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

Monago-Ighorodje Comfort¹, Duru Majesty²*, Adindu Eze³, Nwauche Kelechi⁴, Ezekwe Ahamefula⁵, Nosiri Ijeoma⁶, Odika Prince⁶, Onyeabo Chimaraoke⁷, Ogar Ishade⁸, Berezi Ebitimi Peter⁹, Ugoh Austin Ikenna¹⁰, Eboagwu Ijeoma¹¹ and Otta Emmanuel²

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

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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
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<sub>50</sub>) was carried using the method as described by Lorke (1983), and the toxic dosage (LD<sub>50</sub>) 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 libitum. 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 Bethlöt Searcy’s method (Searcy et al., 1967); creatinine was determined by the method described by Larsen (1971). Sodium ion (Na<sup>+</sup>) and chloride (Cl<sup>-</sup>) ion levels were determined according to the instructions on their diagnostic kits purchased from Randox laboratories (UK). Potassium ion (K<sup>+</sup>) was determined by direct spectrophotometric method. Bicarbonate (HCO<sub>3</sub>) was determined using Forrester et al., (1979) method. Urea estimation was done using Urease-Berthlöt method. Creatinine was estimated using the method described by Heinégard 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 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<sup>12</sup> cell/L (Figure 3); WBC ranged from 2.10-4.53×10<sup>9</sup> 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<sup>+</sup>) ranged from 71.00 to 107.55 mEq/L (Figure 10); potassium ion (K<sup>+</sup>) ranged from 4.11 to 6.36 mEq/L (Figure 11); Chloride ion (Cl<sup>-</sup>) ranged from 60.71 to 92.73 mEq/L (Figure 12), bicarbonate ion (HCO<sub>3</sub>) ranged from 1056 Afr. J. Biotechnol.
16.54 to 21.35 mmol/L (Figure 13); urea ranged from 38.12 to 56.56 mg/dL (Figure 14) and creatinine ranged 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 glycosylated haemoglobin in results in decrease 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.
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.
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 O₂ 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.
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
presence \textit{V. major} leaf extract. Mean cell volume (MCV) and Mean corpuscular haemoglobin 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.
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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). HCO3− 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) HCO3− 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 alloxa 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 7th and 21 days of the study (Figure 18). The observations made on lipid profile parameters in this study could be indicative 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 7th day, the observed reduction became significant (p<0.05) in test groups 2 and 4 rats on the 14th and 21th days whereas the significant reduction in total bilirubin manifested in all the test rats on the 28th 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.

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

Pingdwende Kader Aziz Bamogo¹,²,³, Christophe Brugidou¹,², Fidèle Tiendrébéogo², Drissa Séremé², Florencia Wendkuuni Djigma³, Jacques Simpore³ and Séverine Lacombe¹,²*

¹IRD, CIRAD, Université Montpellier, Interactions Plantes Microorganismes et Environnement (IPME), 911 Avenue Agropolis BP 64501, 34394 Montpellier Cedex 5, France.
²Institut 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.
³Laboratoire 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.

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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...
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) (Thiéméié 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 ORF1 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, F15. 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 ampiclon and VIGS technologies for practical and fundamental exploitations, respectively.

MATERIALS AND METHODS

RYMV and rice material

RYMV amplicon was designed from the RYMVM1 isolate genomic sequence (genbank number: AJ608210). After the stop codon (TAA) of ORF2b, a SnaBI restriction site (TACGTA) was inserted before the Tobacco etch virus (TEV) protease cleavage site, GAAAACCTGATTTTCAGGGA. Right after, Green Fluorescent Protein GFPS-ER sequence (U87974) was inserted. A SpeI (ACTAGT) restriction site was added after the stop codon (TAA) of GFPS-ER sequence. T7 promoter was inserted at the 5’ extremity of the RYMVM1 sequence. T7 promoter sequence was the same as for Fl5 infectious clone: TCTAGACTGCAGTAATACGACTCTATAGG with the T7 RNA polymerase promoter sequence (underlined), an additional G downstream residue and XbaI (TCTAGA) PstI (CTGCAG) restriction sites (Brugidou et al., 1995). A BamHI restriction site was added at the 3’ extremity of the construct. Amplicon::GFPS de novo synthesis was performed by Genecust company as pUC57: amplicon::GFPS clone. RYMV infection clone used is the Fl5 clone developed by Bugidou et al. (1995) from a Côte d’Ivoire isolate. RYMVM1 and RYMVC5 isolates were obtained from infected frozen leaves provided by Pinel-Gaizl, 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

RYVM1 and RYMVC5 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 (NaH2PO4 0.1 M, K2HPO4 0.1 M, pH 7.2). Inoculations were done either by mechanical carborundum abrasion or syringe infiltration.

