedlings were transplanted in plastic pots (9 cm in diameter) filled with soil (star bed and peat moss, 1:1). Plants were grown in a glass greenhouse for 14 days (Gifu Prefectural Agricultural Research Institute, Gifu, Japan). A bottom water supply system was used during cultivation.

Preparation of aqueous silicic acid solution and treatment method

Monomer and polymer sodium silicate aqueous solutions were prepared by dissolving the reagent of sodium silicate (Wako Pure Chemical Industries, Ltd.) in distilled water (DW). The ratio of sodium silicate solution to water (DW) was set to 1 g/1000 mL for the monomer form and 10 g/1000 mL for the polymer form. The sodium silicate was completely dissolved in water (DOWEX 50wx4 100-200 H-form, Muromachi Technos Co., Ltd.), and the ion exchange resin of both forms was adjusted to pH 6.0 to 7.0. Both forms of aqueous sodium silicate solutions were adjusted with DW (pH 5.5), to concentrations of 0.5 and 1.0 mM. The aqueous solution of potassium silicate was prepared using a potassium silicate solution (Wako Pure Chemical Industries, Ltd.). An ion exchange resin was adjusted to pH 6.0 to 7.0 using DW (pH 5.5), and then the concentration was adjusted to 0.5 and 1.0 mM. Each silicate aqueous solution (30 mL) was applied to the plants as a soil drench at three time points within a week (every two days).

Disease severity assessment

Powdery mildew infected plants were used as an inoculum source. In the first test, plants were treated with sodium silicate aqueous solutions 2 weeks before setting the diseased plants as inoculum source. Disease severity was measured in plants treated with both forms of sodium silicate aqueous solution. Treatment continued up to 4 weeks after installation. In the second test, sodium silicate treatments were applied 4 weeks before setting the diseased plants as the inoculum source and were continued in the identical manner of processing up to 4 weeks after the installation. In both the first and second tests, the number of small leaves infected with powdery mildew disease was measured every 7 days after setting the diseased plants as the inoculum source. Disease incidence was measured by counting and removing the percentage of infected leaflets among the complete leaflet. In the second test, in addition to disease incidence, disease severity was measured. The disease severity rate was evaluated in the small leaves as follows: (no disease) 0% infected of the whole leaves, (1) leaves showing 0 to 25% leaf area

infected, (2) leaves showing 25 to 50% leaf area infected, and (3) leaves showing 50 to 100% leaf area infected. The disease severity was measured in both the first and second tests up to 28 days after setting the diseased plants as the inoculum source.

Determination of silicon

Si contents in rose leaflets were analyzed calorimetrically using the molybdenum blue method. Leaflets (samples) from each treatment group were dried for 2 to 3 days in an oven at 65°C; the samples were then ground and subjected to ashing and silica extraction as described by Boone (2007). The Si concentration was the absorbance value at a wavelength of 650 nm.

Root rot experiments

Plants and pathogens

Miniature rose plants (variety: Silk Red) were prepared as described previously. Miniature rose root rot fungus (*Pythium helicoides* B1-21 strain) was used in this experiment. A potassium silicate aqueous solution and monomer and polymer sodium silicate aqueous solutions (Wako Pure Chemical Industries, Ltd.) were prepared by dissolving the salts in distilled water as described previously. The plants were separated in eight groups: (a) water treatment (Si-, P-), (b) silicic acid untreated plot (Si-, P+), (c) 0.5 mM monomer sodium silicate solution treatment (0.5 mM monomer, p+) (d), 1.0 mM monomer sodium silicate solution treatment (1.0 mM monomer, p+), (e) 0.5 mM polymer sodium silicate solution treatment (0.5 mM polymer, p+), (f) 1.0 mM polymer sodium silicate solution treatment (1.0 mM polymer, p+), (g) 0.5 mM potassium silicate aqueous solution treatments (0.5 mM potassium, p+), (h) 1.0 mM potassium silicate aqueous solution treatment (1.0 mM potassium, p+). The experiment was repeated twice with 5 plants per treatment.

Effect of silicate solution on the severity of miniature rose root rot disease

The pathogen inoculum was prepared using autoclaved bentgrass seeds (variety: Highland, Takiishubyo, 1 g in 4 mL DW). The seeds were inoculated in a 300 mL Erlenmeyer flask with 10–15 mycelial discs (5 mm) transferred from the actively growing margin of 3 to 5-day-old potato dextrose agar (PDA; 2% agar) cultures of *P. helicoides*. The seeds that were entirely covered with flora were used as pathogen inoculum. The pathogen inoculum was completely mixed with 9 g of potting medium by hand, and this mixture was used as a contact inoculum source. The pathogen inoculum was added to soil surface of the miniature rose at 10 days from transplanting to plastic pots (30 cm × 20 cm × 10 cm). These pots served as the disease stock. The experiment was conducted in a glass greenhouse (Gifu University, Faculty of Applied Biological Sciences). The miniature roses were planted in plastic pots (30 cm × 20 cm × 10 cm) into the waterlogging state. The pathogen strain and management treatments were cultivated and irrigated using a bottom water supply. The plants were pre-treated with each silicate aqueous solutions at 10 d after cultivation of the miniature rose. The treatments were continued up to 4 weeks (every two days) after installing the diseased plants as the inoculum source. The disease stock was installed in each plastic pot as a post-treatment. Both of the disease incidence and severity of aboveground parts of plants were evaluated every four days after inoculation to observe the progress of disease symptoms of the aboveground plants. The disease incidence and severity of the browning on the roots were examined at 32 days after pathogen inoculation.

Determination of silicon

Si contents in the roots and the leaves of the treated miniature plants were measured by the molybdenum-blue method using wet ashing as mentioned above.

Effect of silicate solutions on the growth of P. helicoides

In total, 100 µl of each silicic acid aqueous solution (1 mM) were dropped onto sterile thick paper discs (8 mm in diameter, produced by Advantec® Japan). The strain *P. helicoides* B1-21 and the treated paper discs were inoculated on PDA medium. Petri dishes were cultured for 3 days (at 25°C in the dark), and the growth of flora was observed.

Data analysis

The data were subjected to an analysis of variance (ANOVA) using EKUSERU-TOUKEI 2010 (Social Survey Research Information Co., Ltd). The experiments were repeated at least three times, and treatment averages were separated using a Fisher's least significant difference (LSD) test. All analyses were conducted at a significance value of $P \leq 0.05$.

RESULTS

Powdery mildew disease

Disease suppression experiment

First test: Twenty-eight days after setting the diseased plants as the inoculum source, the disease incidence of the silicate untreated plot was 51.1, whereas it was 41.3 and 43.2% in the 0.5 and 1.0 mM monomer sodium silicate aqueous solution treatments, respectively. Polymer sodium silicate aqueous solutions in concentrations of 0.5 and 1.0 mM recorded 12.0 and 11.3%, respectively. The polymer sodium silicate aqueous solution treatment displayed a significantly reduced disease incidence on leaflets. Among all treatments throughout the study period, the polymer sodium silicate aqueous solution treatment achieved the lowest incidence of powdery mildew disease on rose leaflets. Both the monomer sodium silicate aqueous solution treatment and silicic acid untreated plot showed approximately equal values of disease incidence without significant difference between them. The experiments were performed using different concentrations (1.0 and 0.5 mM) of both monomer and polymer sodium silicate aqueous solution, but no significant differences were found on disease incidence rate because of differences in concentrations (Table 1). Protection values were calculated using disease incidence. Polymer sodium silicate aqueous solutions showed high protection values compared with monomer sodium silicate aqueous solutions (Figure 1).

Second experiment: Twenty-eight days after setting the infected plants as the inoculum source, the disease

Table 1. Disease incidence of powdery mildew on miniature rose leaves up to 6 weeks after setting the infected plants as inoculum source in experiment I.

Treatments	Days after setting the diseased plant as inoculum source			
	7	14	21	28
Si-	5.0±0.7[b]	10.8±2.2[b]	29.0±7.2[c]	51.1±8.7[b]
0.5 mM monomer	5.3±1.1[b]	11.9±1.0[b]	17.6±3.4[ab]	41.3±4.2[b]
1.0 mM monomer	4.1±0.9[b]	7.8±1.0[ab]	26.7±1.1[bc]	43.2±1.0[b]
0.5 mM polymer	1.0±0.3[a]	6.1±0.5[a]	7.5±0.8[a]	12.0±0.9[a]
1.0 mM polymer	2.1±0.5[ab]	9.0±2.2[ab]	8.8±0.7[a]	11.3±0.8[a]

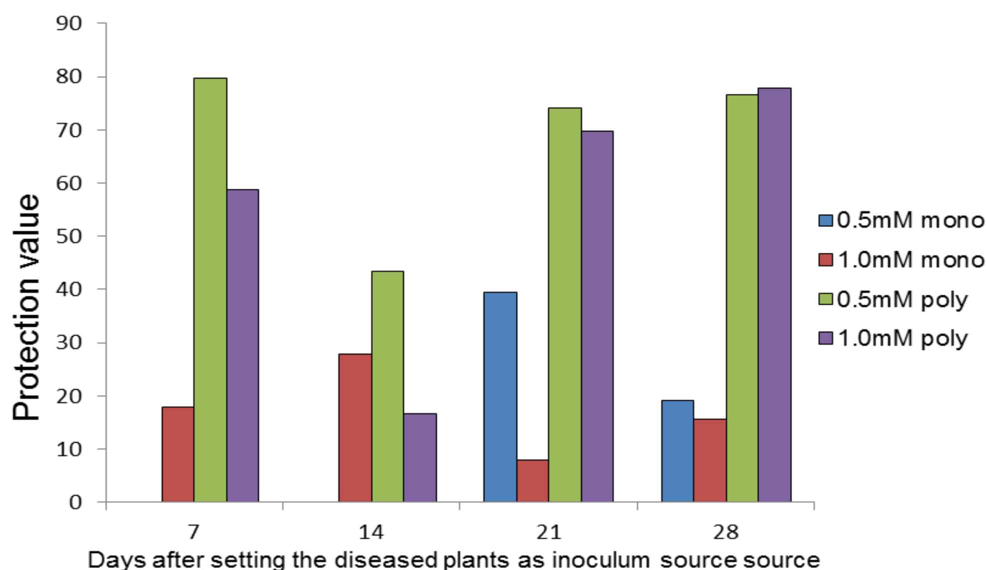

Figure 1. Protection values were calculated based on disease incidence at 7, 14, 21 and 28 days after setting the diseased plants as inoculum source. Si- = non-silicon treated plants; mono = monomer sodium silicate treated plants; poly = polymer sodium silicate treated plants.

incidence was 25.1% in silicate untreated plants. The monomer sodium silicate aqueous solution treatments in concentrations of 0.5 and 1.0 mM recorded 21.6 and 23.6 disease incidences, respectively, whereas 0.5 and 1.0 mM polymer sodium silicate aqueous solution treatments achieved 11.9 and 10.7%, respectively. The disease incidence of 0.5 and 1.0 mM potassium silicate aqueous solutions were 17.8 and 17.9%, respectively. The polymer sodium silicate aqueous solution treatment achieved the lowest incidence rate on the leaflet. The polymer sodium silicate aqueous solution treatments inhibited the onset of disease more than the potassium silicate aqueous solution treatments. The monomer sodium silicate aqueous solution treated plants recorded almost equivalent disease incidence rate as the silicate untreated plot (Table 2). The disease severity of the silicate untreated plot was 13.8, whereas it was 5.6 and 4.7 for the 0.5 and 1.0 mM polymer sodium silicate aqueous solution. The disease severity of polymer sodium silicate aqueous solution treatments showed the lowest value (Figure 2). In addition, no significant differences were observed in disease severity between the two provided concentrations of silicate aqueous solutions in the treated plants (Figure 2). The protection values were calculated from the disease severity and disease incidence rates. The protection values of the polymer sodium silicate aqueous solution calculated from the disease incidence rate ranged from 52.6 to 57.5, whereas the protection values calculated from the disease severity ranged from 59.3 to 65.8. The polymer sodium silicate aqueous solution showed inhibitory effects in both the symptoms and disease development (Figure 3).

Determination of silicon in the small leaves

First experiment: The leaflets were randomly sampled (regardless to disease symptoms on the small leaves) to quantify the silicon from each treatment group. No

Table 2. Disease incidence of powdery mildew on miniature rose leaves until 6 weeks after setting the infected plants as inoculum source in experiment II.

Treatments	Days after setting the diseased plant as inoculum source					
	7	14	21	28	35	42
Si-	1.1±0.3[c]	0.4±0.3[a]	11.4±1.0[b]	25.1±1.8[d]	30.4±1.3[c]	45.9±2.4[d]
0.5 mM monomer	0.7± 0.4[bc]	0.8±0.5a	8.9±2.9[ab]	21.6±2.0[bcd]	27.1±2.6[bc]	35.6±3.9[bc]
1.0 mM monomer	0.2±0.1[ab]	0.7±0.5a	11.9±4.0[b]	23.6±2.8[cd]	32.5±3.1[c]	44.5±4.1[cd]
0.5 mM polymer	0.1±0.1[ab]	0.3±0.1a	3.9±0.6[a]	11.9±0.7[a]	22.1±0.9[ab]	21.5±0.7[a]
1.0 mM polymer	0.1±0.1[ab]	0.3±0.1a	4.0±0.9[a]	10.7±2.1[a]	18.3±4.3[a]	25.5±5.1[a]
0.5 Mm K_2SiO_4	0.1±0.1[a]	0.2±0.1a	7.9±1.7[ab]	17.8±1.7[b]	26.8±2.1[bc]	28.3±0.7[ab]
1.0 Mm K_2SiO_4	0.2±0.1[a]	0.2±0.1a	8.8±1.5[ab]	17.9±1.4[bc]	23.2±1.5[abc]	26.9±1.3[ab]

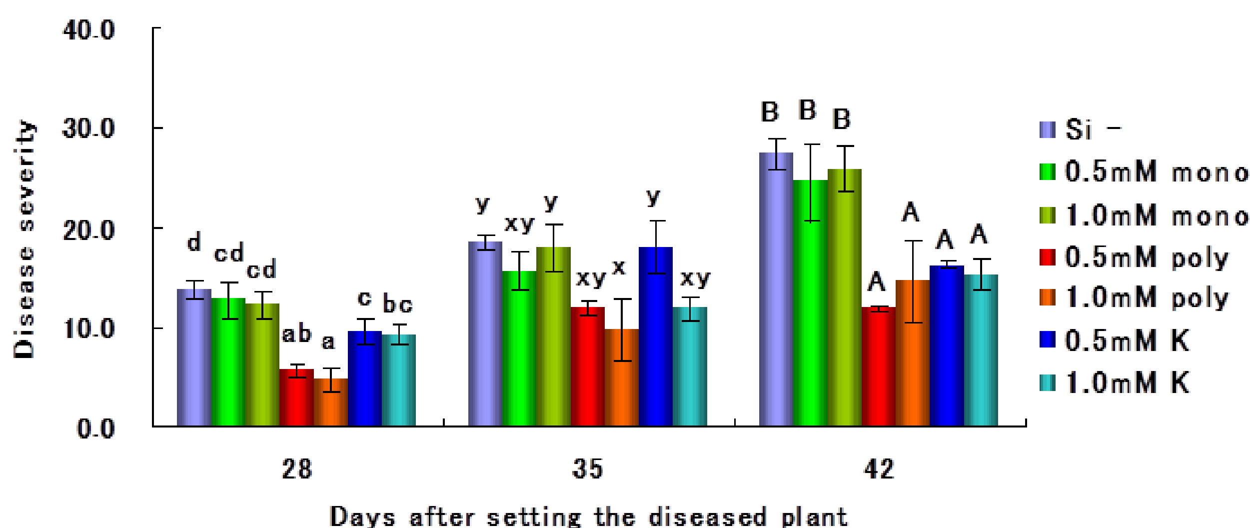

Figure 2. Disease severity of powdery mildew at 28, 35 and 42 d after setting the diseased plants as inoculum source. Disease severity was assessed as index of ratio of symptom area on small leaves using a scale of 0 to 4; 0 = no symptoms, 1 = 0-25%, 2 = 25 to 50%, 3 = 50 to 100%. Disease severity = $\sum (P_{(0\text{-}3)} \times S_{(0\text{-}3)}) \times 100/ (3 \times$ total small leaves), where $P_{(0\text{-}3)}$ = plant number in score 0, 1, 2, and 3, $S_{(0\text{-}3)}$ = score 0, 1, 2, and 3. (Si-) = non-silicon treated plants, (mono) = monomer sodium silicate treated plants, (poly) = polymer sodium silicate treated plants and (K) = potassium silicate treated plants. Bars labelled with the same letters are not significantly different according to LSD test at 5 %. Vertical lines indicate the standard error.

significant differences were found in silicon contents between all treatments and the control (Figure 4).

