IOSR Journal Of Pharmacy And Biological Sciences (IOSR-JPBS)
e-ISSN:2278-3008, p-ISSN:2319-7676. Volume 15, Issue 6 Ser. I (Nov. –Dec. 2020), PP 36-42
www.Iosrjournals.Org

Isolation, Identification and characterization of phyllosphere Bacteria from vegetables

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Abstract: *Diverse group of microorganism colonize phyllopshere and carry out different but specific ecological functions. The phyllosphere of five different vegetables plants; Common Okra)AbelmoschusesiculentusL., (Fluted Pumpkin) Telfariaoccidentalis, (African Spinach)Amaranthuscruentus ,(Jew's Mallow)Corchorusolitorius and (Lagos Spinach) Celosia argentea}, each from two different locations, (Student Union Building and the Fadama of Federal University of Agriculture, Abeokuta, Ogun State) were examined Microbiologically for bacterial growth using culture-dependent techniques. A total of 43 bacterial covering 11 genera were isolated and characterized as Citrobacter, Staphylococcus, Pseudomonas , Streptococcus, Salmonella, Proteus, Shigella, Escherichia, Enterobacter, Klebsiella, and Bacillus*.

Key words: *epiphyte, locations, pathogens, plant types.*

Date of Submission: 08-11-2020 Date of Acceptance: 22-11-2020

I. Introduction

Bacteria are considered to be the dominant microbial inhabitants of the phyllosphere, although archaea, filamentous fungi and yeasts may also be important. These microbes can be found both as epiphytes on the plant surface and as endophytes within plant tissues (Arnold et al. 2000; Inacio et al., 2002; Lindow and Brandl 2003; Stapleton and Simmons 2006). There are three major microhabitats where microorganism can inhabit depending on the one that most support their growth with reference to plants (Montesino, 2003). These microhabitats are the aerial plant part (the phyllosphere), the zone of influence of root system (the rhizosphere) and the internal transport system (the endosphere). Microorganisms which inhabit such microhabitat are called epiphytes, rhizophytes and endophytes respectively (Montesino, 2003). Phyllosphere therefore is a microhabitat on the surface of plant's leaf where diverse group of microorganisms colonize and carry out their different and specific ecological function. The diversity of the microbial composition of phyllosphere includes algae, bacteria, filamentous fungi, yeast and in rare cases nematodes and protozoans (Morris et al., 2002; Lindow and Brandl, 2003). The majority of epiphytic bacteria are commensal. Some provide specific ecosystem services such as phytoremediation of toxic pollutants (Ali et al., 2012) and biogeochemical cycling of important elements (Feurnkranz et al., 2008).

II. Materials and Methods:

The vegetable leaves used in this study were harvested from the mature plants on the point of collection. All the leaves were collected from two different locations, which are FADAMA, Federal University of Agriculture, Abeokuta, (FUNAAB) and Student Union Building (SUB), FUNAAB, Abeokuta. The leaf samples were put separately into sterile bags and transported to Microbiology Laboratory, Federal University of Agriculture, Abeokuta (FUNAAB**)**.

The plant used was identified by DR. AKINTOKUN from the Department of plant science and seed technology, Federal University of Agriculture, Abeokuta (FUNAAB**)**, Nigeria. The experiment was carried out at the Microbiology Laboratory, College of Bio-science, Federal University of Agriculture, Abeokuta (FUNAAB), Ogun State, NIGERIA. The university is situated within the south western part of Nigeria and located on latitude 7° 10° N, longitude 3°2°E and in altitude of 76m above sea level (Google earth, 2014).

