OPEN ACCESS

African Journal of Biotechnology

December 2019
ISSN 1684-5315
DOI: 10.5897/AJB
www.academicjournals.org

ACADEMIC JOURNALS
expand your knowledge

About AJB

The African Journal of Biotechnology (AJB) is a peer reviewed journal which commenced publication in 2002. AJB publishes articles from all areas of biotechnology including medical and pharmaceutical biotechnology, molecular diagnostics, applied biochemistry, industrial microbiology, molecular biology, bioinformatics, genomics and proteomics, transcriptomics and genome editing, food and agricultural technologies, and metabolic engineering. Manuscripts on economic and ethical issues relating to biotechnology research are also considered.

Indexing

CAB Abstracts, CABI's Global Health Database, Chemical Abstracts (CAS Source Index) Dimensions Database, Google Scholar, Matrix of Information for The Analysis of Journals (MIAR), Microsoft Academic, Research Gate

Open Access Policy

Open Access is a publication model that enables the dissemination of research articles to the global community without restriction through the internet. All articles published under open access can be accessed by anyone with internet connection.

The African Journals of Biotechnology is an Open Access journal. Abstracts and full texts of all articles published in this journal are freely accessible to everyone immediately after publication without any form of restriction.

Article License

All articles published by African Journal of Biotechnology are licensed under the Creative Commons Attribution 4.0 International License. This permits anyone to copy, redistribute, remix, transmit and adapt the work provided the original work and source is appropriately cited. Citation should include the article DOI. The article license is displayed on the abstract page the following statement:

This article is published under the terms of the Creative Commons Attribution License 4.0
Please refer to https://creativecommons.org/licenses/by/4.0/legalcode for details
about Creative Commons Attribution License 4.0

Memberships and Standards

Academic Journals strongly supports the Open Access initiative. Abstracts and full texts of all articles published by Academic Journals are freely accessible to everyone immediately after publication.

Crossref is an association of scholarly publishers that developed Digital Object Identification (DOI) system for the unique identification published materials. Academic Journals is a member of Crossref and uses the DOI system. All articles published by Academic Journals are issued DOI.

Similarity Check powered by iThenticate is an initiative started by CrossRef to help its members actively engage in efforts to prevent scholarly and professional plagiarism. Academic Journals is a member of Similarity Check.

CrossRef Cited-by Linking (formerly Forward Linking) is a service that allows you to discover how your publications are being cited and to incorporate that information into your online publication platform. Academic Journals is a member of CrossRef Cited-by.

Academic Journals is a member of the International Digital Publishing Forum (IDPF). The IDPF is the global trade and standards organization dedicated to the development and promotion of electronic publishing and content consumption.

Contact

Editorial Office: ajb@academicjournals.org

Help Desk: helpdesk@academicjournals.org

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

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

Academic Journals
73023 Victoria Island, Lagos, Nigeria
ICEA Building, 17th Floor,
Kenyatta Avenue, Nairobi, Kenya.

Editor-in-Chief

Editorial Board Members

Table of Content

Vol. 18(32), pp. 1054-1068, December, 2019
DOI: 10.5897/AJB2019.16902
Article Number: 50C61F362436
ISSN: 1684-5315

http://www.academicjournals.org/AJB

ACADEMIC JOURNALS
expand your knowledge

African Journal of Biotechnology

Full Length Research Paper

Effect of ethanolic leaf extract of *Vinca major* L. on biochemical parameters and glucose level of alloxan induced diabetic rats

Monago-Ighorodje Comfort[1], Duru Majesty[2]*, Adindu Eze[3], Nwauche Kelechi[2], Ezekwe Ahamefula[4], Nosiri Ijeoma[5], Odika Prince[6], Onyeabo Chimaraoke[7], Ogar Ishade[8], Berezi Ebitimi Peter[9], Ugoh Austin Ikenna[10], Eboagwu Ijeoma[11] and Otta Emmanuel[2]

[1]Department of Biochemistry, Faculty of Sciences, University of Port Harcourt, Choba, Nigeria.
[2]Department of Chemical Sciences (Biochemistry Unit), College of Basic and Applied Sciences, Rhema University, Aba, Nigeria.
[3]Department of Biochemistry, College of Basic and Medical Sciences, University of Calabar, Calabar, Nigeria.
[4]Department of Medical Biochemistry, Faculty of Basic Medical Sciences, Imo State University, Owerri, Nigeria.
[5]Department of Biochemistry, Faculty of Physical and Biological Sciences, Abia State University, Uturu, Nigeria.
[6]Department of Biochemistry, Faculty of Sciences, Imo State University, Owerri, Nigeria.
[7]Department of Biochemistry, College of Natural Sciences, Michael Okpara University of Agriculture, Umudike, Nigeria.
[8]Department of Physiology, College of Basic and Medical Sciences, University of Calabar, Calabar, Nigeria.
[9]Department of Chemistry, Isaac Jasper Boro College of Education, Sagbgama, Bayelsa State, Nigeria.
[10]Department of Biochemistry, Faculty of Sciences, University of Nigeria, Nsukka, Nigeria.
[11]Department of Food Technology, Federal Institute of Industrial Research Oshodi, Nigeria.

Received 26 June 2019; Accepted 28 August, 2019

In view of World Health Organization (WHO) recommendation on the use of medicinal plants for the management of diabetes mellitus, this study evaluated the effect of ethanolic leaf extract of *Vinca major* L. on biochemical parameters and glucose level of alloxan induced diabetic rats as objective. Sixty-four male Wistar albino rats were induced with diabetes by a single intraperitoneal injection and were separated into four groups (1-4) of sixteen rats each. Group 1 served as the control while groups 2 to 4 served as the test groups. Apart from the control, the test groups were treated with different concentrations of the leaf extract, and four rats from each group were sacrificed every seven days for assessment. The treatment period lasted for 28 days. Haemoglobin (Hb) levels in groups 3 and 4 rats significantly increased ($p<0.05$) against the control throughout the number of days of the study. Red blood cell (RBC) levels in groups 2 and 4 increased significantly ($p<0.05$) against the control on the 14th day, while all the test group rats had significantly increased ($p<0.05$) RBC levels on the 21st and 28th days of the study. The observed trend followed by electrolyte ions, urea and creatinine in test rats against the control in the present study, may be attributed to *V. major* leaf extract trying to salvage the excretory organs of alloxan battered diabetic rats. Glucose level, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) liver enzymes reduced significantly ($p<0.05$) in test rats against the control. Diabetic rats treated with *V. major* leaf extract in this study, showed significant improvements on those associate problems of diabetes which include anaemia, dyslipidaemia, and hepatic necrosis and inflammation. Rats treated with the leaf extract also showed reduced glucose level (Hypoglycemia). From the observations of this study, extract from leaf of *V. major* may be effective against diabetes and some of its associate problems.

Key words: diabetes, biochemical parameters, *Vinca major*, leaf extract, Wistar rats.

INTRODUCTION

Diabetes is among the diseases that plagued the existence of man on this planet Earth. According to Demoz et al., (2015), diabetes is a complex and chronic illness characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. Edem (2009) and Ogbonnia et al., (2008) noted that diabetes is a major degenerative disease in the world today, affecting millions of people and having complications which include hypertension, atherosclerosis and microcirculatory disorders. Demoz et al., (2015) reported that over 3 million deaths worldwide are attributed to diabetes every year. Over 380 million people were reportedly living with diabetes in 2014, and the number is projected to rise by more than double by 2035 without any intervention (Shrivastava et al., 2013). Type 2 diabetes mellitus (T2DM) is among the existing types of diabetic disorder with rapidly increasing incidence in the world (Demoz et al., 2015; Islam et al., 2017). According to Kesari et al., (2007), Type 2 diabetes mellitus accounts for about 90% of diabetic population. Islam et al., (2017) and Bastaki (2005) noted that among all the endocrine disorders, diabetes mellitus is the most prevalent one and almost three hundred million will suffer from the disease by 2025. Ethnopharmacological studies revealed that more than 1200 plants are utilized in traditional medicine for their alleged hypoglycemic activity (Marles and Farnsworth, 1995; Nole et al., 2016; Tsabang et al., 2016). Such plants are known as medicinal plants and are the main source of organic compounds such as polyphenols, tannins, alkaloids, carbohydrates, terpenoids, steroids, flavonoids and etcetera (Marles and Farnsworth, 1995). These compounds are collectively addressed as phytochemicals (Marles and Farnsworth, 1995; Amadi et al., 2012; Duru et al., 2018) and represent a source for the discovery and development of new types of antidiabetic molecules (Firdous, 2014). Nawrot et al. (2003) and Agomuo et al., (2017) noted that natural compounds found in plants or their synthetic forms are the basis of modern pharmacopeia.

Vinca major L. an Apocynaceae also known as periwinkle, is one of such medicinal plants with some of these organic compounds addressed as phytochemicals. It is an evergreen perennial trailing vine plant that roots along its stems to form dense masses of groundcover. It can grow up to 25 cm (10 in) high while spreading indefinitely (Blamey and Grey-Wilson, 1989). The leaves of *V. major* can grow up to 9 cm long and 6 cm broad. Its flowers are hermaphrodite in nature (Blamey and Grey-Wilson, 1989). The plant is found along river banks and grows well in full sun and shade at an altitude between 0 and 800 metres (0–2,625 ft) above sea level (Blamey and Grey-Wilson, 1989). Extract of the plant is used against diseases such as malaria, leukemia and Hodgkin's disease. The leaf juice of *V. major* is used in the treatment of treat wasp stings, and sore throats. The flower extract is used for infants' eyewash. The plant is used in the preparation of periwinkle tea, which is used in the treatment of cough (Blamey and Grey-Wilson, 1989). Due to adverse effects and other factors militating against the use of many synthetic anti-diabetic agents, medicinal plants continue to play very vital role in the management of diabetes mellitus in developing country. It has been reported that plants products that are effective against diabetic disease are cost effective and less toxic than synthesised drugs (Jung et al., 2006; Patel et al., 2012). In view of World Health Organization (WHO) recommendation on the use of medicinal plants for the management of diabetes mellitus, there is need to expand the frontiers of scientific research to discover more plants with anti-diabetic properties, especially now that recent findings have shown a rise in number of new cases of type 2 DM with an earlier onset and associated complications in developing countries. Hence upturning the earlier believe that diabetes is mainly found in developed countries of the world.

Through *in vivo* studies using animal models, a number of plants with anti-diabetic activity have been discovered (Bnouham et al., 2006; Kayarohanam et al., 2015; Mussie et al., 2015; Tsabang et al., 2017), but there is paucity of studies on *V. major* in relation to diabetic activity and other biochemical parameters. The present study investigated the effect of ethanolic leaf extract of *Vinca major* L. on biochemical parameters and glucose level of alloxan induced diabetic rats.

MATERIALS AND METHODS

Plant collection, identification and preparation

Fresh leaves of *V. major* L. were collected from the plant between the months of February and March 2018, in Choba, Rivers State, Nigeria. Taxonomic identification was done and voucher specimen was deposited at the Herbarium unit of Rhema University, Nigeria. The identified leaves were air dried for two weeks and then blended into powder.

Plant extraction and toxicity study

The method as described by Adebayo et al., (2006) was used for preparation of plant extract. The powdered leaves of *V. major* (800 g) were soaked in 8 L of 70% ethanol for four days, after which the extract was filtered using a Whatman no. 1 filter paper and a cotton

*Corresponding author. E-mail: mkcduru@rhemauniversity.edu.ng.

wool. It was further concentrated at 50°C using a rotary evaporator and further concentrated using water bath at 48°C. The weight of the extract obtained was 80 g giving a percentage yield of 10%. The toxicity study (LD_{50}) was carried using the method as described by Lorke (1983), and the toxic dosage (LD_{50}) for the leaf extract was found to be above 6,000 mg/kg.

Experimental design, induction of diabetes and treatment of experimental animals

A total of sixty four male Wistar albino rats were procured from the animal colony of Department of Biochemistry, University of Port Harcourt, Nigeria for the present study. The rats were housed in plastic cages covered with wire mesh with facilities for food and water in them. The water and feeds were given *ad libitium*. The rat feed was a brand of commercial grower freshly obtained from a feed dealer along Abayi road, Aba, Nigeria. After acclimatization period, the experimental rats were made diabetic by Induction with alloxan monohydrate which was prepared by dissolving 10 g in 100 mL sterilized water. By single intraperitoneal injection, diabetes was induced in fasting rats by administration of 150 mg/kg body weight alloxan to each rat. Rats with blood glucose level above 200 mg/dL (after three days of post induction) were considered diabetic and chosen for the study.

The induced diabetic rats were then separated into four (1-4) groups of sixteen rats each. Group 1 served as the control, while groups 2 to 4 served as test groups. The treatments given to the groups are shown below

Group 1(Control): Diabetic rats treated normal saline (0.9% (w/v) NaCl)
Group 2: Diabetic rats treated with 100 mg/kg body weight of extract
Group 3: Diabetic rats treated with 250 mg/kg body weight of the extract
Group 4: Diabetic rats treated with 450 mg/kg body weight of the extract

The extract was given orally daily. The groups were placed on the same feed and water for twenty-eight days. The floors of the cages were constantly cleaned at interval of two days, their feed consumption and body weights were taken at interval of seven days.

Experimental handling of animals was in accordance with international guidelines on animal care and uses (NIH, 1985).

Samples collection for analysis

Four rats from each group were sacrificed weekly (7, 14, 21 and 28 days) after subjecting them to overnight fast. The rats were subsequently anaesthetized with diethyl-ether and blood samples were collected by cardiac puncture into clean tubes for liver enzymes, electrolyte ions, urea, creatinine and glucose studies. Blood samples for haematological indices were collected with anticoagulant tubes. The tubes were properly labelled and used for analysis.

Haematology analysis

The autoanalyzer machine was used for hematological analysis. The blood samples contained in the anticoagulant tubes were swirled/rolled on the blood roller each for five seconds and then opened and put under the probe of the autoanalyzer. The probe then collected the blood from the tubes for about 10 s and entered back into the haematology machine. The result was then printed a few seconds later, giving the parameters haemoglobin (Hb), red blood cells (RBC), white blood cells (WBC), lymphocytes, monocytes, Basophils, mean corpuscular volume (MCV), and mean corpuscular haemoglobin concentration (MCHC). Haematocrit (PCV) level was estimated using microhaematocrit methods as described by Alexander and Griffiths (1993).

Biochemical assays

All the biochemical parameters investigated were measured using BUCK 211 spectrophotometer, England. The following biochemical parameters were carried out in serum; urea was analysed using the Bethlot Searcy's method (Searcy et al., 1967); creatinine was determined by the method described by Larsen (1971). Sodium ion (Na^+) and chloride (Cl^-) ion levels were determined according to the instructions on their diagnostic kits purchased from Randox laboratories (UK). Potassium ion (K^+) was determined by direct spectrophotometric method. Bicarbonate (HCO_3^-) was determined using Forrester et al., (1979) method. Urea estimation was done using Urease-Berthlot method. Creatinine was estimated using the method described by Heinegård and Tiderström (1973). Total cholesterol, high density lipoprotein cholesterol (HDL-cholesterol), and triglyceride lipid profiles were assayed enzymatically with their test kits (Randox Laboratories, England). The relations as described by Friedwald et al. (1972) were used for low density lipoprotein cholesterol (LDL-cholesterol) estimation. Liver enzyme such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were estimated using Reitman and Frankel (1957) methods; alkaline phosphatase (ALP) was carried out using the phenolphthalein monophosphate method (Babson et al., 1966); total bilirubin was calculated using Doumas et al., (1973) method; whereas glucose level was obtained using the enzymatic GOD-PAP method as described by Trinder (1969).

Statistical analysis

Results are presented as mean and standard deviations of triplicate determinations. Group comparisons were done using least significant difference (LSD). Significant difference was established at 5% level as described by Onu and Igwemma (2000). Bars of the same day with different letters of alphabet are statistically significant at $p<0.05$.

RESULTS

Figures 1 to 9 show haematology result of rats given *V. major* leaf extract at different concentrations. From the Figures, Hb ranged from 9.11 to 12.85 g/dl (Figure 1); haematocrit or PCV ranged from 25.80 to 38.00% (Figure 2); RBC ranged from 2.75-5.48×10^9 cell/L (Figure 3); WBC ranged from 2.10-4.53×10^9 cell/L (Figure 4); lymphocytes ranged from 35.10-66.00% (Figure 5); monocytes ranged from 14.10-19.95% (Figure 6); basophil ranged from 1.50-4.68% (Figure 7); mean cell volume (MCV) ranged from 71.70-98.30 fl (Figure 8); and mean corpuscular haemoglobin concentration (MCHC) ranged from 26.60-36.58 pg/dl (Figure 9).

Results of electrolyte ions, urea and creatinine as presented in Figures 10 to 15 show that sodium ion (Na^+) ranged from 71.00 to 107.55 mEq/L (Figure 10); potassium ion (K^+) ranged from 4.11 to 6.36 mEq/L) (Figure 11); Chloride ion (Cl^-) ranged from 60.71 to 92.73 mEq/L (Figure 12), bicarbonate ion (HCO_3^-) ranged from

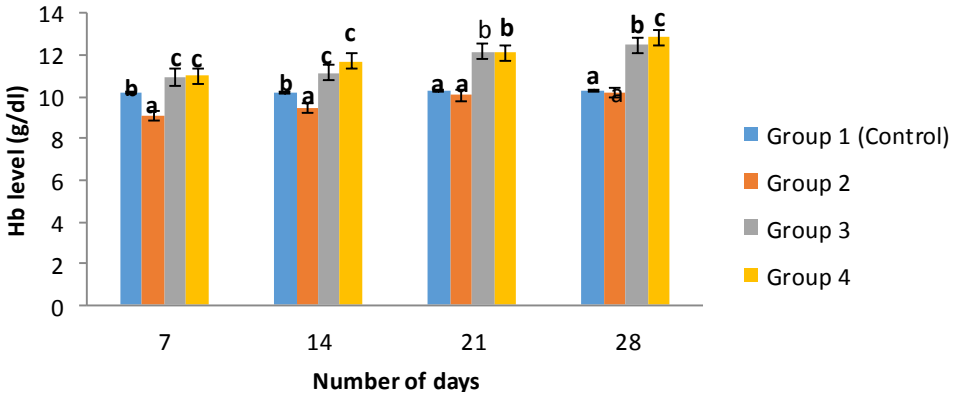

Figure 1. Hb level of the diabetic rats treated with *V. major* leaf extract for 28 days.

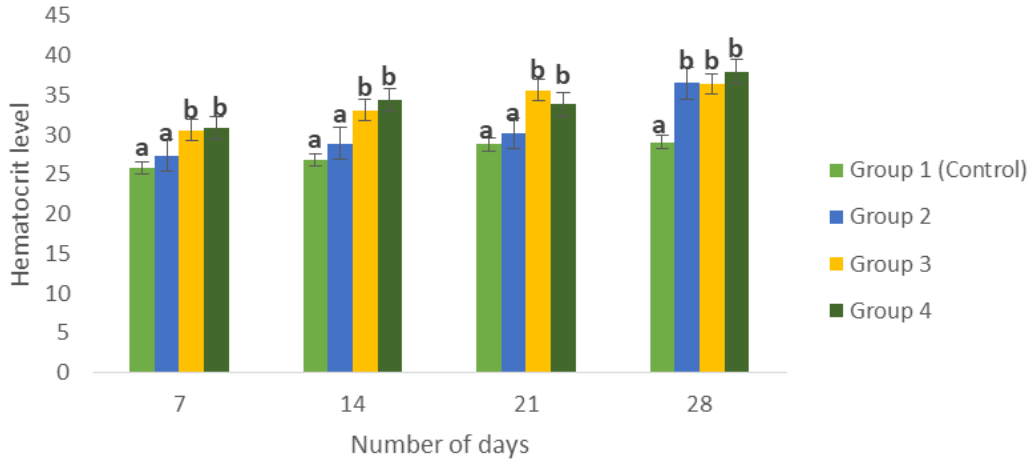

Figure 2. Haematocrit level of diabetic rats treated with *V. major* leaf extract for 28 days.

16.54 to 21.35 mmol/L (Figure 13); urea ranged from 38.12 to 56.56 mg/dL (Figure 14) and creatinine raged from 0.01 to 4.36 mg/dL (Figure 15).

The lipid profiles as presented in Figures 16 to 19 reveal that total cholesterol ranged from 69.67 to 123.10 mg/dL (Figure 16), triglyceride ranged from 98.38 to 147.18 mg/dL (Figure 17); HDL cholesterol ranged from 16.34 to 24.01 mg/dL (Figure 18) and LDL cholesterol ranged from 30.60 to 83.53 mg/dL (Figure 19). Liver enzymes, total bilirubin, protein and glucose levels as presented in Figures 20 to 24 show that AST was between 16.17 to 27.15 U/L (Figure 20); ALT ranged 46.93 to 62.10 U/L (Figure 21); ALP ranged 12.71 to 25.31 U/L (Figure 22); total bilirubin ranged from 0.14 to 0.25 mg/dl (Figure 23); and glucose ranged from 224.76 to 306.37 mg/dl (Figure 24).

DISCUSSION

Assessment of haematological parameters can be used to determine the extent of deleterious effect of foreign compounds in the body (Mohammed et al., 2009; Duru et al., 2012b; Duru et al., 2018). Both plant extracts and free radical from alloxan, on the blood constituents of an animal are amongst the foreign compounds (Mohammed et al., 2009). According to Lebovitz (1994) and Andreoli et al., (1990), blood relating functions are amongst the aberration of diabetes mellitus. Haemoglobin (Hb) levels in groups 3 and 4 rats significantly increased ($p<0.05$) against the control throughout the number of days of the study. However, Hb in group 2 rats reduced significantly ($p<0.05$) when compared to the control (Group 1) on the 7th and 14th days, but increased insignificantly ($p>0.05$) against the control on the 21st and 28th days of the study (Figure 1). Sheela and Augusti (1992) noted that diabetic rats form glyscosylated haemoglobin in results in decreased total haemoglobin. The increase in Hb levels of test rats in the present study could be attributed to the ability of *V. major* leaf extract to induce Hb production in diabetic condition. Haematocrit levels in rats of test groups 3 and 4 increased significantly ($p<0.05$) when compared to the control (Group 1). Haematocrit in group 2 increased insignificantly ($p>0.05$) in test group 2 rats against the control. However, the observed increase

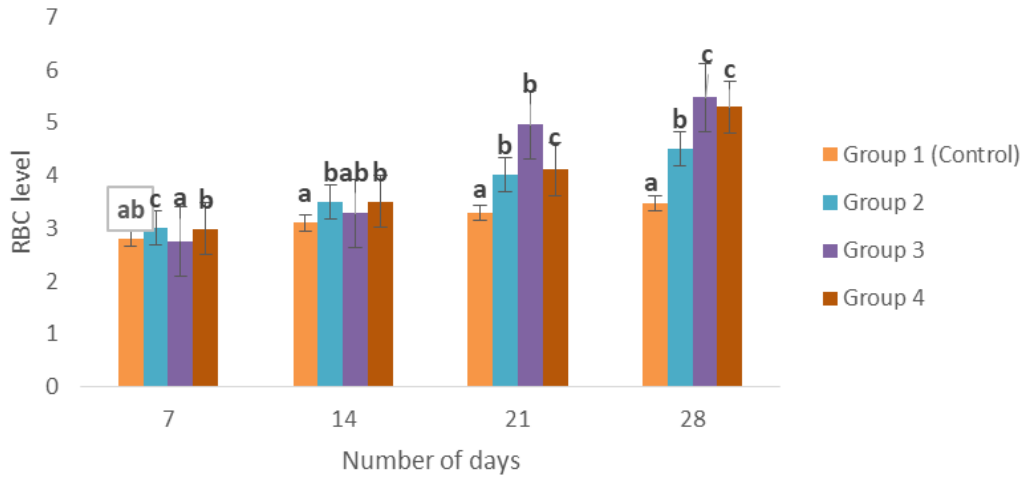

Figure 3. RBC level of diabetic rats treated with *V. major* leaf extract for 28 days.

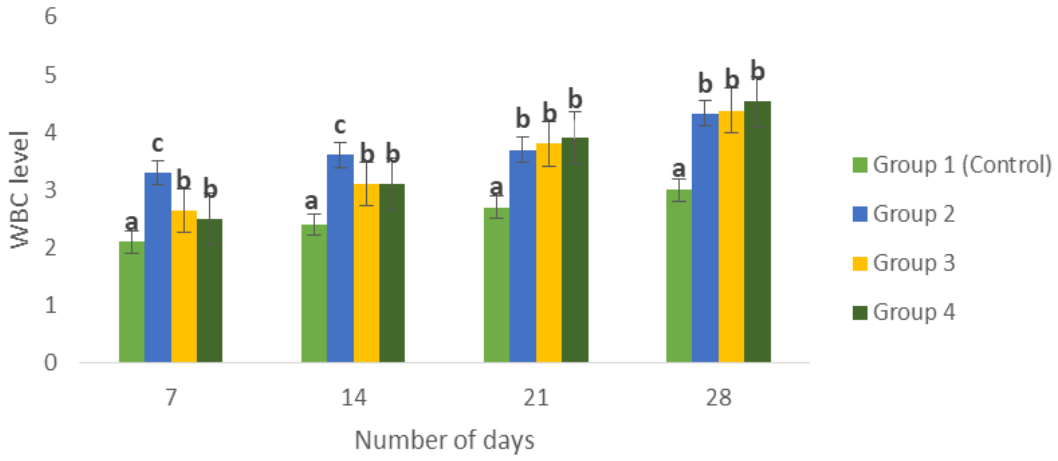

Figure 4. WBC level of diabetic rats treated with *V. major* leaf extract for 28 days.

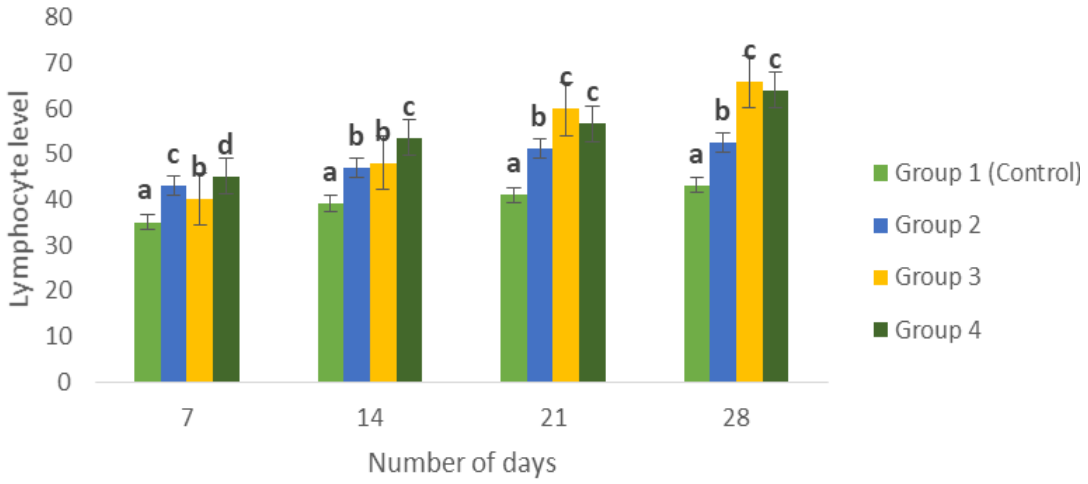

Figure 5. Lymphocyte level of diabetic rats treated with *V. major* leaf extract for 28 days.

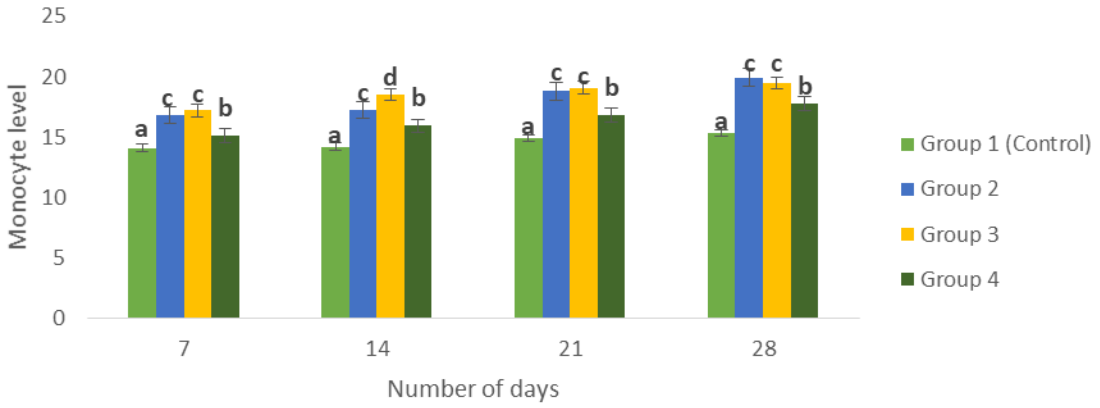

Figure 6. Monocyte level of diabetic rats treated with *V. major* leaf extract for 28 days.

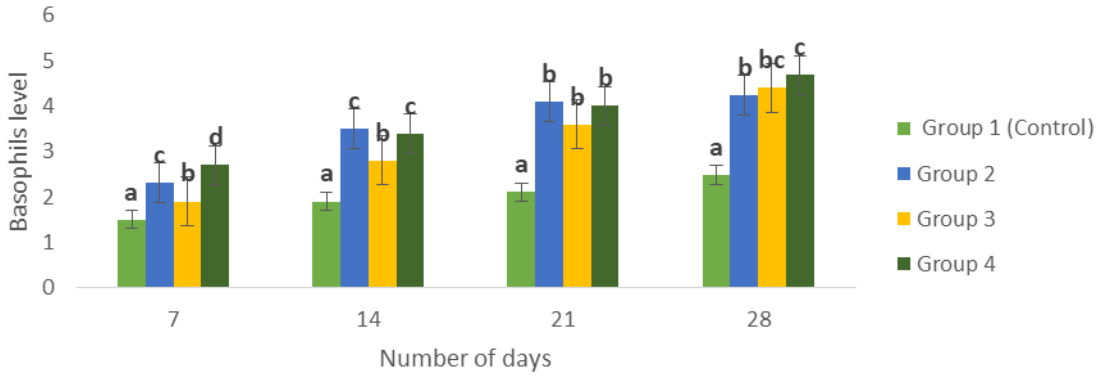

Figure 7. Basophils level of diabetic rats treated with *V. major* leaf extract for 28 days.

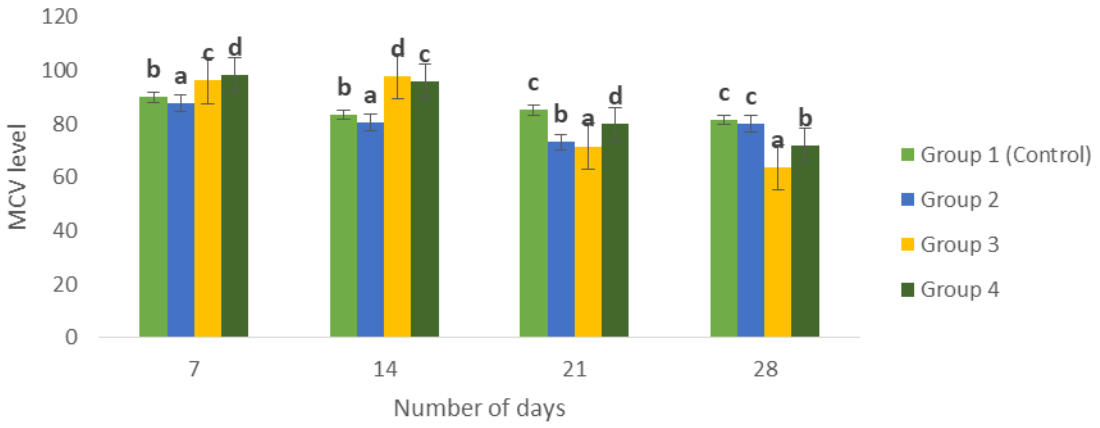

Figure 8. Mean cell volume (MCV) level of diabetic rats treated with *V. major* leaf extract for 28 days.

became significant ($p<0.05$) on the 28th day of the study (Figure 2). Saliu et al., (2012) attributed decrease in haematocrit in diabetic condition to cellular damage on the erythrocyte membrane as a result of oxidative stress by agent of induction. Haematocrit measures the percentage by volume of packed red blood cells (RBCs) in a whole blood sample after centrifugation. Only group 2 rats had significantly increased ($p < 0.05$) RBC level

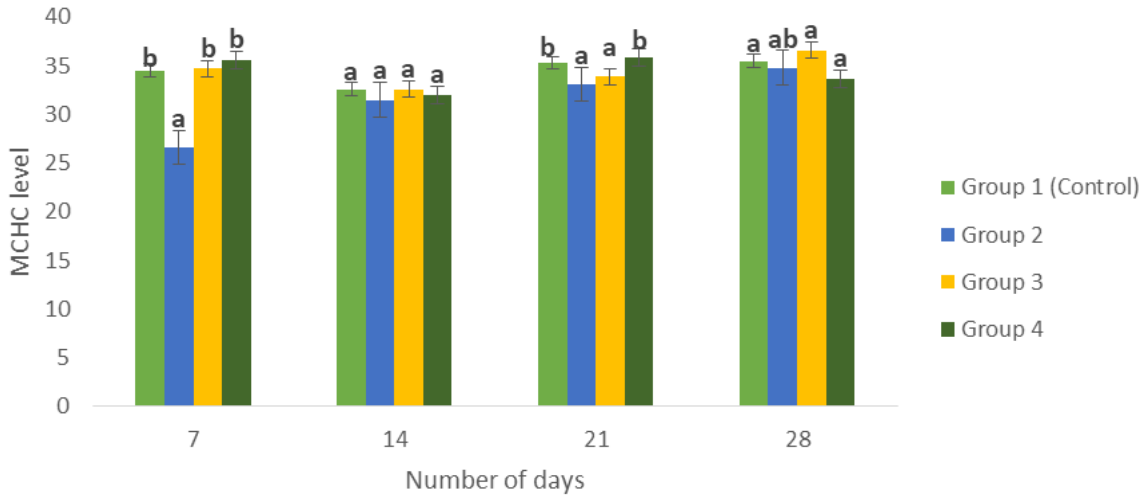

Figure 9. Mean corpuscular haemoglobin concentration (MCHC) level of diabetic rats treated *V. major* leaf extract for 28 days.

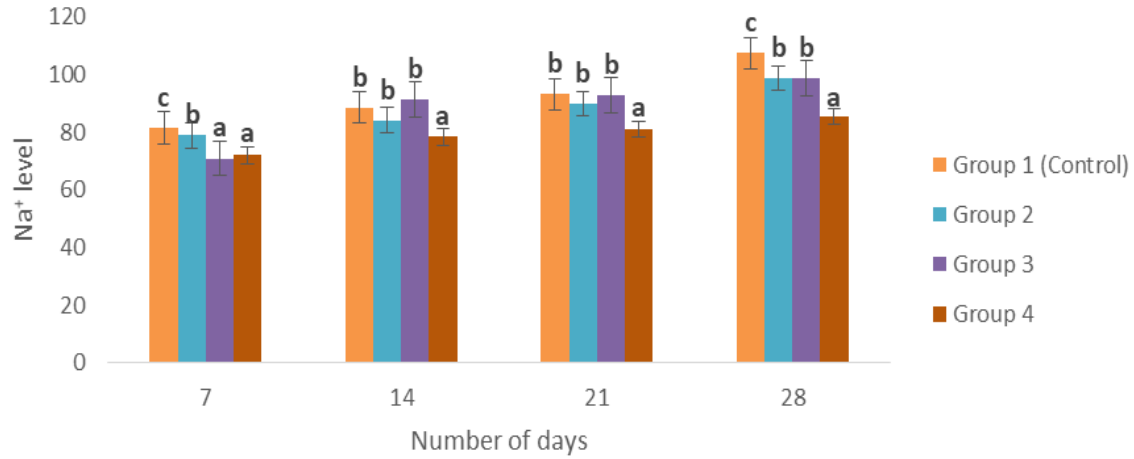

Figure 10. Na^+ level of the rats treated with *V. major* leaf extract for 28 days.

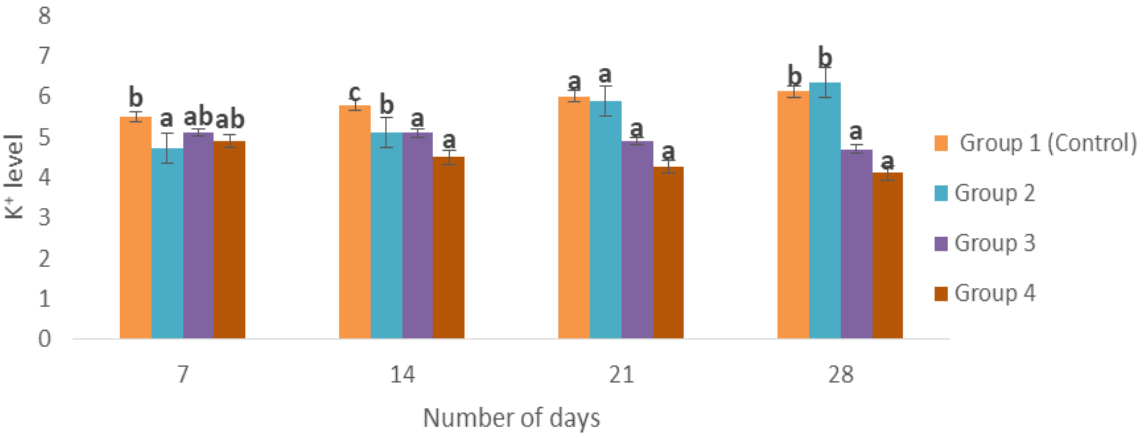

Figure 11. K^+ level of diabetic rats treated with *V. major* leaf extract for 28 days.

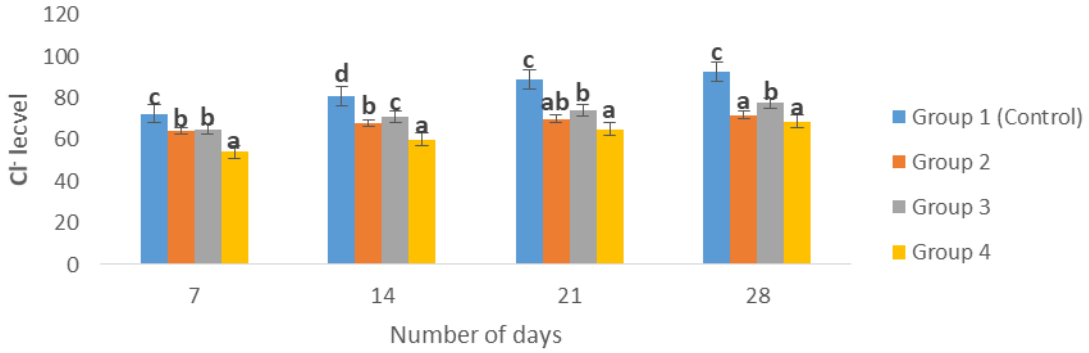

Figure 12. Cl^+ level of diabetic rats treated with *V. major* leaf extract for 28 days.

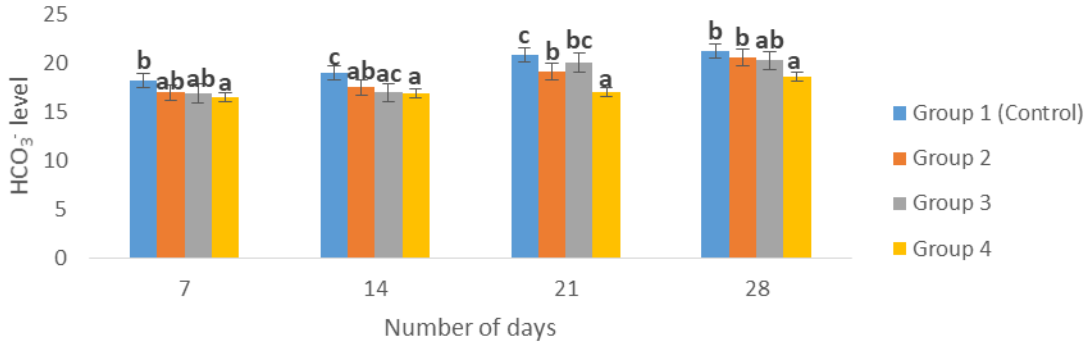

Figure 13. HCO_3^- (mmol/L) of the rats treated with *V. major* leaf extract for 28 days.