Second experiment: Samples were separated into symptomatic leaflets and non-symptomatic leaflets in all treatments, and the Si contents were quantified. The Si contents were higher in sodium silicate treatments when compared to potassium silicate treatments. No significant differences were observed between 0.5 and 1.0 mM of monomer sodium silicate aqueous solution treated plants. In other treatments, significant differences were observed between different concentrations of each treatment in non-symptomatic leaves (Figure 5). Although the disease inhibition effects of Si application were found, no relation was observed between Si content and disease incidence in each experiment. This result suggests that the Si content does not affect the incidence of powdery mildew.

Root rot disease

Effect of each silicate solution on the root rot of miniature rose

The disease incidence of root rot on the aboveground parts and roots of miniature rose was examined. For the aboveground parts, the disease incidence of the pathogen alone treatment (control) was 90%, whereas all silicate solution treatments showed lower incidences than the control. Among all treatments, the lowest incidence

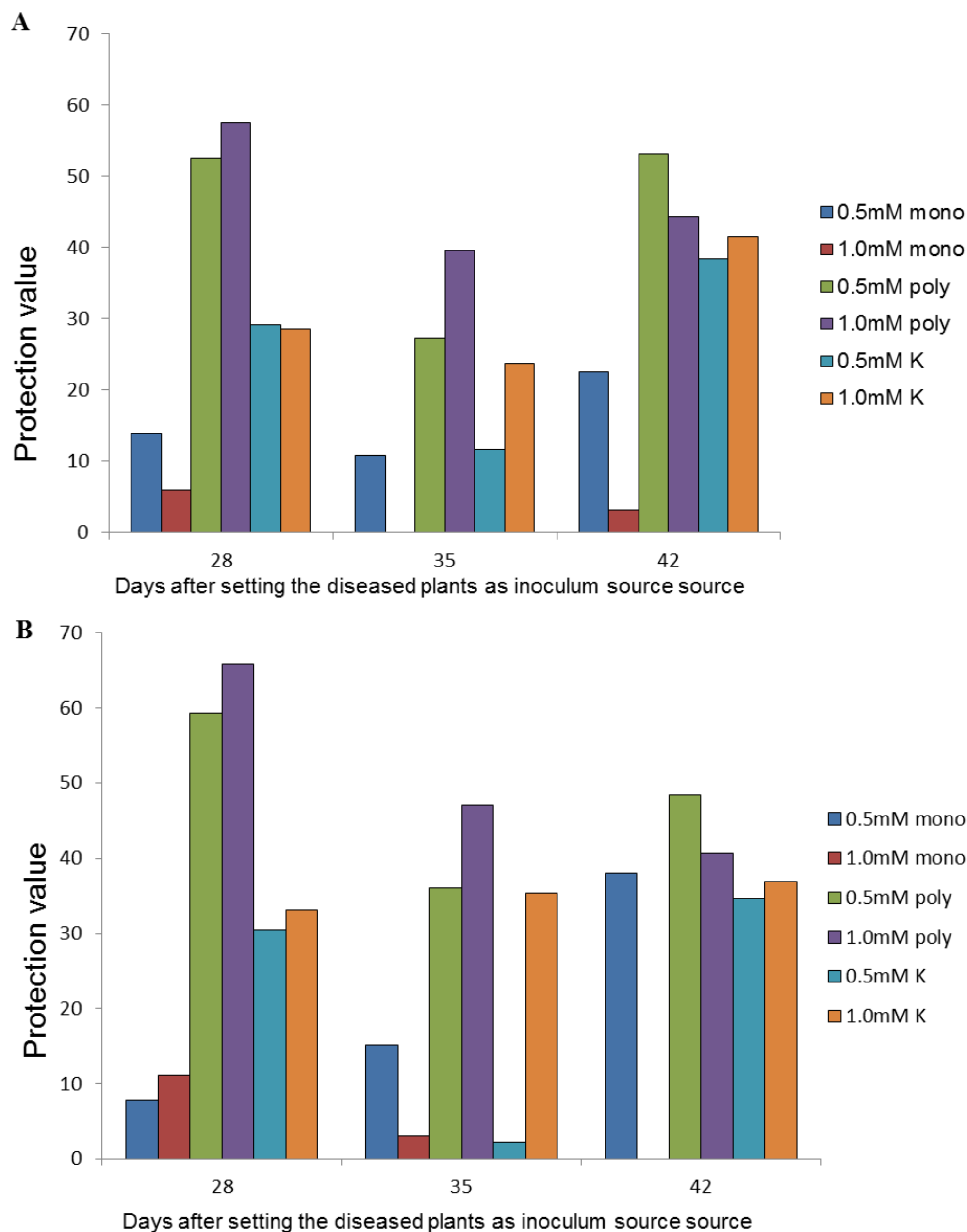

Figure 3. Protection values were calculated based on disease incidence (A) and disease severity (B) at 28, 35 and 42 days after setting the diseased plants as inoculum source. mono = monomer sodium silicate treated plants; poly = polymer sodium silicate treated plants; K = potassium silicate treated plants.

was achieved by the polymer treatments group (60%). The disease severity of the control treatment was 32.5, whereas it was 17.5 and 20.0 for 0.5 and 1.0 mM polymer sodium silicate treatments, respectively. The protection values were 46.2 and 38.5 for 0.5 and 1.0 mM polymer sodium silicate treatments, respectively (Table 3). Although the disease severity was not high in the control treatment, the polymer silicate treatments showed the lowest disease severity values throughout the study period (Table 3). The 1.0 mM polymer sodium silicate treatment recorded the lowest incidence of root rot in the roots of the miniature rose. The root browning degree of the pathogen alone treated plants was 42.5, whereas it was 17.5 for the 1.0 mM polymer sodium silicate treatment, showing a 58.8% protection value (Table 4). The above results showed that the polymer sodium silicate treatment achieved the highest protection values for the aerial and root portions.

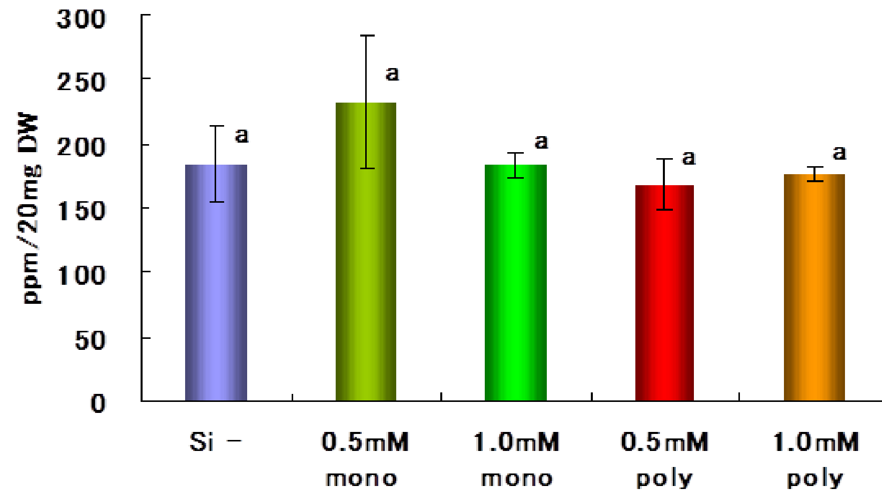

Figure 4. Silicon concentrations in small leaves at 28 d after setting the diseased plant as inoculum source in experiment I. Silicon concentration was measured by spectrophotometer with 650nm. Si- = non-silicon treated plants; mono; = monomer sodium silicate treated plants; poly = polymer sodium silicate treated plant. Bars labelled with the same letters are not significantly different according to LSD test at 5%. Vertical lines indicate the standard error.

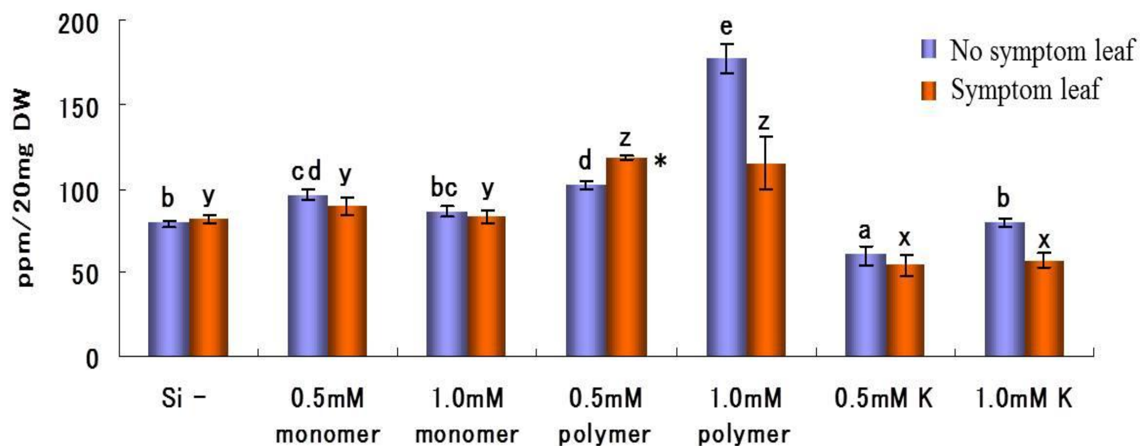

Figure 5. Silicon concentration of small leaves tissue at 42 d after setting the diseased plant as inoculum source. Silicon concentration was measured by spectrophotometer at 650nm. Si- = non-silicon treated plants; mono = monomer sodium silicate treated plants; poly = polymer sodium silicate treated plants; K = potassium silicate treated plants. Bars labelled with the same letters are not significantly different according to LSD test at 5%. Small letters refer to comparison between each treatment in non-symptomatic leaves and capital letter to comparison between each treatment in symptomatic leaves. Mark (*) approve significantly difference between non-symptomatic and symptomatic leaves with t- test at 5 %. Vertical lines indicate the standard error.

Determination of silicon in the leaves and the roots

The Si contents were analyzed in the leaves and the roots. Plants treated with 1.0 mM polymer sodium silicate showed the highest Si contents in the roots compared to the other treatments. However, 1.0 mM monomer sodium silicate, 0.5 mM polymer sodium silicate, 1.0 mM polymer sodium silicate, and 1.0 mM potassium silicate treatment groups were significantly higher in Si contents in the leaves than in the untreated and pathogen alone treated plants (Figure 6).

Effect of each silicate solution on growth of P. helicoides

The inhibition effects of monomer and polymer sodium silicate solutions and potassium silicate solution on the

Table 3. Disease incidence and disease severity on miniature rose plants at 32 d after setting the infected plants as inoculum source.

Treatments	Plant number in score					Disease incidence [1)]	Disease severity [2)]	Protection value [3)]
	0	1	2	3	4			
Control (Si-P-)	10					0	0.0	
Pathogen (Si-P+)	1	6	2	1		90	32.5	
0.5 mM mono	2	4	2	2		80	35.0	0
1.0 mM mono	4	4	2			60	20.0	38.5
0.5 mM poly	4	5	1			60	17.5	46.2
1.0 mM poly	4	4	2			60	20.0	38.5
0.5 mM K_2SiO_4	3	5	2			70	27.5	15.4
1.0 mM K_2SiO_4	3	7				70	17.5	46.2

[1)] Disease incidence is the percentage of yellowed plants out of the total number of plants in the treatment. [2)] Disease severity was assessed as index of ratio of yellowing on plant using a scale of 0 to 4; 0=no yellowing, 1=0~25% yellowing, 2= 25~50%, 3= 50~75%, 4 = 75~100 and dead. Disease severity=$\sum (P_{0\text{-}4} \times S_{0\text{-}4}) \times 100/ (4\times$ total plants), where $P_{0\text{-}4}$= plant number in score 0, 1, 2, 3 and 4, $S_{0\text{-}4}$ = score 0, 1, 2, 3, and 4, protection value was calculated based on disease severity. [3)] Protection value = (pathogen - each treatment) ×100/pathogen. (Control) = non-silicon treated and not inoculated plants, (pathogen) = non-silicon treated and inoculated plants, (mono) = sodium silicate treated plants, (poly) = polymer- sodium silicate treated plants and (K_2SiO_4) = potassium silicate treated plants.

Table 4. Disease incidence and discoloration severity on root at 32 d after setting the infected plants as inoculum source.

Treatments	Plant number in score					Disease incidence [1)]	Disease severity [2)]	Protection value [3)]
	0	1	2	3	4			
Control (Si-P-)	10					0	0.0	
Pathogen (Si-P+)		7		2	1	100	42.5	
0.5 mM mono	1	5	3	1		90	35.0	17.6
1.0 mM mono	3	7				70	17.5	58.8
0.5 mM poly	3	3	4			70	27.5	35.3
1.0 mM poly	4	5	1			60	17.5	58.8
0.5 mM K_2SiO_4	4	5	1			60	17.5	58.8
1.0 mM K_2SiO_4	1	2	3	3	1	90	52.5	0

[1)] Disease incidence is the percentage of discolored plants out of the total number of plants in the treatment. [2)] Disease severity was assessed as index of ratio of discoloration area on root using a scale of 0 to 4; 0 = no discoloration, 1 = 0~25% discoloration, 2 = 25~50%, 3 = 50~75%, 4 = 75~. Disease severity = $\sum (P_{0\text{-}4} \times S_{0\text{-}4}) \times 100/ (4\times$ total plants), where $P_{0\text{-}4}$ = plant number in score 0, 1, 2, 3 and 4, $S_{0\text{-}4}$= score 0, 1, 2, 3, and 4. [3)] Protection value was calculated on discoloration severity. Protection value = (pathogen-each treatment) ×100/pathogen. (Control) = non-silicon treated and not inoculated plants, (pathogen) = non-silicon treated and inoculated plants, (mono) = monomer - sodium silicate treated plants, (poly) = polymer - sodium silicate treated plants and (K_2SiO_4) = potassium silicate treated plants.

growth of *P. helicoides* were tested. No affect was found using the solution itself. Therefore, no direct inhibition effect was observed to pathogen growth (Figure 7).