Isolation of bacteria

Twenty discs each of 10 mm in diameter was cut aseptically from each of the leaf categories using a 10 mm cork borer. Each leaf category was put in a 10 ml sterile distilled water and hand shaken for 20 minutes. Serial dilution up to the second diluent was done using 1 ml from the stock culture. This was repeated for other leaves categories, each time shaking for uniform distribution of the cells (conidia). One mililitre of the aliquots from 10^{-2} diluent of each leaf sample, was transferred to sterile Petri plates, pour plate method was employed. Two replicates for each dilution were made for each of bacteria. Nutrient agar for was used for the isolation of

bacteria. Plates were incubated at 37°C for 24 hours in inverted position. Colony forming units per millilitres (cfu/ml) were counted as described by Mukhtar*et al.,* (2010). The medium (Nutrient agar) used was weighed following the manufacturer's specification and dispensed into separate clean conical flask; the distilled water was poured into the conical flasks and allowed to dissolve. The conical flasks were corked immediately and transferred to the autoclave for sterilization at 121^{o}c for 15 minutes. Serial dilution was done, thus, Ten mls of distilled water was measured and dispensed after sterilization into the first test tube which was the stock solution and a tenfold serial dilutions was done .1ml from the stock solution was pipetted aseptically into the test tube labeled 10^{-1} and mixed. 1ml from 10^{-1} was then transferred to the next test tube (10^{-2}) and mixed and repeated up to the last test tube (10^{-2}). This was done for the ten vegetable leaves samples. Then 1ml each of the diluents 10^{-2} was inoculated on the plates followed by the agar and gently rotated. It was then allowed to cool and gel. Afterwards, the plates were inverted for the vapour to leave. The Nutrient agar plates were incubated at 37^{o}C for 24hours

Microbial Count

Microbial count was carried out to determine the microbial concentration in a given sample from each sample and to also compare the amount of growth of microorganisms under various conditions (Onyeagba, 2004). This was done by counting the growth colonies based on their physiological characteristics and their number on each plate taking note of the serial diluted tube number. Counts were made from plates that contain between 30-300 colonies. For pure culture, distinct colonies were cultured on newly prepared sterile Nutrient agar for bacteria by streaking, using sterile wire loop and incubated at 37^{o}C for 24hours.

III. Results

Bacterial Isolates From the Phyllosphere Samples

Culture–dependent techniques were employed to study the diversity of microbial communities on the phyllopshere of five selected plants: okra, pumpkin, African spinach, Lagos spinach and Jew's mallow. A total of 43 bacterial isolates covering eleven genera were obtained and characterized as *Staphylococcus, Pseudomonas, Escherichia Coli, Bacillus, Salmonella, Citrobacter, Streptococcus, Proteus, Enterobacter, Shigella*and*Klebsiella.*

A total of number of 21 bacterial isolates were isolated and identified from the first location for this research purpose the student union building(SUB). Isolation from the phyllosphere of Pumpkin had the highest number of bacterial isolates of 8 (Table 1), having a percentage of 38.1%, (Table 1), while Lagos spinach and Jew's mallow had the lowest frequency of 1 (Table 1), with percentage of 4.8% each, (Table 1).

*Staphylococcus aureus*had the highest frequency of 5 (Table 1) representing 23.8% (Table 1) of the total number bacterial isolated from the (SUB), while *Citrobacterspp, Enterobacterspp, Pseudomonas fluorescens, Proteus mirabilis, E. coli, Klebsiellaoxytoca, and Streptococcus spp* all had *a* frequency of 1, (Table 1), representing 4.83% (Table 1) of the bacterial populations isolated from (SUB).

A total number of 22 bacterial isolates were isolated and identified from FADAMA, the second location for this research purpose. Isolation from the phyllopshere of Pumpkin had the highest number of 7 bacteria isolates (Table 1) representing a percentage of 31.8% (Table) while Lagos spinach had lowest of 2 isolates (Tables 1) representing 9.1% (Table 1). *Staphylococcus aureus*had the highest frequency of 5 (Table1), representing 22.7% (Table 1) while *Citrobacterfreundii, Shigellaspp*and *Enterobacter species* had the lowest frequency of 1 isolate each (Table 1) representing 4.6% (Table 1). More bacteria isolates 15 (34%) were obtained from the phyllosphere of pumpkin from both sites. There was an even distribution of bacteria isolated from the phyllosphereof pumpkin and African Spinach, this contributed to them having the highest frequency of bacteria isolates, unlike the remaining samples plants. *Staphylococcus aureus*was found to be predominant among the isolates while *Proteus spp*and *Streptococcus spp*had the least frequency of occurrence among the bacteria isolates. It was observed that Gram Negative bacteria (62.8%) predominated over Gram positive (37.2%).