In vitro transcription and rice inoculation of ampiclon::GFPS and Fl5 construct

In vitro transcription of ampiclon::GFPS and FL5 constructs were done as previously described (Brugidou et al., 1995). Basically, pUC57:ampliclon::GFPS and Fl5 clones were multiplied by overnight bacterial cultures. Plasmid DNA were purified (Promega) and linearized by HindIII or BamHI restriction for FL5 and ampiclon::GFPS 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 mG3pppG (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 OD260 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 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 oligo(dT15) primer and a RYMV specific primer designed on the 3’ extremity of RYMV sequence on the reverse complementary orientation: 5’CTCCCCACCCCATCCGGAGA3’. This primer mix allowed reverse transcription of mRNA and non poly(A) RYMV RNA populations. To detect either GFP or CP RNA accumulation from ampiclon::GFPS and Fl5 samples, respectively, RT-PCR were performed as previously described (Lacombre et al., 2010) using RYMV PB 5’CCAGGAGGCGGAAGAAAATC3’ and RYMV JB 5’CAGGGTATCGATCTCTGAG3’ primers. These primers were designed to surround GFP insertion site. They allowed amplification of a 1269 pb and 995 pb fragments from ampiclon::GFPS and Fl5 cDNA, respectively. The endogenous Os EF-1a gene was used as internal loading control with the primer pair 5’GCACGCTCTTCTTGCTTCTCT3’ and 3’CCTATACCAGGGCT3’.

RESULTS

Design of RYMVM1 ampliclon tool

Efficient ampiclon tool has to display optimal replication and production of protein of interest. Consequently, we selected ampiclon genomic background corresponding to a RYMV isolated displaying a strong replication capacity, RYMVM1 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 RYMVM1 viral accumulation in rice

In order to identify tissues that would be the most
appropriated for protein of interest production from a RYMV based tool, we researched tissues displaying a strong RYMV accumulation. We considered natural RYMVMg1 isolate as amplicon tool we produced has a RYMVMg1 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 these 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 RYMVMg1 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, RYMVMg1 sap inoculation were performed in three spots by syringe infiltration (Figure 3A). RYMVMg1 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 RYMVMg1 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 FI5 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. FI5 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 FI5, respectively (Figure 4). Amplicon::GFP RNA accumulation was
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$_{Mgp1}$ (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$^x$ x and 10$^{3x}$ x 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$^3$, 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$^3$, 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

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**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.
Figure 3. Viral accumulation in rice inoculated leaves. A- Inoculated IR64 leaves were split in three distinct areas: Inoculation area (Inoc), upstream area (Up) and downstream 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 infiltration were done with amplicon::GFP transcript. Mock and F15 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 F15 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 appropriate tissues for a high RYMV replication. This finding fit with the amplicon::GFP construct we designed. Indeed, using F15 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 F15 infectious clone control. This observation is in accordance with previous work on F15 mutated on CP coding sequence (Brugidou et al., 1995). Indeed, replication of F15 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 F15 mutants whereas a strong signal was detected for F15 control samples. The results demonstrated that F15 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$_{Gia}$ 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.

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**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**


Full Length Research Paper

An alternative caprine infection model for Mycoplasma mycoides

Ssajjakambwe Paul1,2*, Elise Schieck1, Anne Liljander1, Tveyongyere Robert2, Matovu Enock2, Kazibwe Ann2, Nanteza Ann2 and Mwiine Frank2

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

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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.

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biology of *M. mycoides* subsp *mycoides*, and corresponding host-pathogen interactions. 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 interactions (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 septicemia. The two *Mycoplasma* strains used in this experiment were Mmc GM12 (Wild type strain) and Mmc YCP1.1. Each sample was grown in duplicate and colored to observe a color change from red to orange or yellow as a sign for growth.

**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 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 1). 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 perpendicular into the trachea lumen. Once the needle bevel was within the lumen of the trachea, Mycoplasma culture (10⁶ 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 at least 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
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.

### 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 tracheal 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

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**Table 1.** Blood culture results pre and post infection.

<table>
<thead>
<tr>
<th>Mmc strain inoculated in animal</th>
<th>Animal ID</th>
<th>Pre-infection cultures</th>
<th>Post infection cultures (blood)- time point 1 DPI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mmc GM12 parent</td>
<td>CK043</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>CK048</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>CK034</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>CK032</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>CK046</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>CK040</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>CK051</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>CL002</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>CK047</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>CK049</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mmc YCP 1.1 Δ68</td>
<td>CK045</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>CK035</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>CL003</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>CL001</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
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 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.
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


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

Martina C. Agbo¹*, Ifeoma M. Ezeonu², Maurice N. Odo¹, Chukwuebuka M. Ononugbo², Beatrice O. Onodagu³, Chinelo C. Eze¹, Ezinwanne N. Ezeibe¹ and Chizoba A. Ozioko¹

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

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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,
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-lactamas, efflux pumps and target site or outer membrane modifications (Tam et al., 2010).