DISCUSSION

Rose powdery mildew, caused by the fungus *Podosphaera pannosa*, infects a wide variety of roses. The disease is destructive and occurs wherever roses are grown outdoors and in greenhouses, notably for those grown in dryer climates (Gubler et al., 2011). Under favorable conditions for disease development (hot and dry weather with cool and moist nights) powdery mildew can cause complete defoliation. The disease appears as a white powdery growth on young rose leaves, stems and all other portions of the rose, even buds and flowers. Pythium root rot continually threatens the productivity of several types of crops including cucumbers, sweet peppers, tomatoes, lettuce, spinach, roses, and chrysanthemums (Sutton et al., 2006). The principal causal agent of rose root rot disease is *P. helicoides* (Kageyama et al., 2002; Watanabe et al., 2007). The root rot disease has also been observed in rockwool cultures of cutting rose and has spread in Japan. Integrated pest management (IPM) is an effective and environmentally sensitive approach to crop management to minimize losses from disease and insect pests (Mousa et al.,

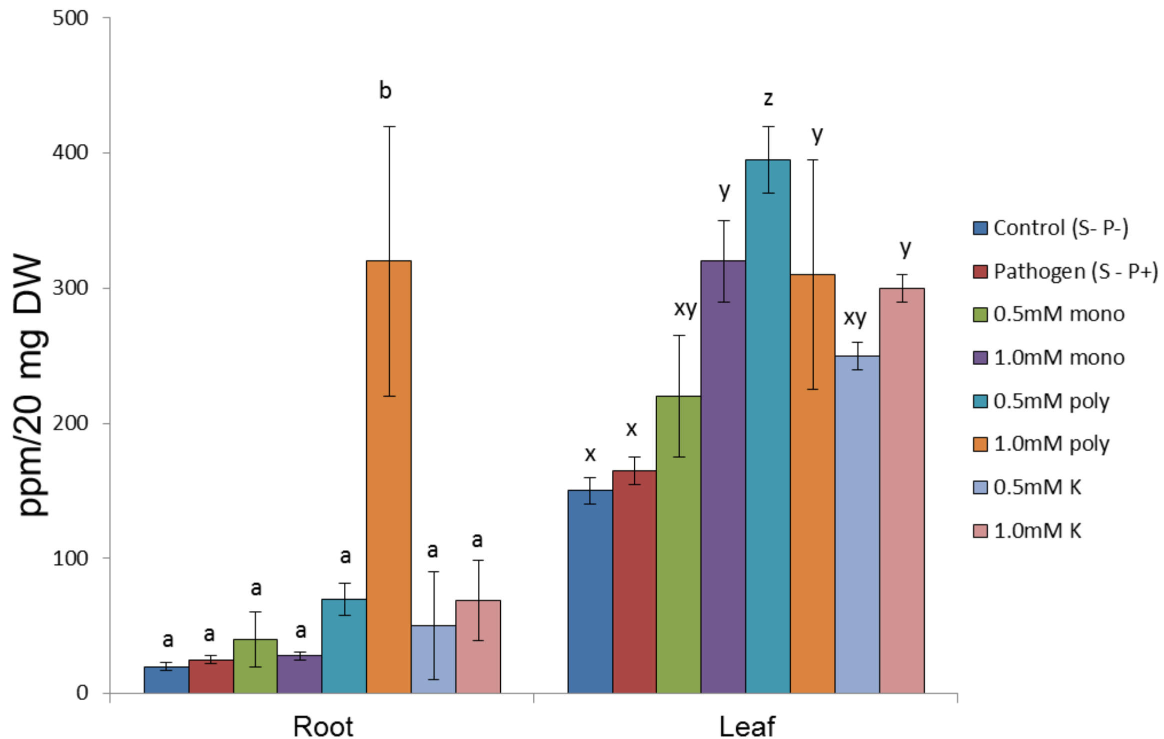

Figure 6. Silicon concentration in roots and leaves tissues at 32 d after setting the diseased plant as inoculum source. Silicon concentration was measured by spectrophotometer at 650 nm. Control = non-silicon treated and not inoculated plants; Pathogen = non – silicon treated plants; K = potassium silicate treated plants. Bars labelled with the same letters are not significantly different according to LSD test at 5%. Vertical lines indicate the standard error.

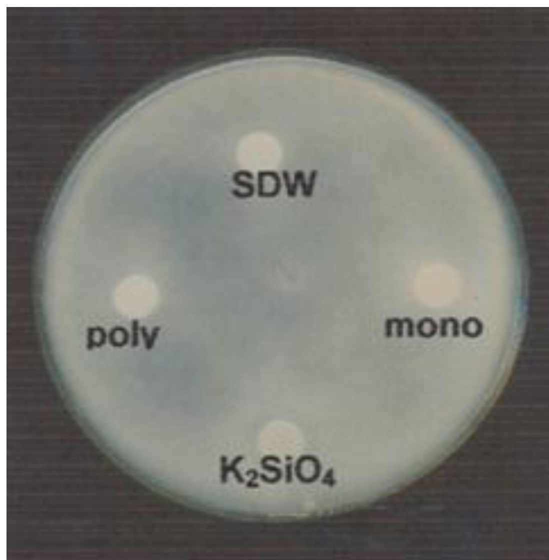

Figure 7. Confirmation of direct inhibition effects of monomer and polymer sodium silicate and potassium silicate on the growth of *Pythium helicoides*. Above: sterile distilled water (SDW), center left: polymer sodium silicate (poly), center right: monomer sodium silicate (mono) and under: potassium silicate (K_2SiO_4).

2014). IPM programs use pest and environmental information with the most economical pest control means and with the least possible hazard to human health and the environment (Elsharkawy and El-Sawy, 2015; Hassan et al., 2014a). An integrated approach to disease control aims to reduce the frequency and the amount of pesticides used. This can be achieved in part by using alternative products when appropriate. Si is known to reduce the severity of a number of plant diseases. This study was performed using both forms (polymer and monomer) of an aqueous sodium silicate solution. The results of the two experiments showed that high inhibitory effects were found against powdery mildew and root rot diseases of miniature roses in polymer sodium silicate treated plants when compared to plants treated with monomer sodium silicate. These results may be because polymers are large-sized molecules or macromolecules, and in select cases, the polymeric phase is more stable than the monomeric (Currie and Perry, 2007). Similarly, cucumber root rot caused by *Pythium* spp. was suppressed by the application of Si in cucumber plants. The pathogenic fungus attacks the root and hypocotyl (Belanger et al., 1995). Although, the roles of providing a physical and/or biochemical defense system have been proposed, the protective effect of Si has yet to be fully

elucidated. The role of Si deposition as a physical barrier to pathogen penetration has been examined (Yoshida et al., 1962). The results show that polymer sodium silicate treated plants recorded higher silicon contents in the roots when compared to monomer sodium silicate treated plants.

Additionally, no antimicrobial effect was found in polymer sodium silicate against *P. helicoides*. Debate remains as to whether this increased physical strength is sufficient in explaining the observed protective effects (Fauteux et al., 2005). Another explanation is the emerging role of Si as a biologically active element capable of enhancing the natural defense response of the plant. Si-treated plants exhibited increased activity of peroxidases, chitinases, polyphenol oxidases and flavonoid phytoalexins, which play an important role in plant resistance against fungal pathogens (Chérif et al., 1994; Fawe et al., 1998). Additionally, increased production of glycosylated phenolics, antimicrobial products such as diterpenoid phytoalexins and a proline-rich protein in Si-treated plants indicated the role of these products in the protection effects of Si against plant diseases (Belanger et al., 2003; Kauss et al., 2003; Rodriques et al., 2003). The bioactivity of Si as a regulator of plant defense mechanisms may be explained through the biochemical properties. Si can bind to hydroxyl groups of proteins strategically involved in signal transduction. Si also may interfere with cationic co-factors of enzymes influencing pathogenesis-related events. Therefore, Si may interact with several key components of plant stress signaling systems leading to induced resistance. In this study, the ability of different concentrations of both polymer and monomer sodium silicate to reduce the severity of the powdery mildew disease of miniature rose has been tested. No significant differences were found in the severity of powdery mildew and root rot diseases between the two concentrations of silicate aqueous solution treated plants.

Cherif et al. (1992) found that the Si application was not physically blocking the entry site of the *Pythium* spp. and that the Si was not accumulating in the entry site. The disease suppression by Si against root rot is not only associated with lignin and the strengthening of physical barrier against infection, but it is associated with the accumulation of phenolic substances (Cherif et al., 1994). The activity of disease resistance related enzymes, peroxidases and polyphenols oxidases, quickly increased after the infection with *Pythium ultimum* in silicon treated cucumber plants compared with the non-treated cucumber (Cherif et al., 1994). Rhamnetin, an O-methylated flavonol, is a phytoalexin and was reported in cucumber plants treated with Si, suggesting the possible role of Si in physiological resistance reactions (Fawe et al., 1998). The size and structure of the elicitors has been reported in disease resistance. Silicic acid was involved in the physiological resistance reaction in cucumbers and could be involved against rose powdery mildew and the root rot disease that has been reported in this study through the silicic acid application. Physiological disease resistance could be considered as a possible mechanism other than the production of antibacterial substances such as phytoalexin.

In conclusion, the inhibitory effects of polymer sodium silicate were significantly higher than monomer sodium silicate. Therefore, according to the results obtained in the present study, polymer sodium silicate can play a role in controlling powdery mildew and root rot infection in rose plants.

Conflict of interests

The author(s) did not declare any conflict of interest.

REFERENCES

Belanger RR, Benhamou N, Menzies JG (2003). Cytological evidence of an active role of silicon in wheat resistance to powdery mildew (*Blumeria graminis* f. sp. *tritici*). Phytopathology 93:402-412.

Belanger RR, Bowen PA, Ehret DL, Menzies JG (1995). Soluble silicon: Its role in crop and disease management of greenhouse crops. Plant Dis. 79:329-336.

Boone CE (2007). Analytical approach to the quantitative analysis of silicon in plants: Its application to plant silica extraction, Master thesis, Oregon State University.

Bowen P, Menzies J, Ehret D, Samuels L, Glass ADM (1992). Soluble silicon sprays inhibit powdery mildew development on grape leaves. J. Am. Soc. Hort. Sci. 117:906-912

Cherif M, Asselin A, Belanger RR (1994). Defense responses induced by soluble silicon in cucumber roots infected by *Pythium* spp. Phytopathology 84:236-242

Cherif M, Belanger RR (1992). Use of potassium silicate amendments in recirculating nutrient solutions to suppress *Pythium ultimum* on long english cucumber. Plant Dis. 76:1008-1011.

Currie H, Perry C (2007). Silica in plants: biological, biochemical and chemical studies. Ann. Bot. 100:1383-1389

El-kazzaz MK, Salem EA, Ghoneim KE, Elsharkawy MM, El-Kot GAN, Kalboush ZAE (2015). Integrated control of rice kernel smut disease using plant extracts and salicylic acid. Archives of Phytopathology and Plant Protection. DOI: 10.1080/03235408.2015.1092202.

Elsharkawy MM, Shimizu M, Takahashi H, Hyakumachi M (2012a). Induction of systemic resistance against *Cucumber mosaic virus* by *Penicillium simplicissimum* GP17-2 in *Arabidopsis* and tobacco. Plant Pathol. 61:964-976

Elsharkawy MM, Shimizu M, Takahashi H, Hyakumachi M (2012b). The plant growth-promoting fungus *Fusarium equiseti* and the arabuscular mycorrhizal fungus *Glomus mosseae* induce systemic resistance against *Cucumber mosaic virus* in cucumber plants. Plant Soil 361: 397-409.

Elsharkawy MM, Shimizu M, Takahashi H, Ozaki K, Hyakumachi M (2013). Induction of systemic resistance against *Cucumber mosaic virus* in *Arabidopsis thaliana* by *Trichoderma asperellum* SKT-1. Plant Pathol. J. 29:193-200.

Epstein E (1994). The anomaly of silicon in plant biology. Proc. Natl. Acad. Sci. U.S.A. 91:11-17.

Exley C (1998). Silicon in life: a bioinorganic solution to bioorganic essentiality. J. Biol. Inorg. Chem. 69:139-144.

Fauteux F, Remus-Borel W, Menzies JG, Belanger RR (2005). Silicon and plant disease resistance against pathogenic fungi. FEMS Microbiol. Lett. 249:1-6.

Fawe A, Abou-Zaid M, Menzies JG, Belanger RR (1998). Silicon-mediated accumulation of flavonoid phytoalexins in cucumber. Phytopathology 88:396-401.

Ghanmi D, McNally DJ, Benhamou N, Menzies JG, Belanger RR (2004). Powdery mildew of *Arabidopsis thaliana*: A pathosystem for exploring the role of silicon in plant-microbe interactions. Phys. Mol. Plant Pathol. 64:189-199.

Gubler WD, Davis UC, Koike ST (2011). Powdery Mildew on Ornamentals. University of California- Agriculture and Natural Resources. University of California state-wide Integrated Pest Management Program, 18 Jan. 2011.

Hassan N, Elsharkawy MM, Shimizu M, Hyakumachi M (2014a). Control of root rot and wilt diseases of roselle under field conditions. Mycobiology 42:376-384.

Hassan N, Elsharkawy MM, Shivanna MB, Meera MS, Hyakumachi M (2014b). Elevated expression of hydrolases, oxidase, and lyase in susceptible and resistant cucumber cultivars systemically induced with plant growth-promoting fungi against anthracnose. Acta Agric. Scand. B-S P 64:155-164.

Hassan N, Elsharkawy MM, Villajuan-Abgona R, Hyakumachi M (2014c). A nonpathogenic species of binucleate Rhizoctonia inhibits the formation of infection structures caused by Rhizoctonia solani on cucumber. Acta Agric. Scand. B-S P 65:208-214.

Hayasaka T, Fujii H, Namai T (2005). Silicon content in rice seedlings to protect rice blast fungus at the nursery stage. J. Gen. Plant Pathol. 71:169-173.

Hodson MJ, White PJ, Mead A, Broadley MR (2005). Phylogenetic variation in the silicon composition of plants. Ann. Bot. 96: 1027-46.

Jones LHP, Handreck KA (1967). Silica in soils, plants, and animals. Adv. Agron. 19:107-149.

Kageyama K, Aoyaagi T, Sunouchi R, Fukui H (2002). Root rot of miniature rose caused by Pythium helicoides. J. Gen. Plant Path. 68:15-20.

Kanto T, Miyoshi A, Ogawa T, Maekawa K, Aino M (2004). Suppressive effect of potassium silicate on powdery mildew of strawberry in hydroponics. J. Gen. Plant Pathol. 70:207-211.

Kanto T, Miyoshi A, Ogawa T, Maekawa K, Aino M (2006). Suppressive effect of liquid potassium silicate on powdery mildew of strawberry in soil. J. Gen. Plant Pathol. 72:137-142.

Kim SG, Kim KW, Park EW, Choi D (2002). Silicon induced cell wall fortification of rice leaves: a possible cellular mechanism of enhanced host resistance to blast. Phytopathology 92:1095-1103.

Ma JF, Mitani N, Nagao S, Konishi S, Tamai K, Iwashita T, Yano M (2004). Characterization of the silicon uptake system and molecular mapping of the silicon transporter gene in rice. Plant Physiol. 136: 3284–3289.

Maekawa K, Watanabe K, Aino M, Iwamoto Y (2001). Suppression of rice seedling blast with some silicic acid materials in nursery box. Jpn. J. Soil Sci. Plant Nutr. 72:56-62.