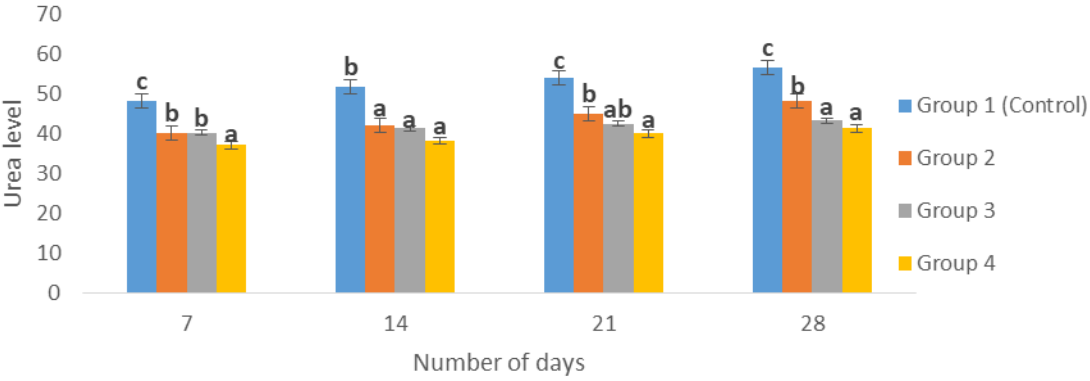

Figure 14. Urea level of diabetic rats treated with *V. major leaf extract* for 28 days.

when compared to the control on the 7th day. RBC levels in groups 2 and 4 increased significantly ($p<0.05$) against the control on the 14th day, while all the test group rats had significantly increased ($p<0.05$) RBC levels on the 21st and 28th days of the study (Figure 3). According to Rao et al., (2003), reactive O_2 species generated during alloxan metabolism is implicated in red cell damage. The increase in RBC levels of test rats could give credence to the increased haematocrit, which may be linked to the influence of *V. major* leaf extract on Hb production in diabetic condition. Edet et al., (2011) noted that alloxan diabetogenesis may cause perturbation in the bone marrow stem cells. This observation was experienced in the present study where the level of WBC in all the test rats significantly increased ($p<0.05$) when compared to the control throughout the study (Figure 4). White blood cell differentials are indicators of the ability of an organism to eliminate infection (Duru et al., 2012a, b;

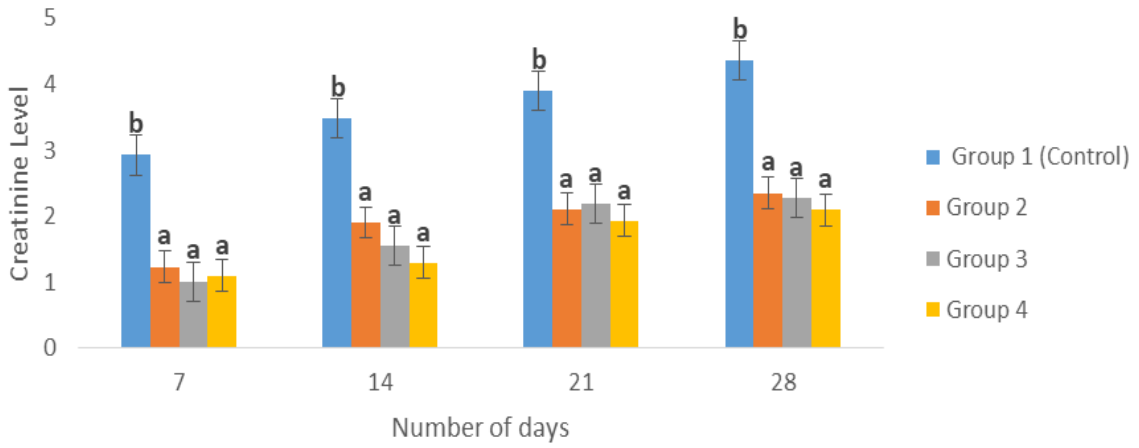

Figure 15. Creatinine level of diabetic rats treated with *V. major* leaf extract for 28 days.

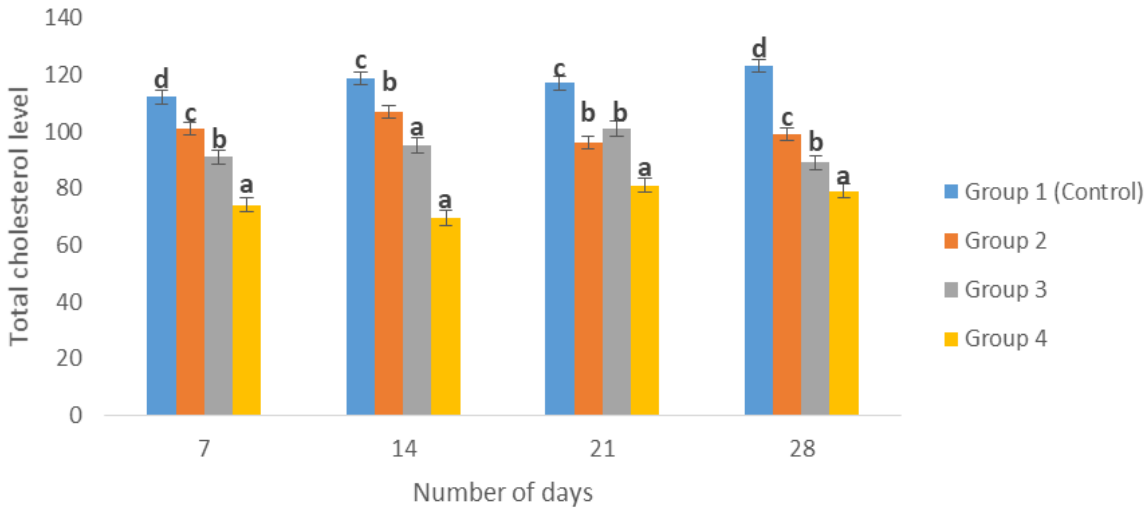

Figure 16. Total cholesterol level of diabetic rats treated with *V. major* leaf extract for 28 days.

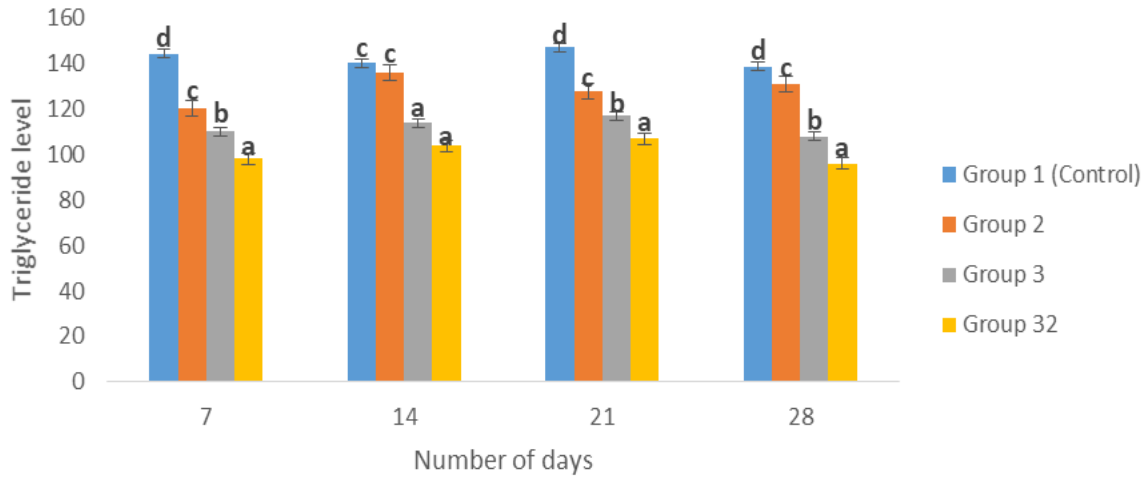

Figure 17. Triglyceride level of diabetic rats treated with *V. major* leaf extract for 28 days.

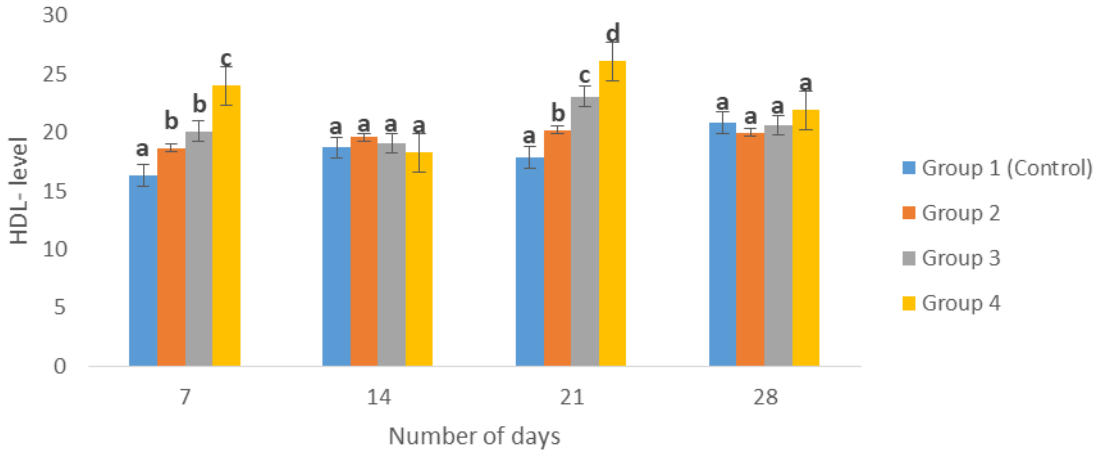

Figure 18. HDL-cholesterol level of diabetic rats treated with *V. major* leaf extract for 28 days.

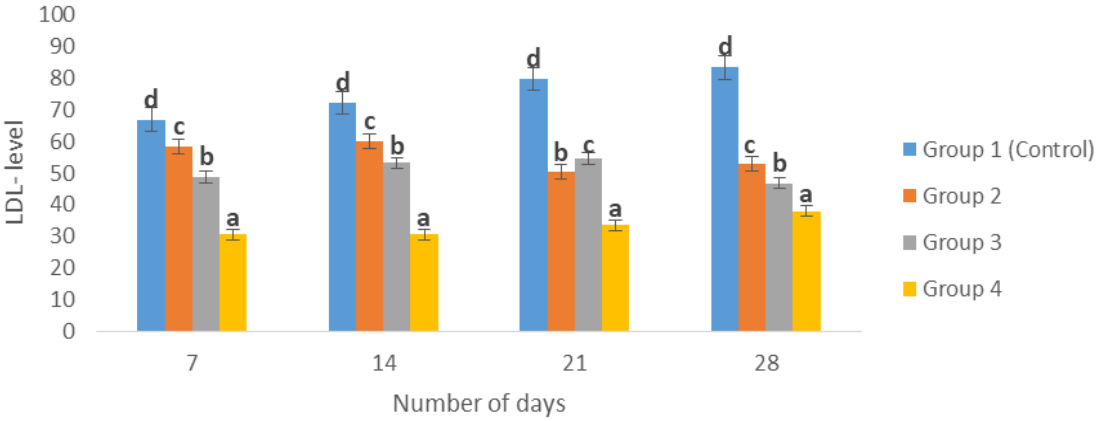

Figure 19. LDL-cholesterol level of diabetic rats treated *V. major* leaf extract for 28 day.

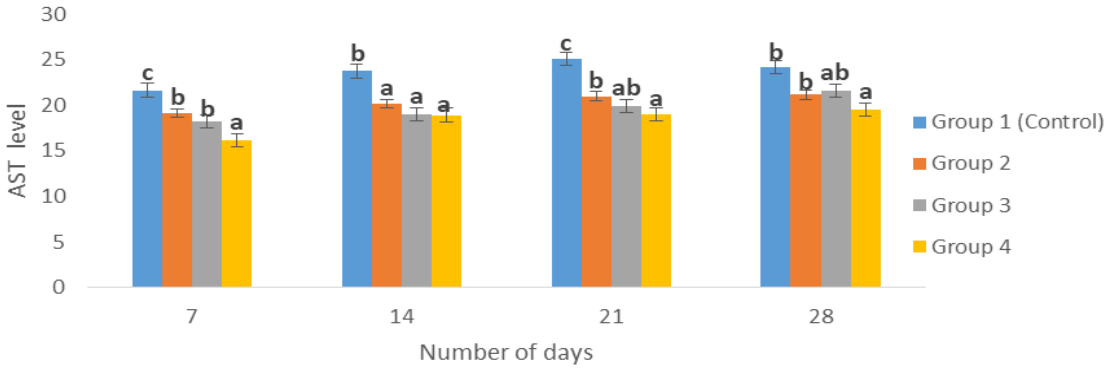

Figure 20. AST level of diabetic rats treated with *V. major* leaf extract for 28 days.

Amadi et al., 2013; Ugbogu et al., 2016). Levels of lymphocytes, monocytes and basophils increased significantly ($p<0.05$) in test rats when compared to the control (Figure 5 to 7). The observed increase in WBC differential could be indication of improved immune defence in test rats, and could be attributed to the

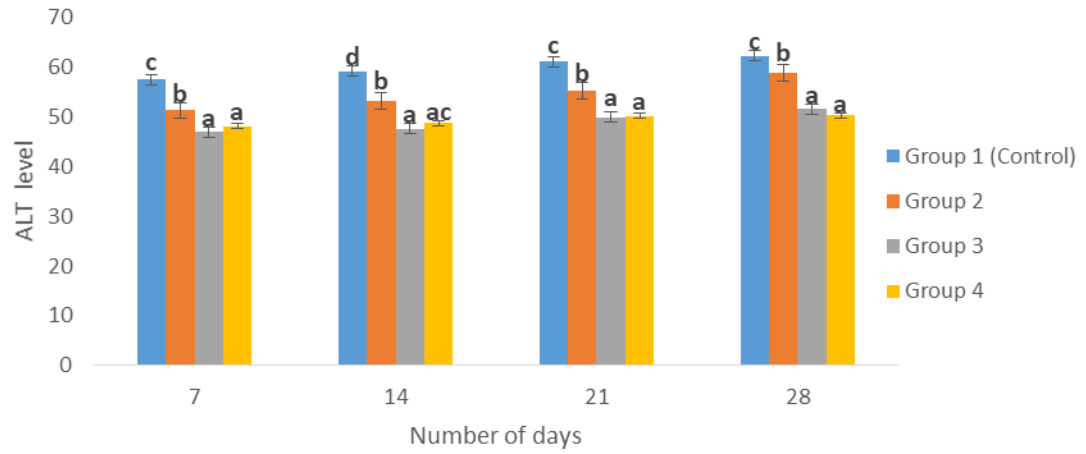

Figure 21. ALT level of diabetic rats treated with *V. major* leaf extract for 28 days.

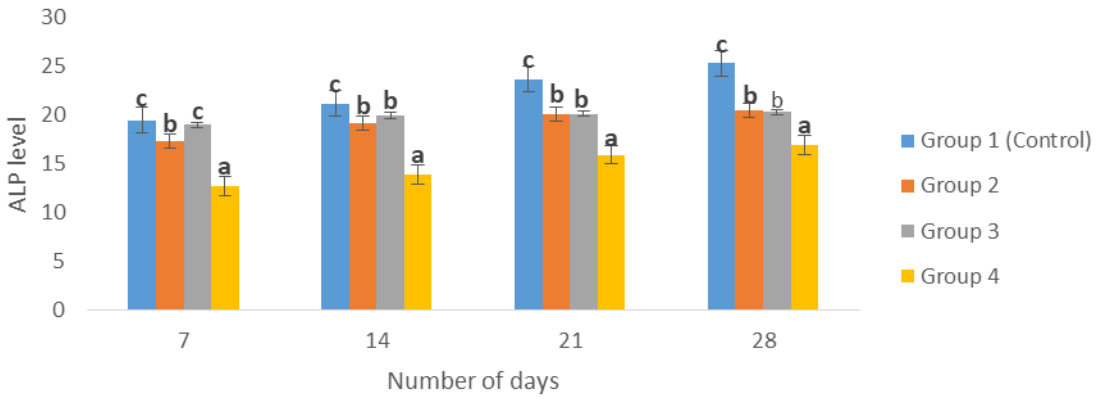

Figure 22. ALP level of diabetic rats after treated with *V. major* leaf extract for 28 days.

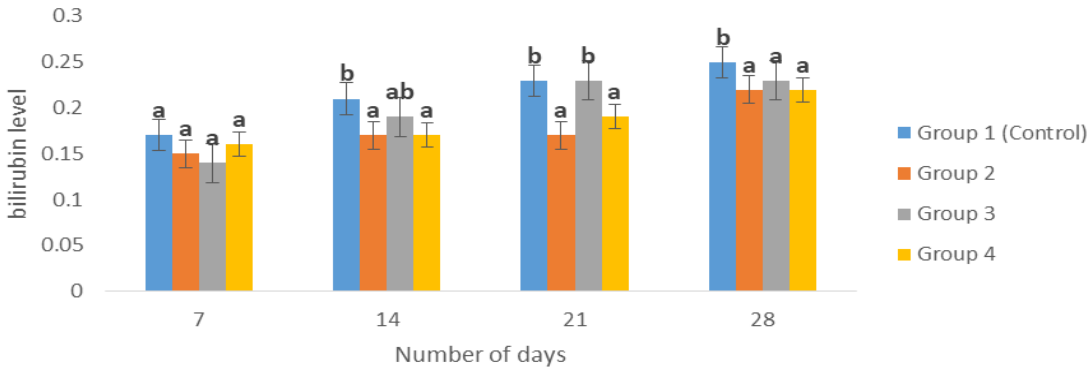

Figure 23. Total bilirubin level of diabetic rats treated with *V. major* leaf extract for 28 days.

presence *V. major* leaf extract. Mean cell volume (MCV) and Mean corpuscular heamoglobin concentration (MCHC) are related to individual red blood cells (Adebayo et al., 2005, 2010, Duru et al., 2018). MCV level of test rats in groups 3 and 4 increased significantly ($p<0.05$) against the control on the 7th and 14th days. MCV of group 2 rats reduced significantly ($p<0.05$) when compared to the control on the 7th and 14th days

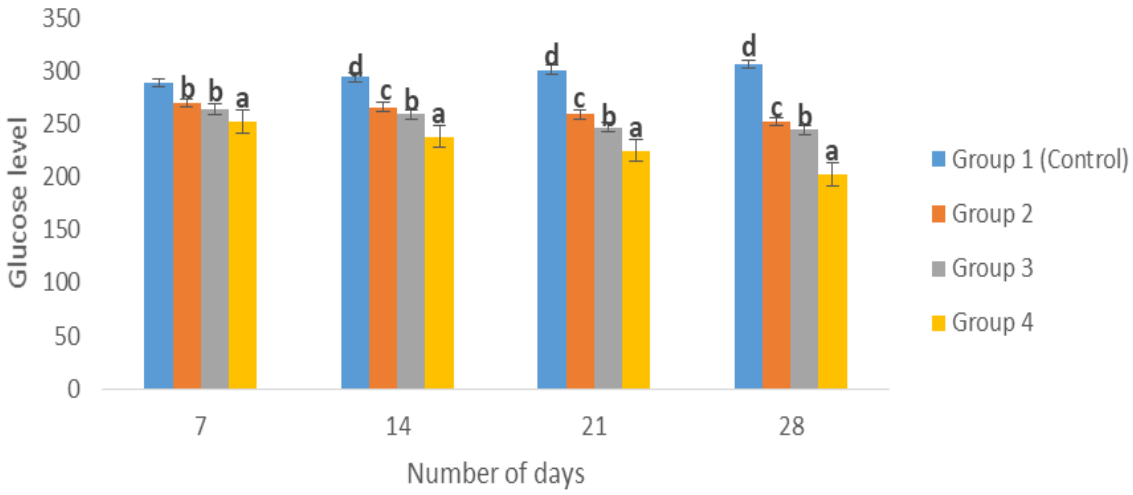

Figure 24. Glucose level of diabetic rats treated with *V. major* leaf extract for 28 days.

(Figure 8). The trend however reversed on the 21st and 28th days of the study. All the test rats had significantly reduced ($p<0.05$) MCV level on the 21st day while only test groups 3 and 4 rats reduced significantly ($p<0.05$) against the control on the 28th day (Figure 8). MCHC increased insignificantly ($p>0.05$) in rats test groups 3 and 4 on the 7th day against the control. MCHC was also insignificantly affected ($p>0.05$) in all the test rats against the control on the 14th and 28th days of the present study (Figure 9). It has been noted that several haematological changes affecting the red blood cells (RBCs), white blood cells (WBCs) and the coagulation factors are directly associated with diabetes mellitus (Wong and Lin, 1998; Bunza and Dallatu, 2017). Mansi and Lahham (2008) revealed that various hematological parameters and the immune system were reported to be altered during the course of diabetes. Hence, all the observed improvements on haematological parameters of the test rats against the control could be attributed the treatment with *V. major* leaf extract.

Electrolyte ions, urea and creatinine are indices for evaluation of excretory organ (Duru et al., 2012b, 2013). Pecoits-Filho et al., (2016) noted that diabetic kidney disease is one of the most frequent and dangerous complications of diabetes mellitus. High level of blood sugar, genetics and blood pressure are among the factors that facilitate renal organ disease in diabetic condition. Na^+ reduced significantly ($p<0.05$) in test rats against the control on the 7th and 28th days; while on the 14th and 21st days, only rats in test group 4 had insignificant reduction when compared to the control (Figure 10). K^+ in test group 2 rats reduced significantly ($p<0.05$) against the control on the 7th day; all the test group rats had significantly reduced K^+ when compared to the control on the 14th day (Figure 11); whereas only test groups 3 and 4 rats had significantly ($p<0.05$) reduced K^+ against the control on the 21st and 28 days of the study (Figure 11). Cl^- in the test rats reduced ($p<0.05$) significant against the control (Figure 12). HCO_3^- significantly reduced ($p<0.05$) in test group 4 rats on the 7th and 28 days against the control (Figure 13); those of test group 2 and 4 reduced significantly ($p<0.05$) on the 4th day against the control; on the 21st day, all the test rats had significantly reduced ($p<0.05$) HCO_3^- when compared to the control (Figure 13). Urea and creatinine are both important parameters of renal function. The levels of urea and creatinine in test rats reduced significantly ($p<0.05$) against the control (Figures 14 and 15). The observed trend followed by electrolyte ions, urea and creatinine in test rats against the control in the present study, may be attributed to *V. major* leaf extract trying to salvage the excretory organs of alloxan battered diabetic rats.

Diabetic dyslipidaemia is a common experience in diabetic condition. It is normally the aetiology of premature coronary heart disease and atherosclerosis. There is increasing evidence that dyslipidaemia in diabetes is associated with increased risk of cardiovascular disease, which is the leading cause of death in patients with type 2 diabetes (Ronald, 2004). Levels of total cholesterol (Figure 16), triglyceride (Figure 17), and LDL-cholesterol (Figure 19) reduced ($p<0.05$) significantly in test rats when compared to the control for the number days of study. Cholesterol is needed by the body to maintain healthy cell status (Njoku et al., 2017). However, its high level in the body leads to coronary artery disease (Njoku et al., 2017). Triglyceride is a type of fat found in the blood and its high levels are related to higher risk of heart and blood vessel disease (Duru et al., 2014). Decrease in LDL-cholesterol, the bad cholesterol has been linked to reduced risk of coronary heart diseases (Glew, 2006; Shen, 2007; Duru et al., 2017); while increase in high levels of HDL-cholesterol are linked to a reduced risk of heart and blood vessel disease

(Shen, 2007). HDL-cholesterol of diabetic treated rats increased significantly ($p<0.05$) against the control on the 7^{th} and 21 days of the study (Figure 18). The observations made on lipid profile parameters in this study could be indication of the salvage power of *V. major* leaf extract on diabetic dyslipidaemia.

Different authors have noted that hepatic dysfunction is among the abnormalities of diabetic condition (Yakhchalian et al., 2018). There are increasing evidence on occurrence of hepatic necrosis and inflammation induced by diabetes mellitus pathogenesis (West, 2000; Kyle et al., 2002). Inflammation and liver injuries that lead to damage of hepatocytes are also ascribed to agent of diabetic induction in experimental animals such as alloxan and streptozotocin (Jacobs et al., 2004; Zafar et al., 2009; Pagana and Pagana, 2013). AST, ALT and ALP liver enzymes reduced significantly ($p<0.05$) in test rats against the control (Figures 20 to 22). Total bilirubin reduced insignificantly ($p>0.05$) in test rats against the control on the 7^{th} day, the observed reduction became significant ($p<0.05$) in test groups 2 and 4 rats on the 14^{th} and 21^{st} days whereas the significant reduction in total bilirubin manifested in all the test rats on the 28^{th} day (Figure 23). Level of glucose in test rats reduced significantly $p<0.05$) when compared to the control (Figure 24). Many studies have reported that single diabetogenic dose of alloxan or streptozotocin showed an increase in glucose, ALT and AST levels (Zafar et al., 2009, Zafar and Naqvi 2010; Ahmed et al., 2012). The observed reduction in AST, ALT, ALP and glucose in test rats in this could be attributed to the ability of *V. major* leaf extract to improve on the hepatic necrosis and inflammable associated with diabetic condition.

Conclusion

Diabetic rats treated with *V. major* leaf extract in this study, showed significant improvements on those associate problems of diabetes which include anaemia, dyslipidaemia, and hepatic necrosis and inflammation. Rats treated with the leaf extract also showed reduced glucose level (Hypoglycemia). From the observations of this study, extract from leaf of *V. major* may be effective against diabetes and some of its associate problems. There is need to urgently extend the scope of the present study to accommodate the isolation of the active ingredients or compounds that could be responsible for these actions in *V. major* leaf.

CONFLICT OF INTERESTS

The Authors declare no conflict of interests and are all aware that the article has been submitted to African Journal of Biotechnology. The Authors also declare that extra authors may be added as the case maybe.

ACKNOWLEDGMENTS

We acknowledgement the management of Links Laboratories, Owerri, Nigeria, and Chief Laboratory Technologist of Rhema University, Nigeria for their input during the bench work of this research.

REFERENCES

Adebayo AH, Abolaji AO, Opata TK, Adegbenro IK (2010). Effects of ethanolic leaf extract of *Chrysophyllum albidum* G. on biochemical and haematological parameters of albino wistar rats. African Journal of Biotechnology9(14):2145-2150.

Adebayo AH, Aliyu R, Gatsing D, Garba IH (2006). The effects of ethanolic leaf extract of *Commiphora africana* (Burseraceae) on lipid profile in rats. International Journal of Pharmacology 2(6):618-622.

Adebayo JO, Adesokan AA, Olatunji LA, Buoro DO, Soladoye AO (2005). Effect of ethanolic extract of *Bougainvillea spectabilis* leaves on haematological and serum lipid variables in rats. BIOKEMISTRI 17(1):45-50.

Agomuo E, Duru M, Amadi B, Amadi P, Ugwokaegbe P (2017). Effect of Caffeine on Some Selected Biochemical Parameters Using Rat Model. https://doi.org/10.1155/2017/9303276

Ahmed O, Mahmoud A, Abdel-Moneim A, Ashour M (2012). Antidiabetic effects of hesperidin and naringin in type 2 diabetic rats. Diabetologia Croatica 41(2):53-67.

Amadi BA, Agomuo EN, Duru MKC (2013). Toxicological studies of *Asmina triloba* leaves on haematology, liver, kidney using rat model. International Science Research Journal 4(2):11-17.

Amadi BA, Duru MKC, Agomuo EN (2012). Chemical profiles of leaf, steam, and flower of *Ageratum conyzoides*. Asian Journal of Plant Science and Research 2(4):428-432.

Alexander RR, Griffths JM (1993). Haematocrit in Basic Biochemical Methods, John Wiley & Sons, New York, NY, USA, 2nd Edition.

Andreoli TE, Carpenter CC, Plum F, Smith LH (1990). Diabetes mellitus. In: Cecil Esssential of Medicine, Dyson, J. (Ed.). 2nd Edn. W. B. Saunders, Philadelphia, PA., USA pp. 496-505.

Babson LA, Greeley SJ, Coleman CM, Philips GD (1966). Serum alkaline phosphatase determination. Clinical Chemistry 12:482-490.

Bastaki S (2005). Review: Diabetes mellitus and its treatment. International Journal of Diabetes and Metabolism. 13(3):111-134

Blamey M, Grey-Wilson C (1989). Flora of Britain and Northern Europe. Hodder and Stoughton.

Bnouham M, Ziyyat A, Mekhfi H, Tahri A, Legssyer A (2006). Medicinal plants with potential anti-diabetic activity- A review of ten years of herbal research (1990-2000). International Journal of Diabetes and Metabolism 14(1):1-25.

Bunza FU, Dallatus MK (2017). Hematological indices in alloxan-Induced diabetic rats: effect of supplementation with the antioxidant dimethyl sulfoxide. Asian Journal of Medicine and Health 8(1):1-7.

Demoz MS, Gachoki KP, Mungai KJ, Negusse BG (2015). Evaluation of the anti-diabetic potential of the methanol extracts of *Aloe camperi*, *Meriandra dianthera* and a Polyherb. Journal of Diabetes Mellitus 5(4):267-276

Doumas BT, Perry BW, Sasse EA, Straumfjord JV (1973). Standardization in bilirubin assays: evaluation of selected methods and stability of bilirubin solutions. Clinical Chemistry 19(9):984-93.

Duru M, Amadi B, Amadike U, Adindu E (2014). Effect of “udu”, an antimalarial herbal preparation on visceral organ weight and blood lipid profiles in wistar rats. JPC 8:1-7. www.arpapress.com/Volumes/JPCS/Vol8/JPCS_8_01.pdf

Duru MKC, Agomuo EN, Amadi BA (2012b). Biochemical studies on “udu” an antimalarial concoction used in Umunchi village, Isiala Mbano L.G.A of Imo State, Nigeria. Continental Journal of Pharmacology and Toxicology Research 5(2): 28-34.

Duru MKC, Amadi BA, Amadi CT, Lele KC, Anudike JC, Chima-Ezika OR, Osuocha K (2012a). Toxic effect of *Carica papaya* bark on body weight, haematology, and some biochemical parameters. Biokemistri 24(2):67-71.

Duru M, Nwadike C, Ozougwu J, Eboagwu I (2017). Bioactive constituents of *Pleurotus squarrosulus* (Mont.) singer and effect of its dietary incorporation on body/organ weights and lipid profile levels of rats placed on high cholesterol diet. Academic Journal Chemistry 2(4):28-37.

Duru M, Constance N, Ahamefula E, Caleb N, Ijeoma E, Odika P, Samuel N, Chieme C (2018). Evaluation of nutritional, anti-nutritional and some biochemical studies on *Pleurotus sqarrosulus* (Mont) singer using rats. African Journal of Biochemistry Research 12(20):7-27.

Duru M, Amadike U, Amadi B (2013). Effect of *Solanum macrocarpon* fruit on haematology, hepatic and renal function. Advances in Biochemistry 1(2):28-32.

Edem OD (2009). Hypoglycemic effects of ethanolic extract of alligator pear seed (*Persea americana* Mill) in rats. European Journal of Scientific Research 33(4):669-678.

Edet EE, Akpanabiatu MI, Uboh FE, Edet TE, Eno AE, Itam EH, Umoh IB (2011). *Gongronema latifolium* crude leaf extract reverses alterations in hematological indices and weight loss in diabetic rats. Journal of Pharmacology and Toxicology 6(2):174-181.

Firdous SM (2014). Phytochemicals for treatment of diabetes EXCLI Journal 13:451-453.

Forrester RL, Watafe LJ, Silverman DA, Pierre KJ (1979). Enzymatic method for the determination of CO2 in serum. Clinical Chemistry 22(2):243–245.

Friedwald TW, Federickson DS, Levy RJ (1972). LDL cholesterol estimation. Clinical Chemistry 18:499-501.

Glew RH (2006). Lipid metabolism II: Pathways of metabolism of special lipids. In: Devlin TM (ed) Textbook of biochemistry with clinical correlations, 6th edn. Wiley Liss, New Jersey pp. 695-741.

Heinegård D, Tiderström G (1973). Determination of serum creatinine by a direct colorimetric method. Clinica Chimica Acta. 43(3):305–310.

Islam D, Huque A, Mohanta LC, Lipy EP, Rahman MN, Sultana SA, Prodhan UK (2017). Studies on the hypoglycemic and hypolipidemic effects of *Nelumbo nucifera* leaf in long-evans rats. Journal of Diabetes Mellitus 7(3):55-70.

Jacobs SD, Demott RW, Oxley KD (2004). Laboratory test handbook. 3ed; Lexi comp.

Jung M, Park M, Lee HC, Kang YH, Kang ES, Kim SK (2006). Anti-Diabetic Agents from Medicinal Plants. Current Medicinal Chemistry 13(10):1203-1218.

Kayarohanam S, Kavimani S (2015). Current Trends of Plants Having Antidiabetic Activity: A Review. Journal of Bioanalysis & Biomedicine 7(2):55-65.

Kesari AN, Kesari S, Singh SK, Gupta RK, Watal G (2007). Studies on the glycemic and lipidemic effect of *Murraya koenigii* in experimental animals. Journal of Ethnopharmacology 112(2):305-311.

Kyle R, Katzmann J, Lust J, Dispenzieri A (2002). Clinical indications and applications of electrophoresis and immunofixation. In Manual of Clinical Laboratory Immunology. 6ed Washington DC; ASM Press.

Larsen K (1971). Creatinine assay by a reaction-kinetic principle. Clinica Chimica Acta 41:209-217.

Lebovitz HE (1994). Oral anti-diabetic agents. In: Joslins diabetes mellitus, Khan, C.R. and G.C. Weir (Eds.). 13th Edn. Lea and Febiger, Philadelphia, PA., USA pp. 508-524.

Lorke D (1983). A new approach to practical acute toxicity test. Institute Fur Toxicologie, Bayer AG, Friedrich – Ebert- Strasse; 217, D-5600.

Mansi K, Lahham J (2008). Effects of Artemisia sieberi Besser (a. herba-alba) on heart rate and some hematological values in normal and alloxan-induced diabetic rats. Australian Journal of Basic and Applied Sciences 4(2):57-62.

Marles RJ, Farnsworth NR (1995). Antidiabetic plants and their active constituents. Phytomedicine 2(2):137-189.

Mohammed A, Adelaiye AB, Bakari AG, Mabrouk MA (2009). Antidiabetic and some hematological effects of ethyl acetate and n-butanol fractions of *Ganoderma lucidum* aqueous extract in alloxan-induced diabetic wistar rats. International Journal of Medicine and Medical Sciences 1(12):530-535.

Mussie SD, Kareru PG, Keriko JM, Berhane GN (2015). Ethnobotanical survey and preliminary phytochemical studies of plants traditionally used for diabetes in Eritrea. European Journal of Medicinal Plants 9(2):1-11.

National Institute of Health (1985). Guide for the care and use of laboratory animals. U.S. Department of health education and welfare. Washington, D.C.: NIH Publication.

Nawrot P, Jordan S, Eastwood J, Rotstein J, Hugenholtz A, Feeley M (2003). Effects of caffeine on human health. Food Additives and Contaminants 20(1):1-30.

Njoku S, Duru M, Akubugwo E, Ozougwu J, Nwadike C (2017). The influence of cigarette smoking and moderate beer consumption on selected biochemical indices in male volunteers. Journal of Biology and Nature 7(4):177-189.

Nole T, Lionel TDW, Cedrix TFS, Gabriel AA (2016). Ethnomedical and Ethnopharmacological Study of Plants Used For Potential Treatments of Diabetes and Arterial Hypertension by Indigenous People in Three Phytogeographic Regions of Cameroon. Diabetes Case Reports 1(2):1-9. doi: 10.4172/2572-5629.1000110.

Ogbonnia SO, Odimegwu JI, Enwuru VN (2008). Evaluation of hypoglycemic and hypolipidemic effects of ethanolic extract of *Treculia Africana* Decne and *Bryophyllum pinnatum* Lam. and their mixture on streptozotocin (STZ)-induced diabetic ratsAfrican Journal of Biotechnology 7(15):2535-2539.

Onu M, Igwemma AA (2000). Applied statistical techniques for business and basic sciences, Skillmark Media, 2nd edition.

Pagana K, Pagana T (2013). Mosby`s manual of diagnostic and laboratory tests. Elsevier Health Sciences.

Patel DK, Prasad SK, Kumar R, Hemalatha S (2012). An overview on anti-diabetic medicinal plants having insulin mimetic property. Asian Pacific Journal of Tropical Biomedicine 2(4):320-330.

Rao G, Kamath U, Raghothama C, Pradeep KS, Rao P (2003). Maternal and fetal indicators of oxidative stress in various obstetric complications. Indian Journal of Clinical Biochemistry 18(2):80-86.

Reitman S, Frankel S (1957). A Colorimetric Method for the Determination of Serum Glutamic Oxalacetic and Glutamic Pyruvic Transaminases. American Journal of Clinical Pathology 28(1):56-63.

Ronald MK (2004). Lipids and lipoproteins in patients with type 2 diabetes. Daibetes Care 27(6):1496-1504.

Pecoits-Filho R, Abensur H, Betônico CCR, Machado AD, Parente EB, Queiroz M, Salles JEN, Titan S, Vencio S (2016). Interactions between kidney disease and diabetes: dangerous liaisons. Diabetology & Metabolic Syndrome 8:50.

Searcy RL, Reardon JE, Foreman JA (1967). A new photometric method for serum urea nitrogen determination. American Journal of Medical Technology 33(1):15-20.

Saliu JA, Elekofehinti OO, Komolafe K, Oboh G (2012). Effects of some green leafy vegetables on the haematological parameters of diabetic rats. Journal of Natural Product and Plant Resources 2(4):482-485.

Sheela CG, Augusti KT (1992). Antidiabetic effects of S-allyl cysteine sulphoxide isolated from garlic *Allium sativum* Linn. Indian Journal of Experimental Biology 30(6):523-526.

Shen GX (2007). Lipid disorders in diabetes mellitus and current management. Current Pharmaceutical Analysis 3(1):17-24.

Shrivastava SR, Shrivastava PS, Ramasamy J (2013). Role of self-care in management of diabetes mellitus. Journal of Diabetes & Metabolic Disorders 12(1):14.

Trinder P (1969). Determination of glucose in blood using glucose oxidase with an alternative oxygen acceptor. Annals of Clinical Biochemistry 6:24-27.

Tsabang N, Fongnzossié E, Keumeze V, Jiofack R, Njamen D, Sonwa DJ Nguelefack BT (2017). Ethnomedical and Ethnopharmacological Study of Plants Used by Indigenous People of Cameroon for The Treatments of Diabetes and its Signs, Symptoms and Complications. Journal of Molecular Biomarkers and Diagnosis 8:310. doi: 10.4172/2155-9929.1000310

Tsabang N, Ngah N, Estella FT, Agbor GA (2016). Herbal medicine and treatment of diabetes in Africa: case study in Cameroon. Diabetes Case Reports 1(2):112. doi: 10.4172/2572-5629.1000112.

Ugbogu AE, Okezie E, Uche-Ikonne C, Duru M, Atasie OC (2016). Toxicity evaluation of the aqueous stem extract of Senna alata in wistar rats. American Journal of Biomedical Research 4(4):80-86.

West I (2000). Radicals and oxidative stress in diabeties. Diabetic Medicine 17(3):171-180.

Wong CK, Lin CS (1998). Remarkable response of lipoid proteinosis to oral dimethyl sulphoxide.. British Journal of Dermatology 119(4):541-

544.
Yakhchalian N, Mohammadian N, Hatami K, Nosrati H, Yousofvand N (2018). Hematological and serum biochemical analysis of streptozotocin-induced insulin dependent diabetes mellitus in male adult wistar rats. https://doi.org/10.1101/359844
Zafar M, Naqvi S (2010). Effects of STZ-induced diabetes on the relative weights of kidney, liver and pancreas in albino rats: a comparative study. International Journal of Morphology 28(1):135-142.
Zafar M, SN-u-H N, Ahmed M, Khani ZAK (2009). Altered liver morphology and enzymes in streptozotocininduced diabetic rats. International Journal of Morphology 27(3):719-25.

Vol. 18(32), pp. 1069-1076, December, 2019
DOI: 10.5897/AJB2019.17000
Article Number: AB613EA62470
ISSN: 1684-5315

http://www.academicjournals.org/AJB

ACADEMIC JOURNALS
expand your knowledge

African Journal of Biotechnology

Full Length Research Paper

Development of a rice yellow mottle virus amplicon biotechnological tool for the production of high value proteins in rice

Pingdwende Kader Aziz Bamogo[1,2,3], Christophe Brugidou[1,2], Fidèle Tiendrébéogo[2], Drissa Sérémé[2], Florencia Wendkuuni Djigma[3], Jacques Simpore[3] and Séverine Lacombe[1,2*]

[1]IRD, CIRAD, Université Montpellier, Interactions Plantes Microorganismes et Environnement (IPME), 911 Avenue Agropolis BP 64501, 34394 Montpellier Cedex 5, France.
[2]Institut de L'Environnement et de Recherches Agricoles (INERA)/LMI Patho-Bios, Laboratoire de Virologie et de Biotechnologies Végétales, 01 BP 476, Ouagadougou 01, Burkina Faso.
[3]Laboratoire de Biologie Moléculaire et de Génétique (LABIOGENE), Ecole Doctorale Sciences et Technologie, Centre de Recherche Biomoléculaire Piétro Annigoni (CERBA), Université Joseph Ki-Zerbo, BP 364, Ouagadougou 01, Burkina Faso.

Received 6 November, 2019; Accepted 29 November, 2019

Lately, advances on biotechnology have led to the emergence of plants as bioreactor for the production of molecules of industrial, pharmaceutical and agribusiness interests. One of the major advances was the exploitation of plant viruses as "amplifier" for the protein production. This amplicon technology relies on viral vector engineered to contain a gene encoding candidate protein that is produced in significant quantities during virus replication. To date, only few of these virus tools are developed for monocot species. Here, we set up experimental approaches to develop the first amplicon tool based on the Rice yellow mottle virus (RYMV) to be used on rice. We showed that inoculated 4-week-old leaves display the most important RYMV initial replication and retained this tissue for protein of interest production from RYMV amplicon. We engineered RYMV tool based on highly replicative $RYMV_{Mg1}$ isolated with GFP gene of interest replacing Coat Protein ORF. Replication of RYMV amplicon was optimized in inoculated rice leaves by adding an RYMV helper to complement RYMV amplicon with CP. This work identify for the first time a viral amplicon tool active in rice offering promising perspectives for the use of rice as bioreactor for the production of high value proteins.

