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Isolation and Identification of Bacteria Associated with Aerial Part of Rice Plant from Kware Lake

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Authors' contributions

This work was carried out in collaboration between all authors. Author AA designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors MTM and JS managed the literature searches, while authors MTM and RMS managed the analysis of the study. All authors read and approved the final manuscript.

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ABSTRACT

A study was conducted to isolate, identify and establish the Pathogenecity test of bacteria associated with diseases affecting the aerial part of rice in kware lake. Bacteria was isolated from the infected parts collected from Kware Lake and identified by microscopy and biochemical tests. The isolated bacterial were *Staphylococcus aureus*, *Xanthomonas oryzae*, *Staphylococcus intermedius*, *Clostridium noryi*, *Pseudomonas putida*, *Corynoebacterium xerosis,* and *Bacillus macerans*. However, *Staphylococcus aureus*, occurred more frequently with 32.93%, followed by *Xanthomonas oryzae* 27.44*%, Corynoebacterium xerosis 20.73%, then Staphylococcus intermedius* 14.63%, *Baccilus macerans* 2.44%, *Clostridium noryi* 1.22% and *Pseudomonas putida* with 0.61%. The biochemical reactions include: citrate test, spore test, indole test, starch hydrolysis, urease test, catalyse test, gas formation, glucose, sucrose and lactose test, motility test, H_2S production and gram reactions, but only two of the identified bacteria caused infection to the plant after undergoing pathogenecity test. They are therefore recommended for the possible infection of the aerial part of rice caused by these pathogenic bacteria.

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Keywords: Kware lake; Xanthomonas oryzae; Corynoebacterium xerosis; biochemical reactions; rice; pathogenecity.

1. INTRODUCTION

Rice (*Oryzae sativa* L.) belongs to the kingdom plantae in the division of Magnoliophyta, class Liliopsida, order poales and family poaceae in the genus oryzea. Rice refers to two species *(Oryzae sativa and O. glaberrima)* of grass, native to tropical and sub-tropical Southern and southeastern Asia and to Africa [1]. It is an annual plant growing up to 1.8 m tall occasionally with long slender leaves 50 -100 cm long and 2-2.5 cm broad. The small wind pollinated flowers are produced in a branch arching to pendulous inflorescence 30-50 cm long. The seed is a grain (caryopsis) 5-12 mm long and 2-3 mm thick [2].

O. sativa is an Asian type while *O. glaberrima* is of West African type. The two varieties can be easily distinguished by their ligules. Also in *O*. *glaberrima* the grains are axe-shaped while in *O. sativa* they are pear shaped. *O. sativa* is further classified geographically in to wetland and upland types [1]. The upland is essentially a dry land crop depending on rainfall. There are many ways to distinguish rice varieties. The most common one is based on the length of the grain. The main groups of varieties are thus, long-grained and short-grained varieties. The short-grained varieties are more adapted to colder climates and are, therefore, commonly grown in Japan and Korea and in Europe. The long-grained varieties are common to warmer climates. Some experts further classify varieties based on the ratio between their length and their width [3].

Rice is one of the most important tropical crops and forms the source of food for nearly half of the world's human population. There are many varieties of rice and they differ in height in the amount of time they take to mature and their water requirement [4]. In various parts of its ranges, rice is grown in different ways but most of the rice in Southeast Asia is grown in unusual condition for a cereal plant. It is grown partly in submerged in water in paddy fields. The fields are flooded and then ploughed. Young rice is planted in the rich mud formed in these paddy fields. The oxygen concentration of this mud fails rapidly after the paddy field has been flooded. The top ten centimetres or so retain some oxygen because it is able to diffuse in but below this depth anaerobic condition exist and there is little or no oxygen present [4].

The main diseases in rice are rice blast and sheath blight. However, a large number of other microbes (fungi, bacteria, nematodes and virus) occur in the rice field which could from time to time cause problems locally. Disease management largely depends on variety selection and good management. A good fertiliser management is important [5].

Rice is one of the most important tropical crops and forms the source of food for nearly half of the world's human population and with the increasing concern about food security, there is need to identify diseases affecting the rice plant and alternative way to overcome the menace using botanical control measures [3]. There are many serious plant diseases of rice, including the ascomycete fungus *Pyricularia grisea* which causes the disease known as rice blast [5]. *Pyricularia grisea* can infect most sections of the plant, but infections of the node or the panicle are the most damaging phases of the disease [6]. Some pesticides have lost their effectiveness because of the development of resistant pathogens [7]. Diseases are considered major constraint in rice production [4]. Rice diseases are mainly caused by fungi, bacteria or viruses [4].