Maekawa K, Watanabe K, Kanto T, Aino M, Iwatomo Y (2002). Accumulation of silicon around penetration sites of *Magnaporthe grisea* and silicon-dependent promotion of superoxide generation after inoculation of rice leaf. In: Matoh T (ed) Second ‘‘silicon in agriculture’’ conference. Press-Net, Kyoto, Japan. pp. 34-38.

Menzies J, Bowen P, Ehret D, Glass DM (1992). Foliar application of potassium silicate reduce severity of powdery mildew on cucumber, muskmelon, and zucchini squash. J. Am. Soc. Hort. Sci. 117:902-905.

Mousa KM, Elsharkawy MM, Khodeir IA, Dakhakhni TN, Youssef AE (2014). Growth perturbation, abnormalilties and mortalilty of oriental armyworm *Mythimna separata* (Walker) (Lepidoptera: Noctuidae) caused by silica nanoparticles and *Bacillus thuringiensis* toxin. Egypt. J. Biol. Pest Control 24: 283-287.

Moyer C, Peres NA, Datnoff LE, Simonne EH, Deng Z (2008). Evaluation of silicon for managing powdery mildew on gerbera daisy. J. Plant Nutr. 31:2131-2144.

Rodriques FA, Benhamou N, Datnoff LE, Jones JB, Belanger RR (2003). Ultrastructural and cytochemical aspects of silicon-mediated rice blast resistance. Phytopathology 93:535-546.

Seebold K, Kucharek T, Datnoff L, CorreaVictoria F, Marchetti M (2001). The influence of silicon on components of resistance to blast in susceptible, partially resistant, and resistant cultivars of rice. Phytopathology 91:63-69.

Sutton JC, Sopher CR, Owen-Going TN, Liu W, Grodzinski B, Hall JC, Benchimol RL (2006). Etiology and epidemiology of Pythium root rot in hydroponic crops: current knowledge and perspectives. Summa Phytopathol. 32:307-321

Taguchi Y, Elsharkawy MM, Hassan N, Hyakumachi M (2014). A novel method for controlling rice blast disease using fan-forced wind on paddy fields. Crop Prot. 63:68-75.

Taguchi Y, Elsharkawy MM, Hyakumachi M (2015). Effect of artificially–generated wind on removing guttation and dew droplets from rice leaf surface for controlling rice blast disease. Afr. J. Biotechnol. 14:1039-1047.

Takahashi E (1987). Revenue and expenditure of silicon in paddy soil. In Siliciphiloul PlAnl and Calciphilous Plant, Ed. E. Takahashi, Noubunkyo, Tokyo (in Japanese). pp. 127-129.

Watanabe H, Taguchi H, Hyakumachi M, Kageyama K (2007). *Pythium* and *Phytophthora* species associated with root and stem rots of kalanchoe. J. Gen. Plant Pathol. 73:81-88.

Yoshida S, Ohnishi Y, Kitagishi K (1962). Chemical forms, mobility, and deposition of silicon in the rice plant. Jpn. J. Soil Sci. Plant Nutr. 8:107-111.

academicJournals

Vol. 14(42), pp. 2928-2936, 21 October, 2015
DOI:10.5897/AJB2015.14590
Article Number: A2EAABF55884
ISSN 1684-5315
Copyright © 2015
Author(s) retain the copyright of this article
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Toxic effects of *Ricinus communis* non-protein trypsin inhibitor on *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera:Noctuidae)

Gislaine A. Carvalho[1]*, Custódio D. dos Santos[2], Dejane S. Alves[3], Geraldo A. Carvalho[3], Maria das G. Cardoso[2] and Marcelo M. de Haro[4]

[1]Departamento de Entomologia, Universidade Federal de Viçosa, Viçosa, MG 36570-900, Brazil.
[2]Departamento de Química, Universidade Federal de Lavras, Lavras, MG 37200-000, Brazil.
[3]Departamento de Entomologia, Universidade Federal de Lavras, Lavras, MG 37200-000, Brazil
[4]Agricultural Research and Rural Extension Agency of Santa Catarina (EPAGRI/EEI), Itajaí, SC 88034-901, Brazil.

Received 24 March, 2015; Accepted 8 October, 2015

In the study reported herein, we aimed to isolate a trypsin inhibitor from *Ricinus communis* leaves through chromatographic and spectrometric techniques and evaluate its toxic effects on the development of *Spodoptera frugiperda* larvae. Plant extracts were submitted to fractionation in adsorption column. The fraction 10, which showed the highest inhibitory activity, were incorporated into an artificial diet at the concentrations of 0, 0.06, 0.12, 0.25 and 0.5%, and offered to *S. frugiperda* larvae. Fresh weight of larvae, food consumed and weight of eliminated faeces were registered. Based on these parameters the following nutritional index were calculated: Relative Consumption Rate (RCR), Relative Metabolic Rate (RMR), Relative Growth Rate (RGR), Approximated Digestibility (AD), Efficiency of Ingested Food Conversion (EIC), Efficiency of Digested Food Conversion (EDC) and the Metabolic Cost (100 - EDC). The inhibitor at 0.5% concentration was deleterious to *S. frugiperda*, extending the larval stage in 11 days, with higher RCR and ECD, and lower RGR, ECI and ECD. Therefore, the trypsin inhibitor from leaves of *R. communis* affected the *S. frugiperda* larval development, being promising in studies of alternative and sustainable control methods for lepidopteran pest species.

Key words: Castor beans, enzymatic inhibition, integrated pest management, plant defense against herbivory.

INTRODUCTION

The fall armyworm, *Spodoptera frugiperda* (Smith, 1797) (Lepidoptera: Noctuidae), is one of the most important insect pest of maize in Brazil, attacking the plant at different growth stages, when the larvae burrow into the plant whorl causing severe defoliation and 37% loss in production (Cruz, 2002). Due to the polyphagous feeding

*Corresponding author. E-mail: gislaine.carvalho.2012@gmail.com. Tel: (+55)(31) 9401-9905.

Abbreviations: RCR, Relative consumption rate; **RMR,** relative metabolic rate; **RGR,** relative growth rate; **AD,** approximated digestibility; **EIC,** efficiency of ingested food conversion; **EDC,** efficiency of digested food conversion.

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

behavior, fall armyworm may attack and damage many other agricultural crops such as rice, sorghum, cotton, soybean and many others (Alvarez et al., 2009). Current control of *S. frugiperda* populations relies mainly on spraying chemical insecticides, which have been overused in recent years, resulting in insect resistance (Yu et al., 2003), environmental contamination (Starner and Goh, 2012) and affecting the human health (Loewenherz et al., 1997). Due to those factors, organic cultivation has been increased, replacing the use of chemical insecticides by alternative methods of pest control (Uchino et al., 2015). A promising alternative to control fall armyworm is the use of plants secondary metabolites (Tavares et al., 2009; Alves et al., 2014). These chemicals compounds produced by plants can induce deleterious effects on insects such as weight loss, fecundity and fertility reduction, increasing duration of immature stages, feeding deterrence, ultra structural modification on tissues, changes in some nutritional parameters, and inhibition of digestive enzymes, which is lethal to some insects (Malau and James, 2008; Nathan et al., 2008; Correia et al., 2009). The use of proteinase inhibitors (PIs), a class of substances involved in plant defense is an example of these new alternative. Levels of PIs in plant leaves are usually low and can be increased to high levels if the plants are attacked by insects, suffer mechanical damage or exposed to plant hormones (Rakwal et al., 2001).

Plant proteinase inhibitors forms stable and complex structures in which proteolysis is limited and extremely slow (Tiffin and Gaut, 2001). Proteinase inhibitors also causes an amino acid deficiency in insects influencing growth, development and eventually causing its death by inhibiting gut proteinases or due to a massive over-production of the digestive enzymes, reducing the availability of essential amino acids for the production of other proteins (Jongsma and Bolter, 1997; Pompermayer et al., 2001). Based on these facts, *Ricinus communis* L. (Euphorbiaceae) was highlighted as a promising plant to be used on integrated pest management. Santiago et al. (2008) observed bioactivity of aqueous extract from green fruits of *R. communis* when added to an artificial diet, reducing the period of immature stages and the larvae weight of *S. frugiperda*. Bigi et al. (2004) identified toxic compounds from *R. communis* to the leaf cutting ant *Atta sexdens rubropilosa* (Forel, 1908) (Hymenoptera: Formicidae) and concluded that the metabolites responsible for this activity were mainly the fat acids and the ricinin. Consequently, the aim of this study was to isolate a trypsin inhibitor from leaves of *R. communis* and evaluate its effect on the development of *S. frugiperda* larvae.

MATERIALS AND METHODS

Insect rearing

The experiment was conducted in a climatized room at 25 ± 2°C with 12 h photofase and 70 ± 10% relative humidity. The insects were reared on the artificial diet developed by Karster-Junior et al. (1978).

Collection of the plant and preparation of extracts

Leaves of *R. communis* were collected in a field located in Lavras County (21°13'29.73'' S; 49°58'43.93'' W) state of Minas Gerais, Brazil, and the voucher specimen was deposited in the herbarium of the Federal University of Lavras (UFLA). All of the plant extracts were prepared in the Biochemistry laboratory at UFLA. Leaves, without petiole, central and secondary nervures, were cut in 1 cm² pieces, and placed on an Erlenmeyer with ethanol (pa > 99.8%) respecting the proportion of 1:7 (weight of leaves/volume of extractor). The remained moisture was stored away from the light for 20 days, and filtered after this period in a Büchner funnel with filter paper and a rotary evaporator connected to a vacuum pump. After filtration, the extract was oven-dried at 45°C to eliminate all of the solvent.

Chromatographic conditions and enzyme inhibition assays

Chromatographic analysis was performed using a 50 cm long column, and 2.5 cm inner diameter packed with silica Kieselgel 60 (0.040 to 0.063 mm; 230 to 400 mesh) Merck®. The eluents were hexane, chloroform, ethyl acetate, ethanol, methanol and acetic acid. All the fractions collected showed different volume (around 100 mL) and the solvent exchange occurred based on the observations of spots in the column. Seventeen fractions were obtained from this column, which were concentrated in rotary evaporator until complete eluent evaporation. All fractions were resuspended separately in 5 mL of ethanol and subjected to trypsin inhibition *in vitro* testing. Inhibitor activity was determined as described by Kakade et al. (1974) by the comparison between a kinetic assay of trypsin activity in the inhibitor presence and absence, over four time intervals (Erlanger et al., 1961). Results, obtained in three replicates, were expressed in milliunits of inhibited trypsin (mUIT). This unit represents the amount of light absorbed by 1 nMole p-nitroanilide produced from the trypsin activity on N – alpha – benzoyl – DL – arginine - p - nitroanilide (BapNA) substrate after one minute of reaction with 1 g leaves of *R. communis*. Fifth instar larvae of *S. frugiperda* were immobilized on ice and dissected. Midgut was removed and macerated in a Potter homogenizer in the proportion of 1 midgut to 4 mL of water at 4°C. The homogenized solution was filtered through 100 µm nylon mesh and centrifuged for 10 min at 14,000 x g (4°C). Supernatant was used in the inhibitor activity assays. The fraction that exhibited higher trypsin inhibition in *in vitro* tests was submitted to chromatographic separations, which were achieved using a High Performance Liquid Chromatographer Shimadzu LC 20A, equipped with an automatic injector (injection volume 20 µL) and a UV-VIS detector. A Agilent – Zorbax Eclipse AAA-C18 column (150 mm × 4.6 mm containing 5 µm spherical particles) and an Agilent – Zorbax Eclipse AAA pre-column (12.5 × 4.6 mm, 5 µm) were used. Oven temperature was 45°C. Analyses were realized through elution gradient, in which Eluent A (Metanol HPLC grade) was added in the eluent B (Water MiliQ) by a linear gradient (20 to 100% in 45 min), returning to initial concentration in the last 5 min. Fractions with 1 mL of volume were collected at every minute in glass tubes for 50 min. Fifty fractions were obtained and used in trypsin inhibition assays following Kakade et al. (1974). Control assays were performed to avoid variation caused by different eluent concentrations. These assays were driven through the injection of ethanol (solvent used to solubilize the inhibitor) in the HPLC in the same conditions of the samples. Fractions that showed enzyme inhibition were injected in a mass spectrometer. Mass spectrometry was performed using Photo-Fenton UV/H_2O_2 process. The spectra

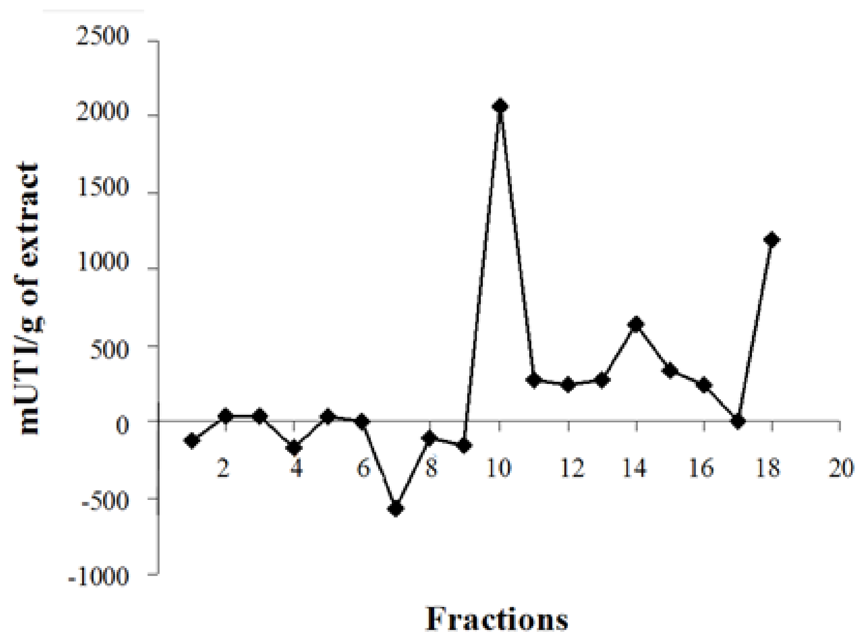

Figure 1. Trypsin Inhibited activity (mUTI) per *g* of *R. communis* leaves extract. Fractions (obtained by silica absorption column): 1 - hexane, 2 - hexane / chloroform, 3 to 6 - chloroform; 7 - chloroform / ethyl acetate, 8 - acetate ethyl, 9-ethyl acetate / ethanol, 10 and 11 - ethanol, 12 - ethanol / methanol, 13 to 15 - methanol, 16 - methanol / acetic acid; 17:18 - acetic acid.

were obtained in the negative mode in a mass spectrometer model LC/MS Trap Agilent 1100 under the following conditions: air flow 5 µL/min; capillary voltage of -3500 V; dry temperature of 325°C; and nebulizer pressure 10 psi. The analysis was carried in triplicates.