Key words: Rice, rice yellow mottle virus, plant bioreactor, viral amplicon, virus induced gene silencing (VIGS).

INTRODUCTION

In recent years, advances have been made in the use of plants as bioreactor to produce proteins of interest such as vaccine antigens, industrial, pharmaceutical and agribusiness proteins. The rapid growth of these technologies is mainly due to their low production cost, their product safety and their easy scale-up compared to

*Corresponding author. E-mail: severine.lacombe@ird.fr. Tel: 00 33 4 67 41 62 39.

others production systems based on bacteria, yeast or mammalian cells. Altogether, plants offer attractive advantages in production of recombinant proteins. Several plant species including tobacco, a related species *Nicotiana benthamiana* and lettuce can be used based on their ability to be stably or transiently transformed (Daniell et al., 2009). While most of the early research works was carried out with stably transformed plants, in the recent years there has been a growing trend towards the use of transient expression systems. Indeed, *Agrobacterium* infiltration based systems (agro-infiltration) offer the chance of getting large amounts of proteins in days after infiltration rather than the months necessary for transgenic expression (Fischer et al., 1999). One of the major constraint in these transient gene expression plant based systems is the relatively limited product yield. Indeed, setting up production systems leading to expression level that is acceptable for economic production (> 50 mg/kg for antibodies) are not trivial process. One of the most effective advances in yield improvement has been made with the development of viral vectors for the expression of proteins in plants: Amplicon technology. This process relies on the direct or indirect delivery of viral RNA replicons to plant cells. Once introduced in a host plant cells, a virus engineered to contain a gene of interest will replicate and the protein of interest can be produced in significant quantities (Gleba et al., 2007). This method has been shown to work with numerous proteins. Viral vectors used have been mostly derived from Tobacco mosaic virus (TMV), Potato virus X (PVX) and Cowpea mosaic virus (CPMV) combined with host plants such as tobacco, *N. benthamiana* and cowpea (Daniell et al., 2009). Due to their high efficiency, most of these amplicon-based technologies are patented (www.pbltechnology.com).

In addition to be highly replicative in plant cells, virus genomes are also the target of RNA silencing set up by plants in response to introduction of foreign nucleic acid. This property has been used to develop an efficient tool for gene function studies: Virus Induced Gene Silencing (VIGS) technology. By inserting a fragment of the gene of interest into VIGS vectors, the corresponding RNA is selectively degraded during virus infection resulting in transient silencing of the targeted gene (Baulcombe, 1999). Consequently, depending on how it is engineered, vectors based on a same virus can be either used as amplicon or VIGS tools such as TMV and PVX based vectors (Kumagai et al., 1995; Turpen at al., 1995; Mallory et al., 2002; Angell and Baulcombe, 1999).

To date, there is no one universally suitable production system or host. Initially, viral based biotechnological tools were almost exclusively developed for uses on dicotyledonous species. Until now, no amplicon vectors exist for routine uses in monocot. VIGS vectors based on Barley stripe mosaic virus (BSMV) and Brome mosaic virus (BMV) were developed for barley, wheat, rice and maize (Holzberg et al., 2002; Tai et al., 2005; Ding et al., 2006). However, these tools are based on multipartite viruses and are consequently not fully adapted for easy and routine uses.

Rice yellow mottle virus (RYMV) is a monopartite positive strand RNA virus belonging to *Sobemoviruses*. RYMV is endemic to Africa where it is one of the major pathogen for rice production. Transmission occurs mainly by contact between infected and uninfected plants and by insect vectors (mainly beetles). It is detected in most rice-growing areas, especially East and West Africa (Kouassi et al., 2005). Natural susceptibility, tolerance and resistance to RYMV have been described in the two cultivated rice species, that is, *Oryza sativa* (Asian rice) and *Oryza glaberrima* (African rice) (Thiémélé et al., 2010, Pidon et al., 2017, Albar et al., 2003, Orjuela et al., 2013). RYMV genome of 4.5 kb is one of the smallest described for RNA viruses. Its 3' end is non poly(A). It encodes five Open Reading Frame (ORF) (Ling et al., 2013). Except for ORFx that has been characterized recently (Ling et al., 2013), functions of the four others are well known. ORF1 encodes for P1 protein that has been initially described to be involved in virus movement (Bonneau et al., 1998) and then in RNA silencing suppression (Voinnet et al., 1999, Siré et al., 2008; Lacombe et al., 2010). ORF2a and 2b encode for polyproteins that are processed to produce the RNA dependent RNA Polymerase (RdRP), the VPg and a serine protease. VPg protein is linked to the viral genome at its 5' extremity and interact with the host translation initiator unit eIF(iso)4G. This interaction would be involved in the ribosome recruitment for translation (Hébrard et al., 2010). ORF3 encoded for the Coat Protein (CP) that is essential for viral particle formation and thus for virus propagation. A RYMV infectious clone has been synthetized under T7 RNA polymerase promoter from an isolate from Côte d'Ivoire, Fl5. Using CP mutants on this RYMV infectious clone, it has been shown that CP is dispensable for local replication but required for systemic infection (Brugidou et al., 1995). ORF1, 2a and 2b are translated from genomic RNA while ORF3 is translated from subgenomic RNA. Because of its relative simple genomic organization, the deep functional characterization and possibility to develop active RYMV infectious clones, RYMV represent a candidate of choice to set up amplicon biotechnological tools.

Here, we set up experimental approaches to develop a RYMV amplicon tool to be used in rice. RYMV amplicon that we developed, carried a GFP sequence as gene encoding protein of interest. To optimize its production, GFP gene was integrated in the highly replicative subgenomic region. It replaced the CP ORF that has been described as dispensable for replication. We first set up experimental conditions to identify tissue displaying an optimal RYMV replication. Based on these finding, RYMV amplicon expression in rice leaves was optimized allowing viral amplicon replication for the first time in rice. These developments allow exciting

perspectives for viral tool uses in rice such as amplicon and VIGS technologies for practical and fundamental exploitations, respectively.

MATERIALS AND METHODS

RYMV and rice material

RYMV amplicon was designed from the $RYMV_{Mg1}$ isolate genomic sequence (genbank number: AJ608210). After the stop codon ($^{5'}$TAA$^{3'}$) of ORF2b, a *Sna*BI restriction site ($^{5'}$TACGTA$^{3'}$) was inserted before the *Tobacco etch virus* (TEV) protease cleavage site, $^{5'}$GAAAACCTGTATTTTCAGGGA$^{3'}$. Right after, Green Fluorescent Protein GFP5-ER sequence (U87974) was inserted. A *Spe*I ($^{5'}$ACTAGT$^{3'}$) restriction site was added after the stop codon ($^{5'}$TAA$^{3'}$) of GFP5-ER sequence. T7 promoter was inserted at the 5' extremity of the $RYMV_{Mg1}$ sequence. T7 promoter sequence was the same as for Fl5 infectious clone: $^{5'}$TCTAGACTGCAGTAATACGACTCACTATAG$^{3'}$ with the T7 RNA polymerase promoter sequence (underlined), an additional G downstream residue and *Xba*I ($^{5'}$TCTAGA$^{3'}$) *Pst*I ($^{5'}$CTGCAG$^{3'}$) restriction sites (Brugidou et al., 1995). A *BamH*I restriction site was added at the 3' extremity of the construct. Amplicon::GFP *de novo* synthesis was performed by Genecust company as pUC57: amplicon::GFP clone. RYMV infection clone used is the Fl5 clone developed by Bugidou et al. (1995) from a Côte d'Ivoire isolate. $RYMV_{Mg1}$ and $RYMV_{Cia}$ isolates were obtained from infected frozen leaves provided by Pinel-Galzi, IRD, Montpellier (France). *Oryza sativa indica* cvs genotype used in this study is the IR64 variety. It was chosen due to its susceptibility for RYMV infection (Ghesquière et al., 1997).

Viral inoculation

$RYMV_{Mg1}$ and $RYMV_{Cia}$ inoculation were performed on two-week old rice plants on the second to the last youngest leaves. Sap inoculum was prepared by grinding infected frozen leaves in 0.1 M inoculation buffer (Na_2HPO_4 0.1 M, KH_2PO_4 0.1 M, pH 7.2). Inoculations were done either by mechanical carborundum abrasion or syringe infiltration.

In vitro transcription and rice inoculation of amplicon::GFP and Fl5 construct

In vitro transcription of amplicon::GFP and FL5 constructs were done as previously described (Brugidou et al., 1995). Basically, pUC57:amplicon::GFP and Fl5 clones were multiplied by overnight bacterial cultures. Plasmid DNA were purified (Promega) and linearized by *Hind*III or *Bam*HI restriction for FL5 and amplicon::GFP constructs, respectively. After phenol: chloroform (1:1, v:v) purification, *in vitro* transcriptions by T7 polymerase (Promega) were performed in presence of the cap analogue $m^7G^{5'}ppp^{5'}G$ (New England Biolab). RNA production and integrity were verified by electrophoresis on agarose gel and UV visualization after Ethidium bromide staining. RNA quantifications were performed by OD_{600} absorption estimation by a NanoDrop spectrophotometer (ThermoFisher Scientific). Inoculation solutions were prepared by dilution of *in vitro* transcripts in 0.1M inoculation buffer to a final transcript concentration of 250 ng/µl. 20 µl were used per inoculation on leaves previously lightly rubbed with carborundum.

RNA extraction and RT-PCR analysis of gene expression

Total RNA was extracted from rice tissues using TriReagent (Euromedex). RNA samples were treated with RQ1 Rnase free Dnase (Promega) and quantified with a NanoDrop spectrophotometer. Reverse transcriptions were performed with M-MLV Reverse Transcriptase (Promega) using a 1:1 (v:v) mix of oligodT(15) primer and a RYMV specific primer designed on the 3' extremity of RYMV sequence on the reverse complementary orientation: $^{5'}$CTCCCCCACCCATCCCGAGA$^{3'}$. This primer mix allowed reverse transcription of mRNA and non poly(A) RYMV RNA populations. To detect either GFP or CP RNA accumulation from amplicon::GFP and Fl5 samples, respectively, RT-PCR were performed as previously described (Lacombe et al., 2010) using RYMV PB $^{5'}$CCAGGAAGGGCAAGAAAATC$^{3'}$ and RYMV JB $^{5'}$CAGGGTAGTCGATCTCTGAG$^{3'}$ primers. These primers were designed to surround GFP insertion site. They allowed amplification of a 1259 pb and 995 pb fragments from amplicon::GFP and FL5 cDNA, respectively. The endogenous *Os* EF-1α gene was used as internal loading control with the primer pair $F_{OsEF\text{-}1\alpha}$ $^{5'}$GCACGCTCTTCTTGCTTTCACTCT$^{3'}$ and $R_{OsEF\text{-}1\alpha}$ $^{5'}$AAAGGTCA-CCACCATACCAGGCTT$^{3'}$.

RESULTS

Design of $RYMV_{Mg1}$ amplicon tool

Efficient amplicon tool has to display optimal replication and production of protein of interest. Consequently, we selected amplicon genomic background corresponding to a RYMV isolated displaying a strong replication capacity, $RYMV_{Mg1}$ isolated from Madagascar. Indeed, it has been shown that this isolate overcome rapidly partial resistance during serial inoculation suggesting a strong replication capacity (Fargette et al., 2002). For initial development, GFP encoding sequence has been chosen for its easy detection. For an optimal replication, it has been integrated into subgenomic part of RYMV genome (Figure 1A). CP was described as dispensable for virus replication (Brugidou et al., 1995). In order to minimize RYMV genome structure modification, substitution of the CP ORF by the GFP coding sequences was proposed. However, CP ORF overlaps with ORF 2b at its 5' end. To maintain the ORF2b integrity, CP ORF replacement has to be partial with no change to the overlapping region. Consequently, GFP sequence insertion lead to a CP-GFP fusion protein production. In order to be able to obtain the expected protein of interest, a TEV cleavage site was inserted between the CP and GFP coding sequences to remove the CP part from the fusion CP-GFP protein (Figure 1A). T7 promoter sequence and insertion has been done as the Fl5 reference clone (Figure 1A and B). As rice genotype host, IR64 *Oryza sativa* genotype was selected based on its susceptibility to RYMV infection (Ghesquière et al., 1997).

Spatio-temporal pattern of $RYMV_{Mg1}$ viral accumulation in rice

In order to identify tissues that would be the most

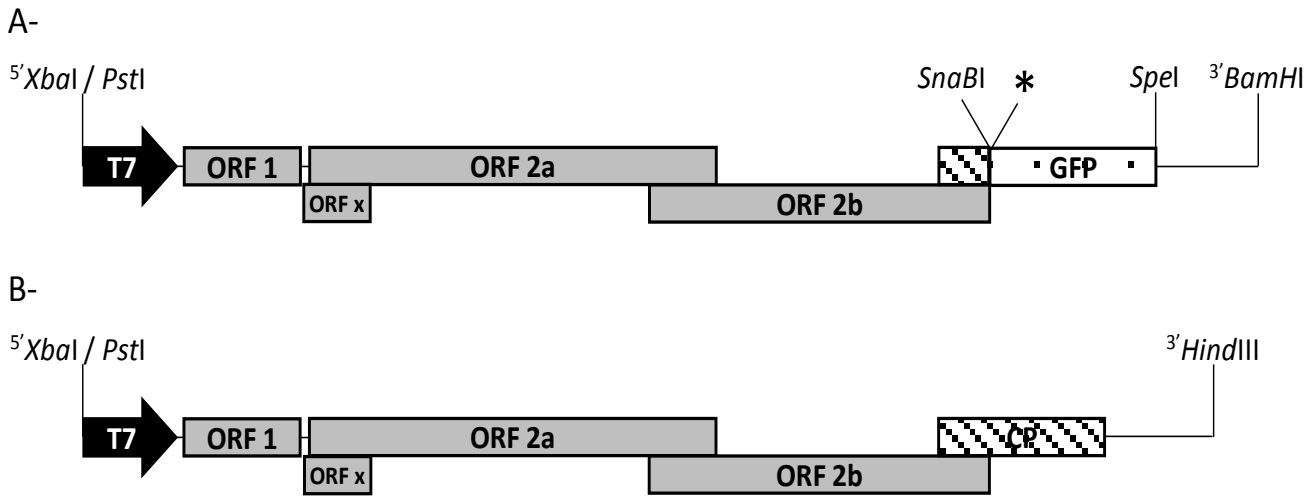

Figure 1. RYMV based construction, Fl5 and amplicon: GFP used in this study. A- Amplicon::GFP construction caring and incomplete CP ORF (noted in hatched box) fused to a GFP encoding sequence (noted in doted box). The star indicates the TEV cleavage site between the CP and the GFP sequences. Restriction sites that have been added are noted. It starts with a XbaI / PstI double restriction site at its 5' extremity and ends with a BamHI restriction site at the 3' extremity. B- Reference infectious clone Fl5 caring a complete CP ORF (noted in hatched box). It starts with a XbaI / PstI double restriction site at its 5' extremity and ends with a HindIII restriction site at the 3' extremity. Both constructions are under the control of a T7 promotor. ORF1, x, 2a and 2b are represented by grey boxes.

appropriated for protein of interest production from a RYMV based tool, we researched tissues displaying a strong RYMV accumulation. We considered natural $RYMV_{Mg1}$ isolate as amplicon tool we produced has a $RYMV_{Mg1}$ backbone. IR64 rice genotype was chosen because of its RYMV sensitive behavior. Sap inoculations were performed on the second to last youngest leaves by mechanical carborundum abrasion. Inoculated and systemic leaves and apical areas were collected from 2 to 20 days post inoculation (dpi) to follow RYMV RNA accumulation by semi-quantitative RT-PCR (Figure 2). In infected leaves, RYMV RNA accumulated increasingly from 2 to 20 dpi. In systemic leaves and apical areas, RYMV was only detected from 8 dpi and 14 dpi, respectively. As in inoculated leaves, viral RNA accumulated increasingly from theses stages. For each sampling time considered here, viral RNA accumulated more in infected leaves compared to others tissues considered. So, in term of quantity and presence during infection time, inoculated leaf tissues were chosen for the following experiments as tissues for protein of interest production from the $RYMV_{Mg1}$ amplicon.

Viral accumulation in rice inoculated leaves

In order to specify areas in inoculated leaves where virus preferentially accumulated, inoculated leaves were virtually divided in three distinct zones: inoculated, upstream and downstream zones. In order to perform inoculation only in the inoculated area, $RYMV_{Mg1}$ sap inoculation were performed in three spots by syringe infiltration (Figure 3A). $RYMV_{Mg1}$ RNA accumulation was followed by RT-PCR on RNA extracted from samples collected at 4 and 8 dpi (Figure 3B). At both 4 and 8 dpi, viral RNA was only detected in the downstream area. This accumulation strongly increased from 4 to 8 dpi. Surprisingly, no viral RNA was detected neither in inoculated and upstream zones (Figure 3B). This result shows that $RYMV_{Mg1}$ virus spread directionally and rapidly to the base of leaves. Consequently, in order to increase this tissue availability for amplicon multiplication, amplicon inoculation has to be done close to the top of leaves.

Amplicon::GFP replication in rice inoculated leaves

To verify if RYMV amplicon we designed is able to replicate in rice leaves, we tried to detect amplicon RNA in IR64 rice inoculated leaves. Viral RNA obtained after *in vitro* transcription such as for amplicon::GFP and Fl5 infectious clone, can be inoculated into rice leaf only by mechanical carborundum abrasion. Leaf infections were performed with amplicon::GFP RNA close to the top of leaves. Fl5 RNA and mock infections were used as positives and negative controls, respectively. Inoculated leaf samples were collected at 4 and 8 dpi. Viral RNA accumulations were followed by RT-PCR using primers amplifying either the GFP (1259 bp) or CP (995 bp) genomic regions for amplicon::GFP and Fl5, respectively (Figure 4). Amplicon::GFP RNA accumulation was

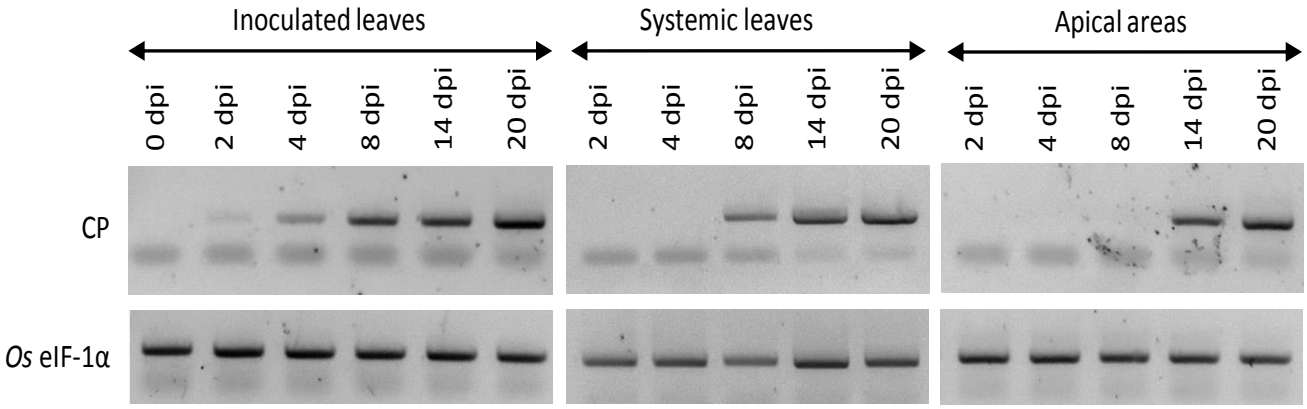

Figure 2. Spatio-temporal pattern of RYMVMg1 viral accumulation in IR64 rice. Inoculated and systemic leaves and apical areas were collected at 2, 4, 8, 14 and 20 days post inoculation (dpi). Leaves before inoculation were also collected (0 dpi). RYMV accumulation in these tissues were followed by semi-quantitive RT PCR of CP region from RNA extracted of each sample. Amplification of Os eIF-1α was used as internal control.

detected at 4 dpi. No amplification was detected at 8 dpi but no amplification was noticed as well for the eIF-1α internal control suggesting a quality problem for this cDNA sample. As expected, amplification was detected for Fl5 samples at 4 and 8 dpi. The intensity of amplifications was much more important in the case of Fl5 compared to amplicon::GFP samples. These data suggest that amplicon::GFP is able to replicate in rice leaves but at low level.

Increasing amplicon::GFP replication by CP complementation through $RYMV_{Cia}$ helper strategy

Using Fl5 infectious clones mutated on CP ORF, Brugidou and collaborators demonstrated that CP was dispensable for virus replication in rice protoplast. However, the level of replication detected by northern blot was reduced compared to the wild type Fl5 (Brugidou et al., 1995). This suggest that even if CP is dispensable for viral replication, it is required for a high level of replication probably by facilitating the cell-to-cell movement. The amplicon::GFP construct we designed does not have a CP gene because it is replaced by GFP gene. Therefore, in order to improve amplicon::GFP replication in rice, we proposed to complement the amplicon with an external CP. To do this, we used a wild type RYMV as a helper to bring its CP *in trans* for the amplicon::GFP. As we suspected a competition for CP availability may occur between amplicon::GFP and RYMV helper, $RYMV_{Cia}$ isolate was chosen as helper as it induced a moderated replication compared to other isolates such as $RYMV_{Mg1}$ (Brugidou, unpublished data). Several dilution factors for $RYMV_{Cia}$ sap inoculum were considerate to favor amplicon::GFP in the case of a competition for CP availability (2x, 10x, 10^2x and 10^3x dilutions). Sap inoculations of $RYMV_{Cia}$ helper were performed by syringe infiltrations of three spots close to the top of leaves. Two days after $RYMV_{Cia}$ helper inoculation, amplicon::GFP *in vitro* transcripts were mechanically brought by carborundum abrasion in helper inoculated leaves above initial spots. Inoculated leaf samples were collected at 4 and 8 dpi and viral RNA accumulation was followed by RT-PCR as previously described. At 4 dpi, $RYMV_{Cia}$ amplification was detected only in the case of helper dilutions 2x and 10x with a decreased intensity in agreement with the dilution factors. At 4 dpi, levels of amplicon::GFP amplification were similar for samples without helper and with helper dilution 2x, 10x and 10^3x. For sample corresponding to helper dilution x10^2, an increased amplicon::GFP amplification was detected. This suggests that at this helper dilution, amplicon::GFP could benefit of CP from the $RYMV_{Cia}$ helper to increase its replication. For lower helper dilution, competition for CP availability would be in favor of the $RYMV_{Cia}$ helper. For dilution 10^3x, helper may be too diluted to allow replication initiation and thus to produce CP. At 8 dpi, helper amplification is only detected for 2x dilution samples. This suggests that in the case of dilution above 2x, viral titer might not be enough to maintain replication. At 8 dpi, no improvement of amplicon::GFP accumulation is observed whatever the helper dilution. This could be explained by the fact that helper is not accumulating at dilutions above 2x and consequently cannot complement amplicon::GFP with its CP. At dilution 2x, helper accumulates strongly suggesting that competition for CP availability would be in favor of the $RYMV_{Cia}$ helper.

DISCUSSION

Work presented here set up experimental procedures to develop an active amplicon tool in rice. We first design the RYMV amplicon based on the previously acquired

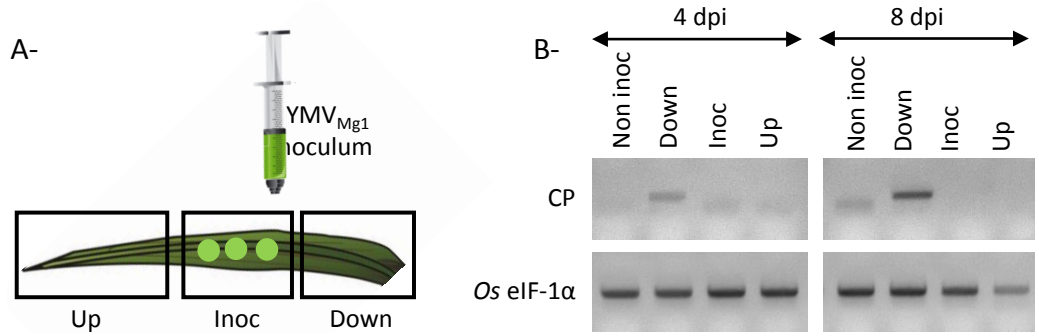

Figure 3. Viral accumulation in rice inoculated leaves. A- Inoculated IR64 leaves were splited in three distinct areas: Inoculation area (Inoc), upstream area (Up) and Dowstream area (Down). RYMV inoculum were injected on the inoculate area by three syringe spots. B- Inoculated, upstream and downstream areas were collected at 4 and 8 dpi. Non inoculated leaves were also collected on the same rice plants (Non Inoc). RYMV accumulation in these tissues was followed by semi-quantitive RT PCR of CP region from RNA extracted of each sample. Amplification of Os eIF-1α was used as internal control.

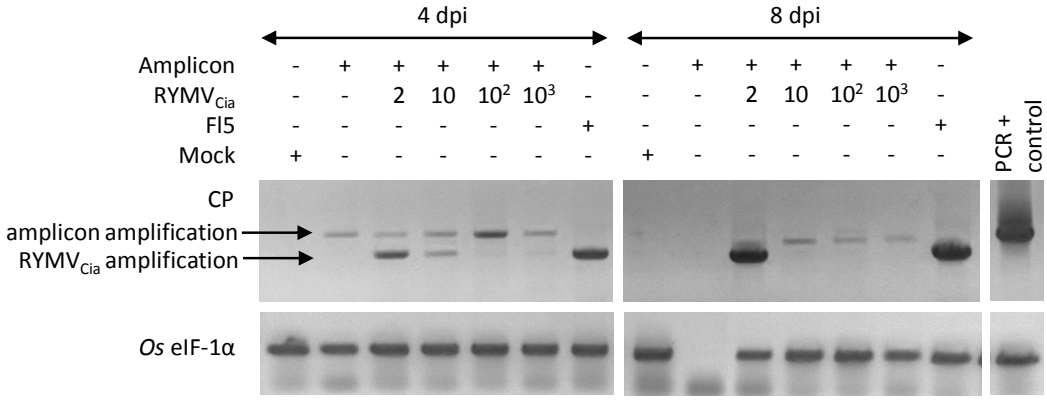

Figure 4. Amplicon and RYMVCia virus helper in co-inoculated IR64 rice leaves. Rice leaves were first inoculated with RYMVCia inoculum. Dilution factors (2, 10, 102, 103) of RYMVCia inoculum are indicated. Two days after this initial inoculation inoculation were done with amplicon::GFP transcript. Mock and Fl5 inoculation samples were used as negative and positive controls, respectively. Tissues were collected at 4 and 8 days after the second inoculation. RYMV accumulation in these tissues was followed by semi-quantitive RT PCR of CP region from RNA extracted of each sample. Arrows indicated bands corresponding to amplifications of amplicon cDNA (1259 pb) and of Fl5 cDNA (995 pb). Amplification of Os eIF-1α was used as internal control. Amplicon vector DNA and rice cDNA were used as PCR positive controls for CP and Os eIF-1α amplification.

knowledge. RYMV$_{Mg1}$ isolate background was chosen because of its expected strong replication behavior (Fargette et al., 2002) and CP genomic region was chosen to be replaced by the gene of interest due to its dispensability for RYMV replication (Brugidou et al., 1995). We then identified infected leaves as the appropriated tissues for a high RYMV replication. This finding fit with the amplicon::GFP construct we designed. Indeed, using Fl5 infectious clone with mutations on CP ORF, Brugidou and collaborator demonstrated that CP is required for long distance movement (Brugidou et al., 1995). As our amplicon::GFP construct is without CP, infected leaves are the most appropriated tissues for its use for protein of interest production. Using amplicon::GFP, we demonstrated that it was able to accumulate in infected leaves. However, the level of replication was reduced compared to Fl5 infectious clone control. This observation is in accordance with previous work on FL5 mutated on CP coding sequence (Brugidou et al., 1995). Indeed, replication of Fl5 mutants was detected in rice inoculated leaves only by southern blotting after reverse transcription and PCR amplification. Classical northern method failed to detect any signal on samples corresponding to Fl5 mutants whereas a strong signal was detected for Fl5 control samples. These results demonstrated that Fl5 mutants on CP region replicated only at a low level suggesting that CP is required for a strong virus replication (Brugidou et al.,

1995).

In order to bring the missing CP to the amplicon::GFP, helper strategy was tested with a wild type isolate. It was previously shown that in the case of co-inoculation between two RYMV isolates, competitions occur between them leading to the exclusion of one of them and fixation of the other one (Poulicard et al., 2012). We hypothesized that competition would be in favor to the most replicative isolate. We previously demonstrated that amplicon::GFP displayed a weak replication in inoculated leaves. In order to favor amplicon::GFP face to the helper, $RYMV_{Cia}$ isolate was chosen as helper due to its low replication behavior compared to other isolates such as $RYMV_{Mg1}$ (Brugidou unpublished data). Because amplicon::GFP need to recruit CP from the helper, an equilibrium has to be found between enough helper to produce the required CP and not too much helper to win the competition against amplicon::GFP. Several helper dilutions were tested. The 2x helper dilution is clearly in favor of the helper that tends to excluded amplicon::GFP. With the 10^3 helper dilution, helper titer may not be sufficient to initiate its own replication and consequently to produce the required CP. Equilibrium was found at helper dilution 10^2 with enough helper to produce CP and not too much to exclude amplicon::GFP. This lead to an improved amplicon::GFP replication at 4 dpi. However, this helper benefit is not maintained at 8 dpi due to an absence of helper replication and consequently of the required CP (Figure 4). All these data demonstrated that the helper strategy is valid to improve amplicon::GFP replication but equilibrium required is extremely delicate.

Transgenic rice lines expressing RYMV CP has been previously developed. It has been shown an increased RYMV accumulation in these transgenic plants compared to the wild type. This finding suggests that CP promote RYMV infection (Kouassi et al., 2006). These transgenic lines could be used for RYMV amplicon exploitation.

Indeed, in this transgenic context, CP would be available for RYMV amplicon without competition as in the case of helper strategy. However, we cannot exclude that improvement of amplicon::GFP replication shown in the helper strategy is not due only to CP complementation but also to a favorable cellular context induced by helper replication. Indeed, it has been shown that RYMV particles stabilize in a specific pH and calcium cellular context (Brugidou et al., 2002). Moreover, RYMV proteins have to recruit host proteins to perform their biological functions (Brizard et al., 2006). Both of these works demonstrated that host cellular context plays a crucial role for an efficient RYMV replication. In the case of amplicon::GFP/helper co-inoculation, helper replication could induce a favorable cellular context that amplicon::GFP could benefit.

Conclusion

The work presented here shows promising results about the development of an amplicon tool based on RYMV to be used in rice. Exploitation of such a tool will be of a great interest for high value protein production such as therapeutics in a new non patented bio-system. Moreover, this RYMV based amplicon could also be exploited as VIGS tool in rice for fundamentals studies. This would be of major interest for the rice scientific community that can only use complex multipartite VIGS tools so far.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors thanked Agnes Pinel for providing RYMV saps, Dr Eugenie Hébrard and Dr Nils Poulicard for supports and suggestions.

ABBREVIATIONS

BMV, Brome mosaic virus; **BSMV,** Barley stripe mosaic virus; **CP,** Coat Protein; **CPMV,** Cowpea mosaic virus; **Dow,** leaf downstream inoculation area; **Inoc.,** leaf inoculation area; **Non Inoc.,** Non inoculated leaves; **ORF,** Open Reading Frame; **PVX,** *Potato virus X;* **RdRP,** RNA dependent RNA Polymerase; **RYMV,** Rice yellow mottle virus; **TEV,** Tobacco etch virus; **TMV,** Tobacco mosaic virus; **Up,** leaf upstream inoculation area; **VIGS,** Virus Induced Gene Silencing.

REFERENCES

Albar L, Ndjiondjop MN, Esshak Z, Berger A, Pinel A, Jones M, Fargette D, Ghesquière A (2003). Fine genetic mapping of a gene required for Rice yellow mottle virus cell-to-cell movement. Theoretical and Applied Genetics 107(2):371-378.

Angell SM, Baulcombe DC (1999). Technical advance: potato virus X amplicon-mediated silencing of nuclear genes. Plant Journal 20(3):357-362.

Baulcombe DC (1999). Fast forward genetics based on virus-induced gene silencing. Current Opinion in Plant Biology 2(2):109-113.

Bonneau C, Brugidou C, Chen L, Beachy RN, Fauquet C (1998). Expression of the rice yellow mottle virus P1 protein in vitro and in vivo and its involvement in virus spread. Virology 244(1):79-86.

Brizard JP, Carapito C, Delalande F, Van Dorsselaer A, Brugidou C (2006). Proteome analysis of plant-virus interactome: comprehensive data for virus multiplication inside their hosts. Molecular and Cellular Proteomics 5(12):2279-2297.

Brugidou C, Holt C, Yassi MN, Zhang S, Beachy R, Fauquet C (1995). Synthesis of an infectious full-length cDNA clone of rice yellow mottle virus and mutagenesis of the coat protein. Virology 206(1):108-115.

Brugidou C, Opalka N, Yeager M, Beachy RN, Fauquet C (2002). Stability of rice yellow mottle virus and cellular compartmentalization during the infection process in Oryza sativa (L.). Virology 297(1):98-108.

Daniell H, Singh ND, Mason H, Streatfield SJ (2009). Plant-made vaccine antigens and biopharmaceuticals. Trends Plant Science 14(12):669-679.

Ding XS, Schneider WL, Chaluvadi SR, Mian MA, Nelson RS (2006). Characterization of a Brome mosaic virus strain and its use as a vector for gene silencing in monocotyledonous hosts. Molecular Plant-Microbe Interactions 19(11):1229-1239.

Fargette D, Pinel A, Traoré O, Ghesquière A, Konaté G (2002). Emergence of resistance-breaking isolates of Rice yellow mottle virus during serial inoculations. European Journal of Plant Pathology 108:585-591.

Fischer R, Vaquero-Martin C, Sack M, Drossard J, Emans N, Commandeur U (1999). Towards molecular farming in the future: transient protein expression in plants. Biotechnology and Applied Biochemistry 30(2):113-116.

Ghesquière A, Albar L, Lorieux M, Ahmadi N, Fargette D, Huang N, McCouch SR, Notteghem JL (1997). A major quantitative trait locus for rice yellow mottle virus resistance maps to a cluster of blast resistance genes on chromosome 12. Phytopathology 87(12):1243-1249.

Gleba Y, Klimyuk V, Marillonnet S (2007). Viral vectors for the expression of proteins in plants. Current Opinion in Biotechnology 18(2):134-141.

Hébrard E, Poulicard N, Gérard C, Traoré O, Wu HC, Albar L, Fargette D, Bessin Y, Vignols F (2010). Direct interaction between the Rice yellow mottle virus (RYMV) VPg and the central domain of the rice eIF(iso)4G1 factor correlates with rice susceptibility and RYMV virulence. Molecular Plant-Microbe Interactions 23(11):1506-1513.

Holzberg S, Brosio P, Gross C, Pogue GP (2002). Barley stripe mosaic virus-induced gene silencing in a monocot plant. Plant Journal 30(3):315-327.

Kouassi NK, Chen L, Siré C, Bangratz-Reyser M, Beachy RN, Fauquet CM, Brugidou C (2006). Expression of rice yellow mottle virus coat protein enhances virus infection in transgenic plants. Archives of Virology 151(11):2111-2122.

Kouassi NK, N'Guessan P, Albar L, Fauquet CM, Brugidou C (2005). Distribution and characterization of Rice yellow mottle virus: a threat to African farmers. Plant Disease 89(2):124-133

Kumagai MH, Donson J, della-Cioppa G, Harvey D, Hanley K, Grill LK (1995). Cytoplasmic inhibition of carotenoid biosynthesis with virus-derived RNA. Proceedings of the National Academy of Sciences of the United States of America 92(5):1679-1683.

Lacombe S, Bangratz M, Vignols F, Brugidou C (2010). The rice yellow mottle virus P1 protein exhibits dual functions to suppress and activate gene silencing. Plant Journal 61(3):371-382.

Ling R, Pate AE, Carr JP, Firth AE (2013). An essential fifth coding ORF in the sobemoviruses. Virology 446(1-2):397-408.

Mallory AC, Parks G, Endres MW, Baulcombe D, Bowman LH, Pruss GJ, Vance VB (2002). The amplicon-plus system for high-level expression of transgenes in plants. Nature Biotechnology 20(6):622-625.

Orjuela J, Thiémélé D, Kolade O, Chéron S, Ghesquière A, Albar L (2013). A recessive resistance to Rice yellow mottle virus is associated with a rice homolog of the CPR5 gene, a regulator of active defense mechanisms. Molecular Plant-Microbe Interactions 26(12):1455-1463.

Pidon H, Ghesquière A, Chéron S, Issaka S, Hébrard E, Sabot F, Kolade O, Silué D, Albar L (2017). Fine mapping of RYMV3: a new resistance gene to Rice yellow mottle virus from *Oryza glaberrima*. Theoretical and Applied Genetics 130(4):807-818.

Poulicard N, Pinel-Galzi A, Traoré O, Vignols F, Ghesquière A, Konaté G, Hébrard E, Fargette D (2012). Historical contingencies modulate the adaptability of Rice yellow mottle virus. PLOS Pathogens 8(1):e1002482.

Siré C, Bangratz-Reyser M, Fargette D, Brugidou C (2008). Genetic diversity and silencing suppression effects of Rice yellow mottle virus and the P1 protein. Virology Journal 5:55-67.

Tai YS, Bragg J, Edwards MC (2005). Virus vector for gene silencing in wheat. Biotechniques 39(3):310-314.

Thiémélé DE, Boisnard A, Ndjiondjop MN, Chéron S, Séré Y, Aké S, Ghesquière A, Albar L (2010). Identification of a second major resistance gene to Rice yellow mottle virus, RYMV2, in the African cultivated rice species, O. glaberrima. Theoretical and Applied Genetics 121(1):169-179.

Turpen TH, Reinl SJ, Charoenvit Y, Hoffman SL, Fallarme V, Grill LK (1995). Malarial epitopes expressed on the surface of recombinant tobacco mosaic virus. Biotechnology (N Y) 13(1):53-57.

Voinnet O, Pinto Y M, Baulcombe DC (1999). Suppression of gene silencing: A general strategy used by diverse DNA and RNA viruses of plants. Proceedings of the National Academy of Sciences of the United States of America 96(24):14147-14152.

Vol. 18(32), pp. 1077-1082, December, 2019
DOI: 10.5897/AJB2019.16922
Article Number: E9C4B0862472
ISSN: 1684-5315

http://www.academicjournals.org/AJB

ACADEMIC JOURNALS
expand your knowledge

African Journal of Biotechnology

Full Length Research Paper

An alternative caprine infection model for *Mycoplasma mycoides*

Ssajjakambwe Paul[1,2]*, Elise Schieck[1], Anne Liljander[1], Tweyongyere Robert[2], Matovu Enock[2], Kazibwe Ann[2], Nanteza Ann[2] and Mwiine Frank[2]

[1]Department of Biotechnology, International Livestock Research Institute, Nairobi, Kenya.
[2]Animal Resources and Biosecurity, College of Veterinary Medicine, Makerere University Kampala, Kampala Uganda P. O. Box 7062, Kampala, Uganda.

Received 25 July 2019; Accepted 25 October 2019

Ruminant infection models for *Mycoplasma mycoides* are invasive and laborious factors that may interfere with the experiment outcomes. This brief review presents an alternative method that may minimize on the mentioned challenges earlier mentioned. *M. mycoides* subsp. *capri* GM12 and *M. mycoides* subsp. *capri* YCP1.1 ∆68 strains were used; the former, causing a septicemia in goats. An intra-tracheal inoculation technique was employed after aseptic preparation as an alternative to the common intubation method. The former is cheaper, less laborious to handlers, with minimal stress to goats and more time-efficient. In conclusion, this may be used as an alternative to the intubation method. The described technique here will be useful in contagious bovine pleuropneumonia and contagious caprine pleuropneumonia research with modifications, diseases for cattle and small ruminants, respectively.

Key words: *Mycoplasma mycoides*, animal infection model, ruminants.

INTRODUCTION

The *Mycoplasma mycoides* cluster, comprising ruminant pathogens existed under a common ancestor (Fischer et al., 2012). These cause notifiable diseases in sub-Saharan Africa and parts of Asia that include contagious bovine pleuropneumonia (CBPP) a disease of cattle caused by *M. mycoides* subsp *mycoides* (*Mmm*) (Food and Agricultural Organization-FAO, 2003) and contagious caprine pleuropneumonia (CCPP) in goats caused by *Mycoplasma capricolum* subsp *capripneumoniae* (Mccp) (Thiaucourt and Bölske, 1996). CBPP has been reported in 27 African countries, with losses estimated at 2 billion United States dollars annually (Otte et al., 2004).

The recommended control measures for CBPP are massive vaccination, quarantine and stamping out policy (March, 2004). However, the efficacy of these interventions has not been well studied in Africa even when it is known that some present immediate challenges. For example, the existing vaccine strain T1/44 is of low efficacy, presents with side effects (Hubschle et al., 2002) and causes clinical disease on experimental infection in cattle (Mbulu et al., 2004). Therefore, there is need for more studies keen on the

*Corresponding author. E-mail: psajja@covab.mak.ac.ug or paul.sajjakambwe@gmail.com. Tel: +256-782-438699.

biology of *M. mycoides* subsp *mycoides*, and corresponding host-pathogen interractions. Related *Mycoplasma* strains of the *M. mycoides* cluster would provide valuable understanding in such studies.