Generally, extended spectrum β-lactamas (ESBLS) are a group of β-lactamas that hydrolyze penicilllin 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 conjuation. Based on Ambler classification these enzymes are grouped into four groups A to D (Livermore, 2000; Shen and Fang, 2015).

Class B comprises metallo-β-lactamas, which use zinc for the facilit reaction and can be inhibited by ethylenediaminetetraacetic acid (EDTA), whereas classes A, C and D comprise serine β-lactamas, 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, taobactam, and sulbactam. Class A-lactamas 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-lactamas 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-lactamas, 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), ceftoxime 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, UK) were used on Mueller Hinton agar (Oxoid, 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) on Amoxicillin-Clavulanic acid disk prior to incubation. The plate was incubated at 37°C 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-lactamas.

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.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence (51 – 31)</th>
<th>PCR Conditions</th>
<th>Amplicon size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bla GES</td>
<td>GES IF</td>
<td>ATGCGCTTCATTACGGCAG</td>
<td>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 and extension at 72°C for 1 min and final extension at 72°C for 5 min</td>
<td>860</td>
<td>Poirel et al. (2001)</td>
</tr>
<tr>
<td></td>
<td>GES IR</td>
<td>CTATTGGTCCGGTCTAC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bla PER</td>
<td>PER IF</td>
<td>AATTGGGCTTAGGCAGA</td>
<td>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 and extension at 72°C for 45S and final extension at 72°C for 10 min</td>
<td>933</td>
<td>Z21957</td>
</tr>
<tr>
<td></td>
<td>PER 1R</td>
<td>ATGAGTGGCTATACAG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OXA-50</td>
<td>OXA-F</td>
<td>GAAAGGCACCTCGCTGTCTAC</td>
<td>Initial denaturation of 94°C for 5 min; 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</td>
<td>400</td>
<td>Accession No.: Am117128</td>
</tr>
<tr>
<td></td>
<td>OXA-R</td>
<td>CAGAAAGTGGGTCTGTCCAC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TEM</td>
<td>F</td>
<td>GAGACAAATAACCTTGTAAT</td>
<td>Initial denaturation of 94°C for 5 min; 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</td>
<td>459</td>
<td>Parajuli et al. (2016)</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>AGAAAGTGGGTGATTCAT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHV</td>
<td>F</td>
<td>GTCAAGGAAAACACCTCTGCG</td>
<td>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</td>
<td>383</td>
<td>Parajuli et al. (2016)</td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>GTCTATTGCGCCGATAACCC</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>CTX-M</td>
<td>F</td>
<td>GAAGGTATCAAGAAGGTTGGCG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>R</td>
<td>GCATTGCGACCGCTTTCATAG</td>
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</tr>
</tbody>
</table>

Clavulanic acid (CAZ/CAC), and cefotaxime-clavulanic acid (CTX/OEC), (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 milliliter 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 MgCl₂, 22 mM NH₄Cl, 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.