Assay with larvae of caterpillars of *S. frugiperda*

Fraction obtained were also utilized *in vivo* bioassays with larvae of *S. frugiperda*. The semi-purified inhibitor (preparative HPLC) was added in the artificial diet (Kasten-Junior et al., 1978), at the concentrations of 0, 0.06, 0.12, 0.25 and 0.5% (mass/volume). Diets were weighted (5 g) and offered to five-days-old larvae in a closed individual acrylic recipient. Bioassay was conducted in a complete randomized design (CRD) with six replicates per treatment. Each replicate contained six individually reared larvae totalizing 36 larvae per treatment. The biological characteristics evaluated were: larvae weight each three days, which were used to develop a growth curve; larvae survival and length of larval and pupae stages. The amount of food consumed was calculated with a correction factor for the water losses ([1-a/2][W-(L+bL)]), where: e = initial diet weight; b = aliquot average loss; W = introduced diet weight; L = non-eaten food weight (Cohen, 2004). Growth curve values were analyzed by the DRC statistical package of R® software (R Development Core Team, 2009), using the logistic model and lack-of-fit test. Amount of food consumed, larvae weight, pupae weight, and length of larval and pupae stages were submitted to Shapiro-Wilk's test, using the Mvnormteste package of R® software ® (R Development Core Team, 2009. Data were submitted to Analysis of Variance (ANOVA) and Scott-Knott test, using the Laercio package of R® software ® (R Development Core Team, 2009).. The mortality data were evaluated counting the number of dead individuals during the assay period and corrected by the Abbott's formula ([$M = m_e - m_b/1 - m_b$] where: M = Mortality, m_e = Treatment mortality, m_b = Control mortality).

RESULTS

Chromatographic and spectrometric analyses

Chromatographic fractions soluble in hexane, chloroform and ethyl acetate (fractions from 1 to 9) showed no activity in the trypsin inhibition tests. The fraction 7, corresponding to the eluent chloroform/ethyl acetate, had one activity peak and the fractions soluble in ethanol, methanol and acetic acid (fractions 10 to 18) showed trypsin inhibition. The higher inhibition activity was registered at the 10th ethanol fraction (ethanol – 10), which was selected for HPLC analysis and *in vivo* bioassays (Figure 1). Fractions originated from HPLC retention time of 26 to 29 min (Figures 2 and 3) inhibited trypsin from the *S. frugiperda* digestive system. Aiming to decrease the influence of eluents in the trypsin inhibition analysis, a control test containing only ethanol was injected in the HPLC. All mass spectra (Figure 4) were obtained from the HPLC fractions (26 to 29 min) that inhibited tryspin from the lepidopteran digestive system. Mass spectrometry did not allow quantifying the inhibitor mass. However, repeated peaks related with trypsin inhibition were identified in all mass spectra.

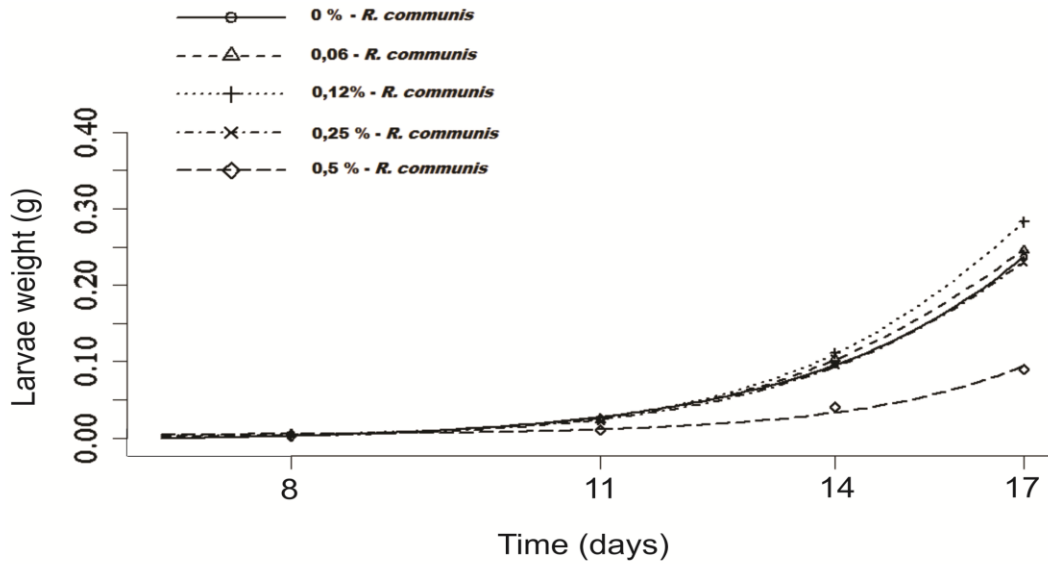

Figure 2. Growth curve of *S. frugiperda* larvae fed on artificial diet containing extract from the leaves of *R. communis*. inhibitor fraction.

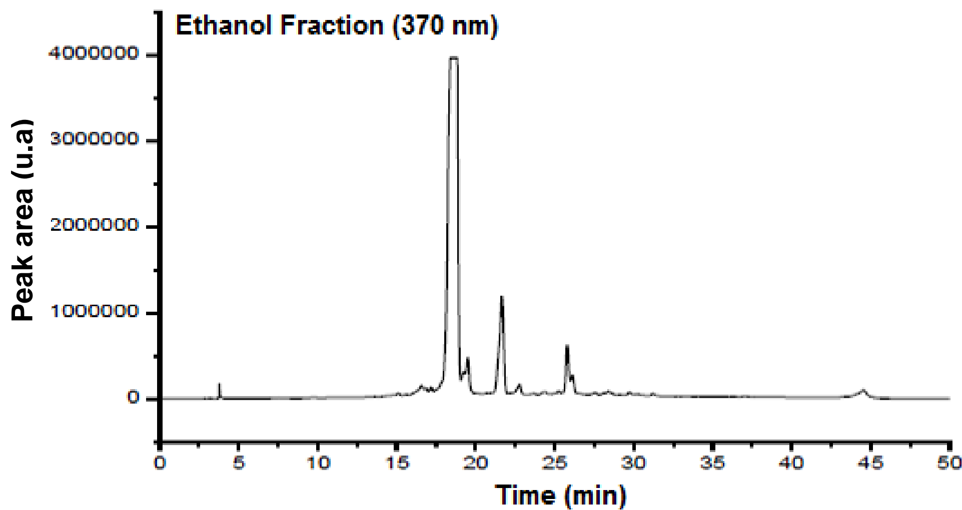

Figure 3. HPLC chromatograms for the ethanolic fraction (370 nm).

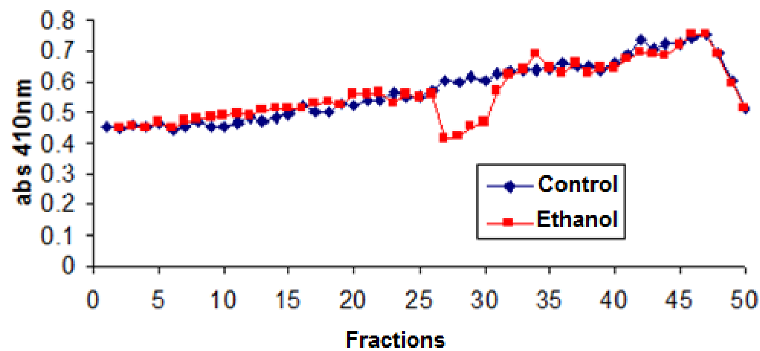

Figure 4. Inhibition of Mass Spectrum of fractions obtained from HPLC (A, 27; B, 28; C, 29; D, 30 min).

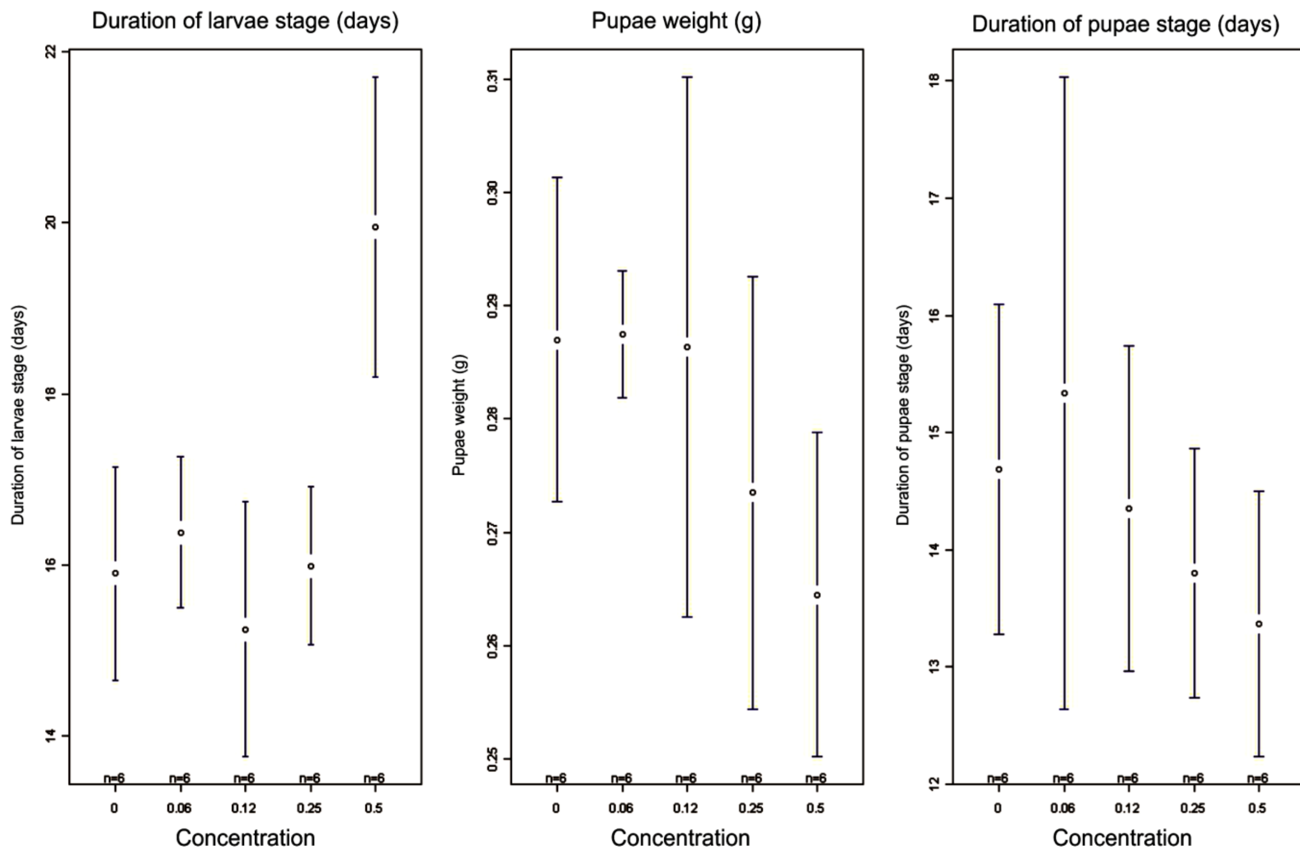

Figure 5. Pupae weight, larval and pupal stage length of *S. frugiperda* fed on artificial diet containing extract from the leaves of *R. communis*.

Biological assay

Castor oil trypsin inhibitor showed toxic characteristic to *S. frugiperda* larvae only at the concentration of 0.5%. Insects fed with 0 and 0.06% of inhibitor developed normally. In the treatments with inhibitor concentrations at 0.12 and 0.25% the larvae reached its maximum weight at the 23rd day, whereas in the treatment with inhibitor concentration at 0.5% the larvae development was problematic, extending the larval stage and decreasing weight average (Figure 5). In the treatment with inhibitor concentration at 0.5%, larval stage was ten days higher than the control treatment. Rates of larvae and pupae survival were not affected by the treatments, ranging from 63.3 to 87.7 and 12.0 to 14.4 days, respectively (Figure 6). Among the nutritional indexes evaluated (Figure 7), 0.5% of inhibitor concentration resulted in higher relative consumption rate (RCR), lower relative growth rate (RGR), efficiency of ingested food conversion (EIC) and efficiency of digested food conversion (EDC). Approximated digestibility (AD) and relative metabolic rate (RMR) showed no significant differences among treatments (Figure 7). The Abbott formula (Abbott 1925) of efficacy for the concentrations of 0.5, 0.25 and 0.12% provided values of 25, 12 and 4.17%, respectively. The concentration of 0.06% showed no efficiency.

DISCUSSION

Trypsin inhibitors are extensively studied in several aspects aiming biochemistry characterization, three dimensional structures, interactions with multiple protease classes and effects on pests and pathogens (Abd El-Latif, 2014; Kuwar et al., 2015; Pontual et al., 2014; Zhu-Salzman and Rensen, 2015). Previous works performed with castor oil leaves suggested that trypsin inhibitor is a non-proteic organic molecule (Rossi et al., 2012) differing from other trypsin inhibitors described in literature. Precipitations with acetone and saturated ammonium solution, high temperature exposition and inhibition assays in the presence of de β-mercapto-ethanol were performed demonstrating the molecule non-proteic characteristic and suggesting different ways to extract and purify this trypsin inhibitor. Aiming to isolate the inhibitor, two chromatographic methods were utilized in the present study. Previously, adsorption chromatography

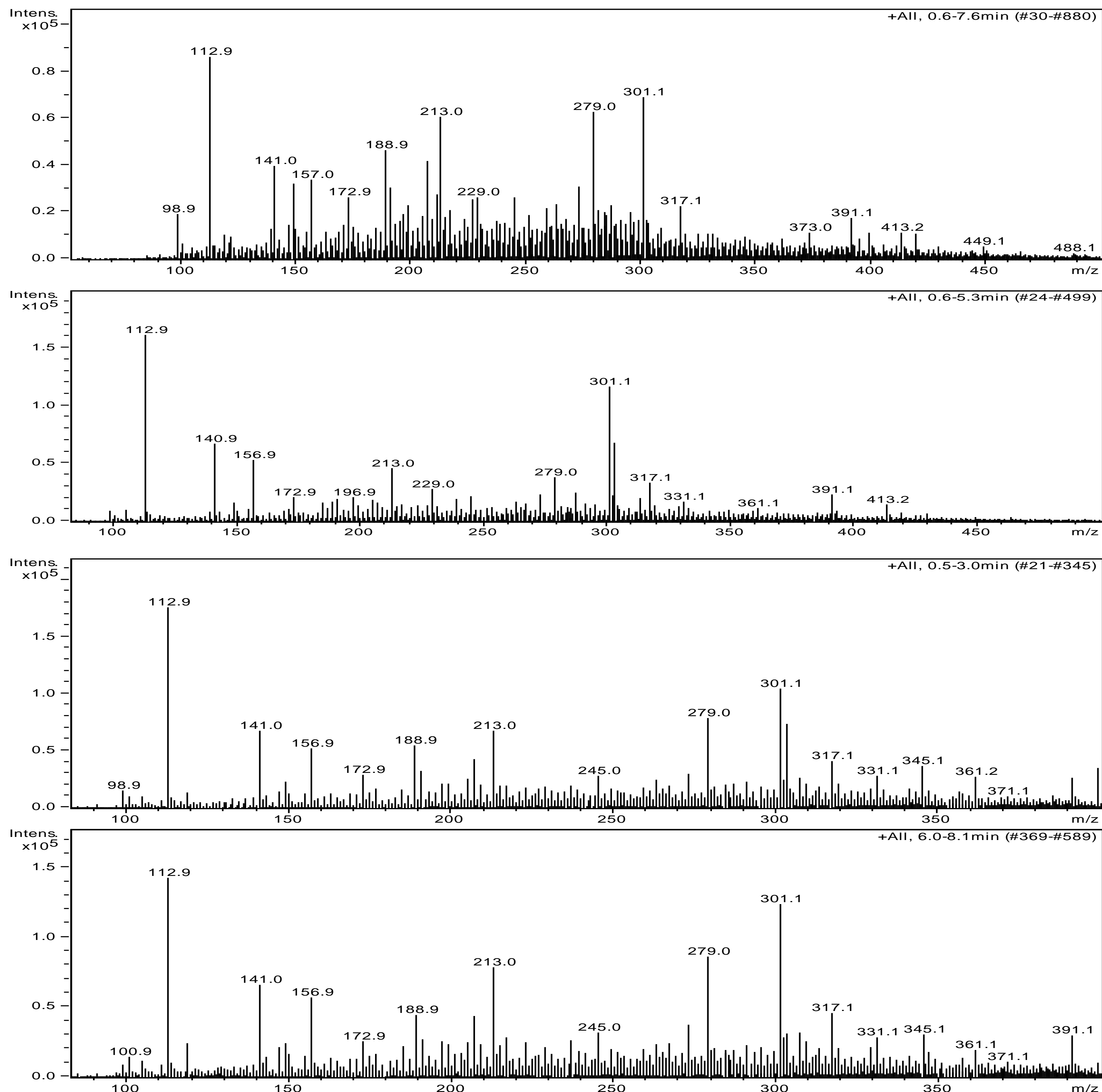

Figure 6. Mass Spectrum of fractions obtained from HPLC (A, 27; B, 28; C, 29; D, 30 min).