However, in these studies, we need to experimentally infect animal subjects. The intra tracheal intubation method commonly used presents with challenges of stress to the animals. In small ruminants, it calls for the usage of sedatives and anesthetics. These drugs have a narrow safety margin with potential to cause ruminal stasis (Taylor, 1991). Consequently, a caprine infection model for *M. mycoides* is prerequisite for systematic investigations with regard to host-pathogen interractions (Jores et al., 2013), taking into consideration animal welfare. This study presents an alternative infection model in goats for *Mycoplasma* research.

MATERIALS AND METHODS

Study design and protocol

Details and major outcomes of this work are well elucidated in the work of Jores et al. (2019). The study was carried out following approval by the ILRI Institutional Animal Care and Use Committee IACUC with a reference number 2011.09.10.

Description of the *Mycoplasma* isolates used

M. mycoides subsp. *capri* GM12 (*Mmc*-GM12) is a pathogen of goats that causes severe septicaemia. The two *Mycoplasma* strains used in this experiment were *Mmc* GM12 (Wild type strain) and *Mmc* YCP1.1 Δ68. The latter is a mutant of *Mmc* GM12, whereby a total of 68 genes were deleted using the Tandem Repeat Endonuclease cleavage (TREC) technique. Among the gene loci deleted are GlpO, GlpF, GlpK, gtsA, gtsB, gtsC and gtsD. The first three facilitate glycerol utilization whereas the latter four are cell membrane transport proteins for *Mycoplasma* metabolites. These metabolites contribute to the establishment of an infection in a susceptible host.

Growth of *Mycoplasma* cultures

Culture of blood samples for isolation of *Mycoplasma* was carried out at 37°C using pleuropneumonia like organism (PPLO) media (that is agar; Difco™, lot no., 4169667 or broth; Difco™, lot no., 3361447, supplemented with 20% horse serum, 0.5% glucose, 0.03% penicillin G, 20 mg/ml thallium acetate and 0.9 g L^{-1} yeast extract). In liquid media, phenol red was used as a pH indicator for color change from red to orange or yellow, as a sign for growth. Each sample was cultured in duplicate and color changing units noted after incubation at 37°C for 10 days (Jores et al., 2019). In addition, the liquid cultures were inoculated on PPLO agar plates and characteristic “fried egg” *Mycoplasma* colonies identified a true reflection of *Mycoplasma* bacterial growths.

Preparation of animals and infection procedure

All animals were screened and declared free of any *Mycoplasma* prior to the start of the experiment (Table 1). In addition, the collected blood samples had no other bacterial growths, which would prejudice the experiment outcomes. A total of eight (8) and six (6) goats received the *Mmc* GM12 and *Mmc* YCP 1.1 Δ68 strains as inoculum, respectively. Each goat was restrained in a standing position; head elevated to expose the ventral cervical area which was shaved using an electric shaver at about the 5-10th cartilaginous ring position (Figure 1i). The shaved area was approximately 4×10 cm in area. The site was disinfected 3 times using 70% ethanol using a sterile piece of gauze. The skin at the prepared site was displaced laterally, about 0.5 cm and the space between cartilaginous rings located by palpation and on confirmation, a sterile hypodermic 18-gauge needle introduced perpendicularly into the trachea lumen. Once the needle bevel was within the lumen of the trachea, *Mycoplasma* culture (10^9) colony forming units were introduced into the trachea space and holding the needle in place, the used needle flushed with 10 ml of a 1X phosphate buffered saline (PBS) solution. Thereafter, the needle was removed and the site disinfected. This method took a time range of 2 to 5 min, a commendable duration that would inflict minimal stress on the experiment subjects as a result of the procedure. Details of the procedure are demonstrated in Figure 1i-iii.

Monitoring of clinical signs, sample collection and handling

Clinical parameters

Monitoring for signs of ill health was done for atleast 30 min per session, three times a day through a wide glass screen without interruption of the animals. These included discerning signs of inappetence or anorexia, depression, respiratory distress, coughing, nasal discharge and ocular discharge. Morning daily rectal temperature was measured using a digital thermometer, once a day. The aberrant findings thereafter were recorded in the data collection forms.

Oxygen concentration

The oxygen concentration was measured using the Edan VE-H100B Oximeter. Once the power button was turned on and the screen display showing the oxygen and heart rate parameter icons, the rectal probe was inserted into the rectum after applying KY jelly. Once a stable reading for the oxygen concentration was obtained after a good signal picked as indicated on the screen and manufacturer’s instructions, the reading was recorded on the data sheet.

Heart rate

The heart rate was measured daily using a stethoscope placed on the left side of the cranial third of the animal’s lateral ventral thoracic area about the 3rd-4th rib position. Once the counts per minute were attained, the value was entered into the data sheet.

Nasal swabs

Once an animal was restrained in a standing position with the head held by the chin and horns, a piece of gauze containing 70% ethanol was wiped around the nasal opening. Thereafter a sterile cotton swab was introduced and moved in a circular motion against the inner most nasal walls. The swab was immediately placed in PPLO broth and incubated at 37°C for 10 days.

Collection of whole blood

An animal was restrained in a standing position and the head

Table 1. Blood culture results pre and post infection.

Mmc strain inoculated in animal	Animal ID	Pre-infection cultures	Post infection cultures (blood)- time point 1 DPI)
Mmc GM12 parent strain	CK043	-	+
	CK048	-	+
	CK034	-	+
	CK032	-	+
	CK046	-	+
	CK040	-	+
	CK051	-	+
	CL002	-	+
Mmc YCP 1.1 Δ68	CK047	-	-
	CK049	-	-
	CK045	-	-
	CK035	-	-
	CL003	-	-
	CL001	-	-

slightly tilted to the lateral side with the head extended. The jugular groove was located and a sterile vacutainer needle and tube used to collect blood for routine red blood and white blood cell profiling. Whole blood was then cultured for any *Mycoplasma* and other bacteria.

Euthanasia

A decision to euthanize (intravenous injection of Lethabarb Euthanasia Injection, Virbac 200 mg/kg body weight) was made once an animal developed severe disease associated with unwarranted moderate to severe suffering (e.g. A fever of 41°C and above for three consecutive days, an oxygen saturation of 92% or less and a lateral recumbence of atleast a day without ability to pick feed or water).

RESULTS

Isolation of *Mycoplasma* from whole blood

As indicated, all samples from the animals that received the parent strain of *Mmc* GM12 had positive cultures (+) in PPLO broth but no growth in the mutant group, which were all negative (-) (Table 1). As shown, no mycoplasma growths were seen post-infection in both groups but identified for the group that received the wild type strain. In addition, no growths were seen for the mutant group after infection, possibly because this strain was potentially attenuated and failed to establish an infection

The alternative infection model in goats

Figure 1 shows an image with the intra-tracheal infection route of a goat with *Mycoplasma mycoides.* (i) Locating the trachea and aseptic introduction of a sterile hypodermic needle; (ii) checking that the needle bevel is within the tracheal lumen by aspirating air using an empty syringe attached onto the introduced needle in (i) and (iii) administering of *Mycoplasma mycoides* culture into the tracheal lumen.

Comparative lung pathology of the two groups

Figure 2 shows a lung presenting a classic case of pneumonia among the animal group that received *Mmc* GM12 Mycoplasma strain (*left image with an arrow*) as compared to another that received the *Mmc* YCP1.1 Δ68 strain (*right image*), the latter looks apparently normal on gross examination.

DISCUSSION

Pathogenesis studies call for the best animal infection model, preferably a natural host of the organism under investigation with strict animal welfare observed.

In this communication, we present an alternative infection model (Figure 1) in goats that took an average implementation time of 2 to 5 min. This involved location of trachea, after proper animal restraint, aseptic preparation of the inoculation site and introduction of the infectious agent into the trachea lumen. Earlier Mycoplasma infection studies in cattle employed the intra-tracheal intubation technique (Nkando et al., 2011, 2016). Advantages of the alternative method are: possible less stress exerted on the animal during the procedure, reduced execution time as well as zero use of

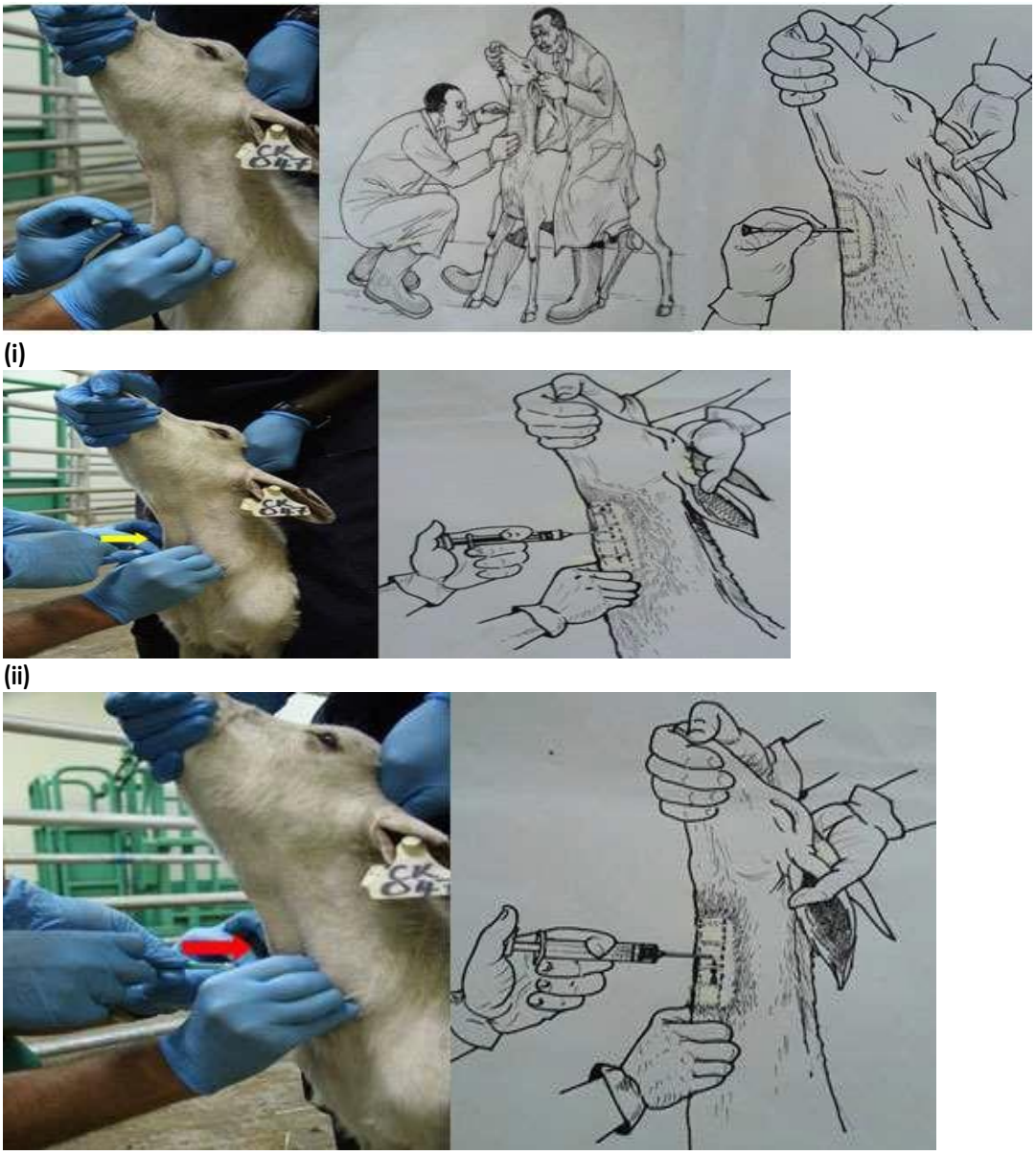

Figure 1. An image showing the intra-tracheal infection route of a goat with Mycoplasma mycoides. (i) Locating the trachea and aseptic introduction of a sterile hypodermic needle; (ii) Checking that the needle bevel is within the tracheal lumen by aspirating air using an empty syringe attached onto the introduced needle in (i) above and (iii) Administering of mycoplasma mycoides culture into the tracheal lumen.

pharmacological agents to calm the animal. In addition, the alternative method does not entail introduction of hot *Mycoplasma* agar down the trachea as shown in Nkando et al. (2011) and Nkando et al., (2016). This is desirable when making a choice for an infection model, which is reproducible while mimicking the natural infection scenarios.

Since *Mycoplasma* bacteria was isolated post infection from the wild type animal group (Table 1), this was evidence that the infection model was a success. Furthermore, much as no *Mycoplasma* growths were identified in the mutant group, this may perhaps be due to the *Mmc* YCP 1.1 Δ68 strain succumbing to the host defence mechanisms that hindered its proliferation without significant pathology (Figure 2). Whereas carrying out a blood culture would aid in the identification of bacteria in blood (Paisley and Lauer, 1994; Spencer, 1988) as this is a septicaemia causing organism, it was observed that no *Mycoplasma* was harvested from the group that received the attenuated strain, *Mmc* 1.1 delta

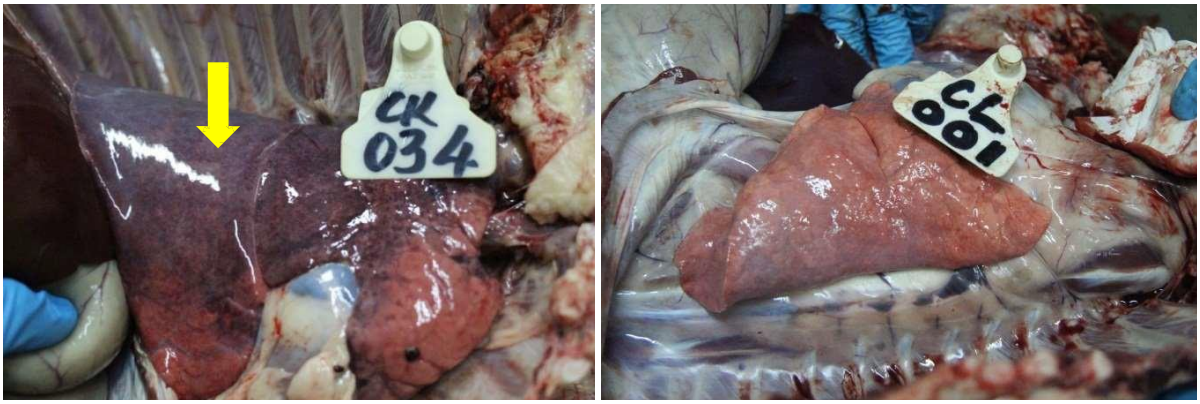

Figure 2. A comparison of the lung pathology from candidates of the two infection groups**.**

68 post infection. This could have been due to the fact that the host immune system countered establishment of an infection, with complete clearance of the bacteria. Another possibility is that the mutant strain could have been maintained at minimal concentrations in circulation to levels that would not be detected on blood culture, although minimal *Mycoplasma* bacterial amounts detectable on culture to our knowledge are yet to be known, a similar observation by Alexander et al. (1999). In comparison to the group that received the wild type *Mmc* GM12 strain, full blown septicaemia and other related clinical signs could be due to a compromised immune system as exemplified by Monserrat et al. (2013), which was not the case for the mutant group. This supports the suggestion that targeted mutagenesis of potential virulence factors led to partial or complete attenuation for the *Mmc* 1.1 delta 68 strain. It is against this background that this work reports the use of an in-vivo experiment, comparing a wild type (Mmc GM12) and Mmc 1.1 delta 68 strains to ascertain whether the mutant after target specific edits led to attenuation. This alternative method was adopted with modifications for other ruminant *Mycoplasma in-vivo* work by Jores et al. (2018) and Liljander et al. (2019), with infection success observed.

Limitations

This study did not take into consideration bred and sex variations in addition to different *Mycoplasma* concentrations for inoculation. This was an initial experiment for proof of principle; however, subsequent experiments may consider some other parameters such as age, sex, breed and varying the inoculation doses of the bacteria under investigation.

Conclusion

The infection model presented here was a success for the *Mycoplasma in-vivo* investigations based on the clear distinction between the results pre and post infection. This could be adopted for other *Mycoplasma* animal infection experiments although with modifications in both large and small ruminants.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors thank the National Science Fund that provided the financial support to carry out this experiment. They are greatly indebted to J. Craig Ventor Institute scientists led by Sanjey Vashee who provided the *Mycoplasma* strain *Mmc* YCP1.1 ∆68 that was included in this animal infection model. They also thank the entire ILRI staff who participated in executing this study.

REFERENCES

Alexander H, Marlies B, Silbylle S, Klaus U, Konrad B, Gerd do R (1999). PCR and Blood Culture for Detection of Escherichia coli Bacteremia in Rats. Journal of Clinical Microbiology 37(8):2479–2482.

Fischer A, Beth S, Cecilia M,Martin H, Christiane S,Erik B, Edy MV, Joachim F, Joerg J (2012). The Origin of the 'Mycoplasma mycoides Cluster' Coincides with Domestication of Ruminants. PLoS ONE 7(4):e36150

Food and Agriculture Organization of the United Nations (FAO) (2003). Contagious bovine pleuropneumonia,. EMPRESS Transboundary Animal Diseases Bulletin, 5612-5621.

Hubschle O, Lelli R, Frey J, Nicholas RAJ (2002). Contagious bovine pleuropneumonia and vaccine strain T1/44. Veterinary Record 150(19):615.

Jores J, Ma L, Ssajjakambwe P, Schieck E, Liljander A, Chandran S, Stoffel MH, Cippa V, Arfi Y, Assad-Garcia N, Falquet L, Sirand-Pugnet P, Blanchard A, Lartigue C, Posthaus H, Labroussaa F,

Vashee S (2019). Removal of a Subset of Non-essential Genes Fully Attenuates a Highly Virulent Mycoplasma Strain. Frontiers in

Microbiology 10:664. doi: 10.3389/fmicb.2019.00664.
Jores J, Schieck E, Liljander A, Sacchini F, Posthaus H, Lartigue C, Blanchard A, Labroussaa F, Vashee S (2018). Capsular polysaccharide in Mycoplasma mycoides is a virulence factor. Journal of Infectious Diseases DOI, 10.1093/infdis/jiy713.
Jores J, Mariner CJ, Naessens J (2013). Development of an improved vaccine for contagious bovine pleuropneumonia: an African perspective on challenges and proposed actions. Veterinary Research 44:122.
Liljander A, Sacchini F, Stoffel MH, Schieck E, Stokar-Regenscheit, Nadine, Labroussaa F, Heller M, Salt J, Frey J, Falquet L, Goovaerts D, Jores J (2019). Reproduction of contagious caprine pleuropneumonia reveals the ability of convalescent sera to reduce hydrogen-peroxide production in vitro. Veterinary Research (50):1-10 https://doi.org/10.1186/s13567-019-0628-0.
March JB (2004). Improved formulations for existing CBPP vaccines — Recommendations for change. Vaccine 22(31-32):4358-64
Mbulu RS, Tjipura-Zaire G, Lelli R, Frey J, Pilo P, Vilei EM, Mettler F, Nicholas RA, Huebschle OJ (2004). Contagious bovine pleuropneumonia (CBPP) caused by vaccine T1/44 Mycoplasma mycoides subsp. mycoides SC. Veterinary Microbiology 98(3-4):229-34.
Monserrat J, de Pablo R, Diaz-Martı´n D, Rodrı´guez-Zapata M, de la Hera A, Prieto A, Alvarez-Mon M (2013). Early alterations of B cells in patients with septic shock. Critical Care 17(3):R105.
Nkando I, Perez-Casal J, Mwirigi M, Prysliak T, Townsend H, Berberov E, Kuria J, Mugambi J, Soi R, Liljander A, Jores J, Gerdts V, Potter A, Naessens J, Wesonga H (2016). Recombinant Mycoplasma mycoides proteins elicit protective immune responses against contagious bovine pleuropneumonia. Veterinary Immunology and Immunopathology 171:103-114.
Nkando I, Ndinda J, Kuria J, Naessens J, Mbithi F, Schnier C, Gicheru M, McKeever D, Wesonga H (2011). Efficacy of two vaccine formulations against contagious bovine pleuropneumonia (CBPP) in Kenyan indigenous cattle. Research in Veterinary Science 93(2):568-73
Otte MJ, Nugent R, McLeod A (2004). Transboundary Animal Diseases: Assessment of socio-economic impacts and institutional responses, in Livestock Policy Discussion paper: Food and Agriculture Organisation; Livestock Information and Policy Branch, AGAL, pp.1-46 http://www.fao.org/3/a-ag273e.pdf
Paisley JW, Lauer BA (1994). Pediatric blood cultures. Clinical Laboratory Medicine 14(1):17-30
Spencer RS (1988). Blood cultures: where do we stand? Journal of Clinica Pathology 41(6):668-670.
Taylor MP (1991). Anaesthesia in Sheep and Goats. In Practice 13(1):31-36.
Thiaucourt F, Bölske G (1996). Contagiuos caprine pleuropneumonia and other pulmonary mycoplasmoses of sheep and goats. Revue Scientifique et Technique 15(4):1397-1414.

Vol. 18(32), pp. 1083-1090, December, 2019
DOI: 10.5897/AJB2019.16974
Article Number: D028ADC62474
ISSN: 1684-5315

http://www.academicjournals.org/AJB

ACADEMIC JOURNALS
expand your knowledge

African Journal of Biotechnology

Full Length Research Paper

Phenotypic and molecular characterization of extended spectrum β-lactamase producing *Pseudomonas aeruginosa* in Nigeria

Martina C. Agbo[1]*, Ifeoma M. Ezeonu[2], Maurice N. Odo[1], Chukwuebuka M. Ononugbo[2], Beatrice O. Onodagu[3], Chinelo C. Eze[1], Ezinwanne N. Ezeibe[1] and Chizoba A. Ozioko[1]

[1]Department of Pharmaceutical Microbiology and Biotechnology, University of Nigeria, Nsukka, Enugu State, Nigeria.
[2]Department of Microbiology, University of Nigeria, Nsukka, Enugu State, Nigeria.
[3]Microbiology Laboratory Unit, University of Nigeria Teaching Hospital, Enugu, Nigeria.

Received 26 September 2019; Accepted 22 November 2019

This present study was undertaken for detection of extended spectrum β-lactamases (ESBLS) enzyme genes among clinical isolates of *Pseudomonas aeruginosa* using phenotypic and molecular techniques. Thirty-four *P. aeruginosa* isolates from different hospitals in Nsukka and University of Nigeria Teaching Hospital (UNTH), Enugu were screened for the presence of ESBL-encoding genes. Phenotypic screening for ESBL producers was carried out using double disk synergy test and combined disk test. Genomic DNA was extracted from the isolates by modified boiling method. Extracted DNA was amplified by polymerase chain reaction (PCR) using ESBL specific primers namely *Bla GES, PER, OXA-50, SHV, CTX-M* and *TEM.* The results revealed that a total of 15 isolates of *P. aeruginosa* were identified as ESBL producer by phenotypic approaches which exhibited varying degrees of resistance to an array of antibiotics tested. While, the PCR screening revealed that 53.33% (n=8) of the isolates that were phenotypically ESBL positive harboured *bla OXA*-50 gene. However, the genes that encode *PER, GES, SHV, TEM* and *CTX-M* were not found in any of the *P. aeruginosa* isolates. This study highlights the need to establish antimicrobial resistance surveillance network to determine the appropriate empirical treatment regimen for *Pseudomonas* infections.

Key words: *Pseudomonas aeruginosa*, antibiotic resistance extended spectrum β-lactamase (ESBL), polymerase chain reaction (PCR), Nsukka.

INTRODUCTION

Pseudomonas aeruginosa is widely known as an opportunistic pathogen, frequently involved in infections of immunosuppressed patients and also causes outbreak of hospital-acquired infections (Wirth et al., 2009). According to the USA nosocomial infection surveillance system, *P. aeruginosa* is the third most common pathogen associated with hospital acquired infections, accounting for 10.1% of all nosocomial infections and is associated with high mortality rate (Moreaus-Marquis et al., 2008). This infection may cause septicaemia,

*Corresponding author. E-mail: martina.agbo@unn.edu.ng. Tel: +234 803 970 5442.

pneumonia, meningitis, wound, urinary tract, surgical wound, burn, and ear infections (Todar, 2008). It can tolerate diverse environmental conditions and resistant to many antimicrobial agents (Todar, 2014). This resistance in *P. aeruginosa* may be mediated via several mechanisms such as the production of β-lactamases, efflux pumps and target site or outer membrane modifications (Tam et al., 2010).

Generally, extended spectrum β -lactamases (ESBLS) are a group of β-lactamases that hydrolyze penicillin and cephalosporin, including oxyamino-β-lactamase (third and fourth generation of cephalosporin) and aztreonam. These ESBL enzymes are known to be inhibited by β-lactamase inhibitors, such as clavulanic acids, sulbactam and tazobactam (Peterson and Bonomo, 2005). These enzymes are most commonly found in *Klebsiella pneumoniae* and *Escherichia coli* and have been recently detected in *P. aeruginosa* at low frequency (Lee et al., 2005). These enzymes are encoded by different genes located on chromosomes or plasmids and transposon and are easily disseminated by conjugation. Based on Ambler classification these enzymes are grouped into four groups A to D (Livermore, 2002; Shen and Fang, 2015).

Class B comprises metallo-β-lactamases, which use zinc for the facility reaction and can be inhibited by ethylenediaminetetracetic acid (EDTA), whereas classes A, C and D comprise serine β-lactamases, which can be resistant to many classes of cephalosporin and oxacillin. Class A is the most diverse class; it comprises enzymes that can inhibit β-lactamase inhibitors such as clavulanic acid, tazobactam, and sulbactam. Class A-lactamases are encoded by many genes such as those that encode *VEB, PER, CTX, SHV* and *TEM* (Bush and Jacoby, 2010). Class C and D β-lactamases can be resistant to cephalosporin and oxacillin, respectively. The enzymes in these classes cannot be inhibited by β-lactamase inhibitors. There are many variants of the genes that encode class D β-lactamases, such as *OXA-1, OXA-2, OXA-10* and *OXA-50* (Poirel et al., 2010).

Most studies on molecular characterization of ESBL producing organisms have been carried out worldwide but only few data are available concerning the genetic characterisation of clinical isolates from Nigeria. Rapid and prompt detection of ESBL producing *P. aeruginosa* is of utmost importance to declare the appropriate antimicrobial therapy and also for preventing cross-transmission to other patients in the hospital. Several phenotypic methods have been proposed for ESBL detection on isolated *P. aeruginosa* strains such as double disk synergy test (DDST), combined disk tests (CDT) methods and the ESBL-E test, but their discrepancy in sensitivity has been reported (Drieux et al., 2008). Moreover, there are specific molecular test for ESBL detection in bacterial isolates which reduce the time of detection and increase the sensitivity and specificity (Sharma et al., 2013). The application of molecular biology technique has enhance the specificity and accuracy in diagnosis of *P. aeruginosa* strains and in the discovery of genotypic form of ESBL *P. aeruginosa* and how these genes disseminate into various isolates. However, there is little information on the molecular characterization of ESBL producing *P. aeruginosa* in Nigeria particularly in Southeastern Nigeria. Therefore, this study aimed to investigate the presence of ESBL production among the clinical isolates of *P. aeruginosa* using phenotypic and genotypic methods.

MATERIALS AND METHODS

The bacterial strains

Thirty four (34) strains of *P. aeruginosa* were collected from Microbiology Laboratories from various hospitals in Nsukka and UNTH, Enugu between May and August, 2018. Ethical approval and informed consent was not required by our Institution Ethics Committee because all bacterial isolates were collected, processed and stored as part of routine diagnosis by the hospitals. No patient information was associated with the data. The isolates obtained from the various laboratories were further characterized using 16S rRNA primer targeting *P. aeruginosa* consensus region (Inqaba biotechnical Company, South Africa).

Antimicrobial susceptibility test

The antimicrobial susceptibility testing of *P. aeruginosa* was performed using the Kirby-Bauer disk diffusion method according to the Clinical and Laboratory Standard Institute (CLSI, 2014) guidelines. Commercially available antimicrobial disks of ceftazidime 30 µg (CAZ), cefepime 30 µg (FEP), ceftriaxone 30 µg (CRO), cefotaxime 30 µg (CTX), gentamicin 10 µg (CN), imipenem 10 µg (IMP), Aztreonam 30 µg (ATM), piperacillin/tazobactam 100/10 µg (TZP), ciprofloxacin 5 µg (CIP), meropenem 10 µg (MEM) and piperacillin 100 µg (PPL) (Oxoid, U.K.) were used on Mueller Hinton Agar (Oxiod, UK) to test susceptibility. Zone of inhibition was recorded as sensitive or resistant according to CLSI (2014) guidelines. *P. aeruginosa* ATCC 27853 was used as positive control.

Detection of ESBL by Double disk diffusion synergy test and combined disk method

ESBL production in all isolates were detected by double disk synergy test (DDST) and combined disk test as described by Jarlier et al. (1998) and CLSI (2014), respectively. 100 µl of the standardized inoculum equivalent to 0.5 McFarland turbidity standards was inoculated onto sterile Mueller Hinton agar. Amoxicillin-Clavulanic acid disk consisting of 20 µg Amoxicillin and 10 µg of clavulanic acid (AMC) (Hi-media) was placed in the centre of the plate and disks of third generation cephalosporin Ceftazidime (CAZ), ceftriaxone (CRO), cefotaxime (CTX) and Aztreonam (ATM) 30 µg each were placed at 20 mm distance (centre to centre) from Amoxicillin-Clavulanic acid disk prior to incubation. The plate was incubated at 37^0C for 24 h. Enhancement of the zone of inhibition of any one of the four drug disks toward Amoxicillin-Clavulanic acid suggested the presence of extended spectrum beta-lactamases.

Combined disk test (CDT) as recommended by the CLSI, for detecting ESBLS in *K. pneumoniae* and *E. coli*, were also performed in all presumed to be ESBL producer by placing disks of ceftazidime (CAZ), cefotaxime (CTX) (30 µg each), ceftazidime-

Table 1. Primer sequences and PCR conditions used to detect ESBL genes.

Gene	Primer	Sequence (51 – 31)	PCR Conditions	Amplicon size (bp)	Reference
Bla GES	GES IF GES IR	ATGCGCTTCATTCACGCAC CTATTTGTCCGTGCTCAGG	Initial denaturation of 94°C for 4 min; 35 cycles of denaturation of 94°C for 1 min; annealing at 50°C for 1 min; extension at 72°C for 1min and final extension at 72°C for 5 min	860	Poirel et al. (2001)
BLa PER	PER-lF PER 1R	AATTTGGGCTTAGGGCAGAA ATGAATGTCATTATAAAAGC		933	Z21957
OXA-50	OXA-F OXA-R	GAAAGGCACCTTCGTCCTCTAC CAGAAAGTGGGTCTGTTCCATC	Initial denaturation of 95°C for 5 min; 35 cycles of denaturation of 95°C for 1 min; annealing at 54°C for 1 min; extension at 72°C for 45S and final extension at 72°C for 10 min	400 -	Accession No.: Am117128 -
TEM	F R	GAGACAATAACCCTGGTAAAT AGAAGTAAGTTGGCAGCAGTC	Initial denaturation of 94°C for 5min; 35 cycles of denaturation of 94°C for 45S; annealing at 55°C for 30S; extension at 72°C for 3 min and final extension at 72°C for 5 min	459 -	Parajuli et al. (2016) -
SHV	F R	GTCAGCGAAAAACACCTTGCC GTCTTATCGGCGATAAACCAG	Initial denaturation of 94°C for 5 min; 35 cycles of denaturation of 94°C for 45 S; annealing at 60°C for 30 S; extension at 72°C for 3 min and final extension at 72°C for 5 min	383	Parajuli et al. (2016)
CTX-M	F R	GAAGGTCATCAAGAAGGTGCG GCATTGCCACGCTTTTCATAG		560 -	Parajuli et al. (2016)

clavulanic acid (CAZ/CAC), and cefotaxime-clavulnaic acid (CTX/CEC), (30/10 µg each) (Hi-Media) on Mueller-Hinton agar plates which was inoculated with test strain at a distance of 20 mm from each other and then incubated at 37°C for 18 to 24 h. Isolate that showed increase of ≥5 mm in the zone of inhibition of the combination disks in comparison to that of the ceftazidime or cefotaxime disk alone was considered as ESBL producer.

DNA extraction and PCR for detection of gene encoding ESBLS

The genomic DNA extraction was performed using the modified boiling method (Katvoravutthichai et al., 2016). First, *P. aeruginosa* isolates were inoculated into a sterile brain heart infusion broth (Oxoid, U.K.) and incubated at 37°C for 72 h. One millilitre of the incubated broth was centrifuged at 12,000×g for 5 min and harvested cells were washed and re-suspended in 50 µl of Nuclease-free water (Norgen, Biotek Corop, Canada). The cells suspension were boiled at 95 to 100°C for 10 min; subjected to cold shock treatment on ice for 10 min and then centrifuged at 12000×g for 10 min.

Fifty microliters of the supernatant was transferred to a new micro centrifuge tube. To the supernatant, 100 µl of ice-cold absolute ethanol was added; mixed to precipitate out the DNA and kept on ice for 30 min. This was centrifuged at 12000×g for 10 min to pellet the DNA. The pelleted DNA was washed using 100 µl of 70% ice-cold ethanol and centrifuged at 12000×g for 2 min. The pellet was washed three times; air dried and dissolved in 50 µl of 1X TE buffer, pH 8.0.

Polymerase chain reaction for detection of ESBL genes

The PCR for detection of ESBL genes was carried out using the new England Bio labs one Taq 2X master mix with standard buffer. Amplification was carried out in a 25 µl total volume of PCR mixture containing 12.5 µl of 1X master mix (England Bio Lab) with standard buffer, 20 µM Tris-Hcl, 1.8 mM $MgCl_2$, 22 mM NH_4Cl, 22 mM KCL, 0.2 mM dNTPs, 5% glycerol, 0.06% GEPAL CA-630, 0.05% Tween 20, 25 units/ml Taq DNA polymerase; 0.5 µl of 10 µM each of primers (Inqaba, Biotech, South Africa) (Table 1); 5 µl of the extracted DNA and 6.5 µl of sterile Nuclease free water (Norgen, Biotek Corop, Canada).

The PCR amplification program for the primers used is shown in Table 1. The PCR was performed in a thermal cycler machine (BIBBY) - Scientific Ltd., UK. The PCR products were separated on 1.5% agarose gel stained with ethidium bromide (5 µg/ml) and electrophoresis was carried out at 70 V for 90 min. The gel was visualized under UV transilluminator (UP Land, USA). A 100 bp DNA Ladder (New England Bio labs) was used as DNA molecular weight marker.

Table 2. Antimicrobial susceptibility profiles of *P. aeruginosa* isolates.

Group of antibiotics	Antibiotic	Sensitive (%)	Resistant (%)
Cephalosporin	Cefepime	16 (47.06)	18 (52.94)
	Ceftriaxone	3 (8.82)	31 (91.18)
	Ceftazidime	16 (47.06)	18 (52.94)
	Cefotaxime	-	34 (100)
Monobactam, Fluoroquinlones, Aminoglycosides, Lipopetide, Carbapenems	Aztreonam	6 (17.65)	28 (82.35)
	Ciprofloxacin	18 (52.94)	16 (47.06)
	Gentamicin	20 (58.82)	14 (41.18)
	Polymyxin B	27 (79.41)	7 (20.59)
	Imipenem	31 (91.18)	3 (8.82)
	Meropenem	29 (85.29)	4 (11.76)
Penicillin, B-lactam inhibitors	Piperacillin	5 (14.71)	28 (82.35)
	Piperacillin/Tazobactam	18 (52.94)	16 (47.06)

Table 3. Resistance patterns of *P. aeruginosa* isolates.

Resistance patterns	No. of isolate
MEM^R, CIP^R, PB^R, CN^R, CEF^R, CRO^R, CAZ^R, CTX^R, ATM^R, PRL^R, TZP^R	-
MEM^R,CIP^R, CN^R, CEF^R, CRO^R, CAZ^R, CTX^R, ATM^R,PRL^R, TZP^R	4
IMP^R, CIP^R, CN^R, CEF^R, CAZ^R, CTX^R, ATM^R ,TZP^R	2
CRO^R, CAZ^R,CTX^R, ATM^R	24
MEM^R ,CIP^R, PB^R, CEF^R, CRO^R, CAZ^R, CTX^R, ATM^R, PRL^R	2
CRO^R, CTX^R, ATM^R	2
Total	34

CIP^R = Ciprofloxacin resistant; CRO^R =Ceftriaxone Resistant; PB^R = polymyxin-B resistant; CAZ^R = Ceftazidime resistant; CN^R = Gentamicin resistant; CTX^R = Cefotaxime resistant; CEF^R = Cefepime resistant; MEM^R = Meropenem resistant; IMP^R = Impenem resistant ; ATM^R = Aztreonam resistant.

RESULTS

Out of 34 clinical isolates of *P. aeruginosa* 15 (44.12%) were found to be potential ESBL producers by preliminary screening. Antimicrobial susceptibility pattern revealed that most of *P. aeruginosa* isolates were resistant to cefotaxime (100%), ceftriaxone (91.18%) and aztreonam (82.35%) while the isolates were generally sensitive to carbapenems group (imipenen 91.18% and meropenem 85.29%), followed by polymyxin B (79.41%) and gentamicin (58.82%) (Table 2). Six different resistance patterns were identified among the *P. aeruginosa* isolates. Twenty four (24) out of thirty-four (34) *P. aeruginosa* isolates were resistant to four antibiotics ceftazidime, cefotaxime, ceftriaxone and Aztreonam shown in Table 3. The information describing the detection ESBL- positive *P. aeruginosa* isolates by different phenotypic tests in relation to the resistance patterns is shown in Table 4. The first group, 24 out of 34 *P. aeruginosa* isolates, were resistant to ceftazidime, cefotaxime, ceftriaxone and Aztreonam while the second group, 2 out of 34 isolates consisted of isolates that were resistant to ceftriaxone, cefotaxime, and aztreonam and at the same time sensitive to ceftazidime.

The highest percentage of ESBL-positive strains among these two groups was detected using the combined disk test (CDT) with emphases on cefotaxime (CTX) alone and cefotaxime-clavulanic acid (CTX/CEC) combination (Figure 1). The PCR method was used to investigate β-lactamase genes in the 15 isolates of ESBL producing *P. aeruginosa* for six genes, namely *bla PER, GES, CTX-M, TEM, SHV* and *OXA-50*. Out of fifteen (15) ESBL producing *P. aeruginosa* isolates that were screened for six ESBL genes. Only *bla OXA*-50 genes was able to show positive amplification in eight (8) isolates (53.33%) (Figure 2).

However, none of the isolates was positive for the *bla-PER, GES, CTX-M, SHV,* and *TEM* genes.

Table 4. Detection of ESBL producing *P. auriginosa* isolates by phenotypic test among the isolates that were resistant to ceftazidime, ceftriaxone, cefotaxime and aztreonam.

Resistant patterns of isolate	No. of positive isolates		
	DDST-AMC	CAZ/CAC	CTX/CEC
CRO, CAZ CTX ATM (24)	3	-	13
CRO, CTX, ATM (2)	-	-	2
Total	3	-	15

CRO^R = Ceftriaxone Resistant; CAZ^R = Ceftazidime resistant; CTX^R = Cefotaxime resistant; ATM^R = Aztreonam resistant.

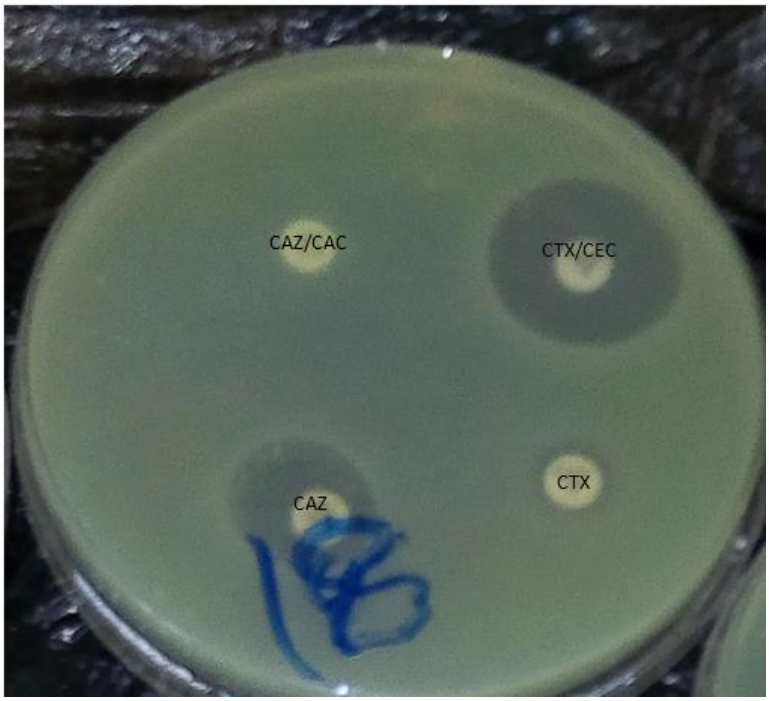

Figure 1. Combined disk test using CAZ/CAC and CTX/CEC.