<table>
<thead>
<tr>
<th>Group of antibiotics</th>
<th>Antibiotic</th>
<th>Sensitive (%)</th>
<th>Resistant (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cephalosporin</td>
<td>Cefepime</td>
<td>16 (47.06)</td>
<td>18 (52.94)</td>
</tr>
<tr>
<td></td>
<td>Ceftriaxone</td>
<td>3 (8.82)</td>
<td>31 (91.18)</td>
</tr>
<tr>
<td></td>
<td>Ceftazidine</td>
<td>16 (47.06)</td>
<td>18 (52.94)</td>
</tr>
<tr>
<td></td>
<td>Cefotaxime</td>
<td>-</td>
<td>34 (100)</td>
</tr>
<tr>
<td>Monobactam, Fluoroquinolones,</td>
<td>Aztreonam</td>
<td>6 (17.65)</td>
<td>28 (82.35)</td>
</tr>
<tr>
<td>Aminoglycosides, Lipopeptide,</td>
<td>Ciprofloxacin</td>
<td>18 (52.94)</td>
<td>16 (47.06)</td>
</tr>
<tr>
<td>Carbapenems</td>
<td>Gentamicin</td>
<td>20 (58.82)</td>
<td>14 (41.18)</td>
</tr>
<tr>
<td></td>
<td>Polymyxin B</td>
<td>27 (79.41)</td>
<td>7 (20.59)</td>
</tr>
<tr>
<td></td>
<td>Imipenem</td>
<td>31 (91.18)</td>
<td>3 (8.82)</td>
</tr>
<tr>
<td></td>
<td>Meropenem</td>
<td>29 (85.29)</td>
<td>4 (11.76)</td>
</tr>
<tr>
<td>Penicillin, β-lactam inhibitors</td>
<td>Piperacillin</td>
<td>5 (14.71)</td>
<td>28 (82.35)</td>
</tr>
<tr>
<td></td>
<td>Piperacillin/Tazobactam</td>
<td>18 (52.94)</td>
<td>16 (47.06)</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Resistance patterns</th>
<th>No. of isolate</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEM\textsuperscript{R}, CIP\textsuperscript{R}, PB\textsuperscript{R}, CN\textsuperscript{R}, CEF\textsuperscript{R}, CRO\textsuperscript{R}, CAZ\textsuperscript{R}, CTX\textsuperscript{R}, ATM\textsuperscript{R}, PRL\textsuperscript{R}, TZP\textsuperscript{R}</td>
<td>-</td>
</tr>
<tr>
<td>MEM\textsuperscript{R}, CIP\textsuperscript{R}, CN\textsuperscript{R}, CEF\textsuperscript{R}, CRO\textsuperscript{R}, CAZ\textsuperscript{R}, CTX\textsuperscript{R}, ATM\textsuperscript{R}, PRL\textsuperscript{R}, TZP\textsuperscript{R}</td>
<td>4</td>
</tr>
<tr>
<td>IMP\textsuperscript{R}, CIP\textsuperscript{R}, CN\textsuperscript{R}, CEF\textsuperscript{R}, CAZ\textsuperscript{R}, CTX\textsuperscript{R}, ATM\textsuperscript{R}, TZP\textsuperscript{R}</td>
<td>2</td>
</tr>
<tr>
<td>CRO\textsuperscript{R}, CAZ\textsuperscript{R}, CTX\textsuperscript{R}, ATM\textsuperscript{R}</td>
<td>24</td>
</tr>
<tr>
<td>MEM\textsuperscript{R}, CIP\textsuperscript{R}, PB\textsuperscript{R}, CEF\textsuperscript{R}, CRO\textsuperscript{R}, CAZ\textsuperscript{R}, CTX\textsuperscript{R}, ATM\textsuperscript{R}, PRL\textsuperscript{R}</td>
<td>2</td>
</tr>
<tr>
<td>CRO\textsuperscript{R}, CTX\textsuperscript{R}, ATM\textsuperscript{R}</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>34</td>
</tr>
</tbody>
</table>

CIP\textsuperscript{R} = Ciprofloxacin resistant; CRO\textsuperscript{R} = Ceftriaxone resistant; PB\textsuperscript{R} = Polymyxin-B resistant; CAZ\textsuperscript{R} = Ceftazidime resistant; CN\textsuperscript{R} = Gentamicin resistant; CEF\textsuperscript{R} = Cefotaxime resistant; MEM\textsuperscript{R} = Meropenem resistant; IMP\textsuperscript{R} = Imipenem resistant; ATM\textsuperscript{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 (imipenem 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 cefotaxide, cefotaxime, ceftaxime 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. aeruginosa* isolates by phenotypic test among the isolates that were resistant to ceftazidime, ceftriaxone, cefotaxime and aztreonam.

<table>
<thead>
<tr>
<th>Resistant patterns of isolate</th>
<th>No. of positive isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DDST-AMC</td>
</tr>
<tr>
<td>CRO, CAZ CTX ATM (24)</td>
<td>3</td>
</tr>
<tr>
<td>CRO, CTX, ATM (2)</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td>3</td>
</tr>
</tbody>
</table>

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

**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. ESBL-producing organisms pose unique challenges to clinical microbiologists, clinicians, and infection control agents.
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 (Crăciună 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 (Kavitvoravuthichai 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 oxacillinase 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 while 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.

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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. Berebon¹*, Kenneth C. Ofokansi², Anthony A. Attama², Christopher O. Eze¹, Restus C. Onwusoba¹, Chukwudi T. Ugwu¹, Charity C. Eze¹ and Somtochukwu A. Evurani¹

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

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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 O₄ resulted in the highest inhibition zone diameter, 13 mm against *P. aeruginosa* while isolates OK₄, UK₁, P₂, P₃ and P₄ did not produce any inhibition zones against any of the pathogens tested. Isolate Y₁ 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.

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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, probiotics 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). Probiotics 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.), Okpkeye (Iron plant - Prosopis 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: O1, O2, O3, O4, O5, O6, OK1, OK2, OK3, OK4, P1, P2, P3, P4, UK1, UK2, UK3, UK4, and Y1, Y2, Y3, Y4.