TABLE 1: Morphological and Biochemical Characteristics of Bacteria Isolated From Five Vegetables Samples for the Student Union Building (SUB) and FADAMA.

Isolate code	Gram	Motility	Glucose	Lactose	Mannitol	Maltose	Indole	Methyl Red	Vogesproskauer	Citrate	H_2S	Sucrose	Urea	Oxidase	Coagulase	Catalase	Probable Identity
AS1	GPC	-	+	+	+	-	-	+	-	+	-	+	+	-	+	+	*Staphylococcus aureus*
AS2	GNB	+	+	-	+	+	-	+	-	+	+	-	-	-	NA	+	*Salmonella specie*
AS3	GNC	-	+	-	+	+	-	+	-	-	-	-	-	-	NA	+	*Shigella sp.*
AS4	GPB	+	+	+	+	-	-	-	+	+	-	+	-	-	NA	+	*Bacillus mycoides*
AS5	GNB	+	+	-	+	+	-	-	-	+	-	-	+	+	NA	+	*Pseudomonas aeruginosa*
AS6	GNB	+	+	+	+	+	+	+	-	-	-	NA	-	-	NA	+	*Escherichia coli*
OS1	GPC	-	+	+	+	-	-	+	-	+	-	+	+	-	+	+	*Staphylococcus aureus*
OS2	GNB	+	+	-	+	+	-	+	-	+	+	-	-	-	NA	+	*Salmonella specie*
OS3	GNB	-	+	+	+	+	-	-	+	+	-	+	-	-	-	+	*Klebsiellaoxytoca*
OS4	GNB	+	+	+	+	+	-	+	-	+	+	-	-	-	-	+	*Citrobacterfreundii*
OS5	GPC	+	+	+	+	-	NA	+	-	+	-	+	+	-	-	-	*Streptococcus specie*
LS1	GPC	-	+	+	+	-	-	+	-	+	-	+	+	-	+	+	*Staphylococcus aureus*
JS1	GPC	-	+	+	+	-	-	+	-	+	-	+	+	-	+	+	*Staphylococcus aureus*
PS1	GPC	-	+	+	+	-	-	+	-	+	-	+	+	-	+	+	*Staphylococcus aureus*
PS2	GNB	+	+	-	+	+	-	+	-	+	+	-	-	-	NA	+	*Salmonella specie*
PS3	GNC	-	+	-	+	+	-	+	-	-	-	-	-	-	NA	+	*Shigella sp.*
PS4	GPB	+	+	+	+	-	-	-	+	+	-	+	-	-	NA	+	*Bacillus mycoides*
PS5	GNB	+	+	-	+	+	-	-	-	+	-	-	+	+	NA	+	*Pseudomonas aeruginosa*
PS6	GNB	+	+	+	+	+	-	-	+	+	-	+	-	-	NA	+	*Enterobacter sp.*
PS7	GNB	+	+	-	-	+	-	+	-	+	+	+	+	-	NA	+	*Proteus mirabilis*
PS8	GNB	+	+	-	+	+	-	+	+	+	+	+	+	+	NA	+	*Pseudomonas fluorescens*
AF1	GPC	-	+	+	+	-	-	+	-	+	-	+	+	-	+	+	*Staphylococcus aureus*
AF2	GNB	+	+	+	+	+	+	+	-	-	-	NA	-	-	NA	+	*Escherichia coli*
AF3	GPB	+	+	+	+	-	-	-	+	+	-	+	-	-	NA	+	*Bacillus mycoides*
AF4	GNB	+	+	-	+	+	-	+	-	+	+	-	-	-	NA	+	*Salmonella specie*
AF5	GNB	+	+	-	+	+	-	-	-	+	-	-	+	+	NA	+	*Pseudomonas aeruginosa*
AF6	GNB	+	+	-	+	+	-	+	+	+	+	+	+	+	NA	+	*Pseudomonas fluorescens*
OF1	GPC	-	+	+	+	-	-	+	-	+	-	+	+	-	+	+	*Staphylococcus aureus*
OF2	GPB	+	+	+	+	-	-	-	+	+	-	+	-	-	NA	+	*Bacillus mycoides*
OF3	GNB	+	+	+	+	+	-	-	+	+	-	+	-	-	NA	+	*Enterobacter sp.*
OF4	GNB	+	+	+	+	+	-	+	-	+	+	-	-	-	-	+	*Citrobacterfreundii*