Isolation and identification of the disease causal pathogen lead to protection and management strategies which will increase yield. Sheath diseases of rice caused by *Xanthomonas oryzae, Corynoebacterium xerosis, Rhizoctonia solani*, *R. oryzae*, *R. oryzae-sativae* and *Sclerotium hygrophilum* are important phytopathogens distributed worldwide and cause yield losses in rice growing countries [8]. However, in Nigeria, there has been relatively little or no investment in identification of the disease affecting rice plant and development of commercial products for botanical control measures [9].

There are vast needs for identifying some of the bacterial pathogens affecting the rice plant in Kware Lake, Sokoto state aimed at isolating and identifying these diseases which cause great losses to the farmers.

2. MATERIALS AND METHODS

2.1 Study Area

The study area is a naturally occurring fresh water lake at Kware, Kware Local Government

Area of Sokoto State and some 20 km north west of Sokoto Town. It is located on longitude 5^0 14^1 E and latitude 13^0 17^1 N. The lake is also linked with River Rima.

The vegetation in Kware town is the Sudan Savannah type with an annual rainfall of about 550- 700mm which peaks in August. Dry season sets in first with cold harmattan wind from October to March and a hot period from April to the end of May when temperature reaches 38°C during the day. Agricultural activity and fishing activities takes place around the lake all year round [10].

2.2 Sample Collection

The samples were collected from Kware Lake in Kware Local Government Area of Sokoto state. Two farms were visited and the samples were collected from each of the farms. The debris was removed from the surface of the leaves. The cut (3x3mm) leaves were sterilised by dipping in absolute alcohol for 2 minutes and rinsed in several changes of sterile distilled water prior to utilisation.

2.3 Sterilisation of Glassware

All the glassware used for this study was sterilised properly. The glassware includes Petri dishes, glass funnel, conical flask, glass slides. They were firstly washed with detergent and rinsed thoroughly with tap water, then with distilled water. They were air dried and sterilised in an oven at 180°C for 45 mins while the sterilisation of conical flask was done by autoclaving [11].

2.4 Preparation of the Media

The nutrient agar medium was prepared according to manufacturer's instructions (28) grams of the media was weighed and dissolved in 1000ml of distilled water. The conical flask containing the media was then plugged with cotton wool and wrapped with aluminium foil. It was then sterilised using steam under pressure of autoclaving at 121°C for 15 minutes). The media was allowed to cool down to 45°C before dispensing in to the sterile plates. The plates were left at room temperature (28°C.) to solidify.

2.5 Preparation of Broth Media

The nutrient broth was weighed on a balance and suspended in a litre of distilled water. The prepared broth media was poured into conical flasks, plugged with cotton wool and capped with aluminum foil. They were autoclaved at 121^0C for 15 minutes. The prepared media were divided into portions of 250ml in the conical flask. After the sterilisation, a portion of the stock cultures were directly inoculated into the conical flask and incubated at temperature of 37^0C for bacterial isolates.

2.6 Inoculation and Incubation

The infected foliar part of rice was surface disinfected with 70% alcohol before weighing. One gram of the infected parts was ground in a sterile mortar with pestle in to 10ml of sterile distilled water. One milliliter of the suspension was obtained using pipette and serially diluted to obtain 10^{-5}, 10^{-6} and 10^{-7} dilutions. These dilutions were singly inoculated in to the fresh nutrient agar plates and the Petri dishes were labeled appropriately. The inoculated Petri dishes were incubated at 37^0C for 48 hours.

2.7 Identification

The pure isolates were subjected to microscopic examination with the view in identifying the organism. The plates were observed under binocular microscope for bacterial scrutiny. The bacterial colonies were first subjected to gram staining prior to microscopic examination. The identification of the isolates was carried out based on their cultural characteristics [11]. The glass slides were made clean with a cotton wool soaked in an alcohol. The inoculation needle was burnt until red hot. A drop of distilled water was placed on the centre of a clean glass slide. The inoculating needle was used to transfer the portion of the bacterial colonies and teased on the glass slide. The slide was covered with a cover slip and mounted on a microscope and examined. The type of the fruiting bodies and spore structures are the basis for which the identification of the isolate was done. However, with the aid of gram staining whereby cultural characteristic of the isolate and biochemical test serve as the basis for the identification [11].

2.8 Biochemical Characterisation

This was done with a view to identify bacteria to species level, according to the procedure of Mansur et al. [10].

2.9 Indole Test

This was done in the determination of the ability of the bacteria to produce indole from tryptophan.

Indole production is detected by kovac's reagent (4 dimethyl amino benzaldehyde). The reaction of the reagent with indole produces a red colored compound. The isolate was grown for 48hrs in test tube containing 5ml peptone water. 0.5ml kovac's reagent was added and shaken gently. The presence of red layer signifies the presence of indole. When there is no indole present, the color remains yellow. This signifies negative results.