DISCUSSION

The *P. aeruginosa* is one of the most important nosocomial pathogens, being responsible for various types of infections with more and more limited therapeutic options. One of the most alarming characteristic of *P. aeruginosa* is its resistance antibiotic susceptibility. This situation is due to expression of various resistance mechanisms such as drug inactivation through the production of β-lactamases, alteration of target site (e.g. alteration of PBP-the target site of penicillin), alteration of metabolic pathway and reduced drug accumulation by decreasing drug permeability or increasing active efflux (pumping out) of the drug across the cell surface.

Extended-spectrum Beta-lactamase-producing bacteria are one of the fastest emerging resistance problems worldwide. Increased global prevalence and dissemination of ESBL genes among pathogenic microorganisms are a serious peril for medical fraternity. The rates of ESBL-positive *P. aeruginosa* (44.12%) found in our study were in accordance with similar studies conducted in Bangladesh, North West of Pakistan and South West of Iran (Ullah et al., 2009; Begum et al., 2013; Mohammadi et al., 2015), although low detection rates of 3.7 to 8.1% were noted in studies conducted by others (Woodford et al., 2008; Lim et al., 2009; Tavajjohi et al., 2011).

The relatively high prevalence of ESBLS recorded in this study might be due to the extreme empirical use of third-generation cephalosporins in clinical settings. The differences in the ESBL rates may be attributable to the geographic difference, antimicrobial stewardship programme and infection control practices. ESBLS producing organisms pose unique challenges to clinical microbiologists, clinicians, and infection control agents

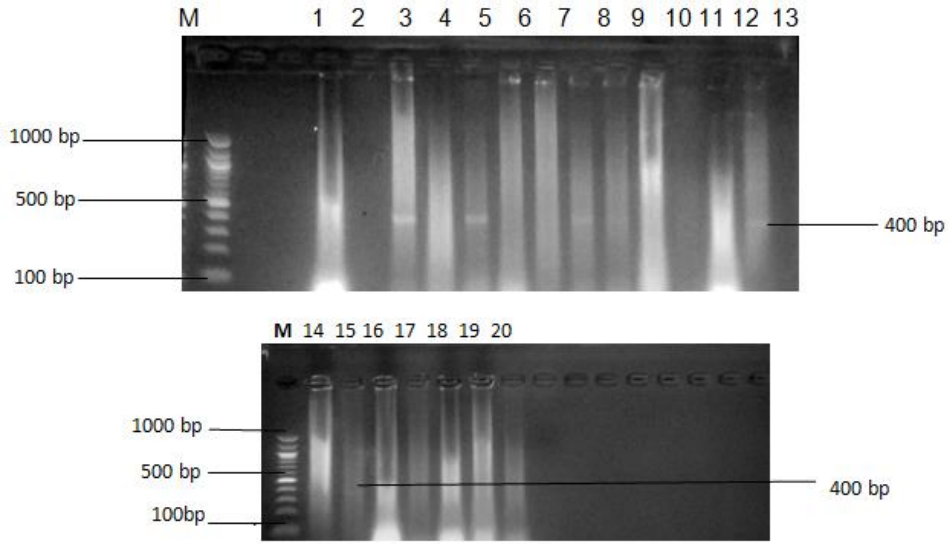

Figure 2. PCR detection of 400 bp amplicons of OXA-50 gene for identification of ESBL. Lane M shows bands for 100 bp molecular weight standard. Lanes 1, 3, 5, 8, 9, 12, 13 and 15 show positive amplification bands indicating the presence of OXA 50 gene in *P. aeruginosa* isolates analyzed. Other lanes show negative amplification and produced no visible band.

ESBL producing bacteria are frequently resistant to many classes of antibiotics resulting in difficult to treat infections. The ESBL producing *P. aeruginosa* isolates exhibited high level of resistance against most of the antibiotics tested (Table 2). Currently carbapenems are regarded as the drugs of choice for treatment of infections caused by ESBL producing organisms. In this study, 91.18 and 85.29% of all the *P. aeruginosa* isolates were sensitive to imipenem and meropenem, respectively. The introduction of carbapenems into clinical practice represented a great advance for the treatment of serious bacterial infection caused by β-lactam resistant bacteria. Due to their broad spectrum of activity and stability to hydrolysis by most beta lactamases, carbapenems have been the drug of choice for treatment of infections caused by penicillin or cephalosporin-resistant Gram-negative bacilli especially ESBL producing strains (Mandiratta et al., 2005). Unfortunately, use of carbapenems has been associated with the emergence of carbapenem-resistant *P. aeruginosa* as observed in this study, 8.82 and 11.76% of isolates were resistant to imipenem and meropenem, respectively. This finding is consistent with the work of Tripathi et al. (2011) and Jayanthi and Jeya (2014) who reported that 5.88 and 9.77% of *P. aeruginosa* isolates were resistant to imipenem. Similar findings have been documented in other countries such as Egypt and India (Senthamarai et al., 2014; Raafat et al., 2016). So, proper infection control practices and antimicrobial susceptibility testing before treatment are essential to prevent the spread and outbreaks of ESBL-producing bacteria. Since *P. aeruginosa,* is the recipient of various genes due to its genetics nature such as plasmids and transposon, therefore it can quickly be resistant to various antibiotics.

With the recent detection of *GES, PES, CTX-M, SHV* and *TEM* producing strains in several countries (Tavajjohi et al., 2011; Ahmed and Asghar, 2017; Laudy et al., 2017), the appearance of ESBL producing clinical isolates of *P. aeruginosa* can be anticipated in Nsukka. Our results suggest the lack of these genes on the *P. aeruginosa* isolates tested. Extended spectrum β lactamase in the bla *GES, PES, CTX-M, SHV* and *TEM* negative isolates is most likely due to other mechanisms such as active drug efflux pumps and cell membrane mechanism and gene mutation. Further studies are necessary to conclude that these genes are not present in the *P. aeruginosa* isolates circulating in this area of the country.

The present study showed that 15 strains of *P. aeruginosa* were identified as ESBL by phenotypic method. Of these 15 phenotypic ESBL isolates, 8 (53.33%) expressed the bla *OXA-50* gene. This gene was identified for first time in *P. aeruginosa* isolates from Turkey and France (Aktas et al., 2005; Peterson and Bonomo, 2005) and in Romania (Crăciunas et al., 2010). In Taiwan, *bla oxa-17* and *bla oxa-10* genes have been detected in *P. aeruginosa* (Du et al., 2010). This finding is in agreement with the work conducted by Porjafari et al., (2013) who reported similar occurrence of *OXA-50* gene among the *P. aeruginosa* strains collected in their hospitals. In Bangkok, Thailand *Oxa-10* was the predominant clone of *P. aeruginosa* clinical isolates (Katvoravutthichai et al., 2016). This raises concern about oxacillinases among *P. aeruginosa* clinical isolates.

The *OXA-50* gene detected in this study belonged to group D β-lactamases. Selective antibiotic pressure that develops in response to over use of β-lactam antibiotics particularly in hospitals can be responsible for the expression and dissemination of these enzymes. The threat of treatment failure is amplified by the evolution of *P. aeruginosa* strains expressing extended spectrum oxacilinase activity.

In conclusion, to the best of our knowledge, this study is the first to report the presence of *bla OXA* gene among clinical isolates of *P. aeruginosa* in Nsukka, Southeast Nigeria. The emergence of extended spectrum group D β-lactamases among *P. aeruginosa* isolates must be taken seriously. There is a need for a comprehensive review in antibiotic prescription and usage to prevent the spread of these pathogens.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors wish to thank the generous assistance of the doctors and nurses of the different hospitals used, for their cooperation, understanding and assistance when this research was going on. The authors gratefully acknowledge the microbiology department of the various hospitals for willingly providing the *Pseudomonas aeruginosa* clinical isolates used in this study.

REFERENCES

Ahmed OB, Asghar AH (2017). Antibiotic susceptibility pattern of *P. aeruginosa* expressing *bla GES* and *bla PER* genes in two different hospitals. African Journal of Biotechnology 16(21):1197-1202.

Aktas Z, Poirel L, Salcioglu M, Ozcan PE, Midilli K, Bal C, Ang O, Nordmann P (2005). PER-1 and OXA-10-like β-lactamases in Ceftazidime-resistant *P.aeruginosa* isolates from intensive Care unit patients in Istandul, Turkey. Clinical Microbiology and Infection 11(3):193-198.

Begum S, Salam MDA, Alam KHF, Begum N, Hassan P,Haq JA (2013). Detection of extended spectrum β-Lactamase in *Pseudomonas* spp.isolated from two tertiary care hospitals in Bangladesh. Biomedical Research Notes 6:7.http://www.biomedcentral.com/1756-0500/6/7.

Bush K, Jacoby GA (2010). Updated functional classification of Beta-lactamase. Antimicrobial Agents and Chemotherapy 54:969-976. http://dx.doi.org/10.1128/AAC.01009-09.

Clinical and Laboratory Standards Institute, CLSI (2014). Performance Standards for Antimicrobial Susceptibility Testing: Twenty-fourth Informational Supplement.M100-S24 (34):1.

Cráciunas C, Butiuc-Keul A, Flonta M, Brad A, Sigarteu M (2010). Application of molecular techniques to the study of *P. aeruginosa* clinical isolates in cluj-napoca, Romania. Analele Universitatii din Oradea-fascicula Biologie 2(17):243-247.

Drieux L, Brossier F, Sougakoff W, Jarlier V (2008). Phenotypic detection of extended-spectrum β-lactamase production in Enterobacteriaceae: review and bench guide. Clinical Microbiology and Infection 14(1):90-103.

Du ST, Kuo HC, Cheng CH, Fel ACY, Wei HW, Chang SK (2010). Molecular Mechanisms of Ceftazidime resistance in *P.aeruginosa* isolates from canine and human infections. Veterinarni Medicina 55(4):172-182.

Jarlier V, Nicolas MH, Fournier G, Philippon A (1998). Extended spectrum β-lactamase conferring transferable resistance to newer β-lactam agents in Enterobacteriacceae: Hospital prevalence and susceptibility patterns. Review and Infection Diseases 10:867-878.

Jayanthi S, Jeya M (2014). Plasmid profile analysis and bla_{VIM} Gene Detection of metallo β-lactamase (MBL) producing *Pseudomonas aeruginosa* isolates from clinical samples. Journal of Clinical and Diagnostic Research 8(6):DC16-DC19.

Katvoravutthichai C, Boonbumrung K, Tiyawisutsri R (2016). Prevalence of β-lactamase classes A, C, and D among clinical isolates of *P. aeruginosa* from a tertiary – level hospital in Bangkok, Thailand. Genetics and Molecular Research 15(3):doi: 10.4238/gmr.15038706.

Laudy AE, Rôg P, Smolińska-król K, ćmiel M, Sloczyńska A, Patzer J, Dzierźanowska D, Wolinowska R, Starościak B, Tyski S (2017). Prevalence of ESBL – producing *P. aeruginosa* isolates in Warsaw, Poland, detected by various phenotypic and genotypic methods. PLOS One 12(6):e0180121.

Lee S, Park Y, Kim M, Lee HK, Han K, Kang CS, Kang MW (2005). Prevalence of Ambler Class A and D beta-lactamase among clinical isolates of *P. aeruginosa* in Korea. Journal of Antimicrobial Chemotherapy 56(1):122-127.

Lim KT, Yasin RM, Yeo CC, Puthucheary SD, Balan G, Maning N, Wahab ZA, Ismail N, Tan EA, Mustaffa A, Thong KL (2009). Genetic finger printing and antimicrobial susceptibility profiles of *P. aeruginosa* hospital isolates in Malaysia. Journal of Microbiology, Immunology and Infection 42:197-209.

Livermore DM (2002). Multiple mechanisms of antimicrobial resistance in *P. aeruginosa*: Our worst nightmare? Clinical infectious Diseases 34:634-640. http://dx.doi.org/10.1086/338782.

Mandiratta DK, Deotale V, Narang P (2005). Metallo beta lactamase producing *P. aeruginosa* in a hospital from rural area. Indian Journal of Medical Research 121:701-703.

Mohammadi AA, Emami A, Bazargani A, Zardosht M, Jafari SMS (2015).Detection of bla $_{PER-1}$ and bla $_{OXA-10}$ among Imipenem resistant isolatesof *P. aeruginosa* isolated from burn patients hospitalized in Shiraz Burn Hospital. Iranian Journal of Microbiology 7(1):7-11.

Moreaus-Marquis S, Stanton BA, O'Toole GA (2008). *P.aeruginosa* biofilm formation in the fibrosis airways. Pulmonary Pharmacology and Therapeutics (21):595-599.

Parajuli NP, Maharjan P, Joshi G, Khanal PR (2016). Emerging Perils of Extended Spectrum β- Lactamase producing Enterobacteriaceae Clinical Isolates in a Teaching Hospital of Nepal. Biomedical Research International. https://www.hindawi.com/journals/bmri/2016/1782835/

Peterson DL, Bonomo RA (2005). Extended Spectrum β-lactamases: A clinical update. Clinical Microbiology Review 18(4):657-686.

Poirel L, Nass T, Nordmann P (2010). Diversity, epidemiology, and genetics of class D. Beta Lactamases. Antimicrobial Agents and Chemotherapy 54:24-38. http://dx.doi.org/10.1128/AAC.01512-08.

Poirel L, Weldhagen GF, Naas T, Champs C, Dove MG, Nordmann P (2001). GES-2, a class A β-Lactamase from *P.aeruginosa* with increased hydrolysis of Imipenem. Antimicrobial Agents and Chemotherapy 45(1):2598-2603.

Porjafari M, Ghane M, Moghadam RG, Azimi Z, Ghaffary H, Kouchaki M (2013). Prevalence of the resistance genes to extended Spectrum β-Lactam antibiotics in *P. aeruginosa* Strains collected from different parts of Tonekabon Shahid Rajai hospital in North of Iran, Using PCR Technique. Annals of Biological Research 4(12):163-168.

Raafat MM, Ali-Tammam M, Ali AE (2016). Phenotypic and genotypic characterization of *Pseudomonas aeruginosa* isolates from Egyptian hospital. African Journal of Microbiology Research 10(39):1645-1653.

Senthamarai S, Suneel K, Peddey A, Sivasankari S, Anitha C, Somasunder V, Kumudharathi MS, Amsharathani SK, Venugopal V (2014). Resistance pattern of *Pseudomonas aeruginosa* in a Tertiary care Hospital of Kanchipuram, Tamilnadu, India. Journal Clinical and Diagnostic Research 8(5):DC30-DC32.

Sharma M, Pathak S, Srivastava P (2013). Prevalence and antibiogram

of Extended spectrum β-lactamase (ESBL) producing Gram negative bacilli and further molecular characterization of ESBL, producing *Escherichia coli and Klebsiella ssp*. Journal of Clinical and Diagnostic Research 7(10):2173-2177.

Shen JL, Fang VP (2015). Detection of drug resistance mechanism of *P. aeruginosa* developing from a sensitive strain to a persister during carbapenem treatment. Genetic and Molecular Research 14:6723-6732.

Tam VH, Chang KT, Abdeiraouf K, Brioso CG, Ameka M, McCaskey KA, Weston JS, Caeiro JB, Garey KW (2010). Prevalence, resistance mechanisms, and susceptibility of multidrug-Resistant blood stream isolates of *P. aeruginosa*. Antimicrobial Agents and Chemotherapy 54:1160-1164.

Tavajjohi Z, Moniri R, Khorshidi A (2011). Detection and characterization of multidrug resistance and extended-spectrum beta-lactamase-producing (ESBLS) *P. aeruginosa* isolates in teaching hospital. African Journal of Microbiology Research 5(20):3223-3228.

Todar K (2008). *Pseudomonas aeruginasa*. In: Online Textbook for Bacteriology.

Todar K (2014). *P. aeruginosa*. Available at [http://textbook of bacteriology.net/pseudomonas.html]. accessed May 30, 2016.

Tripathi P, Banerjee G, Saxena S, Gupta MK, Ramteke PW (2011). Antibiotic resistance pattern of *Pseudomonas aeruginosa* isolated from patients of lower respiratory tract infection. African Journal of Microbiology Research 5(19):2955-2959.

Ullah F, Malik SA, Ahmed J (2009). Antimicrobial susceptibility and ESBL prevalence in *P. aeruginosa* isolated from burn patients in the North West of Pakistan. Burns 35(7):1020-1025.

Wirth FW, Picoli SU, Cantarelli VV, Goncalves AL, Brust FR, Santos LM, Barreto MF (2009). Metallo- β-lactamase-producing *P. aeruginosa* in two hospitals from Southern Brazil. Brazilian Journal of Infectious Diseases 13:170-172.

Woodford N, Zhang J, Kaufmann ME, Yarde , Tomas Mdel M, Faris C, Vardhan MS, Dawson S, Cotterill SL, Livermore DM (2008). Detection of *P. aeruginosa* isolates producing VEB-type extended spectrum β -lactamase in the United Kingdom. Journal of Antimicrobial Agents and Chemotherapy 62:1265-1268.

Vol. 18(32), pp.1091-1097, December, 2019
DOI: 10.5897/AJB2019.16924
Article Number: 6B51AB362476
ISSN: 1684-5315

http://www.academicjournals.org/AJB

ACADEMIC JOURNALS
expand your knowledge

African Journal of Biotechnology

Full Length Research Paper

Evaluation of *Lactobacillus* spp. isolated from locally consumed probiotic food in Nsukka, Enugu State, Nigeria for antimicrobial activity utilizing agar well diffusion and pH tolerance tests

Dinebari P. Berebon[1]*, Kenneth C. Ofokansi[2], Anthony A. Attama[2], Christopher O. Eze[1], Restus C. Onwusoba[1], Chukwudi T. Ugwu[1], Charity C. Eze[1] and Somtochukwu A. Evurani[1]

[1]Department of Pharmaceutical Microbiology and Biotechnology, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka, Enugu State, Nigeria.
[2]Department of Pharmaceutics, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka, Enugu State, Nigeria.

Received 29 July 2019; Accepted 3 October 2019

Antimicrobial activity against entero-pathogens and tolerance to acid stress are crucial characters of probiotic bacteria. *Lactobacillus* spp. isolates were phenotypically characterized using colony observation, catalase test and Gram stain reaction. The pH (1.5, 2.5 and 3.5) tolerance of each isolate was evaluated at 0 and 4 h. The antibacterial activities of the isolates were tested against pathogenic strains of *Bacillus* spp., *Escherichia coli, Pseudomonas aeruginosa, Salmonella typhimurium* and *Staphylococcus aureus.* Among the isolates screened, 20 were Gram positive and catalase negative. In an agar well diffusion, isolate O_4 resulted in the highest inhibition zone diameter, 13 mm against *P. aeruginosa* while isolates OK_4, UK_1, P_2, P_3 and P_4 did not produce any inhibition zones against any of the pathogens tested. Isolate Y_1 showed the broadest inhibitory activity against the pathogens tested inhibiting all the pathogens tested except *S. typhimurium*. The pH tolerability studies showed that the isolates proliferated more at lower acidic pH: 1.5 > 2.5 > 3.5. Food products containing Ogiri, Ukpaka, Okpeye, Akamu and Yoghurt provides useful sources of probiotic bacteria.

Key words: Antimicrobial activity, *Lactobacillus spp*, pH tolerance, entero-pathogens.

INTRODUCTION

There is substantial concern that pharmaceutical industries are not able to develop novel and effective antibiotics at a rate sufficient to compete with the emergence of microbial resistance to antibiotics used in the clinic. Thus, interest in the use of probiotic foods with beneficial microorganisms as an alternative to antibiotic therapy has geometrical increased within the past decades. The antibacterial properties and tolerance to

*Corresponding author. E-mail: dinebari.berebon@unn.edu.ng.

low pH of lactic acid bacteria (LAB) is essential in their applicability as probiotics. To achieve a high concentration (10^9 to 10^{11} CFU/day) and to allow for their beneficial action, LAB must survive the low pH of the gastrointestinal tract (GIT), colonize it and synergistically dislodge pathogenic bacteria via production of defensive metabolites.

Probiotics are 'live microorganisms which, when administered in adequate amounts, confer a health benefit on the host' (FAO/WHO, 2002). Importantly, probionts with probiotic grade must be devoid of any adverse effects (cytotoxicity, antibiotic resistance and hemolysis), and endowed with beneficial traits to inhibit pathogenic bacteria through different mechanisms as reported in different studies (Vieco-Saiz et al., 2019). Probiotics as well as their bacteriocins (produced by some probiotic organisms) are considered to substitute for antibiotics in the food and pharmaceutical sector. Lactic acid bacteria (LAB), commonly regarded as the major group of probiotic bacteria, are rods or cocci, facultative anaerobes, belong to the non-spore forming firmicutes groups with low Guanine (G) and cytosine (C) – G + C (< 50%) with members belonging to the genera - *Enterococci, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus* and *Streptococcus* (Berebon et al., 2019).

There are stringent selection criteria for the development of commercial probiotics. It is based on their unmistakable taxonomic identification, safety assessment, *in vitro* and *in vivo* functional characterization (FAO/WHO, 2006). Importantly, they should be tolerant to acids and bile salts, survival during passage through the gastrointestinal tract (GIT), ability to colonize the intestine, antagonistic properties against pathogenic microorganisms (Shokryazdan et al., 2017). Probiobics should be able to stimulate the immune system, degrade toxic substances and improve absorption of certain nutrients, accompanied by good technological properties (Grajek et al., 2016).

Stefanis et al. (2016) and Kaur et al. (2015) listed basic criteria that a microorganism should fulfill in order to be characterized as a probiotic among which is their ability to withstand and survive physiological stress of acidic pH within the GIT and possession of antimicrobial activity against potentially pathogenic bacteria. The pH tolerance of probiotics is well documented in the literatures (Sahadeva et al., 2011, Hassanzadazar et al., 2012). The aim of this study is to isolate potential probiotic isolates from locally fermented and ready to eat food and evaluate them for probiotic characters.

Antimicrobial metabolites secreted by LAB mitigate the proliferation of pathogens within the GIT. The preservative potentials of LAB in food matrix is attributed to the production of antimicrobial metabolites including organic acids and bacteriocins (Macaluso et al., 2016). Hawaz (2014) reported the antimicrobial activities of LAB against pathogenic bacteria such as: *Bacillus* sp., *Escherichia* sp., *Pseudomonas* sp., *Salmonella* sp. and *Staphylococcus* sp.

LAB-probiotics can reduce the spread of pathogenic bacteria by mechanisms involving production of inhibitory compounds and competitive exclusion (Vieco-Saiz et al., 2019). The antimicrobial effect of LAB may be due to the production of antimicrobial peptides (AMPs) or small organic molecules such as organic acids, ethanol, diacetyl, carbon dioxide, hydrogen peroxide and smaller peptides, that is, bacteriocins (Liao and Nyachoti, 2017). Several bacteriocins have been shown to act in synergy with conventional antibiotics (Cavera et al., 2015, Wolska et al., 2012), thus reducing bactericidal concentrations and reduction in their undesirable side-effects while some produced by Gram positive bacteria are active against viruses (Ben Lagha et al., 2017)

Different techniques had been reported for assaying the antimicrobial activity of LAB. Some examples that were previously reported are delayed antagonism, disc diffusion assay, spot on lawn or agar overlay (immersion bioautography), well diffusion assay and paper disc methods (Soomro et al., 2007; Macaluso et al., 2016; Balouiri et al., 2016; Oliveira et al., 2017), critical dilution assays (Barbosa et al., 2016), flip-streak method (Lewus and Montville, 1991).The aim of this study was to isolate, evaluate the antimicrobial activity and pH tolerance of LAB isolated from locally consumed probiotic food in Nsukka, Enugu State, Nigeria.

MATERIALS AND METHODS

Chemicals and reagents

A 70% ethanol, 3% hydrogen peroxide, dilute sodium hydroxide and dilute HCl solution were prepared from their stock solutions. Gentian violet, Lugol's iodine, safranin, immersion oil, were obtained from their manufacturers and prepared as needed. All chemicals used for the study were of analytical grade.

Food samples and pathogenic organisms

Food samples

Food samples included processed Ogiri (*Sesamum indicum L.*), Okpeye (Iron plant - *Prosopsi africana* endosperm), Pap or Akamu (*Zea mays* steep liquor), Ukpaka (Oil bean - *Pentaclethra macrophylla*) and Yoghurt (Aqua Rapha®) were used. The samples were coded as: O (Ogiri), OK (Opkeye), P (Pap), U (Ukpaka) and Y (Yoghurt) respectively.

Pathogens

Pathogens included Bacillus *Pseudomonas aeruginosa*,

Salmonella sp, *typhimurium, Staphylococcus aureus* and *Escherichia coli.* All pathogenic isolates were obtained from the Department of Pharmaceutical Microbiology and Biotechnology laboratory, UNN.

Media used

Media used in this study includes De Man Rogosa and Sharpe (MRS) agar, De Man, Rogosa and Sharpe (MRS) broth, Nutrient agar.

Collection of food samples

A total of 60 samples comprising of 12 samples per food type (Okpeye, Ogiri, Pap, Ukpaka and Yoghurt) were randomly procured between May and June, 2018 from Ikpa market in Nsukka L.G.A, Enugu State, Nigeria. Samples were transferred in an icebox (-4°C) to the laboratory using sterile containers.

Isolation of potential lactic acid bacteria

A tenfold serial dilution and a spread plate method was used as reported in previous study (Berebon et al., 2019). Based on visual examination a distinct colony was picked with a sterile wire loop and transferring aseptically into a sterile MRS agar plate by quadrant streak method. The plates were incubated anaerobically at 37°C for 24 to 48 h. Each isolate was tested for presence of catalase production. Only isolates which tested negative for catalase production were selected and stocked on MRS slants in a bijou bottle for further studies.

Colony characteristics of lactic acid bacteria

Each colony was observed and the following features were determined: Margin, size, surface, elevation, form recorded. A total of 20 isolates with LAB morphologies on MRS agar plates were selected and coded as: O_1, O_2, O_3, O_4; OK_1, OK_2, OK_3, OK_4; P_1, P_2, P_3, P_4; UK_1, UK_2, UK_3, UK_4 and Y_1, Y_2, Y_3, Y_4.

Preparation of standard inoculum

Each potential probiotic isolate was inoculated into 10 ml MRS broth in test tubes. The cultures were incubated for 24 h at 37ºC. These cultures were used as the standard inoculum for further experiments.

pH tolerance test

The pH of duplicate tubes of 10 ml of MRS broth was adjusted to pH 1.5, 2.5 and 3.5 using 1 M HCl and 1 M NaOH and autoclaved at 121ºC for 15 min. A 100 µl of each probiotic culture was added to each tube and adjusted to 0.5 McFarland standard. The absorbance readings of each isolate was taken at 0 and 4 h using UV/Visible spectrophotometer (Spectrumlab 725s, England) at λ_{max} of 600 nm. Sterile MRS broth pH 7.0 (control) was used as blank.

Antimicrobial activity assay

The antibacterial activity of the LAB isolates against pathogenic pathogens was cultured overnight in Brain Heart Infusion agar. A 100 µl of the pathogenic bacteria (adjusted to 0.5 McFarland standards using sterile saline) were spread on nutrient agar plates and the 100 µl of LAB were added to wells and allowed to diffuse at room temperature into the agar. Subsequently, the inoculated plates were incubated at 37°C for 24 to 48 h. The antimicrobial activity of each probiotic strains was evaluated by measuring the inhibition zone diameter (IZD) around probiotic growth.

RESULTS AND DISCUSSION

Table 1 shows the colonial morphologies of the potential probiotic bacteria isolates belonging to the genus *Lactobacillus* with small circular colonies , Gram positive, non-motile anaerobic and catalase negative.

The survival patterns of each isolate at different acidic pH conditions of 1.5, 2.5 and 3.5 (Figures 1 to 3) indicated that the various potential Lactobacilli survived more generally at low acidic condition with the general decrease in survival at pH: 1.5 > 2.5 > 3.5. This observation corroborated the findings of - Tokatlı et al. (2015) that 35 to 85 % and 33 to 64 % strains of *L. plantarum* and *L. brevis* survived at pH 2.5 for 4 h. However, Succi et al. (2017) noted that *L. plantarum* only exhibited a slight growth at pH: 3.5 and 4.0 but not at 3.0. The isolates are acid tolerant, a property characteristic of probiotic bacteria. Taken together, these results show that pH tolerance is strain - dependent among *Lactobacillus* spp. At pH 1.5, all the probiotic isolates showed increase in growth after 4 h except Y_4 which had remarkable reduction in growth turbidity. Other workers have reported survivability of *Lactobacillus* spp. at acidic pH (Succi et al., 2017; Ngov et al., 2014; Miller et al., 2011). Cotter and Hill (2003) and De Angelis and Gobbetti (2011) reported that acidotolerant observation in lactococci may be due to their innate pH homeostatic system such as: Arginine deiminase (ADI), H^+-ATPase proton pump, and the glutamate decarboxylase gene (GAD) which stabilizes the acid stress.

At pH 2.5, only isolate O_1 showed growth reduction with time while at pH 3.5 all the isolates showed increase in growth with time. According to Sahadeva et al. (2011), the survival pattern of the probiotic isolates at acidic pH conditions is very important because it determines the choice of probiotics used in management of gastrointestinal infections. Isolates that tolerate high acidic condition are good agents used in the management of infections of susceptible bacterial infections of the gastrointestinal tract such as: Diarrhea, peptic ulcer, colitis and salmonellosis (Vantsawa et al., 2017).

The antibacterial activity (Figure 4) of each of the isolates against *Bacillus* sp*., Pseudomonas aeruginosa*, *Salmonella typhimurium, Staphylococcus aureus,* and *Escherichia coli* show that isolate - O_4 had the widest inhibition zone diameter (IZD) of 13 mm against *P. aeruginosa* while isolates: OK_4, UK_1, P_2, P_3, and P_4 did not inhibit any of the pathogens tested. Isolate - Y_1 had the broadest activity inhibiting all the pathogens except

Table 1. Preliminary colony characteristics, catalase test and gram reaction of LAB isolates.

Isolate	Form (shape)	Color	Elevation	Margin	Surface	Gram reaction	Catalase test
OK_1	Circular	Milky	Convex	Entire	Smooth	Positive	Negative
OK_2	Circular	Milky	convex	Entire	Smooth	positive	Negative
OK_3	Circular	Milky	convex	Entire	Smooth	positive	Negative
OK_4	Circular	Milky	convex	Entire	Smooth	positive	Negative
UK_1	Circular	White	convex	Entire	Smooth	positive	Negative
UK_2	Circular	White	convex	Entire	Smooth	positive	Negative
UK_3	Circular	White	convex	Entire	Smooth	positive	Negative
UK_4	Circular	White	convex	Entire	Smooth	positive	Negative
P_1	Circular	Milky	convex	Entire	Smooth	positive	Negative
P_2	Circular	Milky	convex	Entire	Smooth	positive	Negative
P_3	Circular	Milky	convex	Entire	Smooth	positive	Negative
P_4	Circular	Milky	convex	Entire	Smooth	positive	Negative
Y_1	Circular	Milky	convex	Entire	Smooth	positive	Negative
Y_2	Circular	White	Flat	undulate	Smooth	positive	Negative
Y_3	Circular	White	Flat	Entire	Smooth	positive	Negative
Y_4	Circular	White	Flat	Entire	Smooth	positive	Negative
O_1	Circular	Milky	convex	Entire	Smooth	positive	Negative
O_2	Circular	Milky	convex	Entire	Smooth	positive	Negative
O_3	Circular	Milky	convex	Entire	Smooth	positive	Negative
O_4	Circular	Milky	convex	Entire	Smooth	positive	Negative

OK, Okpeye; UK, Ukpaka; P, Pap or Akamu; Y, Yoghurt O, Ogiri.

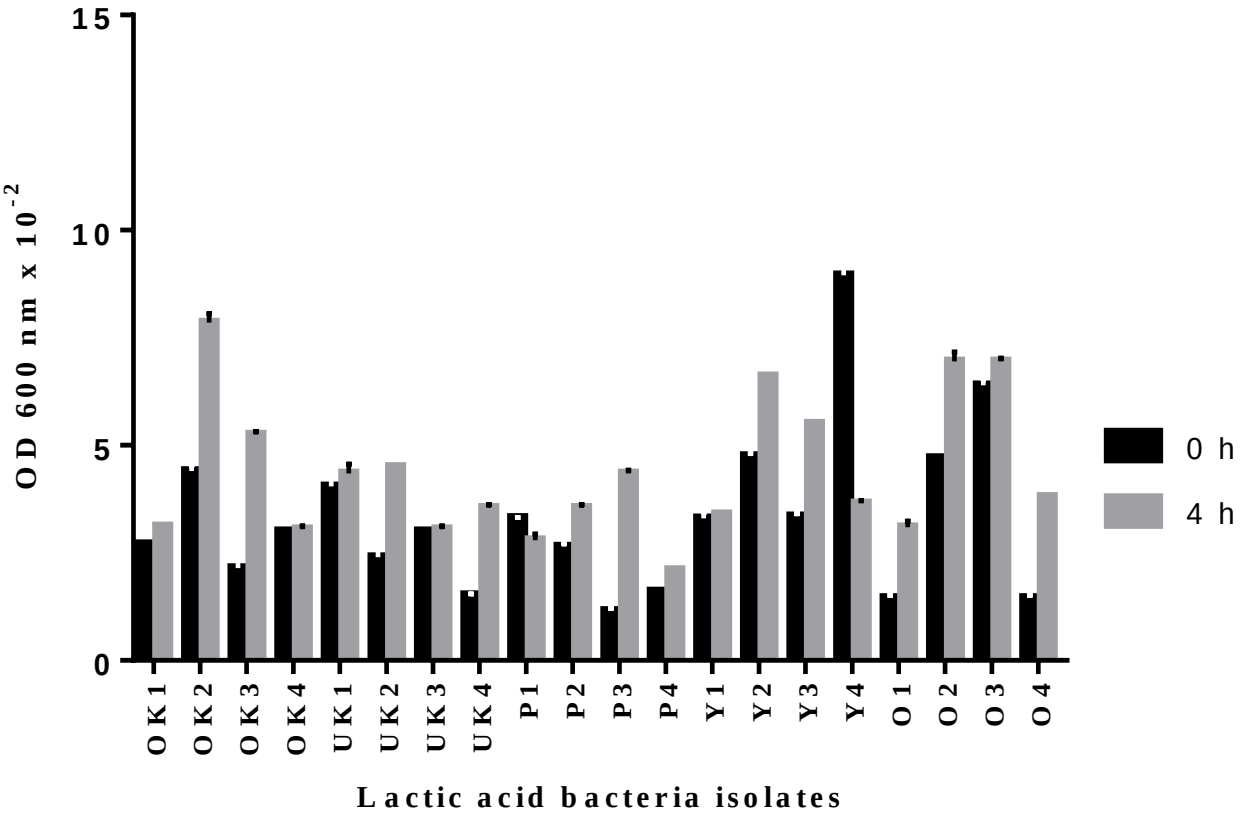

Figure 1. Tolerance of LAB at pH 1.5. OK, Okpeye; UK, Ukpaka; P, Pap or Akamu; Y, Yoghurt O, Ogiri.

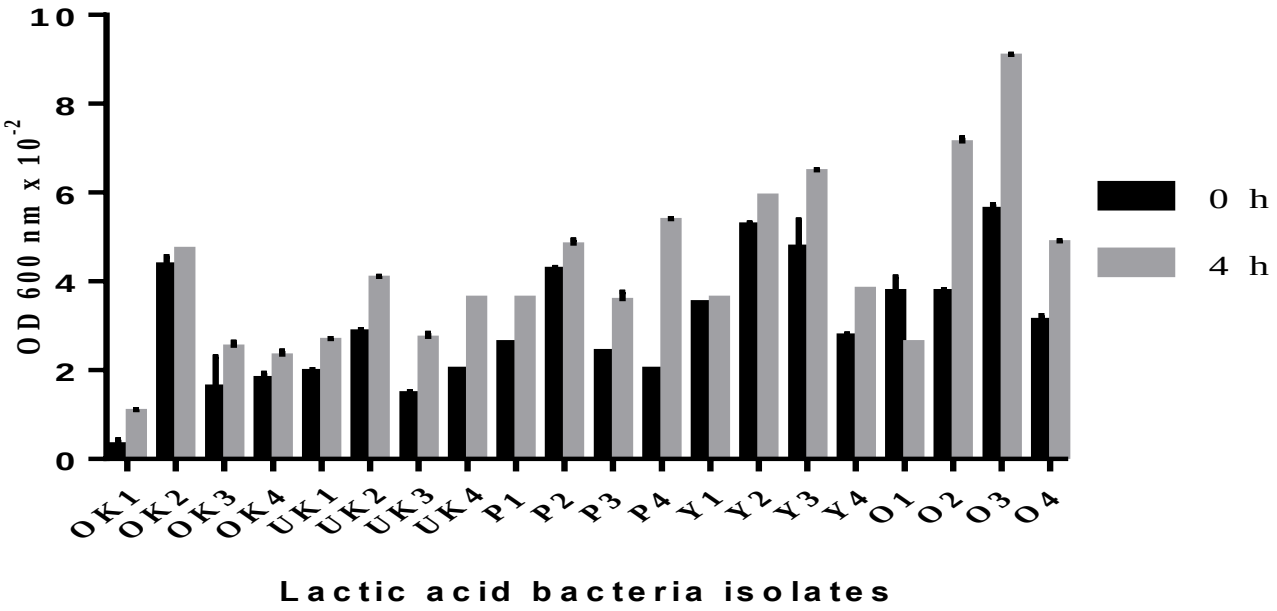

Figure 2. Tolerance of LAB isolates at pH 2.5. OK, Okpeye; UK, Ukpaka; P, Pap or Akamu; Y, Yoghurt O, Ogiri.

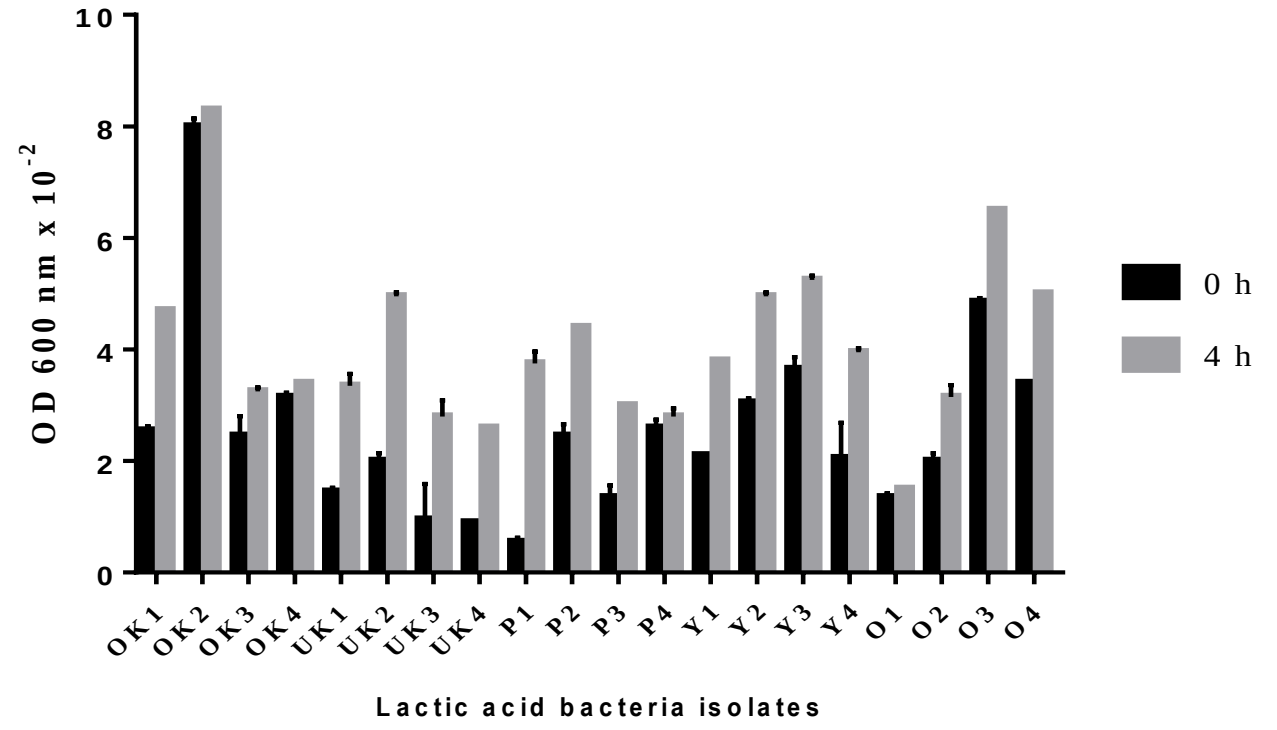

Figure 3. Tolerance of LAB at pH 3.5. OK, Okpeye; UK, Ukpaka; P, Pap or Akamu; Y, Yoghurt O, Ogiri.