LF1	GPC	-	+	+	+	-	-	+	-	+	-	+	+	-	+	+	*Staphylococcus aureus*
LF2	GNC	-	+	-	+	+	-	+	-	-	-	-	-	-	NA	+	*Shigella sp.*
JF1	GPC	-	+	+	+	-	-	+	-	+	-	+	+	-	+	+	*Staphylococcus aureus*
JF2	GNB	+	+	-	+	+	-	+	+	+	+	+	+	+	NA	+	*Pseudomonas fluorescens*
JF3	GNB	-	+	+	+	+	-	-	+	+	-	+	-	-	-	+	*Klebsiellaoxytoca*
PF1	GNB	+	+	+	+	+	+	+	-	-	-	NA	-	-	NA	+	*Escherichia coli*
PF2	GNB	-	+	+	+	+	-	-	+	+	-	+	-	-	-	+	*Klebsiellaoxytoca*
PF3	GPC	-	+	+	+	-	-	+	-	+	-	+	+	-	+	+	*Staphylococcus aureus*
PF4	GPB	+	+	+	+	-	-	-	+	+	-	+	-	-	NA	+	*Bacillus mycoides*
PF5	GNB	+	+	-	+	+	-	+	-	+	+	-	-	-	NA	+	*Salmonella specie*
PF6	GNB	+	+	-	+	+	-	+	+	+	+	+	+	+	NA	+	*Pseudomonas fluorescens*
PF7	GNB	+	+	-	+	+	-	-	-	+	-	-	+	+	NA	+	*Pseudomonas aeruginosa*

KEY NOTE: + = Positive, - =Negative, Na = Not Applicable, Gnb = Gram Negative Bacilli, Gpc = Gram Positive Cocci. Isolate code: the first letter represent the samples African Spinach (A), Okra (O), Lagos Spinach (L), Jew's Mallow (J) and Pumpkin (P), the second letter represent the sample sites, Student Union Building (S) and Fadama (F). While the Arabic Numerals represent the isolate number.