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 37°C. 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 121°C 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 $A_{max}$ 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-motive 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 - Tokati 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 Y4 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 O1 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 - O4 had the widest inhibition zone diameter (IZD) of 13 mm against P. aeruginosa while isolates: OK2, UK1, P2, P3, and P4 did not inhibit any of the pathogens tested. Isolate - Y1 had the broadest activity inhibiting all the pathogens except...
Table 1. Preliminary colony characteristics, catalase test and gram reaction of LAB isolates.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Form (shape)</th>
<th>Color</th>
<th>Elevation</th>
<th>Margin</th>
<th>Surface</th>
<th>Gram reaction</th>
<th>Catalase test</th>
</tr>
</thead>
<tbody>
<tr>
<td>OK&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Circular</td>
<td>Milky</td>
<td>Convex</td>
<td>Entire</td>
<td>Smooth</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>OK&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Circular</td>
<td>Milky</td>
<td>convex</td>
<td>Entire</td>
<td>Smooth</td>
<td>positive</td>
<td>Negative</td>
</tr>
<tr>
<td>OK&lt;sub&gt;3&lt;/sub&gt;</td>
<td>Circular</td>
<td>Milky</td>
<td>convex</td>
<td>Entire</td>
<td>Smooth</td>
<td>positive</td>
<td>Negative</td>
</tr>
<tr>
<td>OK&lt;sub&gt;4&lt;/sub&gt;</td>
<td>Circular</td>
<td>Milky</td>
<td>convex</td>
<td>Entire</td>
<td>Smooth</td>
<td>positive</td>
<td>Negative</td>
</tr>
<tr>
<td>UK&lt;sub&gt;1&lt;/sub&gt;</td>
<td>Circular</td>
<td>White</td>
<td>convex</td>
<td>Entire</td>
<td>Smooth</td>
<td>positive</td>
<td>Negative</td>
</tr>
<tr>
<td>UK&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Circular</td>
<td>White</td>
<td>convex</td>
<td>Entire</td>
<td>Smooth</td>
<td>positive</td>
<td>Negative</td>
</tr>
<tr>
<td>UK&lt;sub&gt;3&lt;/sub&gt;</td>
<td>Circular</td>
<td>White</td>
<td>convex</td>
<td>Entire</td>
<td>Smooth</td>
<td>positive</td>
<td>Negative</td>
</tr>
<tr>
<td>UK&lt;sub&gt;4&lt;/sub&gt;</td>
<td>Circular</td>
<td>White</td>
<td>convex</td>
<td>Entire</td>
<td>Smooth</td>
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<td>P&lt;sub&gt;1&lt;/sub&gt;</td>
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<td>Smooth</td>
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<td>Smooth</td>
<td>positive</td>
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</tr>
<tr>
<td>P&lt;sub&gt;3&lt;/sub&gt;</td>
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<td>Milky</td>
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<td>Negative</td>
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<tr>
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<tr>
<td>Y&lt;sub&gt;1&lt;/sub&gt;</td>
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<td>White</td>
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<td>Smooth</td>
<td>positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Y&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Circular</td>
<td>White</td>
<td>Flat</td>
<td>Entire</td>
<td>Smooth</td>
<td>positive</td>
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<tr>
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<td>Circular</td>
<td>White</td>
<td>Flat</td>
<td>Entire</td>
<td>Smooth</td>
<td>positive</td>
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<tr>
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<td>White</td>
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<td>Smooth</td>
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<td>O&lt;sub&gt;1&lt;/sub&gt;</td>
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<td>Smooth</td>
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<td>O&lt;sub&gt;2&lt;/sub&gt;</td>
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<td>O&lt;sub&gt;3&lt;/sub&gt;</td>
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<td>Entire</td>
<td>Smooth</td>
<td>positive</td>
<td>Negative</td>
</tr>
</tbody>
</table>

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.
S. typhimurium. The cause for antipathogenic activity of isolates O₄ and Y₁ 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 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.
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 probiotics 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.

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http://dx.doi.org/10.1016/j.foodcont.2015.07.029


**Full Length Research Paper**

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

Rita González¹, Manuel Murillo², Hilda Elena Castillo M.¹, Marcelino Jaén³ and Axel Villalobos-Cortés¹

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

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Bovine papillomavirus (BPV) causes benign tumors 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.

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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 (Yaguii 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 Hanson, 2002; Carvalho 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 Chiriquí, 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 crossesbreeds. Each sample collected was immediately stored at -21°C until processing in the laboratory.

DNA extraction was performed using the commercial QuickExtract 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.