S. *typhimurium*. The cause for antipathogenic activity of isolates O_4 and Y_1 against *Bacillus* sp., *Pseudomonas aeruginosa*, *Salmonella typhimurium, Staphylococcus aureus,* and *Escherichia coli* is not known and requires further study. It might be due to formation of either bacteriocins, organic acids (acetic acid, lactic acid) or hydrogen peroxide all having desirable properties as sustainable alternatives to antibiotics. The results of the

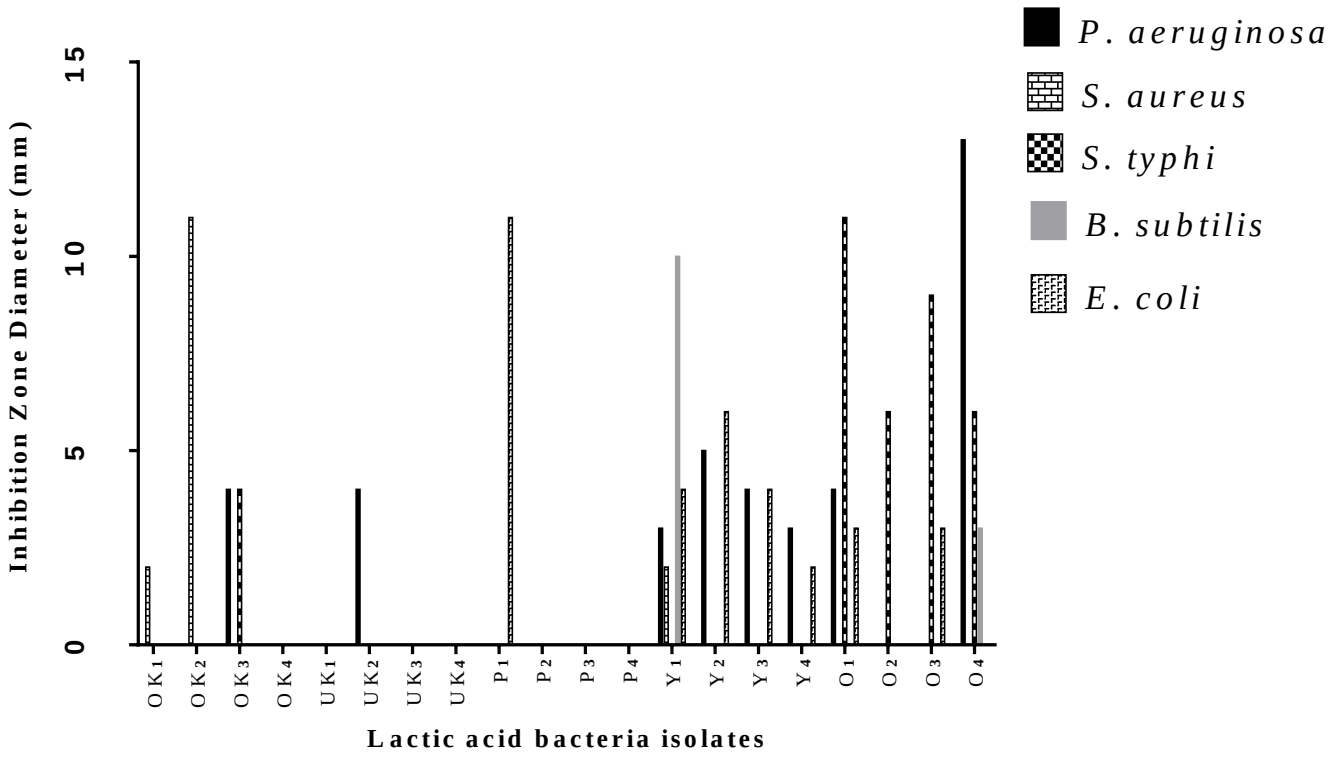

Figure 4. Antibacterial activity of LAB isolates. OK, Okpeye; UK, Ukpaka; P, Pap or Akamu; Y, Yoghurt O, Ogiri.

antimicrobial activity of Lactobacilli against enteropathogens are corroborated by another author who reported their *in vitro* inhibitory activity on *Bacillus* sp*., P. aeruginosa*, *Staph. aureus, E. coli* and *S. typhimurium* (Abubakr, 2018). Among the pathogens tested, *Bacillus* sp*.* had the highest resistance against the probiotic isolates being sensitive to only two isolates. The high resistance profile recorded for *Bacillus* sp*.* may be attributed to their tolerance to bile and acidic pH concentrations, biofilms formation potentials, versatile intrinsic ability to produce protease and lipases that are stable at high temperature. *E. coli* and *P. aeruginosa* showed highest sensitivity with more than eight isolates inhibiting their growth.

Conclusion

Potential *Lactobacillus* species were isolated from the following fermented food products: Ukpaka, Ogiri, Okpeye, Pap and Yoghurt from Ikpa market in Nsukka metropolis. The isolates had morphological characteriztics of known features of *Lactobacillus* bacteria. The *in vitro* antibacterial activity of the isolates showed that some had ability to inhibit selected pathogenic organisms, an indication of their potential relevance in therapeutic treatment of infectious diseases. Survival of these probionts at acidic pH condition validate them to be potentially useful either as adjuvant or active ingredients in preparations that are targeted to the stomach and upper duodenal regions of the gastrointestinal tract (GIT). Further investigations and molecular characterization of the novel potential probiotic isolates and identification to their species level for subsequent pharmaceutical applications are in progress.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors are grateful to both the laboratory technologists of the Departments of Pharmaceutics and Pharmaceutical Microbiology and Biotechnology, University of Nigeria for providing various ancillary materials for the study.

REFERENCES

Abubakr MAS (2018). Antimicrobial Activities of Lactic Acid Bacteria Strains Isolated from Human Breast Milk Against Human Pathogenic Strains. International Journal of Clinical and Developmental Anatomy 4(1):27-31 doi:

10.11648/j.ijcda.20180401.14.
Balouiri M, Sadiki M, Ibnsouda SK (2016). Methods for in vitro evaluating antimicrobial activity: A review. Journal of Pharmaceutical Analysis 6:71-79. http://dx.doi.org/10.1016/j.jpha.2015.11.005
Barbosa MS, Todorov SD, Ivanova IV, Belguesmia Y, Choiset Y, Rabesona H, Chobert JM, Haertle T, Franco BDGM (2016). Characterization of a two-peptide plantaricin produced by *Lactobacillus plantarum* MBSa4 isolated from Brazilian salami. Food Control 60:103-112. http://dx.doi.org/10.1016/j.foodcont.2015.07.029
Berebon DP, Ofokansi KC, Attama AA, Eze CO, Onwusoba RC, Ugwoke IC (2019). Preliminary Studies on Isolation, Bile Tolerance and Antibiogram of Potential Probiotics (Probionts) from Locally Fermented Food Products at Beach Market, Nsukka Metropolis, Enugu State, Nigeria. Biotechnology Journal International 22(3):1-10. doi: 10.9734/BJI/2018/v22i330060
Cavera VL, Arthur TD, Kashtanov D, Chikindas ML (2015). Bacteriocins and their position in the next wave of conventional antibiotics. International Journal of Antimicrobial Agents 46(5):494-501.
Cotter PD, Hill C (2003). Surviving the acid test: responses of Gram-positive bacteria to low pH. Microbiology and Molecular Biology Reviews 67(3):429-453.
De Angelis M, Gobbetti M (2011). "Stress responses of lactobacilli," in Stress Responses of Lactic Acid Bacteria, eds. Tsakalidou E, Papadimitriou K (New York, NY: Springer), 219-249.
FAO/WHO (2002). Guidelines for the evaluation of probiotics in food. London, Ontario, Canada, April 30 and May 1, 2002. ftp://ftp.fao.org/es/esn/food/wgreport2.pdf.
FAO/WHO (2006). Probiotics in food. Health and nutritional properties and guidelines for evaluation. FAO Food and Nutrition Paper 85:1-50. http://www.fao.org/3/a-a0512e.pdf
Grajek K, Sip A, Foksowicz-Flaczyk J, Dobrowolska A Wita A (2016). Adhesive and hydrophobic properties of the selected LAB isolated from gastrointestinal tract of farming animals. Acta Biochimica Polonica 63(2):311-314.
Hassanzadazar H, Ali E, Karim M, Javad H (2012). Investigation of antibacterial, acid and bile tolerance properties of lactobacilli isolated from Koozeh cheese. Veterinary Research Forum 3(3):181-185.
Hawaz E (2014). Isolation and identification of probiotic lactic acid bacteria from curd and in vitro evaluation of its growth inhibition activities against pathogenic bacteria. African Journal of Microbiology Research 8(13):1419-1425.
Kaur IP, Deol PD, Sandhu SK, Rishi P (2015). Pharmaceutical Aspects of Probiotics and Prebiotics. In Koen Venema & Ana Paula do Carmo (Eds), Probiotics and Prebiotics: Current Research and Future Trends. Caister Academic Press. Norfolk, UK. pp. 470-471
Ben Lagha A, Bruno H, Marcelo G, Daniel G (2017). Antimicrobial potential of bacteriocins in poultry and swine production. Veterinary Research 48:22. DOI 10.1186/s13567-017-0425-6.
Lewus CB, Montville TJ (1991). Detection of bacteriocins produced by lactic acid bacteria. Journal of Microbiological Methods 13(2):145-150.
Liao SF, Nyachoti CM (2017). Using probiotics to improve swine gut health and nutrient utilization. Animal Nutrition 3(4):331-343. doi: 10.1016/j.aninu. 2017.06.007.
Macaluso G, Fiorenza G, Gaglio R, Mancuso I, Scatassa ML (2016). In vitro evaluation of bacteriocin-like inhibitory substances produced by lactic acid bacteria isolated during traditional Sicilian cheese making. Italian Journal of Food Safety 5(1):5503.
Miller BJ, Franz CM, Cho GS, du Toit M (2011). Expression of the malolactic enzyme gene (mle) from *Lactobacillus plantarum* under winemaking conditions. Current Microbiology 62:1682-1688. doi: 10.1007/s00284-011-9914-4
Ngov S, Sukboonyasatit D, Paseephol T (2014). Enhancement of Probiotic Survival in Low pH and Bile Salt Condition using Alginate-Hi-maize Starch Encapsulation. Asia-Pacific Journal of Science and Technology19 (Supplement Issue):141-147.
Oliveira LC, Silveira AMM, Monteiro AS, Santos VL, Nicoli JR, Azevedo VAC, Soares SC, Dias-Souza MV, Nardi RMD (2017). In silico Prediction, in vitro Antibacterial Spectrum, and Physicochemical Properties of a Putative Bacteriocin Produced by *Lactobacillus rhamnosus* Strain L156.4. Frontiers in Microbiology 8:876
Sahadeva RPK, Leong SF, Chua KH, Tan CH, Chan HY, Tong EV, Wong SYW, Chan HK (2011). Survival of commercial probiotic strains to pH and bile. International Food Research Journal 18(4):1515-1522.
Shokryazdan P, Jahromi MF, Liang JB, Ho YW (2017). Probiotics: From Isolation to Application. Journal of the American College of Nutrition 36(8):666–676
Soomro AH, Tariq M, Shehla S, Habib AR (2007). Comparison of Different Methods for Detection of Antimicrobial Activity of Lactobacillus spp. Pakistan Journal of Zoology 39(4):265-268.
Stefanis C, Mantzourani I, Plessas S, Alexopoulos A, Galanis A, Bezirtzoglou E, Kandylis P, Varzakas T (2016). Reviewing Classical and Molecular Techniques Regarding Profiling of Probiotic Character of Microorganisms. Current Research in Nutrition and Food Science 4(1):27-47.
Succi M, Pannella G, Tremonte P, Tipaldi L, Coppola R, Iorizzo M, Lombardi SJ, Sorrentino E (2017). Sub-optimal pH Preadaptation Improves the Survival of Lactobacillus plantarum Strains and the Malic Acid Consumption in Wine-Like Medium. Frontiers in Microbiology 8:470. doi: 10.3389/fmicb.2017.00470.
Tokatlı MGG, Elmac SB, İşleyen NA, Özçelik F (2015). In Vitro Properties of Potential Probiotic Indigenous Lactic Acid Bacteria Originating from Traditional Pickles. BioMed Research International 1–8. doi:10.1155/2015/315819.
Vantsawa P, Maryah T, Timothy B (2017). Isolation and identification of lactic acid bacteria with probiotic potential from fermented cow milk (nono) in Unguwar Rimi Kaduna State Nigeria. American Journal of Molecular Biology 7(2): 99-106.
Vieco-Saiz N, Belguesmia Y, Raspoet R, Auclair E, Gancel F, Kempf I, Drider D (2019). Benefits and Inputs from Lactic Acid Bacteria and Their Bacteriocins as Alternatives to Antibiotic Growth Promoters During Food-Animal Production. Frontiers in Microbiology 10:57. doi: 10.3389/fmicb.2019.00057.
Wolska KI, Grzes K, Kurek A (2012). Synergy between antimicrobials and conventional antibiotics or bacteriocins. Polish Journal of Microbiology 61:95-104.

Vol. 18(32), pp.1098-1104, December, 2019
DOI: 10.5897/AJB2019.16975
Article Number: 2A679CB62500
ISSN: 1684-5315

http://www.academicjournals.org/AJB

ACADEMIC JOURNALS
expand your knowledge

African Journal of Biotechnology

Full Length Research Paper

First report of bovine papillomavirus genotypes in cutaneous lesions by polymerase chain reaction (PCR) and direct sequencing in Panama

Rita González[1], Manuel Murillo[2], Hilda Elena Castillo M.[1], Marcelino Jaén[3] and Axel Villalobos-Cortés[1*]

[1]Laboratorio de Análisis y Biología Molecular Aplicada, Instituto de Investigación Agropecuaria, IDIAP, Clayton, Cd Del Saber, Panama.
[2]Laboratorio Biomolecular, IMELCF, Clayton, Cd del Saber, Panama.
[3]Instituto de Investigación Agropecuaria, Laboratorio de Salud Animal, IDIAP, Divisa, Panama.

Received 30 September, 2019; Accepted 20 November, 2019

Bovine papillomavirus (BPV) causes benign tumours in the mucosal and cutaneous epithelium and is characterized by the presence of warts. The present study includes the molecular identification of BPV strains in samples of warts using degenerate polymerase chain reaction (PCR) primers FAP59/64. Skin biopsies were taken from crossbred cattle from two experimental farms of Instituto de Investigación Agropecuaria de Panamá (IDIAP) during the period from July 2016 to February 2018. Fourteen samples were positive by PCR amplification and sequenced at the laboratory of the Institute of Legal Medicine and Forensic Sciences (IMELCF). The analysis of the sequences allowed the identification of strains related to seven viral types; this was the first time that this type of study was carried out in Panama. The present study showed that PCR amplification with the primers FAP59/64, which partially amplify the L1 gene, followed by direct sequencing was useful for genotyping BPV. This study possibly identified local strains of BPV2 and BBA2; however, it is necessary to carry out more studies to establish the diversity and distribution of this virus in the country. The results in this study are important for the development of prophylactic and therapeutic measures that contribute to reducing the economic losses associated with BPV in Panama.

Key words: Livestock, biotechnology, molecular genetics, virology, animal health.

INTRODUCTION

Papillomavirus has been identified as the causative agent of benign and malignant neoplasms that infect epithelial tissue in humans and a wide variety of animals (Campo, 2003; Rector and Van Ranst, 2013; García-Pérez et al., 2014). It can present as a cutaneous papilloma, benign fibroplasia, urinary bladder tumour or oesophageal cancer, causing significant economic losses (Vázquez et al., 2012; Carvalho et al., 2013). In cattle, bovine papillomavirus (BPV) induces exophytic lesions (papillomas, warts) and flat lesions (flat warts, cervical intraepithelial neoplasia) in cutaneous and mucosal epithelia (de Villiers et al., 2004; Dyne et al., 2018).

*Corresponding author. E-mail: villalobos.axel@gmail.com.

Lesions in general are benign and usually revert without causing any serious clinical problem in the host; however, they can persist without being eliminated from the animal. Persistent lesions can be debilitating and can become a focal point for malignant transformation to squamous cell carcinoma, particularly in the presence of environmental or genetic cofactors (Campo, 2003).

Although infections caused by BPV in cattle do not cause much damage, they produce great economic losses due to their impact on aesthetics and the quality of cattle in livestock shows and hinder the commercialization of products derived from animals infected with BPV, such as leather for the production of footwear and other clothing (Catroxo et al., 2013; Araldi et al., 2014). However, superinfections in lesions and milking difficulties when papillomas appear on the udders can cause considerable health and management complications, and finally, some genotypes are associated with the development of carcinogenic lesions (Campo et al., 1992; Borzacchiello et al., 2003).

BPV is usually transmitted by direct contact with infected animals and is introduced into the skin by cutaneous lesions. It is a disease of economic importance because it causes negative effects in cattle judging and sale because bovine papillomatosis causes a loss in body condition, particularly when there is secondary bacterial infection (Salib and Farghali, 2011). Warts present on the udder also interfere with the milking process (Radostitis et al., 2007). This disease affects animals older than 2 years; however, bovine animals of all ages can develop these lesions (Kumar et al., 2013). Immunosuppressive factors play a role in the progression of bovine papillomatosis, as mentioned by Radostitis et al. (2007), including internal and external parasites. Likewise, a high incidence of bovine enzootic leukosis virus has been observed in conjunction with BPV because of a probable co-infection, causing chromosomal aberrations in peripheral blood lymphocytes (Yaguiu et al., 2008)

BPV is a small, non-enveloped virus whose genome consists of a double-stranded DNA molecule (dsDNA), approximately 8 kb, within a T = 7 icosahedral capsid (da Silva et al., 2016); the dsDNA encodes early functional and structural proteins and late proteins, which are expressed in different phases of the viral cycle (Carvalho et al., 2013). The gene encoding the L1 protein is the most conserved within the genome and has been used by many research groups to identify new viral variants (Silva et al., 2013). To classify the types of BPV, the same gene coding for the L1 protein is used; a segment of the gene is amplified and, through sequencing and use of bioinformatic tools, is compared to reconstruct evolutionary relationships, expressed graphically as phylogenetic trees (Chan et al., 1995). Under this classification, the group of BPVs belongs to the C and D supergroup, ungulate fibropapillomas and fibropapillomas that cause true papillomas, respectively (Chan et al., 1995; Antonsson and Hanson, 2002). BPV belongs to the Papillomaviridae family, and several genera and species have been classified in cattle. Of the genus *Deltapapillomavirus* (considered high risk), viral types BPV1, BPV2, BPV13 and BPV14 belonging to species *Deltapapillomavirus 4* have been identified; of the genus *Epsilonpapillomavirus,* viral types BPV5 and BPV8 belonging to species *Epsilonpapillomavirus 1* have been identified; and of the genus *Xipapillomavirus,* viral types BPV3, BPV4, BPV6, BPV9, BPV10, BPV11, BPV15 and BPV23 belonging to species *Xipapillomavirus 1* have been identified and viral type BPV12 belonging to species *Xipapillomavirus 2* has been identified. There are species within this group, such as BPV17, BPV23 and BPV UFPE05BR, that have not been classified. The genus *Dyoxipapillomavirus* has 2 species; however, they have not been classified: BPV7 and BPV22. *Dyokappapapillomavirus,* BPV16, BPV18, and BPV22 are also without classification within this genus.

BPV1 and BPV2 have also been identified from equine sarcoids in horses as a result of transmission between species (Ataseven et al., 2016; Daudt et al., 2018).

Commonly, genetic characterization is performed by polymerase chain reaction (PCR) using degenerate primers (FAP59/FAP64) that amplify the L1 region of the virus, followed by sequencing of the products (Forslund et al., 1999).

In Panama, there are no diagnostic methodologies or information on the genotypes of circulating BPV, and due to the increasing rate of BPV in the world mainly affecting cattle, it is important to have techniques to identify and propose programmes for the prevention, control and eradication of the virus by identifying the circulating genotypes. The use of PCR using degenerate primers, followed by sequencing, has allowed the identification of several types of papillomavirus in humans and animals (Forslund et al., 1999; Antonsson and Hansson, 2002; Carvahlo et al., 2012).

The aim of this study was to develop a methodology for the diagnosis of bovine papillomavirus by PCR and direct sequencing and to identify genotypes present in cutaneous lesions compatible with the presence of the virus during the onset of an outbreak of this disease on 2 farms in 2 regions of the Republic of Panama.

MATERIALS AND METHODS

A total of 14 (S1 to S14) skin biopsy samples were taken from the neck, abdomen and back of animals with warts from the Carlos Manuel Ortega Experimental Station of the Agricultural Research Institute of Panama (Instituto de Investigación Agropecuaria de Panamá - IDIAP), located in Gualaca, province of Chiriqui, and from animals from the Experimental Station El Ejido, province of Los Santos, from July 2016 to February 2018. All samples were obtained from *Bos taurus* × *Bos indicus* crossbreeds. Each sample collected was immediately stored at -21°C until processing in the laboratory.

DNA extraction was performed using the commercial Quick Extract solution (Epicenter, USA) following the manufacturer's instructions, with some modifications. DNA concentration was

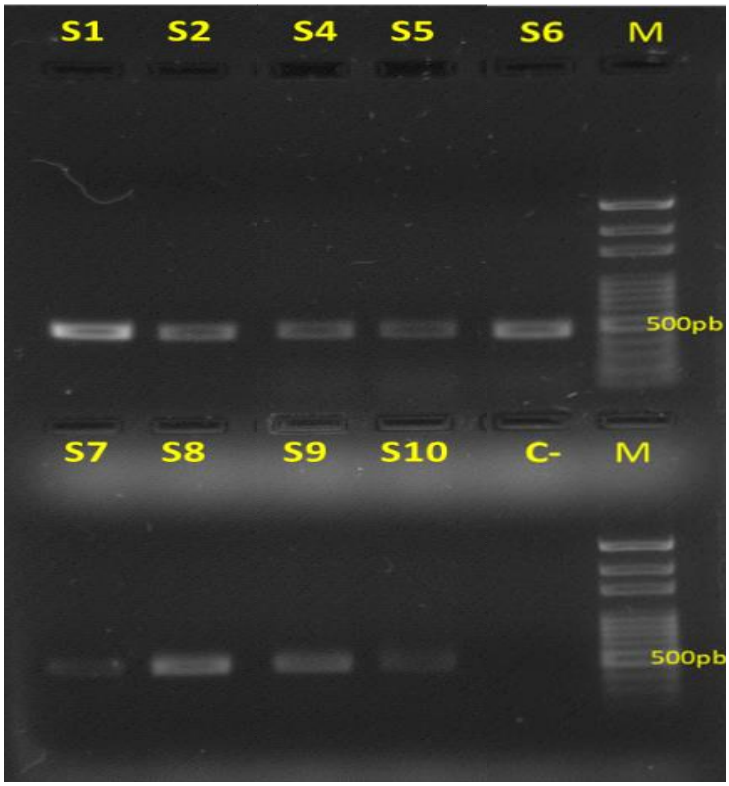

Figure 1. PCR products (478 bp) for the L1 gene of bovine papillomavirus in wart samples using primers FAP59/64. Samples S1, S2, S4 and S5 to S10 represent positive samples; line C represents the negative control, and M represents molecular weight markers from 100 to 1300 base pairs.

measured via absorbance at 260 nm using a spectrophotometer, and DNA quality was verified using a 1% agarose gel. PCR was performed in the region of the FAP gene that encodes the viral protein L1. The protocol used was proposed by Carvalho et al., (2013). The reaction was performed using a final volume of 25 µl, which included 1 to 5 ng of DNA, 0.5 µM each primer, FAP59 (5´-TAACWGTIGGICAYCCWTATT-3´) and FAP64 (5´-CCWATATCWVHCATITCICCATC-3´), 0.2 mM each DNTP, 1X PCR buffer, 1.5 mM $MgCl_2$ and 1 U of Taq DNA polymerase. Amplification consisted of an initial denaturation of 5 min at 95°C, followed by 35 cycles of 60 s at 95°C, 60 seconds at 52°C and 60 s at 72°C, with a final extension of 5 min at 72°C. The amplification products were analysed by electrophoresis in a 1% agarose gel. The presence of a band of 470 base pairs (bp) indicated that the virus was present.

PCR products were purified using the MinElute Gel Extraction Kit (Qiagen). Cycle sequencing was performed directly on the purified PCR products using BigDye Terminator chemistry version 3.1 (Applied Biosystems). All samples were sequenced in both directions using Sanger sequence technology (Sanger et al., 1975) in an ABI 3500 DNA analyser from Applied Biosystems at the Biomolecular Analysis Laboratory of the Institute of Legal Medicine and Forensic Sciences (IMELCF). The quality of the generated sequences was verified using MEGA 7 (Molecular Evolutionary Genetics Analysis version 7 (Kumar et al., 2016). Sequence alignment was performed using Clustal Omega (Goujon et al., 2010) applying the predetermined parameters. Homology analysis of the sequences was performed using Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI). The comparison of the nucleotide sequences was performed with MEGA 7, and for the construction of the phylogenetic tree, maximum parsimony with 2000 bootstraps was used. Depending on the similarities observed after the analysis, sequences of references were included to determine viral genus relations.

RESULTS AND DISCUSSION

Of the total samples examined, 100% were positive for BPV. The presence of the 470 bp fragment established as indicative of infection caused by the virus is shown in Figure 1. This fragment size is similar to that reported by Carvalho et al. (2013), who studied the virus in a herd of Holstein cattle affected by chronic cutaneous papillomatosis. However, Araldi et al., (2014), using the same set of primers, reported a 478 bp fragment in cutaneous papillomas in samples of Angus Red cattle in Sao Paulo, Brazil. Similarly, Claus et al. (2009), in a study conducted in beef cattle in Paraná, Brazil, reported a 480 bp amplicon size using the same sets of primers as in the present study (FAP59/FAP64). However, all amplicons, after subsequent sequencing, were confirmed

Table 1. Comparison of cutaneous lesions, viral types, percentage of similarity, E value of probability and type of accession by region, farm and animal evaluated at 2 locations in the Republic of Panama.

Location	DNA sample	Lesion type	Viral type	Similarity (%)	E	GenBank Accession No.
El Ejido	S1	large cauliflower	BPV2/B160620	98.7	0.0	LC426022.1
El Ejido	S2	small cauliflower	BPV2/B160620	67.6	2e-41	LC426022.1
Gualaca	S3	bulging round	BPV2/B160620	93.9	3e-100	LC426022.1
Gualaca	S4	horny round	BPV2/B160620	98.6	0.0	LC426022.1
Gualaca	S5	bulging round	BPV2/B160620	98.3	0.0	LC426022.1
Gualaca	S6	bulging round	BPV25/14RS13/BR	98.8	0.0	MG252779.1
Gualaca	S7	bulging round	BPV25/14RS13/BR	97.6	0.0	MG252779.1
Gualaca	S8	flat round	BPV2/B160620	98.6	0.0	LC426022.1
Gualaca	S9	leafy horny	BPV UFPE05BR/BPV11	91.2	2e-165	JQ897976.1
Gualaca	S10	bulging round	BPV2/B160620	95.8	0.0	LC426022.1
Gualaca	S11	cauliflower	BPV/ISO 04	99.7	0.0	MF384288.1
Gualaca	S12	cauliflower	BPV6	99.5	0.0	AB845589.1
Gualaca	S13	flat	BAA2	79.1	1e-82	AF485376.1
Gualaca	S14	scaly	BPV2/B160620	98.7	0.0	LC426022.1

as positive for BPV. The variability in the size of base pairs was previously described by Carvalho et al., (2013), who reported sizes ranging between 469 and 484 bp in different viral strains. This fact highlights the importance of using sequencing, in addition to using specific segments of the viral fragment that we wish to amplify because it allows comparative studies of the different genotypes found in an outbreak where the presumptive diagnosis is BPV.

Seven of the sequenced samples, S1, S3, S4, S5, S8, S10 and S14, showed similarities (93.9% to 98.7%) and eigenvalues (E; 0.0 to 3e-100) indicative of the BPV2 strain of the species *Deltapapillomavirus 4* (GenBank Accession No LC426022.1; submitted September 25, 2018, by Nanako Yamashita University of Tokyo, Division of Infection Control and Disease Prevention, Department of Veterinary Medical Science, Graduate School of Agricultural and Life sciences; 1-1-1 Yayoi, Bunkyo-ku, Tokyo, Tokyo 1138657, Japan). The similarity of BPV2 in sample S2 was the lowest among the samples (67.41, E = 2e-41).

Two samples, S6 and S7, showed a similarity of 98.8 and 97.6% (E = 0.0), respectively, with papilloma type 25 (BPV25) isolate 14RS13/BR (unclassified *Xipapillomavirus*; GenBank Accession No MG252779.1; submitted October 23, 2017, by Daudt, C., Da Silva, F.R.C., Cibulski, S.P., Junqueira, D.M. and Canal C.W. Laboratorio de Virología, Universidade Federal do Rio Grande do Sul, Bento Goncalves, 9090, Porto Alegre RS 91540-000, Brazil).

Sample S9 presented a similarity of 91.2% (E = 1e-156) with the Brazil isolate BPV/UFPE05BR (genus unclassified; GenBank Accession No. JQ897976.1 Submitted April 03, 2012, Genética, Universidad Federal de Pernambuco, Av. Moraes Rego S / N, Recife, Pernambuco 50732970, Brazil) and strain BPV11 of the genus *Xipapillomavirus* (species *Xipapillomavirus 1*; GenBank Accession No. AB543507.1; submitted January 25, 2010; Contact: Shinichi Hatama National Institute of Animal Health, Hokkaido Research Station; 4 Hitsujigaoka, Toyohira, Sapporo, Hokkaido 062-0045, Japan) (Hatama et al., 2011; Silva et al., 2013).

Sample S11 presented a similarity of 99.7% (E = 0.0) with isolate 04 as well as UK *Deltapapillomavirus* type 4 (GenBank Accession No MF384288.1; Submitted June 26, 2017, and published by Koch et al. (2017)). Genomic comparison of bovine papillomavirus 1 isolates from bovine, equine and asinine lesional tissue samples; Virus Res. 244, 6-12. Sample S12 presented a similarity of 99.5% (E = 0.0) with BPV6 of the genus *Xipapillomavirus 1* (GenBank accession N° AB845589.1; Submitted August 12, 2013; Contact: Shinichi Hatama National Institute of Animal Health, Hokkaido Research Station; 4 Hitsujigaoka, Toyohira, Sapporo, Hokkaido 062-0045, Japan, unpublished data).

Sample S13 showed a similarity of 79.1% (E = 1e-82) with the BAA2 isolate of the capsid protein (GenBank accession N° AF485376.1; Submitted February 20, 2002, and published by Antonsson and Hansson (2002)). Healthy skin of many animal species harbours papillomaviruses which are closely related to their human counterparts (Table 1).

A total of 57% of the samples analysed showed similarity with viral type BPV2, which is associated with tumours in the urinary tract (Borzacchiello et al., 2007; Balcos et al., 2008; Resendes et al., 2011). This viral type has the greatest geographic distribution and has been reported in countries such as Germany, Brazil, New Zealand, Japan, India, Italy, Turkey and Korea and isolated from cutaneous papillomas, pulmonary fibromatosis, bladder, semen, blood, milk and urine (Daudt et al., 2018). These samples related to BPV2

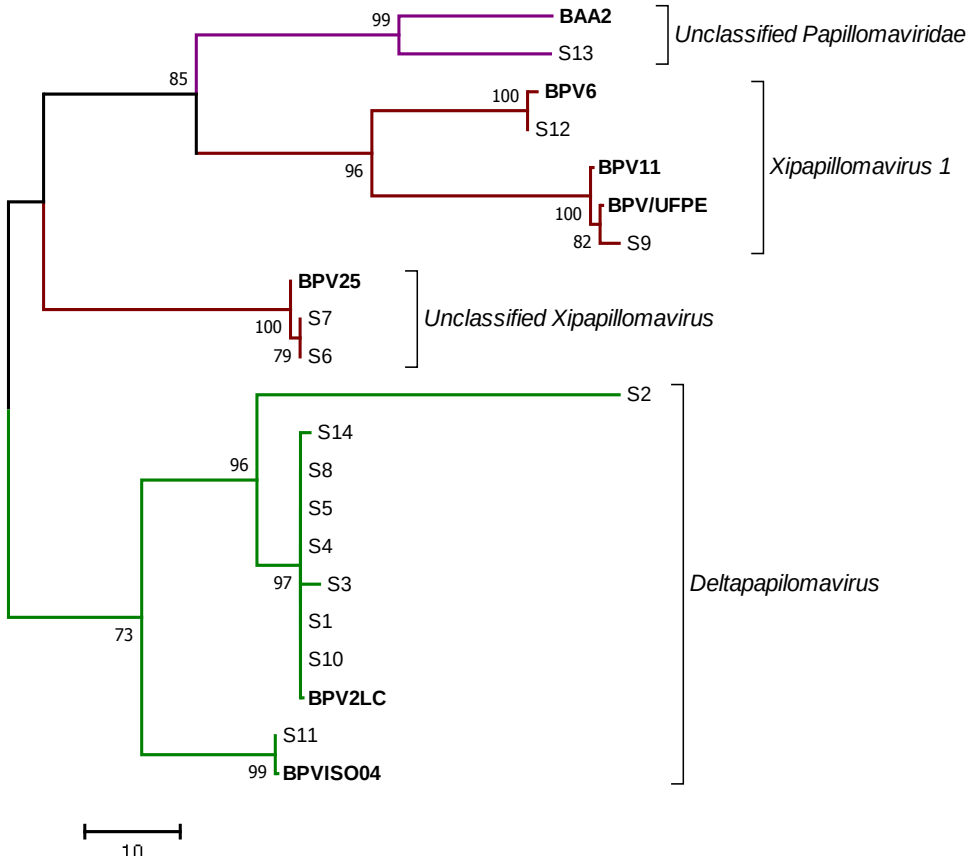

Figure 2. Molecular phylogenetic analysis (MEGA 7) using the maximum parsimony method for sequences of bovine papillomavirus. The percentage of replicated trees in which the taxa were grouped in the bootstrap test (2000 replicates) is shown next to the branches (Felsenstein, 1985).

showed 5 types of lesions: fibropapillomas in the form of large and small cauliflower-like growths observed in El Ejido and flat, bulging and scaly flat forms observed in Gualaca. The samples related to type BPV11 and BPV UFPE05BR presented leafy horny-type lesions, whose presence was reported in South America (Brazil) by Carvalho et al. (2012) and da Silva et al. (2015) and in Japan by Hatama et al. (2011). For isolates with similarity to the BPV25 type (S6 and S7), representing 14.3% of the samples, round bulging lesions were observed. Regarding this viral type, no report has been published since being entered into GenBank in October 2017; therefore, this is the first published article that refers to this viral type. Samples S11 and S12 presented the cauliflower-type form; however, they are related to 2 distinct viral types, *Deltapapillomavirus* and *Xipapillomavirus,* as shown in Figure 2. Sample S12, related to BPV6, showed cauliflower-type lesions, which was also reported by Carvalho et al. (2012) in Brazil and Savini et al. (2016) in Italy and is related to skin, teat and udder lesions (Claus et al., 2009).

Sample S13 was described as flat and was identified as related to BAA2 (Table 1). This particular viral type is related to skin papillomas in humans and was reported by Antonsson and Hansson (2002) in Sweden. This type of BAA2 was not located in any of the supergroups mentioned previously by Chan et al., (1995); therefore, they postulated that this type should be part of another putative supergroup.

The amplification of the fragments by PCR and direct sequencing allowed for the analysis of phylogenetic relationships, as shown in Figure 2, from which several groupings were formed. Most branches were statistically well supported with a minimum of 79% confidence to 100% confidence.

Sample S13 was observed in the upper region of the tree, grouped with the BAA2 isolate of the major protein of the L1 gene capsid, unclassified papillomavirus (bootstrap 99%). Next, sample S12 and viral type BPV6 and sample S9 and types BPV UFPE05BR and BPV11 were observed within the same branch (bootstrap 100%), both related to genus *Xipapillomavirus 1*. However, as it is a single case, it is necessary to perform more isolations; however, at the time of this report, there were

no more animals with papillomatous lesions observed. The subsequent branch included samples S6 (bootstrap 79%) and S7 (bootstrap 100%) and isolate 14RS13/BR of the viral type BPV25, within the genus *Xipapillomavirus* (unclassified). Next, samples related to *Deltapapillomavirus* were observed; within the branch, 3 sub-clusters were observed: sample S2 (bootstrap 96%) and a separate group of samples S14, S8, S5, S4, S3, S1 and S10 with BPV2LC (bootstrap 97%) and sample S11 related to type BPVISO04 (bootstrap 99%).

Conclusion

Before this study, there was no information regarding the subtypes of BPV present in Panama. The molecular characterization described in this report will establish a guide for subsequent studies with a greater number of samples.

The results of this research are important because they contribute to the development of prophylactic and therapeutic measures that minimize economic losses associated with the presence of papillomavirus in cattle in Panama.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests

ACKNOWLEDGEMENTS

The authors thank SENACYT and SNI of Panama for the support in the partial financing in carrying out this work; Engineer Roderick González for the partial contribution of funds from the project “Improving Competitiveness and Sustainability of Intensive (specialized) and Semi-intensive (dual-purpose) Milk Production Systems in Western Panama at the Carlos Manuel Ortega Experimental Station (EEGCMO)"; technologist Lissy Ávila for her collaboration in the IDIAP Animal Health Laboratory; and veterinarians Aristides Villarreal and Ginnette Rodríguez for their support in field sampling.

REFERENCES

Antonsson A, Hansson AB (2002). Healthy skin of many animal species harbors papillomaviruses which are closely related to their human counterparts. Journal of Virology 76(24):12537-12542

Araldi RP, Carvalho RF, Melo TC, Diniz NSP, Ana TAS (2014). Bovine papillomavirus in beef cattle: first description of BPV-12 and putative type BAPV8 in Brazil. Genetics and Molecular Research 13(3):5644-5653.

Ataseven VS, Kanat Ö, Ergün Y (2016). Molecular identification of bovine papillomaviruses in dairy and beef cattle: first description of Xi- and Epsilonpapillomavirus in Turkey. Turkish Journal of Veterinary and Animal Sciences 40(6):757-763.

Balcos LG, Borzacchiello G, Russo V, Popescu O, Roperto S, Roperto F (2008). Association of bovine papillomavirus type-2 and urinary bladder tumours in cattle from Romania. Research in Veterinary Science 85(1):145-148.

Borzacchiello G, Iovane G, Marcante ML, Poggiali F, Franco R, Roperto S, Venuti A (2003). Presence of bovine papillomavirus type 2 DNA and expression of the viral oncoprotein E5 in naturally occurring urinary bladder tumours in cows. Journal of General Virology 84(Pt 11):2921–2926.

Borzacchiello G, Russo V, Spoleto C, Roperto S, Balcos L, Rizzo C, Venuti A, Roperto F (2007). Bovine papillomavirus type-2 DNA and expression of E5 and E7 oncoproteins in vascular tumours of the urinary bladder in cattle. Cancer Letters 250(1):82-91.

Campo MS, Jarrett WF, Barron R, O'Neil BW, Smith KT (1992). Association of Bovine Papillomavirus Type 2 and Bracken Fern with Bladder Cancer in Cattle. Cancer Research 52(24):6898-6904.

Campo M (2003). Papillomavirus and disease in humans and animals. Veterinary and Comparative Oncology 1(1):3-14.

Carvalho CCR, Batista MVA, Silva MAR, Balbino VQ, Freitas AC (2012). Detection of bovine papillomavirus types, co-infection and new BPV11 subtype in cattle. Transboundary and Emerging Diseases 59(5):441-447.

Carvalho RF, Sakata ST, Giovanni DNS, Mori E, Brandao PE, Richtzenhain LJ, Pozzi CR, Arcaro JRP, Miranda MS, Mazzuchelli-de-Souza, Melo TC, Comenale G, Assaf SLMR, Becak W, Stocco RC (2013). Bovine Papillomavirus in Brazil: Detection of Coinfection of Unusual Types by a PCR-RFLP Method. BioMed Research International 2013:270898. https://www.hindawi.com/journals/bmri/2013/270898/

Catroxo MHB, Martins AM, Petrella S, Souza F, Nastari BDB (2013). Ultrastructural Study of Bovine Papillomavirus During Outbreaks in Brazil. International Journal of Morphology 31(2):777-784.

Chan SY, Delius H, Halpern AL, Hans-Ulrich Bernard HU (1995). Analysis of Genomic Sequences of 95 Papillomavirus Types: Uniting Typing, Phylogeny, and Taxonomy. Journal of Virology 69(5):3074-3083.

Claus P, Lunardi M, Alfieri AF, Sartori D, Helena M, Fungaro P, A Alfieri (2009). Identification of the recently described new type of bovine papillomavirus (BPV-8) in a Brazilian beef cattle herd. Pesquisa Veterinária Brasileira 29(1):25-28.

da Silva FRC, Daudt C, Streck A, Weber M, V. Leite F, Driemeier D, Canal CW (2015). Genetic characterization of Amazonian bovine papillomavirus reveals the existence of four new putative types. Virus Genes 51(1):77-84.

da Silva FRC, Cibulski SP, Daudt C, Weber MN, Guimarães LLB, Streck AF, Mayer FQ, Roehe PM, Canal CW (2016). Novel Bovine Papillomavirus Type Discovered by Rolling-Circle Amplification Coupled with Next-Generation Sequencing. PLoS ONE 11(9):e0162345. doi: 10.1371/journal.pone.0162345.

Daudt C, Da Silva FRC, Lunardi M, Alves CBD, Weber MN, Cibulski SP, Alfieri AF, Alfieri AA, Canal CW (2018). Papillomaviruses in ruminants: An update. Transboundary and Emerging Diseases 65(5):1381-1395.

De Villiers EM, Fauquet C, Broker TR, Bernard HU, Zur Hausen H (2004). Classification of papillomaviruses. Virology 324(1):17-27.

Dyne EA, Henley SJ, Saraiya M, Thomas CC, Markowitz LE, Benard VB (2018). Trends in human papillomavirus–associated cancers — United States, 1999–2015. Morbidity and Mortality Weekly Report 67(33):918-924.