with silica was carried out to separate the organic compounds present in the extract, based on the molecule polarity. Subsequently all fractions obtained were subjected to *in vitro* analysis. Trypsin from the *S. frugiperda* digestive system was inhibited by the ethanol and methanol fractions. Several inhibition peaks were observed (Figure 1), suggesting the presence of more than one trypsin inhibitor in castor oil leaves. A trypsin activator molecule was also detected in the fraction chloroform/ethyl acetate. Due to the higher trypsin inhibition activity (Figure 1) only the ethanol fraction – 10 was analyzed in the HPLC. Inhibition assays showed presence of inhibitor molecules

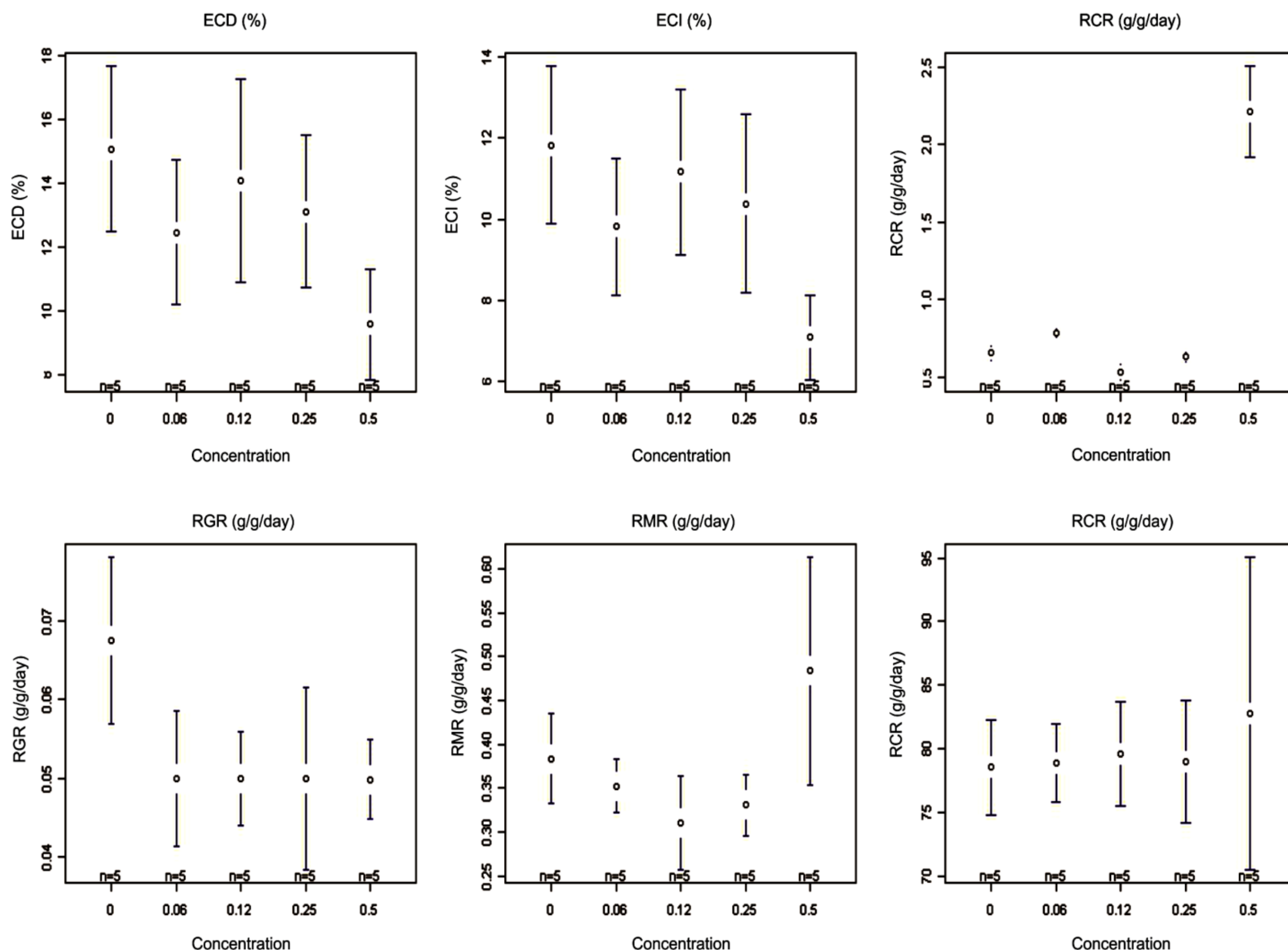

Figure 7. Efficiency of digested food conversion (%EDC), efficiency of ingested food conversion (%EIC), relative consumption rate (RCR), relative consumption rate (RCR) (g/g/day), relative metabolic rate (RMR) (g/g/day), approximated digestibility (AD) of S. *frugiperda* larvae feed on artificial diet containing extract from the leaves of *R. communis*.

at four retention times (26 to 29 min) (Figure 2 and 3). These fractions were submitted to mass spectrometry (Figure 5). More studies are needed to identify the compound responsible for the inhibition. However, based on the repeated peaks observed in the mass spectrometry, this molecule likely has a common main structure, differing only in some ramifications. Inhibitor activity can be evidenced by adding these molecules to artificial diets offered to the target insect. Although *in vitro* assays are initial tests to indicate insecticide activity, many works confirmed their results through *in vivo* assays on larvae of pest insects (Alves et al., 2014; Tavares et al., 2009). Several works on literature reported *in vivo* effect of Kunitz and Bowman-Birk proteinase inhibitors on larval development (Shade et at.,1986), pupae weight (Duan et al., 1996), total viability of insects cycle (Shade et al., 1986) and insect adaptation mechanisms (Brioschi et al., 2007; Paulillo et al., 2000). However, to the best of our knowledge there is no study exploring these responses for a non-proteic trypsin inhibitor.

In this study, the castor oil trypsin inhibitor (COTI) activity was analyzed during the larval development of *S. frugiperda*, an important pest of the Brazilian agriculture. For this, COTI was added in artificial diet in crescent concentrations. Among all the biological parameter analyzed only the length of larval stage and pupae weight showed significant results when compared to the control (Figure 6). An increase of ten days was registered in *S. frugiperda* larval stage length for the treatment with inhibitor concentration at 0.5%, proving the deleterious effect of this inhibitor. Probably, an essential amino acid deficiency may have affected the insect development, delaying the stage conclusion (Ryan, 1990). Decreased pupae weight was registered in the treatments with inhibitor concentration of 0.25% and 0.5%. This parameter

suggested reduced food ingestion, probably caused by the inhibitor presence. Parameters as length of the pupae stage, food ingestion, feces weight, larvae and pupae survival rates showed no variations with the inhibitor inclusion on the diet (Figure 6). Differences between *in vitro* and *in vivo* assays are frequently reported in the literature (Bolter and Jongsma, 1995). In this study, COTI reached 66% of trypsin inhibition *in vitro*, whereas *in vivo* assays showed deleterious effect in few biological parameters. This result is completely understandable due to the pest capacity to adapt to the inhibitor presence on diets or genetically modified plants (Bolter and Jongsma, 1995; Ishimoto et al., 1996; Jongsma and Boulter, 1997). Pest adaptation capacity can be explained by two hypotheses: 1) Higher quantity of digestive enzyme expressed in presence of inhibitor; 2) different proteinase classes expressed to overcome the inhibitor effect. According to Broadway et al., (1986), insect larvae can overproduce protease in the presence of proteinase inhibitor in the digestive system. In the inhibitor concentration of 0.5%, the growth curve presented to the larval stage 12 days was higher compared to the control treatment, showing an evident deficit in larvae development. Pupae formation was also affected, delaying in three days compared with control treatment. Remaining treatments were not affected comparing with control, demonstrating a regular development. However, at the concentration of 0.12 and 0.25% presented larval stage three days higher compared with control treatment (Figure 5).

According to Martinez and Endem (2001) increasing the larval stage and growth inhibition can be triggered by reduced food ingestion and lower food conversion, caused by the presence of one or more inhibitor in the diet. Larvae of *S. frugiperda* fed with concentration of 0.5% also showed alterations in the nutritional indexes (Figure 7) as higher relative consumption rate (RCR), lower relative growth rate (RGR), lower efficiency of ingested food conversion (EIC) and lower efficiency of digested food conversion (EDC) with more metabolic cost. These results attest the potential of COTI, since similar studies on larvae of *H. virescens* fed on tobacco plants expressing potato proteinase inhibitors (PIN-2), showed no variation in nutritional parameters evaluated (Brito et al., 2001). Approximated digestibility (AD) and relative metabolic rate (RMR) showed no significant differences among treatments (Figure 7). The efficacy at the concentrations of 0.5, 0.25 and 0.12% were 25, 12 and 4.17%, respectively. The concentration of 0.06% showed no efficiency. In this study, the non-proteic castor oil trypsin inhibitor at the concentration of 0.5% impaired *S. frugiperda* larvae development changing their nutritional parameters. These results can be used to develop an alternative and more sustainable method to control lepidopteran pest species through trypsin inhibition. Future works should investigate the inhibitor molecule characteristics, activities on enzyme catalysis and evaluate its kinetic properties.

Conflict of interests

The author(s) did not declare any conflict of interest.

ACKNOWLEDGEMENT

The authors gratefully acknowledge the financial support provided by the Coordination for the Improvement of Higher Education Personnel (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - CAPES, Brazil), National Council for Scientific and Technological Development (Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq, Brazil) and State of Minas Gerais Research Support Foundation (by Fundação de Amparo à Pesquisa do Estado de Minas Gerais - FAPEMIG, Brazil).

REFERENCES

Abbott, WS (1925). A method of computing the effectiveness of an insecticide. J Econ Entomol, 18:265-266.

Abd El-latif AO (2014) In vivo and in vitro inhibition of *Spodoptera littoralis* gut-serine protease by protease inhibitors isolated from maize and sorghum seeds. Pest Biochem. Physiol. 116(1):40-48.

Alvarez A, Pera Lm, Loto F, Virla Eg, Baigori, Md (2009). Insecticidal crystal proteins from native *Bacillus thuringiensis*: numerical analysis and biological activity against *Spodoptera frugiperda*. Baigori Biotechnol. Lett. 31(1):77-82.

Alves APC, Corrêa AD, Alves DS, Saczk AA, Lino Jéssica BR, Carvalho GA (2014). Toxicity of the phenolic extract from jabuticabeira (*Myrciaria cauliflora* (Mart.) O. Berg) fruit skins on *Spodoptera frugiperda*. Chil. J. Agric. Res. (Online). *74*(2):200-204.

Bigi MFM, Torkomian VLV, Groote STCS, Hebling MJA, Bueno OC, Pagnocca FC, Fernandes JB, Vieira PC, Silva MFGF (2004). Activity of *Ricinus communis* (Euphorbiaceae) and ricinine against the leaf-cutting ant *Atta sexdens rubropilosa* (Hymenoptera: Formicidae) and the symbiotic fungus *Leucoagaricus gongylophorus*. Pest Manag. Sci. 60(9):933-938.

Bolter CJ, Jongsma MA (1995). Colorado potato beetles (Leptinotarse decemlineata) adapt to proteinase inhibitors induced in potato leaves by methyl jasmonate. J. Insect Physiol. 41(12):1071-1078.

Brioschi D, Nadalini LD, Bengtson MH, Sogayar MC, Moura DS, Marcio Silva-Filho C (2007) General up regulation of *Spodoptera frugiperda* trypsins and chymotrypsins allows its adaptation to soybean proteinase inhibitor. Insect Biochem. Mol. Biol. 37(12):1283-1290.

Brito LO, Lopes AR, Parra JRP, Terra WR, Silva-Filho MC (2001). Adaptation of tobacco budworm *Heliothis virescens* to proteinase inhibitors may be mediated by the synthesis of new proteinases. Comp. Biochem. Physiol. B. 128(2):365-375.

Broadway RM, Duffey SS, Pearce G, Ryan CA (1986). Plant proteinase inhibitors: a defense against herbivorous insects? Entomol. Exp. Appl. 41:33-38.

Cohen AC (2004). Insect diets science and technology. CRC press, Boca Raton, Florida, USA.

Correia AA, Teixeira VW, Teixeira AAC, Oliveira JV, Torres JB (2009). Morphology of the alimentary canal of *Spodoptera frugiperda* (J E Smith) Larvae (Lepidoptera: Noctuidae) fed on neem-treated leaves. Neotrop. Entomol. 38(1):83-91.

Duan X, Li X, Xue Q, Abo-El-Saad M, Xu D, Wu R (1996). Transgenic rice plants harboring an introduced potato proteinase inhibitor II gene are insect resistant. Nat. Biotechnol. 14:494-498.

Erlanger BF, Kokowsky N, Cohen W (1961). The preparation and properties of two, new chromogenic substrates of tripsina. Arch. Biochem. Biophys. 95(1): 271-278.

Ishimoto M, Sato T, Chrisperls MJ, Kitamura K (1996). Bruchid resistance of transgenic azuki bean expressing seed α-amilase inhibitor of common bean. Entomol. Exp. Appl. 79:309-315.

Jongsma, MA, Bolter C (1997). The adaptation of insects to plant protease inhibitors. J. Insect Physiol. 43(10): 885-895.

Kakade ML, Rackis JJ, Mcghee JE, Puski G (1974). Determination of trypsin inhibitor activity of soy bean products: a collaborative analysis of an improved procedure. Cereal Chem. 51(1): 376-382.

Kasten-Junior P, Precetti AACM, Parra JPP (1978). Dados biológicos comparativos de *Spodoptera frugiperda* (J.E. Smith, 1797) em duas dietas artificiais e substrato natural. Rev. Agric. 53(2):68-78.

Kuwar SS, Pauchet Y, Vogel H, Heckel DG (2015). Adaptive regulation of digestive serine proteases in the larval midgut of *Helicoverpa armigera* in response to a plant protease inhibitor. Insect Biochem. Mol. 59:18-29.