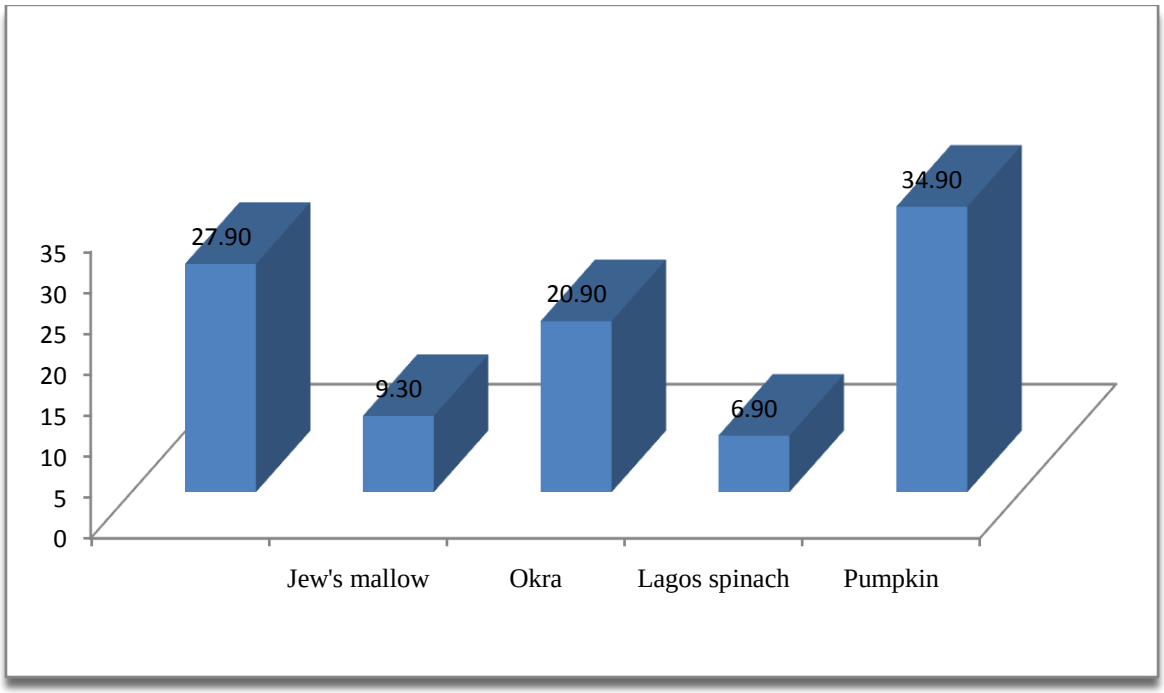

Fig 1. Frequency of bacterial isolates from the two phyllosphere samples.