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...
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 Virologia, 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 No 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 No 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 (Dorzaciello 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

### 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.

<table>
<thead>
<tr>
<th>Location</th>
<th>DNA sample</th>
<th>Lesion type</th>
<th>Viral type</th>
<th>Similarity (%)</th>
<th>E</th>
<th>GenBank Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>El Ejido</td>
<td>S1</td>
<td>large cauliflower</td>
<td>BPV2/B160620</td>
<td>98.7</td>
<td>0.0</td>
<td>LC426022.1</td>
</tr>
<tr>
<td>El Ejido</td>
<td>S2</td>
<td>small cauliflower</td>
<td>BPV2/B160620</td>
<td>67.6</td>
<td>2e-41</td>
<td>LC426022.1</td>
</tr>
<tr>
<td>Gualaca</td>
<td>S3</td>
<td>bulging round</td>
<td>BPV2/B160620</td>
<td>93.9</td>
<td>3e-100</td>
<td>LC426022.1</td>
</tr>
<tr>
<td>Gualaca</td>
<td>S4</td>
<td>horny round</td>
<td>BPV2/B160620</td>
<td>98.6</td>
<td>0.0</td>
<td>LC426022.1</td>
</tr>
<tr>
<td>Gualaca</td>
<td>S5</td>
<td>bulging round</td>
<td>BPV2/B160620</td>
<td>98.3</td>
<td>0.0</td>
<td>LC426022.1</td>
</tr>
<tr>
<td>Gualaca</td>
<td>S6</td>
<td>bulging round</td>
<td>BPV25/14RS13/BR</td>
<td>98.8</td>
<td>0.0</td>
<td>MG252779.1</td>
</tr>
<tr>
<td>Gualaca</td>
<td>S7</td>
<td>bulging round</td>
<td>BPV25/14RS13/BR</td>
<td>97.6</td>
<td>0.0</td>
<td>MG252779.1</td>
</tr>
<tr>
<td>Gualaca</td>
<td>S8</td>
<td>flat round</td>
<td>BPV2/B160620</td>
<td>98.6</td>
<td>0.0</td>
<td>LC426022.1</td>
</tr>
<tr>
<td>Gualaca</td>
<td>S9</td>
<td>leafy horny</td>
<td>BPV UFPE05BR/BPV11</td>
<td>91.2</td>
<td>2e-165</td>
<td>JQ897976.1</td>
</tr>
<tr>
<td>Gualaca</td>
<td>S10</td>
<td>bulging round</td>
<td>BPV2/B160620</td>
<td>95.8</td>
<td>0.0</td>
<td>LC426022.1</td>
</tr>
<tr>
<td>Gualaca</td>
<td>S11</td>
<td>cauliflower</td>
<td>BPV/ISO 04</td>
<td>99.7</td>
<td>0.0</td>
<td>MF384288.1</td>
</tr>
<tr>
<td>Gualaca</td>
<td>S12</td>
<td>cauliflower</td>
<td>BPV6</td>
<td>99.5</td>
<td>0.0</td>
<td>AB845589.1</td>
</tr>
<tr>
<td>Gualaca</td>
<td>S13</td>
<td>flat</td>
<td>BAA2</td>
<td>79.1</td>
<td>1e-82</td>
<td>AF485376.1</td>
</tr>
<tr>
<td>Gualaca</td>
<td>S14</td>
<td>scaly</td>
<td>BPV2/B160620</td>
<td>98.7</td>
<td>0.0</td>
<td>LC426022.1</td>
</tr>
</tbody>
</table>
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*. 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 *Xpapillomavirus* (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 BPVIS004 (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.

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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 subtropical 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...
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 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}/A_{230} and A_{260}/A_{280} 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 spinnned 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: ATGAAATCGTCGGTGGTTAGG / HRP93: TTTGGGTTTTGGACTTGGG) and GRAV2 (HRP110: GAGGGGTCTGGCGAAACATT / HRP111 CCCTTGTAAAGGAAACCGGAAT), 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 analyzing 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 ranging from 23.1 to 35.7% were observed in 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≥.039, α= 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.