Loewenherz C, Fenske RA, Simcox NJ, Bellamy G, Kalman D (1997). Biological monitoring of organophosphorus pesticide exposure among children of agricultural workers in central Washington State. Environ. Health Perspect. 105(12):1344-53.

Malau MB, James DB (2008). Evaluation of larvicidal properties of some plant On *Simulium Damnosum* Complex. Internet J. Toxicol. 4:2.

Martinez SS, Emden HF (2001). Growth disruption, abnormalities and mortality of Spodoptera littoralis (Boisduval) (Lepidoptera: Noctuidae) caused by Azadirachtin. Neotrop. Entomol. 30(1):113-125.

Nathan SS, Choi, MY, Seo HY, Paik CH, Kalaivani K (2008). Toxicity and behavioral effect of 3beta,24,25-trihydroxycycloartane and beddomei lactone on the rice leaffolder *Cnaphalocrocis medinalis* (Guenée) (Lepidoptera: Pyralidae). Ecotoxicol. Environ. Saf. 72(4):1156-1162.

Paulillo LCMS, Lopes AR, Cristofoletti PT, Parra JRP, Terra WR, Silva-Filho MC (2000). Changes in midgut endopeptidase activity for *Spodoptera frugiperda* (Lepidoptera: Noctuidae) are responsable for adaptation to soybean proteinase inhibitors. J. Econ. Entomol. 93(3):892-896.

Pompermayer P, Lopes AR, Terra WR, Parra JRP, Falco MC, Silva-Filho MC (2001). Effects of soybean proteinase inhibitor on development, survival and reproductive potential of the sugarcane borer, *Diatraea saccharalis*. Entomol. Exp. Appl. 99(1):79-85.

Pontual EV, Napoleão TH, Assis CRD, Bezerra RS, Xavier HS, Navarro DMAF, Coelho LCBB, Paiva PMG (2012). Effect of *Moringa oleifera* flower extract on larval trypsin and acethylcholinesterase activities in*Aedes aegypti* Arch. Insect Biochem. Physiol. 79:135-152.

R Development Core Team (2009). R - A language and environment for statistical computing, version 2.10.1, 1st edition. R Foundation for Statistical Computing.

Rakwal R, Agrawal GK, Jwa NS (2001). Characterization of a rice (*Oryza sativa* L.) Bowman-Birk proteinase inhibitor: tightly light regulated induction in response to cut, jasmonic acid, ethylene and protein phosphatase 2A inhibitors. Gene 263(1-2):189-198.

Rossi GD, Santos CD, Carvalho GA, Alves DS, Pereira LLS, Carvalho GA (2012) Biochemical Analysis of a Castor Bean Leaf Extract and its Insecticidal Effects Against *Spodoptera frugiperda* (Smith) (Lepidoptera: Noctuidae). Neotrop. Entomol. 41:503-509.

Ryan CA (1990) Proteinase inhibitors in plants: genes for improving defenses against insects and pathogens. Annu. Rev. Phytopathol. 28:425-449.

Santiago GP, Pádua LEM, Silva PRR, Carvalho EMS, Maia CB (2008). Effects of plant extracts on the biology of S*podoptera frugiperda* (J. E. Smith, 1797) (Lepidoptera: Noctuidae) maintained under artificial diet. Cienc. Agrotec. 32(3):792-796.

Shade RE, Murdock LL, Foard DE, Pomeroy MA (1986). Artificial seed system for bioassay of *Cowpea weevil* (Coleoptera: Bruchidae) growth and development. Environ. Entomol. 15(6):1286-1291.

Starner K, Goh KS (2012). Detections of the Neonicotinoid Insecticide Imidacloprid in Surface Waters of Three Agricultural Regions of California, USA, 2010-2011. Bull. Environ. Contam. Toxicol. 88(3): 316-321.

Tavares WS, Cruz I, Petacci F, Assis Júnior SL, Freitas SS, Zanuncio JC, Serrão JE (2009). Potential use of Asteraceae extracts to control *Spodoptera frugiperda* (Lepidoptera: Noctuidae) and selectivity to their parasitoids *Trichogramma pretiosum* (Hymenoptera: Trichogrammatidae) and *Telenomus remus* (Hymenoptera: Scelionidae). Ind. Crop Prod. 30(3):384-388.

Tiffin P, Gaut BS (2001). Molecular evolution of the wound-induced seine proteinase inhibitor wip1 in zea and related genera. Mol. Biol. Evol. 18(11):2092-2101.

Uchino H, Iwama K, Jitsuyama Y, Yudate T, Nakamura S, Gopal J (2015). Interseeding a Cover Crop as a Weed Management Tool is More Compatible with Soybean than with Maize in Organic Farming Systems. Plant Prod. Sci. 18(2):187-196.

Yu SJ, Nguyen SN, Abo-Elghar GE (2003). Biochemical characteristics of insecticide resistance in the fall armyworm, *Spodoptera frugiperda* (J.E. Smith). Pestic. Biochem. Physiol. 77(1):1-11.

Zhu-Salzman K, Zeng R (2014). Insect Response to Plant Defensive Protease Inhibitors. Annu. Rev. Entomol. 60:233-252.

academicJournals

Vol. 14(42), pp. 22937-2942, 21 October, 2015
DOI:10.5897/AJB2015.14561
Article Number: 008F55655887
ISSN 1684-5315
Copyright © 2015
Author(s) retain the copyright of this article
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

The efficacy of palm oil sludge in reducing ruminal methanogenesis using rumen simulation technique

L. C. Ugwuowo[1]*, A. G. Ezekwe[1], A. O. Ani[1], S. I. Eze[2], C. N. Anyanwu[2] and A. Ofomatah[2]

[1]Department of Animal Science, University of Nigeria, Nsukka, Enugu State, Nigeria.
[2]National Centre for Energy Research and Development, University of Nigeria, Nsukka, Enugu State, Nigeria.

Received 11 March, 2015; Accepted 8 October, 2015

The effect of including palm oil sludge in cattle diet (silage) was conducted *in vitro* in order to ascertain the suppressing effect on ruminal methanogenesis. The silage used for the study was *Panicum maximum, Centrocema pubscens, Bracharia decumbens* and *Andropogon gayanus.* The silage were cut into an average size of 3 to 5 cm and ensiled for 21 days. Four treatment diets were formulated with the silage by including palm oil sludge at 0, 10, 20 and 30%, respectively. Treatments were subjected to anaerobic digestion in 10 L bio-digesters. Total gas production, percentage methane production, volatile fatty acids and microbial population of the substrate were measured. Total gas production was significantly suppressed in the treatment having 30% palm oil sludge for about 25 days while a lag phase of 16 days was observed before a significant amount of methane was detected. Acetic acid production increased only in T1 on the first week while T2, T3 and T4 decreased after which their production remained irregular until the end of the experiment. Propionic acid production also increased in T1 and T4 on the first week while T2 and T3 decreased after which their production maintained an undefined pattern as the experiment progressed. Butyric acid production increased in T1, T2, and T4 on the first week while T2 decreased after which an undefined trend of production was established. Microbial population especially methanogens also reached its peak around the same period when there was maximum gas production. It was concluded that ruminal methanogenesis can be suppressed *in-vitro* by the inclusion of 30% palm oil sludge in the silage.

Key words: Bio-digesters, fermentation, *in-vitro*, methane, silage.

INTRODUCTION

Methane emission from ruminant animals has become a serious issue that attracts global attention. Methane remains one of the greenhouse gases that cause climate change. Report shows that enteric methane is the most important greenhouse gas emitted (50 to 60%) at the farm scale in ruminant production systems (Ogino et al., 2007). Methane has the global warming potential of 25 and with longer residence time when compared to carbon

*Corresponding author. E-mail: chidilu2002@yahoo.com.

Abbreviations: MCA, MacConkey agar; **CRD,** completely randomized design; **NA,** nutrient agar.

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

dioxide with higher global warming potential of 82 and shorter residence time (Wuebbles and Hayhoe, 2002; Forster et al., 2007). Human activities accounts for 70% of methane production and its rising concentration is strongly correlated with increasing population of ruminants (Moss et al., 2000; IPCC, 2007).

Murray et al. (1976) stated that about 90% enteric methane produced by ruminants has its origin in the rumen. Methane mitigation strategies in ruminants have been reviewed critically by several authors. Mitigation strategies notably on animal phenotype, biotechnologies and additives were discussed but nutrition strategies were described as the most developed and ready to be applied in the field. Smink et al. (2004), Van Zijderveld and Van Straalen (2004) and Van Laar and Van Straalen (2004) noted that several Rob-Agro studies have been performed and the effect of nutrition and feed additives on methane emission evaluated. Lin et al. (2013) had reported promising effect of different essential oils in modulating rumen function *in vitro*. According to Hristov et al. (2013) the inclusion of lipids in the diets of ruminants is an effective strategy for reducing enteric methane emission, but the applicability of this practice will depend on its cost effectiveness and its effects on feed intake, productivity and milk production. Hence, the inclusion of palm oil sludge in ruminant diets may have a great potential in reducing enteric methane emission since it contains more phyto-nutrients (vitamin E, vitamin K and alpha carotene, beta-carotene, lycopene and 20 other carotenes) than any other dietary oil. These nutrients are powerful antioxidants that protect the oil from oxidation. It is a waste and constitutes nuisance around households and palm oil processing factories. This study was therefore conducted to ascertain the efficacy and effective levels of palm oil sludge in reducing ruminal methanogenesis *in vitro*.

MATERIALS AND METHODS

Experimental diets and design

A mixture of forage materials which comprises of *Panicum maximum, Centrocema pubscens, Bracharia decumbens* and *Andropogon gayanus* were mixed in equal proportion for the production of silage. The forage materials were cut to a size of between 3 to 5 cm and ensiled for 21 days. The silage were mixed with palm oil sludge at 0, 10, 20 and 30% inclusion levels to form four treatment diets with four replicate each. The treatment diets were subjected to anaerobic digestion in 10 L bio-digesters as the *in vitro* rumen simulation. 5.8 kg of water, 2 kg of treatment diets and 0.2 kg of rumen liquor were put in a 10 L biodigesters while the remaining space was left for gas accumulation. The treatments were replicated four times in a completely randomized design (CRD). Total gas production and percentage methane obtained were recorded using the Sperian gas analyzer; model number SN 66429, USA. Total gas volume was obtained through downward displacement of water in gasometers. The volume of water displaced therefore equals volume of gas produced. Sperian gas analyser recorded the percentage of methane in the total gas and that percentage was used to calculate the volume of methane from total gas volume. Gas collection and analysis were done every 24 h for 90 days.

Table 1. The proximate composition of treatment diets.

Components	Dietary treatments			
	T1	T2	T3	T4
Protein (%)	9.63	9.61	9.49	9.49
Fat (%)	4.03	5.20	5.90	6.35
Moisture (%)	66.73	61.85	62.60	63.50
Fibre (%)	5.77	5.82	6.48	5.88
Ash (%)	8.73	9.13	8.98	9.47

The diets were analyzed for crude protein, ash, fats, moisture and fibre contents before and after the fermentation process according to AOAC (2000). Changes in the concentration of volatile fatty acids (acetic, propionic and butyric) were also determined biweekly during the fermentation period for 90 days using Spectrophotometer (BB Bran England model 7804 C) in a diluted volumes of 0.1 ml in 10 ml samples. Changes in total microbial population of the anaerobic fermentation medium were determined through laboratory methods in food microbiology by Harrigan and McCance P.25-28.

Preparation of diluent and media

Diluent (peptone water) and media (Nutrient agar) were prepared according to manufacturer's specification.

Microbiological analysis of samples

One milliliter (1 ml) of each sample was serially transferred into 9 ml of the sterile diluent (peptone water) with a sterile pipette and shaken vigorously. Serial dilution was continued until 106 dilution was obtained.

Aliquot portion (0.1 ml) of the 106 and was inoculated onto freshly prepared, surface-dried nutrient agar (NA). The inoculi were spread with a sterile (hockey stick-like) glass spreader to obtain even distribution of isolates after incubation. Nutrient agar and MacConkey agar plates were incubated for 24 to 48 h at 37°C. Anaerogen was used during the incubation process.

Enumeration of microbial population

Total plate counts for the nutrient and MacConkey Agar were done by counting colonies at the reverse side of the culture plates. Total colony count was expressed in colony forming units per millilitre (cfu/ml) (Harrigan and McCance, 1990).

RESULTS AND DISCUSSION

The proximate composition of the treatment diets that were subjected to anaerobic fermentation is presented in Table 1. Figure 1 shows the methane production pattern during the experiment. Methane production rate from T4 decreased significantly at the beginning of the curve probably as a result of the inhibitory effect of palm oil sludge. This inhibitory effect was maintained for about

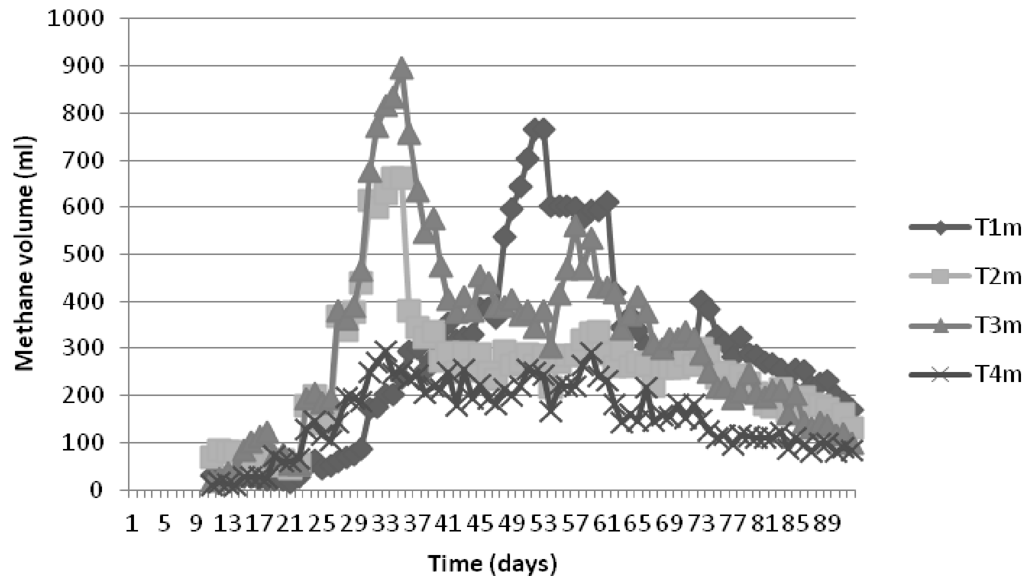

Figure 1. Volume of methane produced during the fermentation process.

nine days before it declined. Though, the rate of methane production from T4 was still relatively lower until after 72 days before it started rising above others and reached its peak around 87th day. This was similar to the ones reported by Broughton et al. (1988), Salminen et al. (2000) and Cirne et al. (2007).