2.10 Triple Sugar Iron Agar Test

This medium contains three sugars; glucose, sucrose and galactose. Some organisms can ferment all the three sugars present and produce acid with changes of color of the 3 sugars from red to yellow. The sugars and proteins are attached oxidatively to release ammonia. Through this media, the production of H_2S can be detected by presence of black color in the media along the stabbed line. Mortality can be detected by presence of growth along the area being stabbed by the sterile wire loop. Gas production was detected by the presence of gas bubbles or crack on the agar in the test tube.

Colonies from the sub cultured plates were picked with a sterile straight wire loop and stab on the butt, streaked on the surface of the slope. This was incubated at 37°C for 24 hrs.

2.11 Urease Test

The test was used to detect the organism's ability to produce the enzymes urease. When the strain was urease producing, the enzyme breaks down urea by hydrolysis to produce ammonia and carbon dioxide. Ammonia produce changes in the pale yellow color of urea to pink red. This was carried out in a sterile slant bottle and incubated for 24 hrs at 37°C.

2.12 Citrate Test

The test was base on the ability of an organism to use citrate as its source of carbon. Some citrate agar was inoculated with the isolate and incubated at 37°C for 48 hrs. The presence of bright blue color indicate citrate positive. While when there is no change of color of the media, it indicates citrate negative.

2.13 Methyl Red and Voge's Proskaur Test

It was used in differentiating bacteria that ferment glucose with production of acetyl methyl cevibinol (acetone). The media contains peptone salt and glucose. Colonies from the stock cultures was inoculated in to methyl red medium and incubated at 37°C for 48 hrs. Two drops of methyl red solution was added and shaken. The presence of red color indicate positive for MR, while a yellow color indicate negative for MR.

Results obtained from the biochemical test were tabulated and compared with the standard bergey's table, in order to identify a particular organism. The 1-day old bacteria culture under incubation at 37°C was subsequently were used in the evaluation of the in vitro in this research.

2.14 Starch Hydrolysis Test

This test shows whether an isolate was capable of utilising starch or not. Each isolate was inoculated in to the starch hydrolysis medium and incubated for 24 hrs. Iodine solution was added to each of the resultant growth and the blue black color observed. Development of blue black color signify the presence of starch (isolate could not utilise it), while absence of starch of starch was indicated by the failure to develop blue black coloration (positive value).

2.15 Catalase Test

This was used to differentiate the bacteria that produce the enzyme Catalase, from non Catalase producing bacteria. Catalase acts as catalyst in breaking down of hydrogen peroxide to oxygen and water. A drop of hydrogen peroxide was placed on a clean glass slide, colonies from the nutrient slant was fetched and emulsified in the H_2O_2 drop and observed immediately for gas bubbles. The presence of active bubbles indicate Catalase positive, while negative shows no bubbles.

2.16 The Frequency of Occurrence of the Isolated Bacteria

The frequency of occurrence of the bacteria associated with the diseases of foliar part of rice was determined. The number of times each of the microbes was encountered was recorded. The frequency of occurrence was calculated using the formula below:-

$$\frac{\text{Number of times a bacterium was encountered}}{\text{Total number of bacterial isolations}} \times 100$$

2.17 Pathogenecity Test

Healthy and fleshy leaves of rice were smeared with the identified organisms appropriately. Stock culture of the selected organisms was serially diluted to serve as the size of inocula used. 10^{-3} dilution was used for the isolates. Sterile camel hair-brushes were used in smearing the isolates on the leaves. All the inoculated leaves were incubated at 37^0C in the incubator. They were observed daily for symptoms caused by the organisms according to the procedure of Mansur et al. [10]. Re-isolation procedure was again carried out to verify the authenticity of the pathogenic activity.

3. RESULTS AND DISCUSSION

3.1 Isolated Bacteria

The bacteria isolated and identified from foliar part of rice were *Staphylococcus aureus*, *Xanthomonas oryzae*, *Staphylococcus intermedius*, *Clostridium noryi*, *Pseudomonas putida*, *Corynoebacterium xerosis,* and *Bacillus macerans*. Cottyn et al. [12], Oh et al. [13], Hong et al. [14] isolated genera which are, *Pantoea* and *Pseudomonas* in stored rice. However, Cottyn et al. [12], Oh et al. [13,15], Hong et al. [14] also isolated *Lactococcus*, *Lactobacillus*, *Leuconostoc*, and *Clostridium,* this genera specifically present or enriched in stored rice, have not been previously identified in any studies associated with rice. Also, Bacteria belonging to the genera *Bacillus, Pectobacterium, Pantoea, Microbacterium, Sphingomonas,* and *Methylobacterium* have been isolated in studies of stored rice in Korea which used culture-based methods such as Biolog and fattty acid methyl ester (FAME) analyses by Oh et al. [13,15] for identification. This result may be due to different environment and different method used during identification. Furthermore, a couple of studies which were conducted using raw rice-straw and freshly harvested rice grains, respectively, have found the following genera to be present: *Pantoea, Bacillus, Microbacterium, Enterococcus, Pseudomonas, Rhodococcus Enterobacter, Xanthomonas, Cellulomonas, Clavibacter, Burkholderia,* and *Paenibacillus* by Hong et al. [14], Cottyn et al. [12]. However, *Staphylococcus aureus*, occurred more frequently with 32.93%, followed by *Xanthomonas oryzae* 27.44%, *Corynoebacterium xerosis 20.73%, then Staphylococcus intermedius* 14.63%, *Bacillus macerans* 2.44%, *Clostridium noryi* 1.22% and *Pseudomonas putida* with 0.61% (Table 1).