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

*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
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 2. Mean GRD severity scores for the five surveyed Counties in western Kenya.](image)

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

<table>
<thead>
<tr>
<th>Locations/sub-counties</th>
<th>County</th>
<th>Scores ranging from 1 to 5 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Teso South, Butula, Nambale, Samia</td>
<td>Busia</td>
<td>12.6</td>
</tr>
<tr>
<td>Rangwe, Ndhiwa, Rachuonyo</td>
<td>Homa Bay</td>
<td>16.7</td>
</tr>
<tr>
<td>Gem</td>
<td>Siaya</td>
<td>16.3</td>
</tr>
<tr>
<td>Webuye, Sirisia</td>
<td>Bungoma</td>
<td>16.2</td>
</tr>
<tr>
<td>Emuhaya</td>
<td>Vihiga</td>
<td>15</td>
</tr>
<tr>
<td><strong>Mean</strong></td>
<td></td>
<td>15.4</td>
</tr>
</tbody>
</table>
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.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

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REFERENCES


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 (GA₃), 6-benzylaminopurine (BAP) and thidiazuron (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 GA₃ (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 GA₃ (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 GA₃ 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 GA₃ 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 4 to 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...
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 been 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 Majuko 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), Mmlka (3°58'24.36''S, 39°44'00.95''E), and Kinaroni (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 scalp. 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 (GA3) (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) gelrite.

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

Effect of plant growth regulators on shoot induction and elongation

The explants were transferred to shoot induction media containing half 2, 4-D (50-125 µM) auxin concentrations alone (A), and in combination with other PGRs including 0.35 µM GA3 (B), 0.5 µM GA3 (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 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 GA3. All the media was supplemented with 4% (W/V) sucrose, 1 g/l activated charcoal and 0.28% (w/v) gelrite. The cultures were incubated in the culture room under 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 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 GA3 (B), 0.5 µM GA3 (C), 5 µM BAP (D) and 9 µM TDZ (E). The cultures were then kept for 12 weeks in dark at 25±2°C for embryo formation (Antonova, 2009; Vidhanaarachchi et al., 2013).

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 eighth 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 GA3 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 GA3 (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
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 embry 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 GA3 (*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

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 GA3; F, Multiple shoot induction from friable embryogenic calli in media containing 10 μM kinetin, 10 μM BAP, 200 μM NAA and 0.5 μM GA3; 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.
Table 1. Effect of 2,4-D alone and in combination with 0.35 µM GA3, 0.5 µM GA3, 5 µM BAP and 9 µM TDZ on callus induction from embryo explants after 12 weeks.

<table>
<thead>
<tr>
<th>Concentration of 2,4-D (µM)</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0±0c</td>
<td>0±0d</td>
<td>0±0d</td>
<td>0±0d</td>
<td>0±0d</td>
</tr>
<tr>
<td>100</td>
<td>33.3±0.21bcd</td>
<td>16.7±0.17cd</td>
<td>50.0±0.22abc</td>
<td>33.3±0.21bcd</td>
<td>66.7±0.21bcd</td>
</tr>
<tr>
<td>125</td>
<td>16.7±0.17cd</td>
<td>16.7±0.17cd</td>
<td>66.7±0.21abc</td>
<td>50.0±0.22a</td>
<td>33.3±0.21bcd</td>
</tr>
<tr>
<td>150</td>
<td>16.7±0.17cd</td>
<td>16.7±0.17cd</td>
<td>83.3±0.17ab</td>
<td>100a</td>
<td>66.7±0.21bcd</td>
</tr>
<tr>
<td>175</td>
<td>16.7±0.17cd</td>
<td>16.7±0.17cd</td>
<td>33.3±0.21bcd</td>
<td>50.0±0.22a</td>
<td>50.0±0.22a</td>
</tr>
<tr>
<td>200</td>
<td>16.7±0.17cd</td>
<td>50.0±0.22abc</td>
<td>33.3±0.21bcd</td>
<td>33.3±0.21bcd</td>
<td>33.3±0.21bcd</td>
</tr>
<tr>
<td>250</td>
<td>16.7±0.17cd</td>
<td>0d</td>
<td>33.3±0.21bcd</td>
<td>100a</td>
<td>33.3±0.21bcd</td>
</tr>
</tbody>
</table>