The initial lag phase in methane production as was observed may be attributed to the variation in the concentration of palm oil sludge. Significant methane production was first detected on the 16th day of the digestion process. The volume of methane produced after the lag phase was lowest in treatment 4 (30% palm oil sludge) than in the other treatments. The lowest level of methane production in T4 was maintained for almost 9 days, after which the activities of the methanogens increased and methane volume started to rise just as it was observed in other treatments. The periodic volume of methane production was almost the same in T1, T2 and T3. The variability in methane production was attributed to the different levels of palm oil sludge used in the study. This tends to show that palm oil sludge is capable of suppressing methane production *in vitro*. Kramer et al. (2013) reported that numerically longer retention time of grass silage-based rations (46.9 ± 5.68 h) compared with corn silage-based rations (36.9 ± 4.28 h) was in accordance with Lund (2002). The lag phase of 16 days implies that the inhibitory effect of the treatments might also be high if applied *in vivo*. Treatment 4 (30% palm oil sludge) produced the highest volume of methane toward the end of the fermentation period when the level of palm oil sludge in diet 4 could no longer suppress methane production since the bacteria have acclamatized themselves and can no longer be suppressed by oil. The highest volume of methane produced towards the end of the fermentation may not affect the applicability of the research result since it is assumed to be the period when the silage must have been digested in the animal and will be in form of manure if it were to be in vivo. Therefore, the inclusion of palm oil sludge in cattle diet can help to suppress methane production *in vitro*. Peak gas production was observed between 3 to 4 weeks of fermentation for all the treatments and this was also the period when microbial population reached its peak as shown in Figure 2. This shows that the volume of gas produced depends on the microbial load present. The variation in microbial population present in the different substrates also showed that the palm oil sludge had an effect on the preponderance of the microbes since the highest population was recorded in treatment 1(0% palm oil sludge) and lowest in treatment 4 (30% palm oil sludge). It is clear that oil coating can make a barrier to microbial attachment to feed particles in the rumen thereby reducing their activities (Narimani-Rad et al., 2012). Methanogens co-exist with several anaerobes to produce methane from carbon dioxide and hydrogen. Hence, the major part of the hydrogen formed in the rumen is converted into methane (Mills et al., 2001). Murphy et al. (1982), Argyle and Baldwin (1988), Pitt et al. (1996), Friggens et al. (1998) and Bannink et al. (2006a) observed that different types of fermented carbohydrate give different profiles of volatile fatty acid production and hence methane yield. Figures 3, 4 and 5 show the production of volatile fatty acids during the fermentation process. The acetic acid concentration followed the same trend except in treatment 1(0% palm oil sludge) that contain no palm oil sludge. As shown by Figure 3, treatment 4 had the highest concentration of acetic acid as compared to the three treatments that

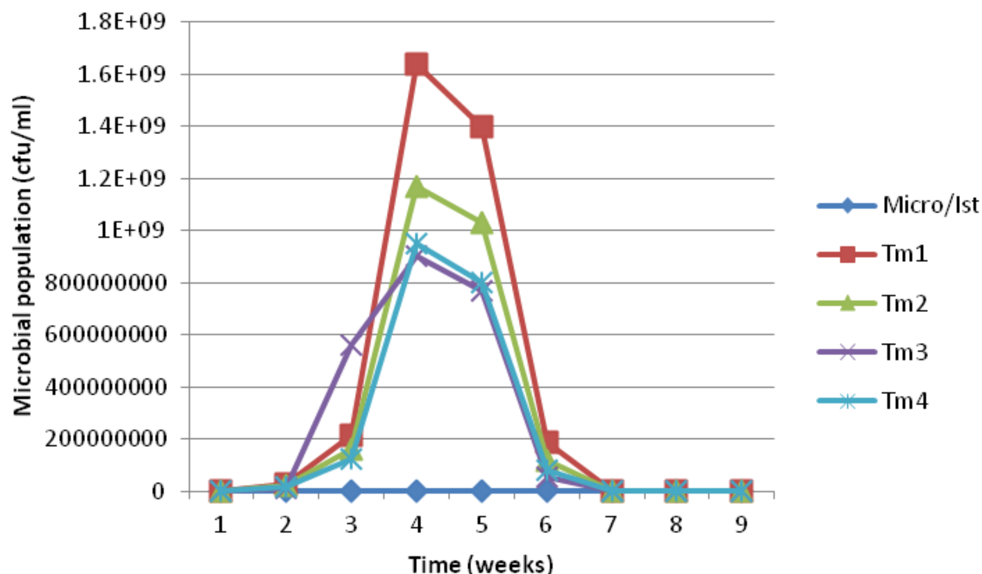

Figure 2. Total anaerobic microbial population

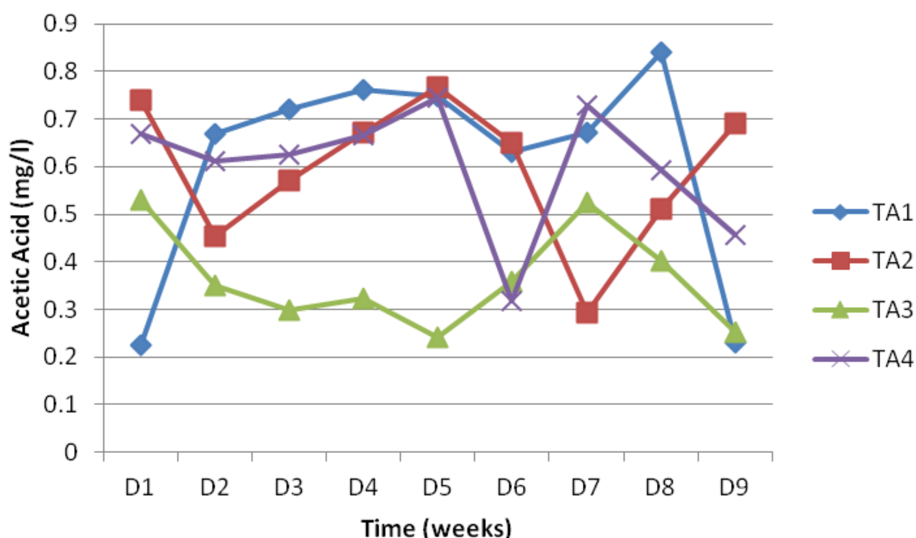

Figure 3. Acetic acid production from the fermenting substrate.

contain palm oil sludge. However, the concentration dropped around 50th day of the fermentation process. Kohn and Boston (2000) and Offner and Sauvant (2006) noted that during acetogenesis, acetate rather than methane is formed from CO_2 and H_2. Therefore, the removal of H_2 by acetogenesis reduces methane yield since H_2 and CO_2 can be converted to acetate by acetogens which are also present in an anaerobic environment (Johnson and Johnson, 1995; Demeyer and Fievez, 2000; Moss et al., 2000). The production pattern of propionic acid was slightly different from that of acetic acid since their production from different treatments did not behave alike by either increasing or decreasing at the same time. Propionic acid concentration in T1 and T_4 followed the same trend of production by increasing and decreasing at the same time while the trend in concentration of propionic acid in T_2 and T_3 were the same. However, butyric acid concentration followed the same trend. There was relatively enhanced propionic acid production in the first 2 weeks of production and a concurrent reduction in the concentration of acetic acid. This is in line with the earlier assertion that enhanced propionic acid concentration reduces methane production and at the same time reduces acetic and butyric acid

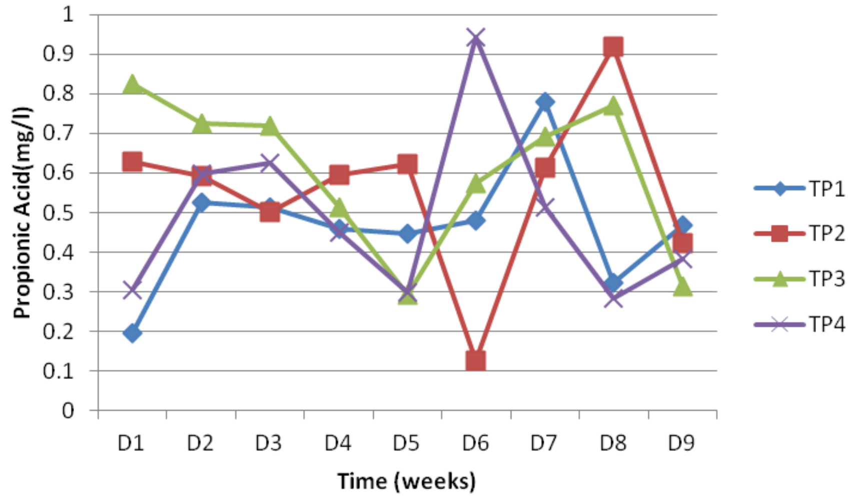

Figure 4. Propionic acid production from the fermenting substrate.

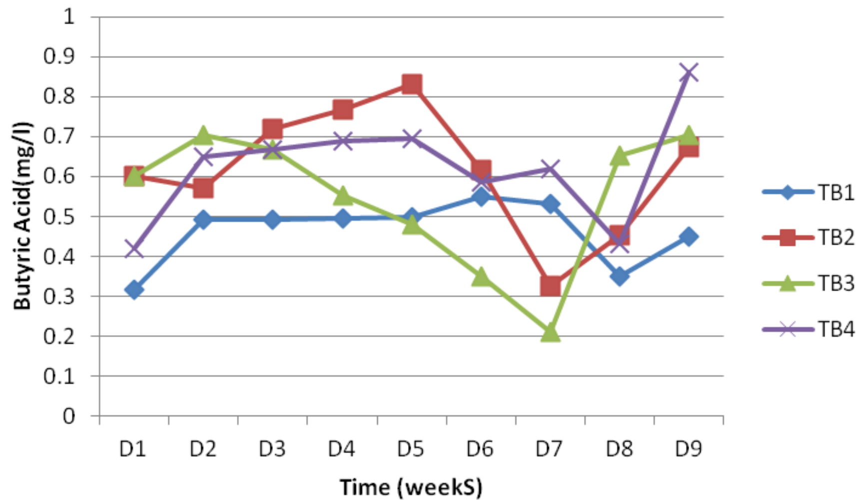

Figure 5. Butyric acid production from the fermenting substrate.

concentration.

Conclusion

An *in vitro* rumen simulation technique was conducted to determine the effect of including palm oil sludge on methane production in the diet (silage) of Cattle. The results of the experiment have shown that inclusion of 30% palm oil sludge in silage is capable of suppressing methanogenesis *in vitro*. The increase in the concentration of acetic, propionic acid and butyric acid on the first week shows that palm oil sludge in the diet of cattle affect the concentration of volatile fatty acids. The reduction in methane production for a period of 9 days at 30% inclusion of palm oil sludge as was observed in this experiment also supports the applicability of this result since there is an average retention period of 45 h in animals.

Conflict of interests

The author(s) did not declare any conflict of interest.

REFERENCES

Argyle JL, Baldwin RL (1988). Modeling of rumen water kinetics and effects of rumen pH changes. J. Dairy Sci. 71:1178-1188.

Bannink A, Kogut J, Dijkstra J, Kebreab E, France J, Tamminga S, Van Vuuren AM, (2006a). Estimation of the stoichiometry of volatile fatty acid production in the rumen of lactating cows. J. Theor. Biol. 238: 36-51

Broughton MJ, Thiele JH, Birch EJ, Cohen A (1998). Anaerobic batch digestion of sheep tallow. Water Res. 32(5):1423-1428.

Cirne DG , Paloumet X, Bjo¨ rnsson L, Alves M M, Mattiasson B (2007). Anaerobic digestion of lipid-rich waste-Effects of lipid concentration. Renew. Energy 32 (6): 965–975.

Demeyer D, Fievez V (2000). Ruminants et environment: la méthanogenèse. Ann. Zootech 49:95-112

Friggens NC, Oldham JD, Dewhurst RJ, Horgan G (1998). Proportions of volatile fatty acids in relation to the chemical composition of feeds based on grass silage. J. Dairy Sci. 81:1331-1344.

Hristov AN, Oh J, Lee C, Meinen R, Montes F, Ott R, Firkins J, Rotz A, Dell C, Adesogan A, Yang W, Tricarico J, Kebreab E, Waghorn G, Dijkstra J, Oosting S, Gerber PJ, Henderson B, Makkar H (2013). Mitigation of Greenhouse Gas Emissions in Livestock Production: A Review of Technical Options for Non-CO_2 Emissions. FAO, Rome, Italy (in press).

IPCC (Intergovernmental Panel on Climate Change) (2007). Climate Change 2007: Mitigation of Climate Change. Contribution of Working Group III to the Fourth Assessment. Report of the Intergovernmental Panel on Climate Change, eds Metz B, Davidson OR, Bosch PR, Dave R, Meyer LA (Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA).

Johnson KA, Johnson DE (1995). Methane emissions from cattle. J. Anim. Sci. 73:2483-2492.

Kohn RA, Boston RC (2000). The role of thermodynamics in controlling rumen metabolism. In: Modelling Nutrient Utilization in Farm Animals. Eds. J.P. McNamara, J. France & D.E. Beever. CAB International, Wallingford, UK. pp. 11-24.

Kramer M, Lund P, Weisbjerg MR (2013). Rumen Passage Kinetics of Forage and Concentrate Derived Fiber in Dairy Cows. J. Dairy Sci. 96(5):3163-3176.

Lin B, Wang JH, Lu Y, Liang Q, Liu JX (2013). *In vitro* rumen fermentation and methane production are influenced by active component of essential oils combined with fumarate. J. Anim. Physiol. Anim. Nutr. 97(1): 1-9.

Mills JAN, Dijkstra J, Bannink A, Cammell SB, Kebreab E, France J (2001). A mechanistic model of whole-tract digestion and methanogenesis in the lactating dairy cow: Model development, evaluation, and application. J. Anim. Sci. 79:1584-1597.

Moss Angela R, Jean-Pierre J, Newbold J (2000). Methane production by ruminants: Its contribution to global warming (Review article). Ann Zootechn. 49:231-253.

Murphy MR, Baldwin RL, Koong LJ (1982). Estimation of stoichiometric parameters for rumen fermentation of roughage and concentrate diets. J. Anim. Sci. 55:411-421.

Murray RM, Bryant AM, Leng RA (1976). Rates of production of methane in the rumen and large intestine of sheep. Br. J. Nutr. 36:1-14.

Narimani-Rad M, Habib AS, Mortaza KN, Alireza L (2012). Effect of sunflower oil supplementation on in *in vitro* fermentation patterns of forage based diets for ruminants. Bull. Environ. Pharmacol. Life Sci. 1(12): 73-77.

Offner A, Sauvant D (2006). Thermodynamic modelling of ruminal fermentations. Anim. Res. 55:1-23.

Ogino A, Orito H, Shimadad K, Hirooka H (2007). Evaluating environmental impacts of the Japanese beef cow–calf system by the life cycle assessment method. Anim. Sci. J. 78:4240-432.

Pitt RE, Van Kessel JS, Fox DG, Pell AN, Barry MC, Van Soest PJ (1996). Prediction of ruminal volatile fatty acids and pH within the net carbohydrate and protein system. J. Anim. Sci. 74:226-244.

Salminen E, Rintala J, Lokshina LYA, Vavilin VA (2000). Anaerobic batch degradation of solid poultry slaughterhouse waste. Water Sci. Technol. 41:33-41.

Smink W, Pellikaan WF, Van der Kolk LJ, Van de, Hoek KW (2004). Methane production as a result from rumen fermentation in cattle calculated by using the IPCC-GPG Tier 2 method. FIS report, FS 04 12 E.

Van Laar H, van Straalen WM (2004). Ontwikkeling van een rantsoen voor melkvee dat de methaanproductie reduceert. Schothorst Feed Reseatrch.

Van Zijderveld S, van Straalen WM (2004). Validatie van de IPCC methaan conversiefactor voorомstandigheden waaronder Nederlands melkvee wordt gehouden. Schothorst Feed Research proefverslag BET. 2004-29. www.robklimaat.nl

Related Journals Published by Academic Journals

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

academicJournals