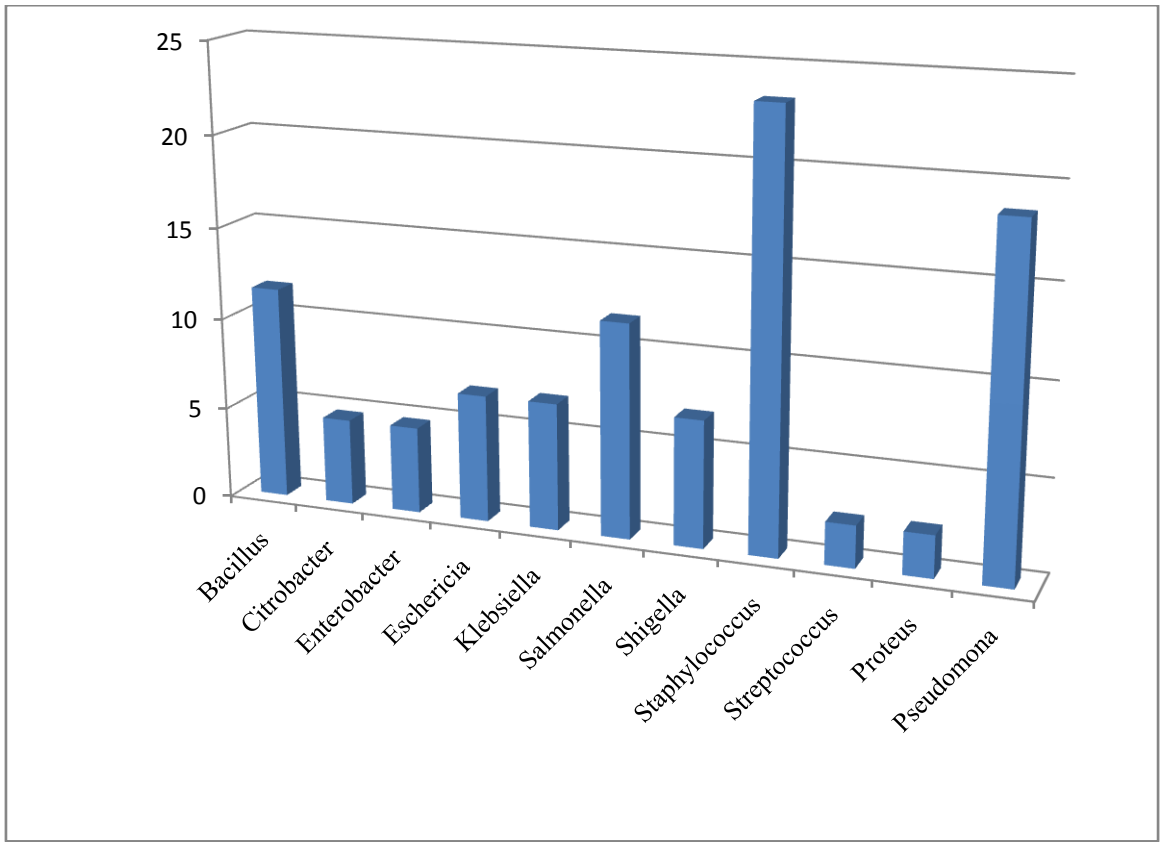

Fig 2 : Frequency of bacterial genera from all the phyllosphere samples collected from the two sampled sites.

IV. Discussion

The highest percentage 38.1% (table 2) and 31.8% (table 2) of bacteria isolated from the phyllosphere of Pumpkin obtained from the STUDENT UNION BUILDING (SUB) and FADAMA respectively within the sampled sites was attributed to the plant species which appear to influence microbial carrying capacity of the leaf. Pumpkin leaves are broad and less waxy; it is also located close to the ground surface compared to other leaves used in this study and tends to have more surface area for bacteria to thrive (Lindow and Brandi, 2003). Researchers have shown that the total number of culturable bacteria recovered from broad-leaf plant were significant greater than that recovered from grasses of waxy broad-leaf (Kinkel *et al.*, 2000); Lindow and BrandI, 2003).

The less frequency of bacteria isolated from Lagos spinach (6.9%), Jew's mallow (9.3%) as obtained from the sampled sites compared to pumpkin was as a result of the waxy nature of the plant (Lindow and BrandI, 2003). Waxy layer of plant leaf limit passive diffusion of nutrient and water vapour from the plants interior on to the surface and defines the hydrophobicity of the leaf (lindow and BrandI, 2003). Thick waxy cuticles have been implicated to interfere with bacteria colonization of plants by limiting diffusion of nutrients and inhabitating the wetting of the leaf surfaces (Lindow and BrandI, 2003).

Also, some phyllospere bacteria have been shown to increase the permeability of the cuticle enhancing water nutrient availability by producing toxins which affect ion transport across plant cells plasma membrane (Hirano and Upper, 2000). These findings supported the result from other studies which show that pronounced inter-species variability in phyllosphere communities exist (Whipps*et al.*, 2008). Many studies have also shown that environmental conditions can have important effects on phyllosphere community structure (Lindow and BrandI, 2003). The variation in microbial load from the different samples site was attributed to the natural and human activities in the various sites. Sample sites Fadama have more trees, crops and plants planted in the site well compared to sample site Student's union building which is dominated by cooking and selling food-related activities.

V. Conclusion

The findings from the study show that phyllosphere are microhabitats which support the growth of various groups of microorganisms including human pathogen with bacteria being the most abundant of the groups. Most of the edible leafy vegetables which are consumed in human have less waxy phyllosphere, which permits microbial growth. It is necessary that they may be washed and cooked properly before consumption to ensure healthy living. It is recommended that they are washed and cooked properly before consumption to avoid ingestion of possible food pathogens that causes food-borne diseases.

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