% Frequency 16.7±0.63b 19.44±0.67b 16.7±0.17cd 61.11±0.82a 47.22±0.84a

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 GA3); C = (100-250 µM 2,4-D + 0.5 µM GA3); 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 GA3, 0.5µM GA3, 5µM BAP and 9µM TDZ on embryo formation after 8 weeks.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>*A</th>
<th>*B</th>
<th>*C</th>
<th>*D</th>
<th>*E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0±0c</td>
<td>0±0c</td>
<td>0±0c</td>
<td>0±0c</td>
<td>0±0c</td>
</tr>
<tr>
<td>50</td>
<td>16.7±0.17c</td>
<td>16.7±0.17c</td>
<td>50.0±0.22abc</td>
<td>33.3±0.21bcd</td>
<td>50.0±0.22abcd</td>
</tr>
<tr>
<td>62.5</td>
<td>16.7±0.17c</td>
<td>16.7±0.17c</td>
<td>50.0±0.22abc</td>
<td>50.0±0.22abcd</td>
<td>33.3±0.21bcd</td>
</tr>
<tr>
<td>75</td>
<td>16.7±0.17c</td>
<td>16.7±0.17c</td>
<td>83.3±0.17ab</td>
<td>83.3±0.17ab</td>
<td>33.3±0.21bcd</td>
</tr>
<tr>
<td>87.5</td>
<td>16.7±0.17c</td>
<td>16.7±0.17c</td>
<td>33.3±0.21bc</td>
<td>50.0±0.22ab</td>
<td>33.3±0.21bc</td>
</tr>
<tr>
<td>100</td>
<td>16.7±0.17c</td>
<td>33.3±0.21abc</td>
<td>33.3±0.21abc</td>
<td>100a</td>
<td>33.3±0.21abc</td>
</tr>
<tr>
<td>125</td>
<td>16.7±0.17c</td>
<td>0c</td>
<td>33.3±0.21abc</td>
<td>100a</td>
<td>16.7±0.17c</td>
</tr>
</tbody>
</table>

% Frequency 13.89±0.59g 16.7±0.63c 47.22±0.84ab 58.33±0.83a 30.56±0.78bc

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 GA3); *C = (50-125 µM 2,4-D + 0.5 µM GA3); *D = (50-125 µM 2,4-D + 0.5 µM BAP) and *E = (50-125 µM 2,4-D + 9 µM TDZ), e.g. 50A will be Y3 media supplemented with 50 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).

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 GA3. The shoots were also white 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 GA3 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 GA3 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 GA3 (Figure 1H). The explant
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 3. Shoot elongation of coconut on Y3 medium supplemented with BAP and GA3, after 8 weeks.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>5 µM BAP</th>
<th>10 µM BAP</th>
<th>15 µM BAP</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shoot height (cm)</td>
<td>1.178±0.19^a</td>
<td>1.667±0.22^a</td>
<td>0.811±0.19^c</td>
<td>0.1±0.03^c</td>
</tr>
<tr>
<td>Number of leaves</td>
<td>2.67±0.33^a</td>
<td>2.67±0.33^a</td>
<td>2.67±0.33^a</td>
<td>0^b</td>
</tr>
</tbody>
</table>

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).
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 Verdei, 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 (Eeuwens, 1976) supplemented with 2,4D alone, GA3 BAP which were compact and TDZ which formed friable calli. The explants in medium

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**Table 4. Effect of IBA in combination with 0.5µM GA3 on root induction after 8 weeks.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>IBA (µm)</th>
<th>5</th>
<th>10</th>
<th>15</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Root length height (cm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.10±1.99&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.83±0.72&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>3.27±0.43&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Number of roots</td>
<td></td>
<td>8.33±2.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.33±0.88&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>7.33±2.18&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0c</td>
</tr>
</tbody>
</table>

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).**

<table>
<thead>
<tr>
<th>Media</th>
<th>Media components</th>
<th>Shoot height (cm)</th>
<th>Survival rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Soil: Sand: Manure (3:1:1)</td>
<td>9.20±1.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25</td>
</tr>
<tr>
<td>B</td>
<td>Vermiculate</td>
<td>7.63±0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8</td>
</tr>
<tr>
<td>C</td>
<td>Control</td>
<td>0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0</td>
</tr>
</tbody>
</table>

Means (± SE) followed by the same alphabets in each column were not significantly different at P≤0.05 using Fisher’s test.
supplemented with various levels of 2,4D and 0.5 \( \mu M \) 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. 

\( \text{G}A_3 \) was added to the culture medium to promote the germination of somatic embryos. However, the effect of \( \text{G}A_3 \) has not been tested on the formation of somatic embryos (Perera et al., 2009).

In this study, the addition of 0.5 \( \mu M \) \( \text{G}A_3 \) proved to be better in the induction of both callus (50%) and embryogenic calli (47.2%) as compared to 0.35 \( \mu M \) \( \text{G}A_3 \) which resulted in callus induction frequency of 16.7% and embryogenic calli frequency of 13.9%. Higher callus induction in \( \text{G}A_3 \) 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 \( \mu M \) 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 \( \mu M \) 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 \( \text{I}B\text{A} \) and \( \text{G}A_3 \). The use of a high concentration of \( \text{I}B\text{A} \) 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 \( \mu M \) \( \text{I}B\text{A} \) induced the highest rooting as compared to 15 \( \mu M \) \( \text{I}B\text{A} \) though not significantly different. No rooting was observed in the treatment without the hormones meaning that the \( \text{I}B\text{A} \) 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 (Vidhanaraarachchi et al., 2013). \( \text{G}A_3 \) 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 \( \text{I}B\text{A} \) 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 also 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 propagation. 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 GA3, 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 kininin, 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