The biochemical reactions include: citrate test, spore test, indole test, starch hydrolysis, urease test, catalyse test, gas formation, glucose, sucrose and lactose test, motility test, H_2S production and gram reactions (Table 2). This result is the same with that of Matsumoto [16] who found *Xanthomonas oryzae, Corynobacterium xerosis Rhizoctonia solani*, *Rhizoctonia oryzae*, *Rhizoctonia oryzae-sativae* and *Sclerotium hydrophilum* and believed is caused by sheath diseases of rice and are important phytopathogens distributed worldwide and cause yield losses in rice growing countries.

Table 1. Frequency of occurrence of bacteria from foliar part of rice

Identified bacteria	Gram reaction	Shape of cell	Number of time isolated	%Frequency
S. aureus	+ve	Spherical in shape and are in chains but long chains	54	32.93%
X. oryzae	-ve	Rod like in shape and some are in chains but not too long chains	45	27.44%
S. intermedius	+ve	Spherical in shape some are in chain, also some are in long and short chain	24	14.63%
C. noryi	+ve	Rod like shape, some are in chains, also some are in long and short chains	2	1.22%
P. putida	-ve	Rod like shape, but long and short chains	1	0.61%
C. xerosis	+ve	Rod like shape, many are in chains but some have longer chains	34	20.73%
B. Macerans	+ve	Rod like in shape, many are in chains and are very long	4	2.44%

Table 2. Biochemical characterisations of the isolated bacteria

s/no	Gram reaction	MR	VP	H_2S Production	Motility Test	Glucose	Sucrose	Lactose	Gas Formation	Catalase test	Urase test	Starch Hydrolysis	Indole test	Spore test	Citrate test	Identified organisms
1	+ve	-	+	+	-	+	+	-	-	+	+	+	+	-	-	*Staphylococcus aureus*
2	-ve	+	-	-	+	+	+	-	-	-	+	+	-	-	-	*Xanthomonas oryzae*
3	+ve	+	-	+	-	+	+	-	-	+	+	+	+	-	-	*Staphylococcus intermedius*
4	+ve	+	-	+	+	+	-	-	-	-	-	+	-	+	-	*Clostridium noryi*
5	-ve	+	-	-	+	+	+	-	-	-	-	+	-	-	-	*Pseudomonas putida*
6	+ve	+	-	-	-	+	+	-	-	+	-	-	-	-	+	*Corynebacterium Xerosis*
7	+ve	-	+	+	+	+	+	-	+	+	-	+	-	+	+	*Bacillus macerans*

3.2 Pathogenecity of the Isolated Bacteria

The Pathogenecity test showed that *Xanthomonas oryzae* and *Corynoebacterium xerosis* showed considerable tissue maceration within 2-3days. These bacterial are therefore toxic to the areal part of the plant. *Clostridium noryi* is also another species of bacteria that is pathogenic to areal part of the plant after pathogenecity test was carried out. This result corresponds with research conducted by Tare, 2014 [17] who observed that *Clostridium* was the only genus associated with stored rice whose species are known to be pathogenic, some producing extremely harmful neurotoxins. However, *Staphylococcus aureus, S. intermedius* were not found to be pathogenic on the rice leaves after swabbing for 2-5days.

4. CONCLUSION

Based on the study, it is concluded that a number of bacteria affect the foliar part of rice. These include, *Staphylococcus aureus, Xanthomonas oryzae, Staphylococcus intermedius, Clostridium noryi*, *Pseudomonas putida, Corynoebacterium xerosis,* and *Bacillus macerans,* but only two of the identified bacteria caused infection to the plant.

Based on the findings of this research, it is recommended that:-

1. The research can be further exploited for formulating integrated disease management schedules for pathogens of rice (foliar part of rice) and other plants at large.
2. Plants extract should be prepared in order to manage these pathogens.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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