Felsenstein J (1985). Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39(4):783-791.

Forslund O, Antonsson A, Nordin P (1999). A broad range of human papillomavirus types detected with a general PCR method suitable for analysis of cutaneous tumours and normal skin. Journal of General Virology 80(Pt 9):2437-2443.

García-Pérez R, Ibáñez C, Godínez JM, Aréchiga N, Garin I, Pérez-Suárez G, de Paz O, Juste J, Echevarria JE, Bravo IG (2014). Novel papillomaviruses in free-ranging Iberian bats: no virus–host co-evolution, no strict host specificity, and hints for recombination. Genome Biology and Evolution 6(1):94-104.

Goujon M, McWilliam H, Li W, Valentin F, Squizzato S, Paern J, Lopez RA (2010). New bioinformatics analysis tools framework at EMBL-EBI. Nucleic Acids Research 38(Web Server issue):W695-699.

Hatama S, Ishihara R, Ueda Y, Kanno T, Uchida I (2011). Detection of

a novel bovine papillomavirus type 11 (BPV-11) using xipapillomavirus consensus polymerase chain reaction primers. Archives of Virology 56(7):1281-1285.
Kumar P, Jangir B, Saikumar G, Somvanshi R (2013). Co-infection of bovine papillomavirus type-1 and -10 in teat warts of dairy cattle. Veterinarni Medicina 58(12):605-608.
Kumar S, Stecher G, Tamura K (2016). MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Molecular Biology and Evolution 33(7):1870-1874.
Radostitis OM, Gay CC, Hinchcliff KW, Constable PD (2007). textbook of Veterinary Medicine,10th edition, 2008 print.; printed by Elsevier, Spain 2:1421-1423.
Rector A, Van Ranst M (2013). Animal papillomaviruses. Virology 445(1-2):213-223.
Resendes A, Roperto S, Trapani F, Urraro CH, Rodrigues A, Roperto F, Giuseppe B (2011). Association of bovine papillomavirus type 2 (BPV-2) and urinary bladder tumours in cattle from the Azores archipelago. Research in Veterinary Science 90(3):526-529.
Salib FA, Farghali HA (2011). Clinical, epidemiological and therapeutic studies on Bovine Papillomatosis in Northern Oases, Egypt in 2008. Veterinary World 4(2):53-59.
Savini F, Mancini S, Gallina L, Donati G, Casa G, Peli A, Scagliarini A (2016). Bovine papillomatosis: First detection of bovine papilllomavirus types 6, 7, 8, 10 and 12 in Italian cattle herds. The Veterinary Journal 210:82-84.
Silva MAR, Batista M, Pontes NE, Santos EUD, Coutinho LCA, Castro SR, Balbino BQ, Freitas AC (2013). Comparison of two PCR strategies for the detection of bovine papillomavirus. Journal of Virological Methods 192(1-2):55-58.
Vázquez R, Escudero C, Doménech A, Gómez-Lucia E, Benítez L (2012). Review. Papilomatosis Bovina: Epidemiología y Diversidad de Papilomavirus Bovinos (BPV). Revista Complutense de Ciencias Veterinarias 6(2):38-57.
Yaguiu A, Dagli MLZ, Birgel Jr EH, Alves Reis, O.P. Ferraz BCA, Pituco EM, Freitas AC, Beçak W, Stocco RC (2008). Simultaneous presence of bovine papillomavirus and bovine leukemia virus in different bovine tissues: in situ hybridization and cytogenetic analysis. Genetics and Molecular Research 7(2):487-497.

Vol. 18(32), pp.1105-1112, December, 2019
DOI: 10.5897/AJB2018.16644
Article Number: 310AA4562522
ISSN: 1684-5315

http://www.academicjournals.org/AJB

ACADEMIC JOURNALS
expand your knowledge

African Journal of Biotechnology

Full Length Research Paper

Survey of the incidence and distribution of groundnut rosette disease in major groundnut-producing regions of Western Kenya

Eric O. Were[1*], Evans N. Nyaboga[2] and Elijah M. Ateka[3]

[1]Kenya Plant Health Inspectorate Service (KEPHIS), Kenya.
[2]Department of Biochemistry, School of Medicine, University of Nairobi Kenya, Kenya.
[3]Department of Horticulture, School of Agriculture and Environmental Sciences, Jomo Kenyatta University of Agriculture and Technology Juja, Kenya.

Received 8 September, 2018; Accepted 24 May, 2019.

Groundnut rosette disease (GRD) is the most important viral disease of groundnuts in sub-Saharan Africa. In Kenya, GRD infection especially before flowering results in 100% loss in pod yield. Surveys were conducted in 2016 and 2017 to determine the incidence and distribution of GRD in five major groundnut growing Counties of western Kenya. A structured questionnaire was used to assess GRD incidence and severity and farmers' awareness about management of GRD. Reverse transcription (RT)-polymerase chain reaction (PCR) was used for the detection of GRD agents in collected symptomatic samples. Results revealed that GRD was prevalent in all the fields of the five counties. The highest mean disease incidence was in Busia County (35.7%) while the lowest incidence was in Siaya (23.1%). The most conspicuous symptoms observed in all the fields inspected were yellow/chlorotic rosette and green rosette. The highest mean disease severity was observed in farmers' fields in Busia (3.1) and Bungoma (3.0) Counties, while the lowest was observed in Siaya (2.8). RT-PCR detected GRD agents in all the symptomatic samples. This study demonstrated the widespread occurrence of GRD in major growing regions of western Kenya and warrants the implementation of effective virus disease control strategies.

Key words: *Arachis hypogaea* L., field survey, groundnut rosette disease, occurrence, severity.

INTRODUCTION

Groundnut (*Arachis hypogaea* L.) is an annual self-pollinated legume, widely grown in tropical and sub-tropical regions of the world. Asia and Africa account for 95% of global groundnut area where it is cultivated under rainfed conditions with low inputs by resource poor farmers. Groundnut is an important cash crop, an affordable source of edible oil rich in omega-3 fatty acids, protein and vitamin E and its stover provides nutritious feed for livestock (Pandey et al., 2012; FAOSTAT, 2014). As a cash crop, groundnut has the potential for improving income and reducing poverty in the rural households (Diop et al., 2004). The crop can also be used to improve

*Corresponding author. E-mail: osewo.were@gmail.com.

soil fertility by fixing atmospheric nitrogen (Andima et al., 2006). In Kenya, groundnut is a principal source of protein and major source of cash income for small-holder farmers. However, groundnut production in Kenya has continued to decline with farmers attaining less than 50% of the yield potential (FAOSTAT, 2014). A major constraint to achieving the yield potential of groundnuts in sub-Saharan Africa has been the presence of Groundnut rosette disease (GRD), fungal rust and Early Leaf Spot (ELS) diseases (Janila et al., 2013; Okello et al., 2013). Groundnut rosette disease is the most important disease of groundnut in Kenya (Okello et al., 2013; Janila et al., 2013).

GRD is the most destructive viral disease of groundnut, exclusively endemic to sub-Saharan Africa (SSA) and its off-shore islands (Deom et al., 2000; Waliyar et al., 2007). The disease was first described in Tanzania by Zimmerman in 1907 (Naidu et al., 1998) and since then epidemics have been reported in all groundnut growing regions of SSA and in Madagascar (Naidu et al., 1999). Groundnut rosette disease is caused by synergistic interactions of three viral agents, namely, *Groundnut rosette assistor virus* (GRAV), *Groundnut rosette virus* (GRV) and satellite RNA (satRNA) (Deom et al., 2000). All agents of GRD are persistently transmitted by aphids (*Aphis craccivora* Koch), may persist in the aphids for more than 10 days (Lynch, 1990), and there is no evidence of seed transmission to-date. Two distinct forms of GRD symptoms occur, chlorotic and green rosette (Waliyar et al., 2007) with variations due to diversity among the causal agents (sat-RNA variants), differences in genotype response, climatic conditions and mixed infections with other viruses (Naidu et al., 1999). Chlorotic rosette is the predominant form throughout sub-Saharan Africa, whereas green rosette has been reported in western and southern Africa regions (Naidu et al., 1998). Groundnut rosette can cause serious morphological disturbances to infected plants which take on bushy appearance and are stunted. Other symptoms include mottling, yellowing, leaf mosaic, and distortion of the shoots. GRD infection especially before flowering of the crop results in 100% loss in pod yield when susceptible varieties are used (Naidu et al., 1999; Waliyar et al., 2005; Okello et al., 2010).

The effects of rosette disease can be devastating on groundnuts if not prevented (Waliyar et al., 2005; Okello et al., 2010). Successful development of effective GRD management strategies depends on a sound understanding of the distribution and incidence of the disease in different agro-ecologies and cropping systems. Although there have been reports of GRD in Kenya (Wangai et al., 1999; Wangai et al., 2001), there is no updated information on the distribution, incidence, and severity of the disease. The only survey on the occurrence of GRD (Wangai et al., 2001) was conducted several years ago and the evolving polyculture/cropping system in Kenya may have greatly impacted on the disease dynamics over the years. Therefore, in this study, an extensive survey was conducted to determine the distribution, incidence, and severity of GRD in five major groundnut growing Counties of western Kenya. Farmers' awareness on the disease and management options were also investigated. This information is crucial in development of appropriate control interventions of the disease.

MATERIALS AND METHODS

Groundnut rosette virus disease (GRD) survey and sampling areas

A diagnostic field survey for GRD was conducted in major groundnut growing areas of Western Kenya from July 2015 to February 2016. The regions surveyed were the Counties of Homa Bay, Siaya, Busia, Bungoma, and Vihiga. Groundnut fields were selected at regular intervals of between 5-10 km along the major and feeder roads. The number of fields surveyed per county depended on the availability of groundnut farms at the time of survey. Farmers' groundnut fields were visited when the crop was between pod pegging and physiological maturity. A total of 76 symptomatic leaf samples were collected. Sampling was done on plants along diagonals and the number of samples collected per field depended on the variability of symptoms and filed size. Coordinates were taken at each sampling site using a global positioning system (GPS) device (Magellan Triton 'Windows CE Core 5.0 X11-15302). During the surveys, a questionnaire was used to capture farmers' experience with GRD and to document current management practices being employed by farmers in addressing the problem. This was captured as a series of responses by asking farmers if they knew about GRD, if they recognized it on their farms, the frequency of the problem on their farms, rate of spread of GRD on their farms and how they described the symptoms of the disease. Data were also collected on respondents' efforts to control the disease. Farmers were shown colored photographs and also groundnut plants affected by GRD on their own farms to ensure they made the correct identification of the disease.

Disease assessment

Assessment of GRD incidence and severity was based on virus symptoms of plants in the field as described by Okello et al. (2014). Disease incidence was calculated by expressing the number of plants with virus symptoms as a percentage of the total number of plants in quadrants of each sampled field. The severity of GRD symptoms were assessed visually and recorded based on a scale of 1 to 5 where; 1 = no symptoms, 2 = mild symptoms on leaves, little distortion of leaf shape, apparent but negligible stunting, 3 = moderate symptoms on leaf, moderate distortion of leaf shape, moderate stunting, 4 = severe symptoms on leaf, severe leaf distortion with reduced size, plant partially stunted, 5 = very severe symptoms on leaf, severe leaf distortion, reduced size, plant severely stunted. Symptomatic leaf samples were collected and placed in 50 ml falcon tubes containing silica gel and kept at room temperature for subsequent molecular diagnosis.

Virus detection by RT–PCR

RNA extraction from groundnut leaves

Total RNA was extracted from groundnut leaf samples using a Plant

RNA MiniPrep (Zymo Research, Irvine, CA, USA) according to the manufacturers' instructions. The quantity and purity of the extracted RNA was assessed by determination of $A_{260/A280}$ and $A_{260/A230}$ absorbance ratio by NanoDrop spectrophotometer (Thermo Fisher, Wilmington MD). The integrity of the extracted RNA was verified in 0.8% agarose gel stained with ethidium bromide. The integrity of RNA was verified in 1.2% denaturing agarose gel stained with Gel Red (Biotium, Hayward, CA, USA), and visualized under UV transilluminator. Visual inspection of the gel images was used to confirm the presence of bands corresponding to the 28S and 18S ribosomal subunits, providing qualitative assessment of RNA integrity.

cDNA synthesis

First-strand cDNA was synthesized in a 20 µl reaction containing approximately 5 µg of total RNA using a RevertAid Premium First-Strand Synthesis Kit (Thermo Scientific, Waltham, MA, USA). Five µl of RNA template was gently mixed with 4 µl of 5x reaction mix, 0.5 mM dNTP, 0.5 µl oligo $dT_{(18)}$ primers, 2 µl enzyme mix, and made up to 20 µl volume using nuclease free water. The mix was gently spinned down for 30 s, then incubated in thermo cycler at 25°C for 10 min followed by 50°C for 60 min and terminated by heating at 85 °C for 2 min.

Conventional reverse transcription PCR (RT-PCR)

The cDNA was amplified by PCR using corresponding 3′ and 5′ primers specific to coat protein sequence of *Groundnut rosette assistor virus* (GRAV) (Table 2) as described by Wangai et al. (2001). Two pairs of primers were used namely GRAV1 (HRP92: ATGAATACGGTCGTGGTTAGG / HRP93: TTTGGGGTTTTGGACTTGGC) and GRAV2 (HRP110: GGAGGGTCTGGCGAAACATT / HRP111 CCCTTGTAAAGGAACCGGAAT), amplifying products of 547 and 890 base pairs, respectively.

The PCR amplifications were performed in a reaction of 20 µl in Bioneer® premix, 2.0 µl forward and reverse primers and 2.0 µl cDNA. The PCR was performed in a MJ Mini™ Thermal Cycler (BIO-RAD, Singapore). The cycling profile was an initial denaturation at 94°C for 2 min followed by 36 cycles of 94°C for 45 s (denaturation), 53°C of 1 min (annealing) and 72°C for 1 min (extension) and a final extension of 5 min. The PCR products were electrophoresed on a 1% (w/v) agarose gel in 1X TAE buffer containing 0.5 µg/ ml of Gel Red and visualized in a UV transilluminator.

Data analysis

The data were analyzed through descriptive statistics (frequencies, percentages and mean values) for all continuous variables to generate summaries and tables. Data analysis was performed analyzed using the Statistical Package for Social Scientists (SPSS) version 16.0.

RESULTS

GRD symptoms and incidence

A total of 76 groundnut leaf samples were collected from farms in five counties of western Kenya. Groundnut rosette disease symptoms were observed in all the fields surveyed in all the 5 Counties. Symptoms observed in the field were manifested mainly in the form of chlorotic rosette symptoms and stunting of severely affected plants (Figure 1). Other GRD symptoms observed ranged from green rosette, leaf curling, and bright mosaic. GRD was present in all the five counties surveyed. The disease incidence varied from one farm to another with the highest incidence of 60% recorded in some farms in Busia County. GRD incidences ranging from 23.1 to 35.7% were observed in the five counties. The mean disease incidence was highest in Busia (35.7%) while Siaya had the lowest (23.1%). Overall, the mean incidence for all the counties surveyed was 29.4% (Table 1).

GRD severity was assessed on infected plants using a scale range of 1 to 5. The scores of 3 to 4, which are characterized by chlorotic of leaves to a stunted growth, were more frequent than the scores of 2 which represent the initial symptoms manifested by mild chlorotic leaves with no stunting. Disease severity ranged from 2.86 to 3.14 across the five counties, with an overall mean of 2.96. The highest GRD severity (3.14) was scored in farmers' fields in Busia County while the lowest was in Siaya County with a score of 2.86 (Figure 2). The disease severity of 3 and 4 had the highest occurrence across the five counties while scale of 5 recorded the least occurrences at 9.9% (Table 2). There was no significant difference in viral disease severity between the counties ($p \geq .039$, $\alpha = 0.05$).

Conventional RT-PCR

Results of the RT-PCR tests conducted using primers specific to the coat protein sequence of GRAV showed that all the samples (100%) collected from the fields in all the five counties of western Kenya tested positive for GRD (Figure 3).

Distribution of GRD in western Kenya

Based on the survey and RT-PCR results, a distribution map of GRD occurring in western Kenya was drawn (Figure 4). GRD was found occurring in all the surveyed groundnut growing regions of western Kenya.

Farmers' knowledge and management strategies against GRD

Most of the 76 interviewed farmers (90%) were able to recognize plants infected with GRD in their fields, while the remaining 10% did not follow the disease symptoms. All of the interviewed farmers reported that the disease is systemic and, overtime, it spreads to all plants in the field. Ninety two percent of farmers reported that no

Figure 1. (a) GRD-infected plant showing chlorotic rosette; (b) GRD-infected plant showing stunted growth and; (c) and (d) healthy plants in the field.

Table 1. GRD incidence in five surveyed counties of western Kenya.

County	Sub-counties	No. of farms surveyed	GRD incidence (%)
Busia	Teso South, Butula, Nambale, Samia	27	35.7 ± 3.24 a*
Homa Bay	Rangwe, Ndhiwa, Rachuonyo	26	30.3 ± 2.23 b
Siaya	Gem	8	23.1 ± 3.16 c
Bungoma	Webuye, Sirisia	9	29.4 ±1.87 b
Vihiga	Emuhaya	6	26.7 ± 1.77 b
Total/Mean		**76**	**29.04**

*Means in the same column followed by the same letter are not significantly different from one another ($p < 0.05$).

groundnuts are produced after infection. A small proportion of the farmers (7%) were not aware of the effect of GRD infection on groundnut production. Regarding the occurrence of the disease in the fields, 45.8 and 19% of the farmers attributed the cause of GRD to excess rainfall and presence of striga weeds, respectively. Other factors attributed to the disease by the farmers were soil fertility and drought stress. Eleven percent of the farmers said that the disease becomes more severe during dry season, although the disease is omnipresent during the whole year. However, ten percent of farmers had no idea as to what the causes GRD in the fields. Among the interviewed farmers, 65.1% reported to have used planting seeds from nearby (<10 km) local markets to establish the crop, 25.3% used their own seeds in the establishment of new fields, 6% obtained the seeds from the agricultural extension office, while 3.6% obtained seeds from their neighbours (home villages). In terms of GRD management efforts, 44.6% of the farmers carried out rouging for disease control. The diseased plants were mainly removed two months after planting and during weeding. About 16.9% of the farmers interviewed sprayed the crop with pesticides for disease control. Thirty two percent of farmers were not aware about disease transmission and believed that the disease can be spread through the soil, while eight percent attributed new infections to the use of infected planting seeds.

DISCUSSION

Field surveys generate knowledge on the current status of GRD prevalence, incidence, and severity, which forms the basis of priority setting in the integrated disease management. Such knowledge is limited for GRD

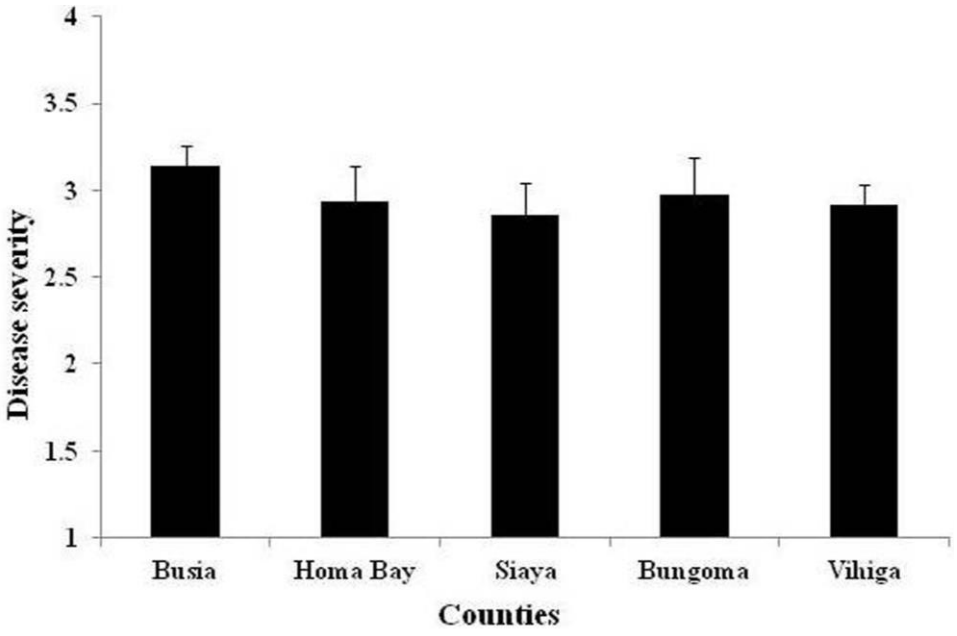

Figure 2. Mean GRD severity scores for the five surveyed Counties in western Kenya.

Table 2. GRD severity ranging from 1 to 5 scores (%) for the five surveyed counties in western Kenya.

Locations/sub-counties	County	Scores ranging from 1 to 5 (%)				
		1	2	3	4	5
Teso South, Butula, Nambale, Samia	Busia	12.6	16.8	28.3	28	14.3
Rangwe, Ndhiwa, Rachuonyo	Homa Bay	16.7	20.8	24.8	27.7	10
Gem	Siaya	16.3	21.3	28.1	28.1	6.2
Webuye, Sirisia	Bungoma	16.2	17.8	28.3	29.4	8.3
Emuhaya	Vihiga	15	23.3	27.5	23.3	10.9
Mean		15.4	20	27.4	27.3	9.9

pathosystem in Kenya. Although GRD is prevalent in Kenya, reports on its incidence and severity are scanty. This study confirms earlier reports on the occurrence of GRD in western Kenya (Wangai et al., 1999, 2001). The plants showing symptoms of GRD were easily identified due to the symptoms they exhibited. Although chlorotic rosette was predominant symptom type, isolated cases of green rosette, leaf curling, and mosaic symptoms were observed on groundnut plants. Similar symptoms were previously reported in Kenya, Uganda, and Nigeria (Wangai et al., 2001; A'Brook, 2007; Okello et al., 2014, 2017). However, green rosette symptom type is described as the most common symptom of GRD in West Africa (Naidu et al., 1998; Appiah et al., 2016).

The results reported in this study shows substantial incidence and severity of GRD during the survey carried out in the five-groundnut major growing counties of western Kenya. There were significant differences in disease incidence among the counties surveyed. According to Hull (2002), variability in the pathological incidences of a viral disease is often attributed to several factors such as susceptibility and age of the host plant and environmental factors such as solar radiation and temperature. The variations in incidences observed in this study may be attributed to the fact that the surveyed counties are in different agro-ecological zones and as such the different environmental conditions prevailing may have influenced vector multiplication and spread. This result could also be due to intrinsic factors of the crops including susceptibility and agricultural practices employed within the different regions (Juarez et al., 2013). Studies by Edema et al. (1997) and Shoyinka et al. (1997) also indicated that weather conditions within seasons and cropping systems can affect distribution of viruses in different environments. The incidences were high in Busia, Siaya, and Homa Bay Counties compared to Bungoma and Vihiga. This confirms past reports that identified Homa Bay as one of the hotspots for GRD in

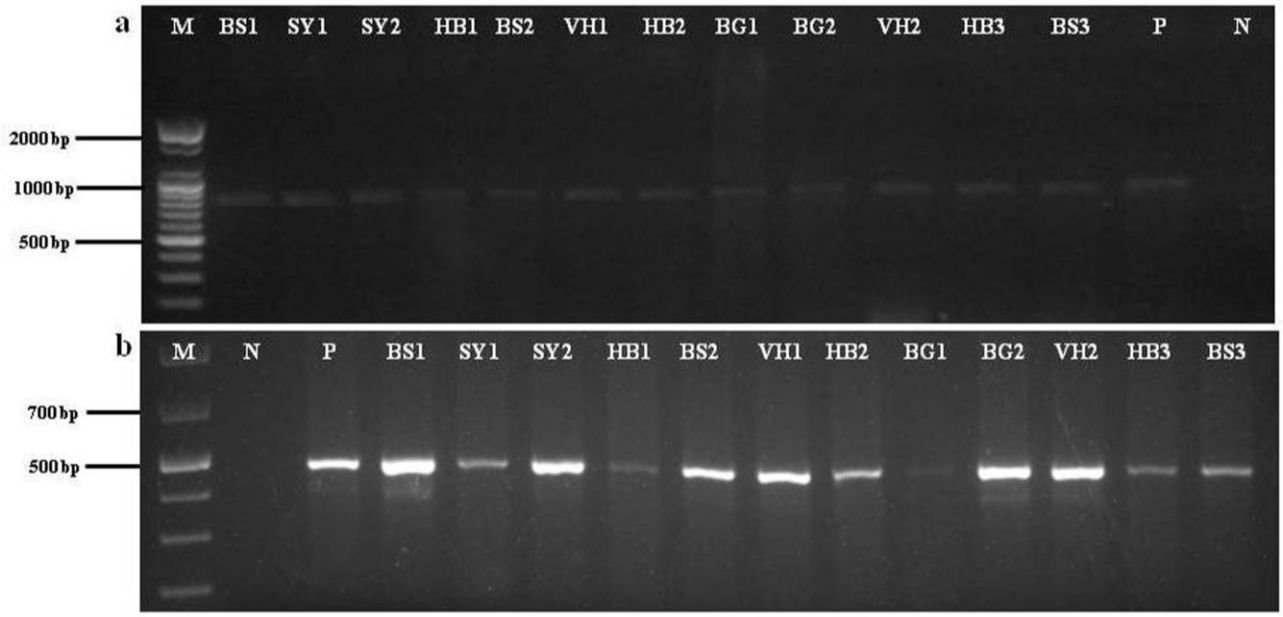

Figure 3. Gel electrophoresis of amplified RT-PCR products using (a) *Groundnut rosette assistor virus* (GRAV2) and; (b) *Groundnut rosette assistor virus* (GRAV1) primers. Lanes P = positive control; N = negative control (healthy plant); BS, SY, HB, BG and VH represent samples from Busia, Siaya, Homa Bay, Bungoma and Vihiga Counties, respectively.

Kenya (Wangai et al., 2001). The variations in incidences in this study can be due to the fact that the low virus incidences in Bungoma and Vihiga may suggest presence of low virus inoculums source to cause high incidence in groundnut fields.

A striking feature of this study was that there was no farm with 0% GRD incidence, an indication that all groundnut varieties in the surveyed area were susceptible to the viruses and that all farms were infected. RT-PCR detected GRAV in all GRD symptomatic plant leaves collected from all the surveyed counties in western Kenya. These results demonstrate a wide distribution of the disease in all groundnut growing regions. This confirms previous reports by Waliyar et al. (2007) that GRD is an important disease of groundnut across all groundnut growing regions in Sub-Saharan Africa.

Although most of the farmers could recognize the disease, they associated the symptoms to abiotic stresses such as drought, too much rain and poor soil fertility. None of the groundnut farmers associated the disease with viruses and aphid vector. Although most farmers observed that the susceptibility of the disease varied among cultivars, they continued growing susceptible cultivars because of their superior agronomic traits such as high yield. The farmers' perception and lack of knowledge on the disease could be some of the factors that might be contributing to the high prevalence of GRD in major groundnut growing regions in western Kenya. The study demonstrated that despite the several strategies used by farmers for the management of GRD, including rouging, cultural practices, and chemical sprays (Naidu et al., 1999), the disease continues to be a major constraint to groundnut production in Kenya.

The study revealed that groundnut rosette disease is prevalent and widely distributed in all major groundnut growing counties in western Kenya and therefore control interventions are urgently required. The results of this survey reflect the need for creation of awareness to enhance farmers' knowledge on GRD in Western Kenya. Emphasis should be on accurate disease identification, sources of seeds for planting and feasible management practices such as rouging and use of resistant varieties. Farmers use their own uncertified seeds or those from neighbors most of which are from susceptible landraces and serve as source of virus. Therefore, for effective control of GRD, farmers need to be sensitized on the benefits of using seeds of improved groundnut varieties. Further work on molecular characterization of GRD agents identified in this study will facilitate the understanding of diversity of viruses infecting groundnut in western Kenya. There is a need to promote production and dissemination of seeds of improved varieties to reduce virus disease incidence in major groundnut growing regions of Kenya. The determination of the areas with high insect vectors and virus disease incidences indicates where resistance breeding and other control strategies are urgently needed. The study has demonstrated the need for breeding to develop improved varieties that have high levels of resistance to GRD. Host plant resistance will be the best long-term solution to the problem of virus diseases, as they will reduce yield losses even if farmers lack knowledge about the spread

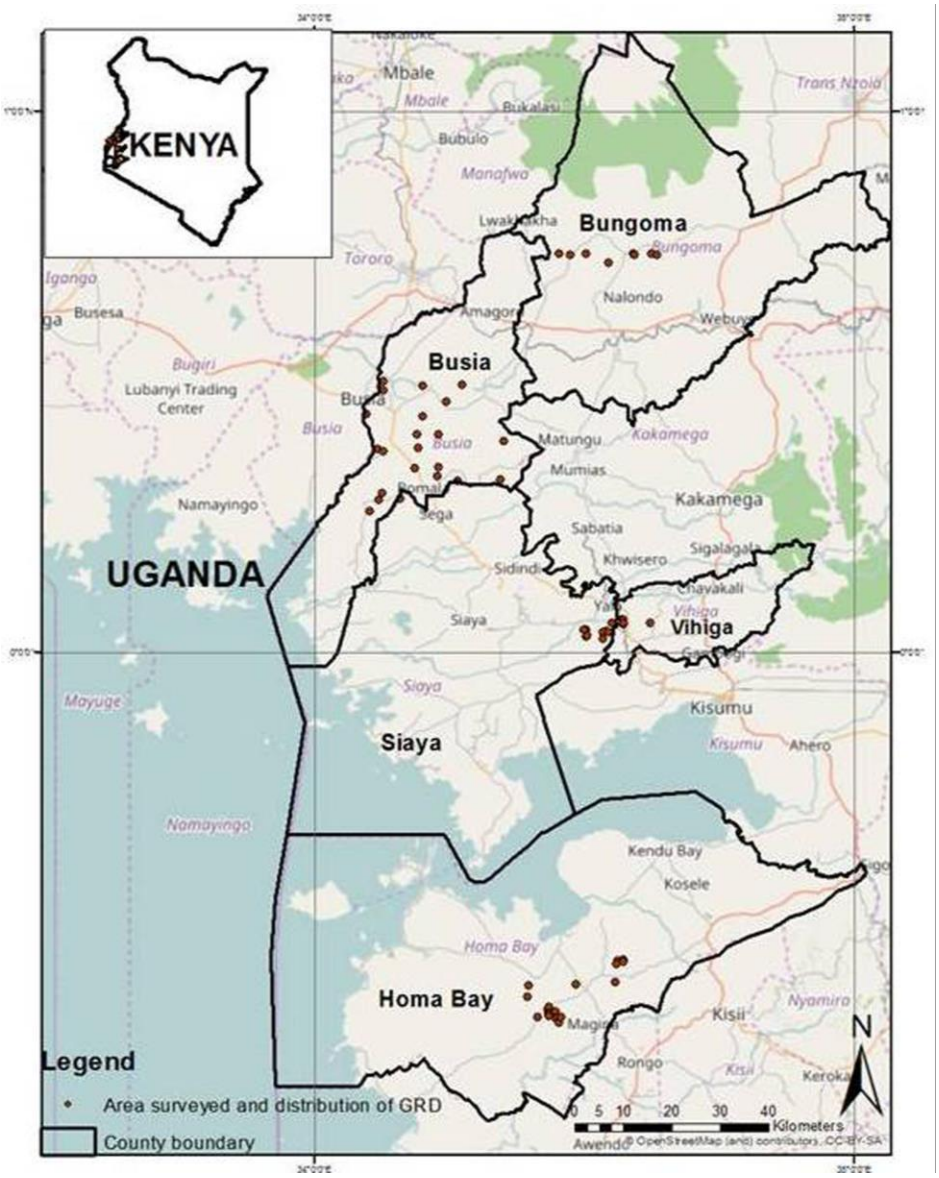

Figure 4. Map of western Kenya showing surveyed counties and distribution of GRD.

of plant virus diseases.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors wish to appreciate the support from the Department of Biochemistry, University of Nairobi, Kenya for providing the laboratory facilities and also thank Daniel Ajaku (ICRISAT, Alupe), Patrick Wandera (ICRISAT, Alupe) and Philip Odero (Jaramogi Oginga Odinga University, Homa Bay) for the help rendered in data and sample collection in the field.

REFERENCES

A'Brook J (2007). The effect of planting date and spacing on the incidence of groundnut rosette disease and of the vector, *Aphis craccivora* Koch, at Mokwa, Northern Nigeria. Annals of Applied Biology 54(2):199-208.

Andima D, Kidula N, Makini F (2006). Research Priorities for KARI Kisii Mandate Area.

Appiah AS, Offei SK, Tegg RS, Wilson CR (2016). Varietal response to groundnut rosette disease and the first report of Groundnut ringspot virus in Ghana. Plant Disease 100(5):946-952.

Deom CM, Naidu RA, Chiyembekeza AJ, Ntare BR, Subrahmanyam P (2000). Sequence diversity within the three agents of Groundnut rosette disease. Phytopathology 90(3):214-219.

Diop N, Beghin J, Sewadeh M (2004). Groundnut policies, global trade dynamics and the impact of trade liberalization, World Bank Policy Research Working Paper 3226, World Bank, P 36.
Edema R, Adipala E, Florini DA (1997). Influence of season and cropping system on occurrence of cowpea diseases in Uganda. African Crop Science Journal Plant Disease 81(5):465-468.
FAOSTAT (2014). Food and Agriculture Organization of the United Nations.
Hull R (2002). Mathew's plant virology, 4th ed. Elsevier Academic Press, New York.
Janila P, Nigam SN, Pandey M, Nagesh P, Varshney RK (2013). Groundnut improvement: use of genetic and genomic tools. Frontiers in Plant Science 4:23.
Juarez M, Legua P, Mengual CM, Kassem MA, Sempere RN, Gómez P, Truniger V, Aranda MA (2013). Relative incidence, spatial distribution and genetic diversity of cucurbit viruses in eastern Spain. Annals of Applied Biology 162(3):362-370.
Lynch RE (1990). Resistance in peanut to major arthropod pests. Florida Entomologist 73(3):422-445.
Naidu RA, Bottenburg H, Subrahmanyam P, Kimmins FM, Robinson DJ, Thresh JM (1998). Epidemiology of groundnut rosette virus disease: current status and future research needs. Annals of Applied Biology 132(3):525-548.
Naidu RA, Kimmins FM, Deom CM, Subrahmanyam P, Chiyembekeza AJ, Van der Merwe PJA (1999). Groundnut rosette a virus disease affecting groundnut production in Sub-Saharan Africa. Plant Disease 83(8):700-709.
Okello DK, Akello LB, Tukamuhabwa P, Odong TL, Ochwo-Ssemakula M, Adriko J, Deom CM (2014). Groundnut rosette disease symptoms types distribution and management of the disease in Uganda. African Journal of Plant Science 8(3):153-163.
Okello DK, Ugen MA, Tukamuhabwa P, Ochwo-Ssemakula M, Odong TL, Adriko J, Kiconco F, Male A, Deom CM (2017). Molecular diagnostics of groundnut rosette disease agents in Uganda: Implications on epidemiology and management of groundnut rosette disease. Journal of Plant Breeding and Crop Science 9(5):63-70.
Okello DK, Biruma M, Deom CM (2010). Overview of Groundnut research in Uganda: Past, Present and Future. African Journal of Biotechnology 9(39):6448-6459.
Okello DK, Monyo E, Deom CM, Ininda J, Oloka HK (2013). Groundnuts production guide for Uganda: Recommended practices for farmers. National Agricultural Research Organisation, Entebbe. ISBN: 978-9970-401-06-2.
Pandey MK, Monyo ES, Ozias-Akins P, Liang X, Guimaraes P, Nigam SN, Upadhyaya HD, Janila P, Zhang X, Guo B, Cook DR., Bertioli DJ, Michelmore R, Varshney RK (2012). Advances in *Arachis* genomics for peanut improvement. Biotechnology Advances 30(3):639-651.
Shoyinka SA, Thottappilly G, Adebayo GG, Anno-Nyako FO (1997). Survey on cowpea virus incidence and distribution in Nigeria. International Journal of Pest Management 43(2):127-132.
Waliyar F, Kumar PL, Ntare BR, Monyo ES, Nigam SN, Reddy AS, Osiru M, Diallo AT (2007). A Century of Research on Groundnut Rosette Disease and its Management. Information Bulletin no. 75. International Crops Research Institute for the Semi-Arid Tropics, Patancheru, Andhra Pradesh, India 40 p.
Waliyar F, Ntare BR, Traore A, Diarra B, Kodio O, Lava Kumar P (2005). Pre- and postharvest management of aflatoxin contamination in groundnut in west and central Africa Reducing impact of mycotoxin in tropical agriculture with emphasis on health and trade, 13-16 September 2005, IITA and Myco-globe, Accra, Ghana pp. 20-21.
Wangai AW, Pappu SS, Pappu HR, Okoko N, Deom CM, Naidu RA (1999). First report of the green rosette variant of groundnut rosette disease in Kenya. Plant Disease 83(8):782.
Wangai AW, Pappu SS, Pappu HR, Deom CM, Naidu RA (2001). Distribution and Characteristics of groundnut rosette disease in Kenya. Plant Disease 85(5):470-474.

Vol. 18(32), pp.1113-1122, December, 2019
DOI: 10.5897/AJB2019.16867
Article Number: BC3973062524
ISSN: 1684-5315

http://www.academicjournals.org/AJB

ACADEMIC JOURNALS
expand your knowledge

African Journal of Biotechnology

Full Length Research Paper

In vitro regeneration of coconut (*Cocos nucifera* L) through indirect somatic embryogenesis in Kenya

Caren C. Bett*, Cecilia M. Mweu and Aggrey B. Nyende

Institute of Biotechnology Research, Jomo Kenyatta University of Agriculture Technology, P.O Box 62000-00200, Nairobi, Kenya.

Received 20 May, 2019; Accepted 20 November, 2019

Regeneration of the coconut (*Cocos nucifera* L) through indirect somatic embryogenesis using 9 to 12 months embryos explants was established in Y3 medium supplemented with 100 to 250 µM 2,4 - dichlorophenoxyacetic acid (2, 4-D) alone and in combination with gibberellic acid (GA_3), 6-benzylaminopurine (BAP) and thiadiazuron (TDZ). The highest callus induction (100%) was observed in medium containing 150 and 250 µM 2, 4-D + 5 µM BAP while the least was in 2, 4-D alone (16.7%) and in combination with 0.35 µM GA_3 (0). The highest embryogenic calli were observed in medium with 125 µM 2, 4-D + 5 µM BAP (100%) and the least was in medium with 2, 4-D alone (16.7) and in combination with 0.35 µM GA_3 (0). Multiple shoot induction was observed in medium supplemented with 10 µM kinetin, 10 µM BAP, 0.5 µM GA3, and 200 µM NAA. Maximum shoot elongation (1.667 cm) was in medium containing 10 µM BAP and the least (0.811 cm) was in medium with 5 µM BAP. Rooting was done in Y3 medium containing three levels of indole-3-butyric acid (IBA). The highest response was observed in Y3 medium supplemented with 5 µM IBA+ 0.5 µM GA_3 both with respect to the number of roots 8.33 and root length 5.10 cm while the least was with 10 µM IBA+ 0.5 µM GA_3 with regard to the number of roots 3.33 and root length 2.83 cm. Acclimatization was achieved in media prepared with soil: sand: manure ratio (3:1:1) with 25% survival rate and vermiculate medium with 8.3% survival rate. Hence, *in vitro* regeneration of coconut through somatic embryogenesis is a viable alternative for mass propagation.

Key words: *Cocos nucifera* L., somatic embryogenesis, zygotic embryos.

INTRODUCTION

Cocos nucifera L. belongs to family Arecaceae and lone species in the genus *Cocos*. It is a large palm growing to a height of up to 30 m tall with leaves 4to 6 m long. The life span of coconut varies depending on the variety; the tall variety can last even between 80 and 90 years while the dwarf variety can last to 50 years. In Kenya, coconut is almost exclusively found in the coastal areas which include Mombasa, Kwale, Kilifi, Lamu Tana River, and Taita Taveta counties. However, recent studies have shown that there is potential for coconut farming in other parts of the country. Piloting trials have indicated encouraging results in Tharaka Nithi, Makueni, Rift valley, Nyanza and western regions which do not belong to the coastal regions. Coconut is both heterozygous (tall variety) and homozygous (dwarf variety) and is so far propagated mainly through seeds (Bandupriya et al., 2016).

Coconut is an important palm in tropics and it provides

*Corresponding author. E-mail: chepcaren@gmail.com. Tel: +254715356833.

a source of livelihood directly and indirectly to more than 50 million smallholder farmers worldwide. It is grown in more than 12 million ha in 90 countries mainly in the Asian Pacific regions where Indonesia has been ranked as the largest coconut growing region followed by the Philippines and thirdly India (Exposomics, 2017). It has a variety of traditional uses such food, beverage, oil, fiber, timber, thatch, mats, fuel, and domestic utensils (Batugal and Oliver, 2005). The copra and its products produce food and non-foods which are environmentally friendly and are used locally or exported hence becoming a source of foreign exchange. The virgin coconut oil is a very rich source of vitamins and enzymes which are used in the cosmetic and pharmaceutical industries (Mansor et al., 2016).

Despite all these, there are also challenges facing the coconut industry which include poor infrastructure, industrialization, utilization of coconut farms for other high-value cash crops, natural disasters, droughts, lack of quality planting material pest and diseases. In Kenya, according to the report released by the Agricultural Business Development (ABD) program of the Danish International Development Agency (DANIDA) which was carried out in collaboration with the Coast Development Authority (CDA) and others on a survey of coconut trees, the main challenge facing farmers at the production level was inaccessibility of quality planting materials and also pest and diseases which hence hindered the realization of its full potential. Farmers generally rely on their current crop to obtain seed nuts for raising seedlings. This conventional breeding approach using the nuts to replant land is very expensive due to the slow multiplication rate of seeds and even when elite germplasm is available it takes decades to multiply. Furthermore, the linkage between the farmer and the processor has not been fully exploited (Muhammed et al., 2013), because the existing aged orchards are poorly managed, high pest infestations and disease and inadequate technologies for mass production (Baudouin et al., 2018; Saha and Mat, 2018; Sathana, 2018).

Considering the economic importance of coconut, crop improvement is of high priority. Tissue culture of coconut is of prime importance for rapid multiplication and distribution of the best genotypes obtained through conventional breeding such as selected parental exhibiting resistance to biotic and/or abiotic stresses and for increasing the yield (Muhammed et al., 2013).

Direct and indirect organogenesis using plumules has often be reported in coconut as compared to somatic embryogenesis (Das et al., 2014). Direct organogenesis has been reported to be more effective in many plant species. This technique is preferred due to minimal soma clonal variation and also cost effective. However, regeneration of coconut through tissue culture technique is still a major challenge because it is a recalcitrant species. On the other hand, SE is a process by which somatic cells develop callus from an explant under induction. Bipolar structures are formed without connection with the original tissue. Then somatic embryos developed from the callus can convert into whole plantlets (Pérez-Núñez et al., 2006). Different coconut explants have been used and each giving diverse results whereas immature inflorescence and plumule have shown the ability of callogenesis, formation of embryos, maturation and plantlet formation (Pérez-Núñez et al., 2006). Somatic embryogenesis allows rapid multiplication of genotypes with the abiotic and abiotic resistant traits, high yielding and fast-growing nature. Through this technique, high quality and quantity of plantlets obtained can be used for establishing uniform orchards which are easy to maintain and even harvesting

Hence, the objective of this study was to develop an efficient protocol using 2,4D in combination with other plant growth regulators for effective *in vitro* regeneration of Kenya coconut and hence can be used for producing improved planting materials for mass production.

MATERIALS AND METHODS

Plant

Plant materials used in this study were obtained from mature nuts (10-11 months old) harvested from the clean coconut mother-plants which had been identified to be highly productive by the Kenya Coconut Development Authority, a Kenya Government Authority in charge of the coconut sector. By using a purposive sampling method, the nuts were collected from South Coast including Msambweni Majikuko Village (4°28'11.34''S, 39°28'46.68''E) and the North Coast including Birini, (3°45'04.14''S, 39°35'27.27''E), Mmleka (3°58'24.36''S, 39°44'00.95''E), and Kinarani (3°43'35.35''S, 39°34'21.66''E) region of Mombasa.

The nuts were packaged in netting bags containing 60 to 100 nuts and transported to Jomo Kenyatta University of Agriculture and Technology (JKUAT), Institute of Biotechnology Research (IBR).

Embryo extraction

The nuts were first dehusked and cleaned thoroughly by gently brushing under running tap water. A cylinder of endosperm surrounding an embryo was carefully extracted from each mature nut using a 2 mm diameter sterile cork borer (Sukendah and Cedo, 2005) and then washed with distilled water (Molla et al., 2004).

Surface sterilization of endosperms extracts and embryo excision

The endosperms containing embryos were soaked in 10% savlon with 100 µl of Tween® 20 for 30 min and rinsed with double distilled water. The endosperm portions with embryos were then placed in 95% ethanol with gentle swirling for 1 min and rinsed with double distilled water then soaked in 10% sodium hypochlorite containing 100 µl of Tween® 20 for 20 min and then rinsed with double distilled water.

Under aseptic conditions inside the clean bench, the embryos were excised from the endosperm cylinders using a sterile scalpel. The embryos were finally sterilized using 1% sodium hypochlorite for 1 min and rinsed thrice with double distilled water.

Effect of plant growth regulators on callus induction from coconut embryo explants

The sterilized embryos were initiated on the Y3 medium (Eeuwens, 1976) containing Morel and Whites vitamins (White, 1951) for callus induction. Six concentrations of 100-250 µM 2,4-dichlorophenoxyacetic acid (2, 4-D) were tested alone (A) and in combination with 0.35 µM gibberellic acid (GA_3) (B), 0.5 µM GA3 (C), 5 µM 6-benzylaminopurine (BAP) (D) and 9 µM thiadiazuron (TDZ) (E) which were combined in 5 different treatments and a control (without plant growth regulators) (Perera et al., 2009; Vidhanaarachchi et al., 2013). All the media were supplemented with 4% (w/v) sucrose, 1 g/l activated charcoal and 0.28% (w/v) gel rite.

The cultures were kept for 12 weeks in dark at 25±2°C for callus induction.

Effect of plant growth regulators somatic embryo formation

Callus formed were transferred to embryo formation media containing half 2, 4-D (50-125 µM) auxin concentrations alone (*A), and in combination with other PGRs including 0.35 µM GA_3 (*B), 0.5 µM GA_3 (*C), 5 µM BAP (*D) and 9 µM TDZ (*E). The cultures were kept in dark for 2 months at 25±2°C for embryo formation (Antonova, 2009; Vidhanaarachchi et al., 2013).

Effect of plant growth regulators on shoot induction and elongation

The embryos formed were transferred to a shoot induction media containing 5 µM BAP+ 0.5 µM GA_3 during 4 weeks and then subcultured into Y3 medium supplemented with 10 µM kinetin + 10 µM BAP + 0.5 µM GA_3 and 200 µM NAA (Vidhanaarachchi et al., 2016) for 8 weeks and, after that, the induced shoots were subcultured on Y3 medium supplemented with three concentrations of BAP (5, 10 and 15 µM) in combination with 0.5 µM GA_3 for shoot elongation. All the cultures were then kept under the light with a photoperiod of (16 h/8 h) provided by cool white fluorescent tubes in the culture room at 25±2°C for 8 weeks (Perera et al., 2008).

Effect of plant growth regulators rooting induction

The elongated shoots (5-6 cm long) were transferred to rooting media containing Y3 medium supplemented with 5, 10 and 15 µM IBA in combination with 0.5 µM GA_3. All the media was supplemented with 4% (W/V) sucrose, 1 g/l activated charcoal and 0.28% (w/v) gel rite. The cultures were incubated in the culture room under a photoperiod of16/8 h of light and darkness and the temperatures were maintained at 28±2°C for 8 weeks to induce roots.

Acclimatization in the greenhouse

The fully developed plantlets with three leaves and at least 5 roots which were at least 3 cm were de-flasked, rinsed with double distilled water and drenched in a mixture of Y3 nutrients and a mixture of BAP and NAA hormones for 1 min before transfer to clear polypropylene bags containing sterilized potting mixtures. Two different substrates that were tested were sterilized soil: sand: manure (3:1:1) (A) and a vermiculate (B). The plantlets were acclimatized at 28±2°C and relative humidity ranging from 70 to 80%. The plants were watered regularly and data on the survival rate was recorded every 2 weeks for 3 months.

Experimental design, data collection, and analysis

Experiments on callus induction, somatic embryo formation shooting, rooting and acclimatization were all set up in a completely random design in 3 replicates. Parameters taken on callus and somatic embryo formation were color texture and period took for callus and embryo induction. All these were done by observation and counting the explants which formed callus and embryos and each was estimated to be 100%. For the shoot induction and elongation, the parameters taken were period taken for shoots to form and the color change which was done by observation, counting the number of leaves and measuring the height of the plantlets (cm). The rooting parameters were root length taken using a ruler (cm) and the numbers of roots formed were counted. The length and number of roots were analyzed using the statistical software to give the means. All the data recorded were analyzed using Minitab 17 Statistical software. Analysis of variance (ANOVA) was used to test the significant differences between the various means (%) and Fisher's test at $P<0.05$ was used to separate means. The variability in data was expressed as the percentage mean ± standard error (SE).

RESULTS

Effect of plant growth regulators on callus induction

The first signs of callusing which include swelling of the embryo and the color change were observed from the eight week. When the explants were subcultured into the same media for an additional four weeks, the callus became more profound and could easily be differentiated from the swollen embryos (Figure 1). The texture of the calli became more visible after 12 weeks where both friable and compact calli were seen and also the color changed from white to cream-white was visible. The calli formed in media containing 2,4-D alone and in combination with GA_3 and some in BAP were compact while those formed in a media containing 2,4-D in combination with TDZ were friable. Data analysis on callus induction after 12 weeks revealed that all the embryos in Y3 media supplemented with 150D and 250D formed calli (100%). Both compact and friable textures of callus were observed in the treatments which were supplemented with BAP (D). To check the optimum hormone combination for callus induction (Table 1 on % frequency) combinations of 2,4-D plus 5 µM BAP (D), the highest % frequency of 61.1%, 2,4-D plus 0.5 µM GA_3 (C) with a % frequency of 50% and those in 2,4-D plus 9 µM TDZ (E) with a % frequency of 47%. While the least frequency was recorded in 2,4-D alone with 16.7% (A) (Table 1).

However, some of the embryos remained dormant even after 16 weeks in the callus induction media.

Effect of PGRs on the formation of embryos

When the formed calli were subcultured into embryo formation media, the majority of the calli became embryogenic after 8 weeks. Data analysis after 8 weeks

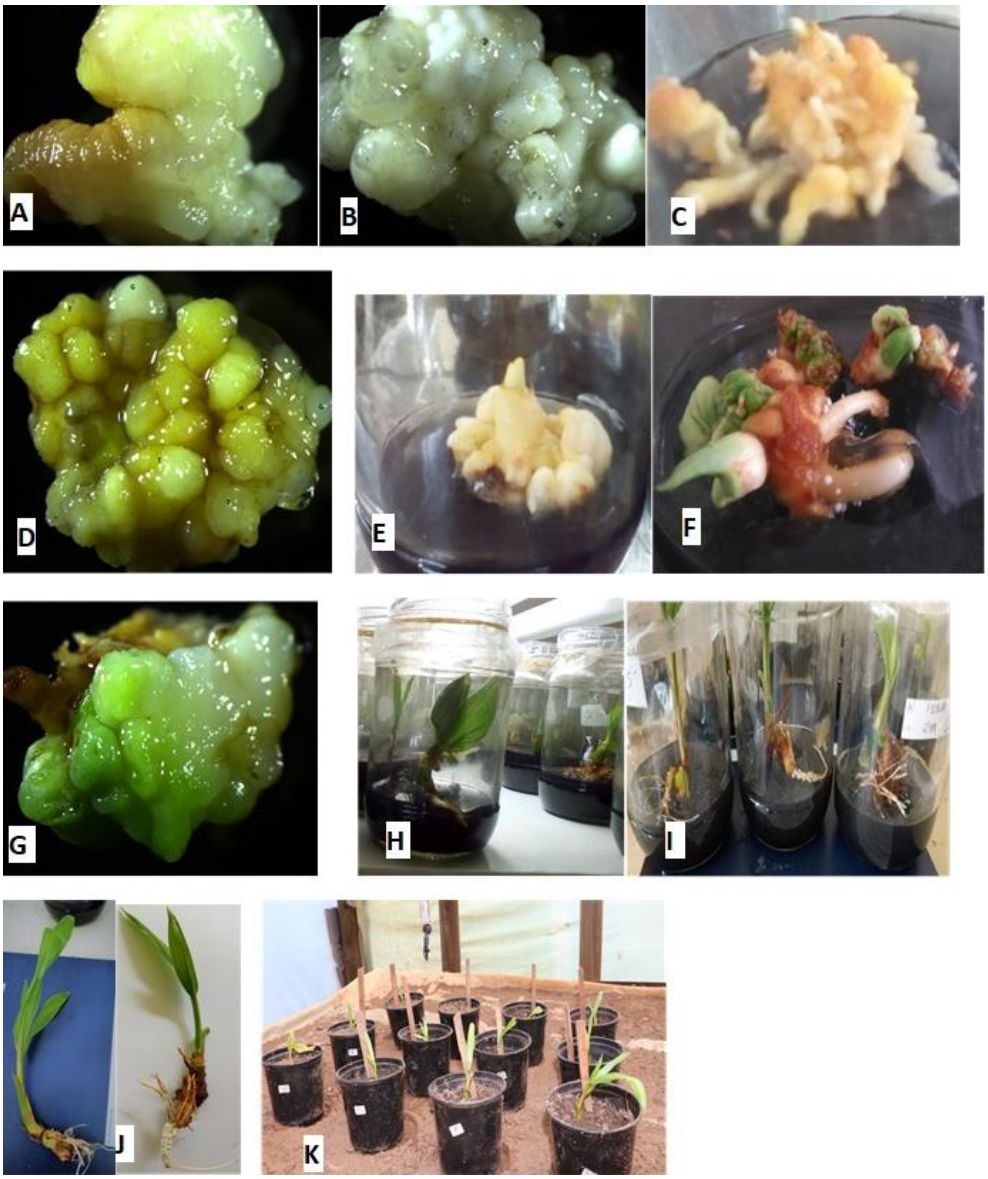

Figure 1. (A) Primary callus formed after 12 weeks in callus induction media; B and C, Compact and friable embryogenic calli, respectively formed after 8 weeks in embryo formation media; D, Embryogenic calli with all stages of embryogenic callus development that is g-globular, H-heart shape and T-torpedo stages; E shows shoot induction from compact embryogenic calli after 8 weeks in maturation media containing BAP and GA_3; F, Multiple shoot induction from friable embryogenic calli in media containing 10 µM kinetin, 10 µM BAP, 200 µM NAA and 0.5 µM GA_3; G, Elongated shoots from green embryogenic calli; H, I and J, Rooted after 8 weeks in root induction media; and K shows the acclimatization of plantlets in the greenhouse.

of subculture in embryo formation media revealed that all the calli in media which were supplemented with 125 µM 2, 4-D+5 µM BAP became embryogenic (100%).

To check the optimum hormone combination and concentration for embryogenic callus induction, (Table 2 on % frequency) a combination of 2, 4-D plus 5 µM BAP (*D) was the highest with 58.3% and the least embryo induction frequency was recorded in the hormonal combination of 2,4-D alone with 13.9% (*A). Just as in callus induction experiments, all the embryogenic calli formed in treatment with 24-D alone and in combination with GA_3 (*B and *C) and some in BAP (*D) were compact while for treatment containing TDZ (*E) and some in BAP media (*D) were friable Table 2.

Also to note is that, embryogenic calli induced after 8 weeks in embryo formation media were visible under the microscope (Figure 1B). Different stages of embryogenic calli development were observed in the same explants

Table 1. Effect of 2,4-D alone and in combination with 0.35 µM GA_3, 0.5µM GA_3, 5 µM BAP and 9 µM TDZ on callus induction from embryo explants after 12 weeks.

Concentration of 2,4-D (µm)	Plant growth regulators (µM)				
	A	B	C	D	E
Control	0^{d}	0^{d}	0^{d}	0^{d}	0^{d}
100	33.3 ± 0.21^{bcd}	16.7 ± 0.17^{cd}	50.0 ± 0.22^{abcd}	33.3 ± 0.21^{bcd}	66.7 ± 0.21^{abc}
125	16.7 ± 0.17^{cd}	16.7 ± 0.17^{cd}	66.7 ± 0.21^{abc}	$50.0\pm0.22a^{bcd}$	33.3 ± 0.21^{bcd}
150	16.7 ± 0.17^{cd}	16.7 ± 0.17^{cd}	83.3 ± 0.17^{ab}	100^{a}	66.7 ± 0.21^{abc}
175	16.7 ± 0.17^{cd}	16.7 ± 0.17^{cd}	33.3 ± 0.21^{bcd}	$50.0\pm0.22a^{bcd}$	$50.0\pm0.22a^{bcd}$
200	16.7 ± 0.17^{cd}	50.0 ± 0.22^{abcd}	33.3 ± 0.21^{bcd}	33.3±0.21bcd	33.3 ± 0.21^{bcd}
250	16.7 ± 0.17^{cd}	0^{d}	33.3 ± 0.21^{bcd}	100^{a}	33.3 ± 0.21^{bcd}
% Frequency	16.7 ± 0.63^{b}	19.44 ± 0.67^{b}	16.7 ± 0.17^{cd}	61.11 ± 0.82^{a}	47.22 ± 0.84^{a}

Means (± SE) followed by the same alphabets in each column were not significantly different at P≤0.05 using Fisher's test (n=186). A = (100-250 µM 2,4-D alone); B=(100-250 µM 2,4-D +0.35 µM GA_3); C=(100-250 µM 2,4-D+0.5 µM GA_3); D=(100-250 µM 2,4-D +0.5 µM BAP) and E=(100-250 µM 2,4-D +9 µM TDZ), e.g. 100A will be Y3 media supplemented with 100 µM 2,4-D alone and 125E will be Y3 media supplemented with 125 µM 2,4-D +9 µM TDZ. % Frequency =average frequency % for hormone combinations (A B C D and E).

Table 2. Effect of 2,4-D alone and in combination with 0.35µM GA_3, 0.5µM GA_3, 5µM BAP and 9µM TDZ on embryo formation after 8 weeks.

Concentration	Plant growth regulators in (µM)				
	*A	*B	*C	*D	*E
Control	0^{c}	0^{c}	0^{c}	0^{c}	0^{c}
50	0^{c}	16.7 ± 0.17^{c}	50.0 ± 0.22^{abc}	33.3 ± 0.21^{bc}	50.0 ± 0.22^{abc}
62.5	16.7 ± 0.17^{c}	16.7 ± 0.17^{c}	50.0 ± 0.22^{abc}	50.0 ± 0.22^{abc}	33.3 ± 0.21^{bc}
75	16.7 ± 0.17^{c}	16.7 ± 0.17^{c}	83.3 ± 0.17^{ab}	83.3 ± 0.17^{ab}	33.3 ± 0.21^{bc}
87.5	16.7 ± 0.17^{c}	16.7 ± 0.17^{c}	33.3 ± 0.21^{bc}	50.0 ± 0.22^{abc}	33.3 ± 0.21^{bc}
100	16.7 ± 0.17^{c}	33.3 ± 0.21^{bc}	33.3 ± 0.21^{bc}	33.3 ± 0.21^{bc}	16.7 ± 0.17^{c}
125	16.7 ± 0.17^{c}	0^{c}	33.3 ± 0.21^{bc}	100^{a}	16.7 ± 0.17^{c}
% Frequency	13.89 ± 0.59^{c}	16.7 ± 0.63^{c}	47.22 ± 0.84^{ab}	58.33 ± 0.83^{a}	30.56 ± 0.78^{bc}

Means (± SE) followed by same alphabets in each column were not significantly different at P ≤0.05 using Fisher's test (n=186). *A = (50-125 µM 2,4-D alone);*B= (50-125 µM 2,4-D +0.35 µM GA_3); *C= (50-125 µM 2,4-D +0.5 µM GA_3);*D=(50-125 µM 2,4-D +0.5 µM BAP) and *E=(50-125 µM 2,4-D +9 µM TDZ), e.g. 50*A will be Y3 media supplemented with 50 µM 2,4-D alone and 125*E will be Y3 media supplemented with 125µM 2,4-D +9 µM TDZ. % Frequency = average frequency % for hormone combinations (A B C D and E).

which include the globular stage was round and shiny protrusions, torpedo and heart shape stages (Figure 1D).

Effects of PGRs on shoot induction

The compact embryogenic calli matured to form shoots after 4 weeks in Y3 media containing 5 µM BAP and 0.5 µM GA_3. The shoots were also white cream in color since they were still in darkness (Figure 1E). When all the embryogenic explants were further subcultured into a fresh media containing 10 µM kinetin, 10 µM BAP, 200 µM NAA and 0.5 µM GA_3 in light, multiple shoots were induced from friable embryogenic calli after 5 weeks in and the color changed from cream white to pale green (9 days) and finally to green (18 days) (Figure 1F). Due to the long juvenile stage of coconut development, some embryogenic calli had not induced shoots but were green and some white cream visible embryos (Figure 1G). Also, to note is that all explants which were initially initiated in Y3 media supplemented with 2.4-D alone eventually matured to form shoots, though at a low rate. It was also very important to note that most explants which were first initiated in Y3 media supplemented with 2,4-D and 0.5 µM GA_3 formed shoots after various subcultures in the shooting media.

Effect of PGRs on shoot elongation

The green, well germinated shoots with an average height of 2.5 cm successfully elongated when they were subcultured in Y3 medium supplemented with BAP in combination with 0.5 µM GA_3 (Figure 1H). The explant

Table 3. Shoot elongation of coconut on Y3 medium supplemented with BAP and GA_3, after 8 weeks.

Parameter	BAP (µm)			
	5	10	15	Control
Shoot height (cm)	1.178±0.19[b]	1.667±0.22[a]	0.811±0.19[c]	0.1±0.03[c]
Number of leafs	2.67±0.33[a]	2.67±0.33[a]	2.67±0.33[a]	0[b]

Means (± SE) followed by the same alphabets in each column were not significantly different at P ≤0.05 using Fisher's test n=36.

which exhibited the highest mean length were those in 10 µM BAP with a significant mean of 1.67 cm and the lowest mean among the three concentrations was at 15 µM BAP with a mean of 0.81 cm. However, the control experiment showed the least mean length of 0.1 cm (Table 3).

Effect of PGRs on the root formation

The shoots with at least 5 cm in height and 2 developed leaves were evident in Y3 media supplemented with all three concentrations of IBA in combination with GA_3 after 8 weeks (Figure 1I). The highest number of roots induced was in the treatment containing 5 µM IBA + 0.5 µM GA_3 while the treatment with 10 µM IBA + 0.5 µM GA_3 had the least number of roots induced as shown in Table 4. However, the control did not induce roots. To check the effect of different PGRs and their concentration on root length, analysis of data revealed that the concentration with significant longer roots were 5 µM IBA + 0.5 µM GA_3 with 5.10 cm while the lowest among the three concentrations was at 10 µM IBA with 2.83 cm (Table 4).

Notably, 5 µM IBA + 0.5 µM GA_3 recorded the highest mean both in length and number of roots induced and the media supplemented with 10 µM IBA + 0.5 µM GA_3 recorded the least mean both in the number of roots induced and root length. Nonetheless, all three levels of IBA were able to induce roots with no difference in their morphological appearances Figure 1I and J.

Effect of different media on acclimatization of coconut plantlets in the greenhouse

After 1 week of acclimatization in the greenhouse, there was 100% survival of all the plantlets with no change in both plant height and number of leaves. The mean height of plantlets in soil media was 8.25 and 6.9 cm in vermiculate with 100% survival. However, during the first 4 weeks of greenhouse acclimatization, there was no or minimal changes in the plantlets. Some died while others experienced a very slow growth rate. To also note is that the majority of the plantlets which were lost began to rot from the roots upwards irrespective of the media. By the end of 12 weeks, the mean height of plantlets in soil media was 9.20 and 7.63 cm in vermiculate. The survival rate for plantlets in both media was 25 and 8.3% in soil and vermiculate media respectively, and since then the remaining plantlets successfully acclimatized with a continued increase in height and new leaves had not formed at that early stage of acclimatization (Table 5).

DISCUSSION

Somatic embryogenesis enables mass production of plants all year round and can be used for genetic improvement of all plants irrespective of the species (Bhansali, 1990). Many trials have been attempted since 1954 by different authors to propagate *C. nucifera in vitro* (Cutter Jr and Wilson, 1954), but still, none of the protocols was found to be efficient. This is because coconut is one of the recalcitrant species to tissue culture and the existing protocols at times cannot be repeated (Solís-Ramos et al., 2012). For somatic embryogenesis to be achieved in coconut there must be a callus intervening stage where callus is first formed and then the regeneration of plantlets from the embryogenic callus (Fernando et al, 2004).

The plant growth regulators concentration, explant source, age, and the variety determines the regeneration efficiency of plants (Kumar et al., 2010). Though callusing can be achieved with or without cytokinins, 2,4-D which is an endogenous auxin is very critical for callogenesis to be achieved and also its concentration varies depending on the type of explants (Solís-Ramos et al., 2012). However, there are several factors including *in vitro* conditions, genotype and mother plant maturity and these may affect the *in vitro* performance (Hernandez-Fernandez and Christie, 1989; Basra, 1995). The gradual reduction in endogenous auxin with a corresponding increase in cytokinins level permits initiation and further development of somatic embryos.

The mature zygotic embryos explants showed better callusing and embryo development, as compared to immature inflorescence which was reported by various authors (Hornung, 1995). Dormancy was also experienced in some embryos and other studies by (Cutter Jr and Wilson, 1954), dormancy was attributed to

Table 4. Effect of IBA in combination with 0.5µM GA_3 on root induction after 8 weeks.

Parameter	IBA (µm)			
	5	10	15	Control
Root length height (cm)	5.10±1.99[a]	2.83±0.72[ab]	3.27±0.43[ab]	0[b]
Number of roots	8.33±2.0[a]	3.33±0.88[ab]	7.33±2.18[a]	0c

Means (± SE) followed by the same alphabets in each column were not significantly different at P≤0.05 using Fisher's test n=36.

Table 5. Effect of media on greenhouse acclimatization of coconut plantlets after 3 months (n=20).

Media	Media components	Shoot height (cm)	Survival rate (%)
A	Soil: Sand: Manure (3:1:1)	9.20±1.5[a]	25
B	Vermiculate	7.63±0.5[a]	8
C	Control	0[b]	0

Means (± SE) followed by the same alphabets in each column were not significantly different at P≤0.05 using Fisher's test.

the use of nuts with mature embryos and endosperm which are likely to contain inhibitory substances leading to dormant cells. The use of young/juvenile explants has also been reported in palms to be more responsive for callus formation (Steinmacher et al., 2007) because their cells are rapidly dividing unlike the mature explants which causes dormancy when subjected to *in vitro* propagation. On subjecting the explants to various combinations and concentrations of plant growth regulators, profuse callus induction was achieved after 8 weeks. And at 12 to 20 weeks, this callus became more profound and even developed embryos. The use of the Y3 medium (Eeuwens, 1976) has been reported to be more favorable for tissue of coconut as compared to the use of MS medium (Murashige and Skoog, 1962). The ammonium and nitrate nitrogen content are half in the Y3 medium as compared to MS medium while the concentrations of microelements such as cobalt copper and iodine are tenfold greater in Y3 medium as compared to MS. This thus reflects those found in the coastal soil providing a favorable habitat for coconut germination (Nguyen et al., 2015).

However, the culture conditions and culture media may vary from one species to another. The addition of 0.4% (w/v) sucrose was essential for callusing and even to germination of the embryos *in vitro.* 0.28% (w/v) gel rite was used as a solidifying agent in this study. Callus induction and regeneration of coconut plantlet *in vitro* was done in darkness at 25 to 28°C. These conditions are required to optimize the germination of the embryo and also reflects the *ex vitro* requirements.

Another component that has shown to be essential in initiating coconut somatic embryos is the presence of activated charcoal (Fernando et al., 2004; Perera et al., 2007; Pérez-Núñez et al., 2006). The role of the activated charcoal is that it has the adsorptive properties hence the ability to adsorb phenols and other growth inhibitory substances. In coconut zygotic embryo culture, the activated charcoal also reduces callus necrosis. It has been known to remove toxic substances released by non-reactive tissues and hence permitting more embryogenic cells to grow (Kumar et al., 2010).

For best callusing to be achieved, it requires the ideal combination of 2, 4-D and activated charcoal in the tissue culture medium. The optimum concentration of 2,4-D in the tissue culture medium may vary with the adsorption capacity of the activated charcoal which also adsorbs 2,4-D (Perera et al., 2007).

Though the specific mechanism involved is less certain. The elimination of growth-inhibitory chemicals such as those produced on autoclaving the medium including 5-hydroxymethylfurfural or from dehydration of sucrose, or by the explanted tissue as toxic metabolites has also been reported. Ethylene gas accumulation especially in closed culture jars/vessels in response to wounding either by forceps or scalpels used during extraction and surface sterilization and also elevated concentrations of exogenous auxin can also be lessened by the activated charcoal and finally controlling browning. Besides the absorption properties of the activated charcoal, in the process of coconut somatic embryogenesis it acts as an "auxin slow-release agent" (Antonova, 2009; Hornung and Verdeil, 1999). Hence, it is an essential component for the successful formation of coconut somatic embryos.

Callusing was evident in all the experiments performed in the Y3 medium (Euwens, 1976) supplemented with 2,4D alone, GA_3 BAP which were compact and TDZ which formed friable calli. The explants in medium

supplemented with various levels of 2,4D and 0.5 µM BAP yielded an average percentage of 61% and the least was those in Y3 media supplemented with 2,4D alone with 16.7%. In previous studies, various authors have also reported different callusing frequencies for example (Hornung, 1995) with 75% (Chan et al., 1998) with 60% and (Fernando et al., 2004) and with 54.3% all-in Euwens Y3 media.

GA_3 was added to the culture medium to promote the germination of somatic embryos. However, the effect of GA_3 has not been tested on the formation of somatic embryos (Perera et al., 2009).

In this study, the addition of 0.5 µM GA_3 proved to be better in the induction of both callus (50%) and embryogenic calli (47.2%) as compared to 0.35 µM GA_3 which resulted in callus induction frequency of 16.7% and embryogenic calli frequency of 13.9%. Higher callus induction in GA_3 containing medium was also reported using coconut ovary explants (Perera et al., 2009). This phytohormone also was used for breaking dormancy at every stage of embryo development hence improving the performance of coconut micropropagation.

TDZ on the other hand as compared to other plant growth regulators when incorporated in the initiation media also revealed to be a potential plant growth hormone for somatic embryogenesis (Perera et al., 2009) reported the use of 9 µM TDZ to be effective in callusing frequency using the ovary explants which resulted in a callusing frequency of 76.4%. The callus induction frequency in this study from embryo explants in the media supplemented with 9 µM TDZ was 47% and the frequency of embryogenic calli was 30.6% which is lower compared to the previous studies. However, the friable and creamy white translucent mass of globules developed in TDZ containing media later induced multiple shoots when subcultured into shooting media (Figure 1F). Similarly, Mweu et al., (2016) in their study also revealed that TDZ is vital for high-frequency induction of callus and multiple shoots which was in agreement for this study.

However, the explants initiated in hormone-free media which were used as a control did not induce callus or embryogenic callus. The hormone-free media without phytohormonal additives serves occasionally in germination of shoots but not in the whole process of tissue culture because in one way or another a plant established *ex vitro* uses the endogenous hormones produced in different pathways aided by the natural environment and hence the *in vitro* plants produced in the lab also requires the phytohormones in their pathways for a complete plantlet formation and also the rapid growth for normal tissues.

Substances that stimulate growth can be applied in form of natural fluids such as coconut water which contains various hormones that break its dormancy, auxin, and cytokinins, and also various vitamins (Fernando and Gamage, 2000) and sugars or chemical compounds required by plants.

Successful initiation of roots is one key factor that determines the survival rate of the tissue culture plantlets upon acclimatization and field transfer. Successful rooting was achieved by supplementing Y3 media with IBA and GA_3. The use of a high concentration of IBA induced rooting in micropropagation of sandalwood which is also one of the recalcitrant plants as well as coconut palm (Solís-Ramos et al., 2012) and for this study, 5 µM IBA induced the highest rooting as compared to 15 µM IBA though not significantly different. No rooting was observed in the treatment without the hormones meaning that the IBA hormone was solely responsible for root induction. This study also revealed that a combination of plant growth regulators in tissue culture media has more affirmative results as compared to using only one hormone (Vidhanaarachchi et al., 2013). GA_3 has been reported by other researchers in various topics to promote normal growth, root development, and axis development and also elongation which were also evident in this study. The quantity of IBA auxin reaching the cambial activity is adequate for initiating root primordia. The maximum number of roots formed could also be due to the hormonal effect resulting to the buildup of internal substances and their downward movement which result in more cell division and hence success in root induction (Muthan et al., 2006).

Tissue cultured plantlets were able to acclimatize in soil: sand: manure (ratio 3:1:1) and vermiculate after 12 weeks though with low survival rate. When tissue cultured plantlets were transplanted, their growth rate in the greenhouse was slow and this may be contributed by many factors one being the leaves. Normally when *in vitro* raised plants are taken for acclimatization their leaves are not fully developed and hence a low level of the photosynthesis and undeveloped cuticular wax impairs stomatal mechanisms. Despite the addition of minerals and hormones to aid in successful acclimatization, yellowing of leaves was still observed after 3 months and the plantlet started withering gradually. This is also in agreement with Rival (2000) who attributed the same to the photosynthetic mechanism and/or poor nutrition in the potting media used in the acclimatization process. Due to the aforementioned characteristics, the nature of the tissue-cultured plantlets during the *ex vitro* transfer causes them prolonged acclimatization duration and the same was also reported by Fernando et al., (2004) and Gunathilake et al. (2004) using plumule as the explants. Also, in some explants rotting from the roots upwards was evident. The same was reported by Steinmacher et al. (2007) and this might have been a result of a poor formation of the root system resulting in a low rate of survival. Acclimatization is a key challenge in most of the tissue cultured plants due to the shock and stress experienced upon transfer from the *in vitro* environment to the *ex vitro* conditions in the greenhouse. In spite of the recalcitrance of coconut palms to tissue culture, a high rate of plantlet loss upon acclimatization

hinders mass production. After the successful hardening of the plantlets, they steadily overcome the morphological stresses and adapted to *ex vitro* conditions which were also in agreement with Fernando et al., (2004).

Conclusion

The present study indicated the viability of regeneration of *C. nucifera* L using embryo explants via indirect somatic embryogenesis where zygotic embryos were initiated in 2,4D (100 to 250 µM) in combination with GA_3, BAP, and TDZ. Lowering the concentration of 2,4D to half while retaining the concentrations of other growth regulators induced embryogenic calli and finally subculturing them to a shoot induction media containing kinetin, BAP and NAA caused multiple shoot induction. This is a key milestone of coconut regeneration in Kenya using Eeuwens Y3 medium with distinct callus becoming embryogenic and eventually developing multiple shoots. This protocol, however, is reproducible and can be used in the mass propagation of coconut.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

Antonova I (2009). Somatic embryogenesis for Micropropagation of coconut *(Cocos nucifera L.)*.

Bandupriya HDD, Femando SC, Vidhanaarachchil YRM (2016). Micropropagation and Androgenesis in coconut: An assessment of Sri Lankan implication. Cocos 22(1):31. https://doi.org/10.4038/cocos.v22i1.5810

Batugal P, Oliver JT (2005). Poverty reduction in coconut growing communities, Volume III: Project achievements and impact. https://cgspace.cgiar.org/handle/10568/104700

Baudouin L, Armero Villanueva AA, Bocs S, This D, Issali AE, Yang Y (2018). Facing the challenges of coconut genetic improvement: The coconut genome [W736]. http://agritrop.cirad.fr/587268/

Bhansali RR (1990). Somatic embryogenesis and regeneration of in plantlets in pomegranate. Annals of Botany 66(3):249-253.

Chan JL, Sáenz L, Talavera C, Hornung R, Robert M, Oropeza C (1998). Regeneration of coconut (*Cocos nucifera* L.) from plumule explants through somatic embryogenesis. Plant Cell Reports 17(6-7):515-521.

Cutter Jr VM, Wilson KS (1954). Effect of coconut endosperm and other growth stimulants upon the development in vitro of embryos of *Cocos nucifera*. Botanical Gazette 115(3):234-240.

Das A, Kumar S, Nandeesha P, Yadav IS, Saini J, Chaturvedi SK, Datta S (2014). An efficient in vitro regeneration system of field pea (*Pisum sativum* L.) via. Shoot organogenesis. Journal of Plant Biochemistry and Biotechnology *23*(2):184-189.

Eeuwens CJ (1976). Mineral requirements for growth and callus initiation of tissue explants excised from mature coconut palms (*Cocos nucifera*) and cultured *in vitro*. Physiologia Plantarum 36(1):23-28.

Exposomics (2017). Final Report. https://doi.org/10.1115/1.802915.ch1

Fernando SC, Gamage CKA (2000). Abscisic acid-induced somatic embryogenesis in immature embryo explants of coconut (*Cocos nucifera* L.). Plant Science 151(2):193-198.

Fernando SC, Weerakoon LK, Gunathilake TR (2004). Micropropagation of coconut through plumule culture. Cocos16:1-10.

Hornung R (1995). Micropropagation of *Cocos nucifera* L. from plumular tissue excised from mature zygotic embryos. Plantations, Recherche, Developpement (France) 2(2):38

Hornung R, Verdeil JL (1999). Somatic embryogenesis in coconut from immature inflorescence explants. Current Advances in Coconut Biotechnology pp. 297-308.

Kumar N, Anand KV, Reddy MP (2010). In vitro plant regeneration of non-toxic *Jatropha curcas* L.: Direct shoot organogenesis from cotyledonary petiole explants. Journal of Crop Science and Biotechnology 13(3):189-194.

Mansor N, Wan Abdullah WA, Bahari A, Hassan Shukri AF (2016). Palm Oil Sustainability Certification and Firm Performance: Is There a Conflict Between RSPO and MSPO? Proceedings, the European Conference on the Social Sciences. https://papers.iafor.org/submission31542/

Molla MMH, Bhuiyan MSA, Dilafroza KM, Pons B (2004). In vitro coconut (*Cocos nucifera* L.) embryo culture in Bangladesh. Biotechnology 3(1):98–101.

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

Muthan B, Rathore TS, Rai VR (2006). Micropropagation of endangered Indian sandalwood (S*antalum album* L.). Journal of Forest Research 11(3):203-209.

Mweu CM, Nyende A, Onguso J (2016). Efficient somatic embryogenesis of *Jatropha curcas* L. from petiole and leaf discs. International Journal of Biotechnology and Molecular Biology Research 7(3):29-35.

Muhammed N, Nyamota R, Hashim S, Malinga JN (2013). Zygotic embryo in vitro culture of *Cocos nucifera* L. (sv. East African Tall variety) in the coastal lowlands of Kenya. African Journal of Biotechnology 12(22):3435-3440, 29 May 2013. https://doi.org/10.5897/AJB2013.11940

Nguyen QT, Bandupriya HD, López-Villalobos A, Sisunandar S, Foale M, Adkins SW (2015). Tissue culture and associated biotechnological interventions for the improvement of coconut (*Cocos nucifera* L.): A review. Planta 242(5):1059-1076.

Perera PI, Hocher V, Verdeil JL, Doulbeau S, Yakandawala DM, Weerakoon LK (2007). Unfertilized ovary: A novel explant for coconut (*Cocos nucifera* L.) somatic embryogenesis. Plant Cell Reports 26(1):21-28.

Perera PI, Hocher V, Verdeil JL, Bandupriya HDD, Yakandawala DMD, Weerakoon LK (2008). Androgenic potential in coconut (*Cocos nucifera* L.). Plant Cell, Tissue and Organ Culture 92(3):293-302.

Perera PI, Vidhanaarachchi VRM, Gunathilake TR, Yakandawala DMD, Hocher V, Verdeil JL, Weerakoon LK (2009). Effect of plant growth regulators on ovary culture of coconut (*Cocos nucifera* L.). Plant Cell, Tissue and Organ Culture 99(1):73-81.

Pérez-Núñez MT, Chan JL, Saenz L, González T, Verdeil JL, Oropeza C (2006). Improved somatic embryogenesis from *Cocos nucifera* (L.) plumule explants. In Vitro Cellular & Developmental Biology-Plant 42(1):37-43.

Rival A (2000). Somatic embryogenesis in oil palm. In Somatic embryogenesis in woody plants. Springer pp. 249-290.

Saha GC, Mat RC (2018). A Study of Coconut Plantation Management Practice: Problems and Status. SMMTC Postgraduate Symposium 2018, 220.

Sathana V (2018). Problems and challenges associated with value addition: With special reference to coconut-based productions in Jaffna District. Kelaniya Journal of Management 6. DOI: http://doi.org/10.4038/kjm.v6i0.7538

Solís-Ramos LY, Andrade-Torres A, Carbonell LAS, Salín CMO, de la Serna EC (2012). Somatic embryogenesis in recalcitrant plants. In *Embryogenesis*. InTech. https://www.intechopen.com/books/embryogenesis/somatic-embryogenesis-in-recalcitrant-plants

Steinmacher DA, Clement CR, Guerra MP (2007). Somatic embryogenesis from immature peach palm inflorescence explants: Towards development of an efficient protocol. Plant Cell, Tissue and Organ Culture 89(1):15-22.

Sukendah S, Cedo ML (2005). In vitro conservation of coconut (*Cocos Nucifera* L.) embryos in culture media. BIOTROPIA-The Southeast Asian Journal of Tropical Biology 25:11-21.

Vidhanaarachchi VRM, Fernando SC, Perera PIP, Weerakoon LK (2013). Application of unfertilized ovary culture to identify elite mother palms of *Cocos nucifera* L. with regenerative potential. Journal of the National Science Foundation of Sri Lanka 41(1):29-34.

Vidhanaarachchi VRM, Suranjith WC, Gunathilake TR (2016). Effect of genotype, embryo maturity and culture medium on in vitro embryo germination of Sri Lankan coconut (*Cocos nucifera* L.) varieties. Journal of the National Science Foundation of Sri Lanka 44(3):273-278

White PR (1951). Nutritional requirements of isolated plant tissues and organs. Annual Review of Plant Physiology *2*(1):231-244.

Related Journals: