International Journal of Advances in Engineering and Management (IJAEM)
Volume 3, Issue 8 Aug 2021, pp: 1234-1240 www.ijaem.net ISSN: 2395-5252

Biocontrol abilities of six selected rhizospheric bacteria from edible fruit bearing trees against phytopathogenic (Xanthomonas campestris and Pseudomonas syringae)

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Submitted: 05-08-2021 Revised: 18-08-2021 Accepted: 20-08-2021

ABSTRACT:A total of seven bacterial isolates comprising of P. flourescens, E.coli, Enterobacter aerogenes, Micrococcus sp, Agrobacterium sp, Bacillus sp and Klebsiella sp. Were isolated from the rhizospheric soil of six edible fruit bearing trees (Mango (Mangifera indica, guava (Psidium guajava), cashew (Anacardium occidentale), pawpaw (Carica papaya), fruit tree (Indian almond) and hog plum (Spondias mombin). The highest cfu/g recorded was 6.03 $x10^8$ while the least recorded was $2.17x10^8$ from Hog plum and Indian almond respectivcely. The screening for plant growth promoting characteristics revealed a wide pattern of activities with P. flourescens, Enterobacter aerogenes, Bacillus sp and Klebsiella sp having a positive result for Hydrogen cyanide production. All of the six selected microorganisms showed a positive result for Ammonia, phosphate and indole acetic acid production. Bacillus sp produced the highest zone of clearance during amylase and protease production test while Micrococcus sp had the least zone during protease production test. In the antagonistic assay against the two selected phytopathogenic bacteria (Xanthomonas campestris and Pseudomonas syringae), Bacillus sp and P. flourescens displayed the most antagonistic activities of 22mm and 20mm against Xanthomonas campestris and 21 and 18mmagainst Pseudomonas syringae. The two organisms also displayed a bactericidal action against the two selected pathogenic bacteria tested.

KEYWORDS:cfu/g, amylase, protease, hydrogen cyanide, rhizospheric, indole acetic acid production.

I. INTRODUCTION

There is a pressing need to protect planet earth, although the plan has been ongoing since 1970 when WCED (World Commission on Environment and Development) first defined sustainable development as the type of development that meets the need of the present generation without endangering the future of the coming generation. In other for a sustainable development to work effectively in a country like Nigeria, the rates at which we dispose chemicals indiscriminately into the environment have to be reviewed, reduced or bring to an end. The campaign to reduce the amount of effluents containing chemicals, fertilizers and heavy metals which tends persists in the environment for longer period (Rajkumar et al., 2010).

[1].Currently, the biological approaches use for improving crop production are gaining momentum among Agriculturist and environmentalists following effective crop management practices and integrated plant nutrient management. Bacteria are ubiquitous in nature and they occur in different soil types and environment. They are essential part of soils. They play major roles in various biotic activities of the soil ecosystem to make it rich in nutrient turn over and sustainable for crop production (Ahemad et al., 2015). They encourage plant growth through

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production of numerous plant growth regulators, mobilizing nutrients in soils, protecting plants from phyto-pathogens by controlling or inhibiting them, improving soil structure and bio-remediating the polluted soils by neutralising toxic heavy metal species and degrading xenobiotic compounds (Li et al., 2016).

[2].Therefore, the bacteria lodging in the plant root area popularly called the rhizobacteria play better roles in transforming, mobilizing, solubilizing the nutrients compared to those from other soil areas (Hayat et al., 2010). Therefore, rhizobacteria are the predominantly the mobilizing forces in soil nutrients recycle, resulting in soil fertility (Glick, 2014) and as such the need to protect the ecosystem in which they call home. However, in other to protect the planet earth, we have to go back to the use of natural control agents instead of using hazardous chemical agents, which have harmful effects on nature and human health. This cannot be addressed effectively without talking about the use of biocontrol agents as they leave no residues as inorganic pesticides do.

[3].The focus of this work is to isolate, identify and characterize bacteria from the rhizosphere of fruit trees and subsequent in-vitro analysis of the microorganisms as a potential biopesticide against two selected disease causing plant pathogenic bacteria.

II. MATERIALS AND METHOD

Sources of materials

The soil around the root area (rhizosphere) of 6 (six) selected fruit trees were collected into sterile bottle for analysis. Selected fruit tree species used include: Mango (Mangifera indica, guava (Psidium guajava), cashew (Anacardium occidentale), pawpaw (Carica papaya), fruit tree (Indian almond) and hog plum (Spondias mombin) trees.

Collection of the soil sample

Soil samples were collected around the root rhizosphere, rhizoplane and non-rhizosphere areas of five (5) different selected plant species into a sterile bottle and were immediately taken to the laboratory for immediate analysis.

Collection of the pathogenic bacteria specimen

Phyto-pathogenic bacteria of beans (Xanthomonas campestris and Pseudomonas syringae) were collected as stock culture from botany Department of Federal University of Agriculture, Abeokuta.

Serial dilution

Serial dilutions were carried out on each soil sample from (rhizosphere soil) and three dilutions (10^{-4}, 10^{-6} and 10^{-8}) were set aside for use. This was achieved by weighing 1g of each soil sample into test-tubes containing 9ml of distilled water and subsequent dilutions were made from this preparation.

Preparation of the media

Nutrient agar medium, peptone broth, Nutrient broth and Yeast malt dextrose broth (YMD) were prepared following the manufacturer's instructions. Triplicates of the medium were made into petri dishes and incubated for 24hours to check for sterility.

Inoculation of the medium

Tree dilutions were chosen from the serially diluted soil samples. From the three (3) dilutions of each soil sample, 0.1ml of the soil suspension from the three dilutions (10^{-4}, 10^{-6} and 10^{-8}) selected were inoculated into a sterilized nutrient medium and incubated at 37+/- 2^{o}c for 48 hrs. The plates were sealed with paper tape and inverted before incubation. The colony forming unit of each of the bacteria isolate was calculated as cfu/g of soil sample used.

$$\frac{\text{No of colonies counted x dilution factor}}{\text{Amount plated}} \quad \textbf{(1)}$$

Sub- cultivation of the isolates

Isolates that developed from each plate were separated into different plates according to their morphological and cultural characteristics. Each discreet colony was transferred unto a fresh medium of nutrient agar.

Physical identification use for the characterization of the isolates

This was done using Berges manual for identification of microorganisms using their sizes shapes, elevation, surface area, margin, color, odour and pigmentation.

Purification of the bacterial isolates

All pure culture of each isolate was transferred to nutrient agar slant and incubated at 35^{o}c for 24-48hrs and then transferred to a refrigerator at 4^{o}c for proper storage.

Biochemical identification of the isolates

The isolates were subjected to gram staining techniques, starch hydrolysis, citrate test, indole test, oxidase test, catalase test, casein hydrolysis, hydrogen sulphide test and Sugar fermentation.

Evaluation of plant growth promoting characteristics of bacterial isolates

Indole acetic acid production test

The potential of the bacterial isolates to produce Indole acetic acid was carried out according to the modified colorimetric estimation procedure of

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Salkowski method with Van Urk Salkowski reagent as described by B. mohite (2013).

Phosphate solubilization test

The potential of the bacterial isolate to solubilize phosphate was carried out using modified method of V. Mohan and Ayswarya (2012). This was achieved by suspending 1g of rhizosphere soil sample 100ml of distilled water. This was serially diluted and plated on Pikovskaya's medium modified by Rao and Sinha (1963) for obtaining microorganisms capable of dissolving phosphate. The plates were incubated for 4-5days and transparent zones of clearance around microbial colonies were recorded as positive result.

Hydrogen Cyanide Production test

Screening for the production of hydrogen cyanide by the bacterial isolates was determined by method described by Rajni Dev and Richa Thakur (2018). Approximately 4.4g glycine/L was used to amend Nutrient broth and isolates were later streaked on this amended medium. Whatmann filter paper No 1 was soaked in 0.5% picric acid and 2% sodium carbonate were used to cover the plates, the lids were replaced and sealed with paper tape before incubation at 37^0c for 72hours. The development of colour change was observed and noted from 48hours. Colour change from orange to red color was recorded as a positive result for HCN production.

Production of ammonia test

The bacterial isolates were screened for their ability to produce ammonia using the method of Nedege et al., (2015). This was done by growing the isolates in peptone broth (10mL), it was later incubated at 37oc for 48- 72 hours. After incubation, 0.5 ml of Nessler's reagent was added to the bacterial suspension. Development of colour change was observed and recorded. Colour change from brown to yellow color was recorded as a positive result.

Antagonistic assay against phyto-pathogenic bacteria (Co-inoculation method)

This was done using co-inoculation method. The stock cultures were subcultured on a fresh media to check for viability. They were later incubated at 37^0c for 24 hours. The growth were subcultured as a slant in a macartney bottle and kept for further use. Subsequently, the revived and viable cultures of Xanthomonas campestris and Pseudomonas syringae stored on slants were used to prepare the 0.5 Mcfarland stardard of each of the organisms. This was done to adjust the turbidity of the soil suspensions to a standard. Approximately 0.1ml of each standard was inoculated into holes bored in a fresh medium that was inoculated with microorganisms from the soil samples.

Bacteriostatic action

This was done to determine the mode of action of the isolates used. Overnight culture of each isolate was co-inoculated with each of pathogenic bacteria. This was to test whether the growth of the isolates will in one way or the other suppress or inhibit the growth of the pathogenic bacteria. This was to measure the susceptibility of the two selected pathogenic isolates to the isolated rhizospheric microorganisms.

Bactericidal action

This was done by subculturing from the test tubes showing no visible growth (under bacteriostatic action) on to a fresh medium to check if growth would resume or not. Plates showing positive growth after 24 hours of been transferred to a fresh mediun were recorded as resistance and the action of the isolate was termed as bacteriostatic, while plates with no visible growth after 24- 48 hours on a fresh medium were taken as susceptible and the action was recorded as bactericidal.

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III. RESULTS AND DISCUSSION

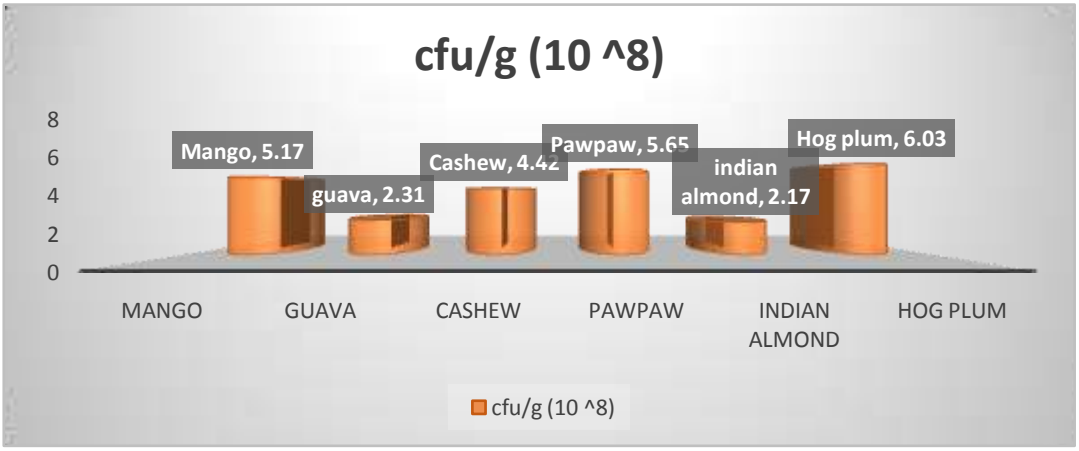

Fig 1: Colony forming unit of isolates per gram of soil cultured from each tree type.

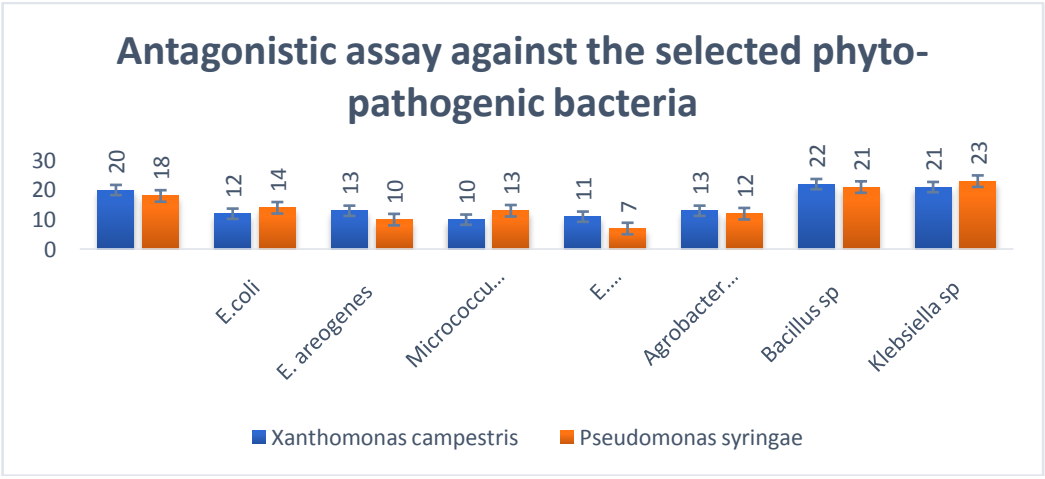

Fig 2: Antagostic assay of the selected isolates against the two selected phytopathogenic bacteria

Table 1: Morphological characteristics of the selected rhizospheric microorganisms

Morphology	Colonies isolated from soil samples						
characteristics	**P. flourescens**	**E.coli**	**Enterobacter aerogenes**	**Micrococcus sp**	**Agrobacterium sp**	**Bacillus sp**	**Klebsiella sp**
Shape	rod	rod	rod	spherical	Rod	Rod	short rod
Colour	creamy	bluish black	grey	creamy	Pink	creamy	pink
Size	2-3mm	0.5um-1um	1-3um	1-2um	1-1.5mm	spherical	0.5um
Arrangement	single	single /clump	clump	pairs	single/pairs	single cell	pairs
Capsule	positive	negative	positive	negative	Negative	positive	positive
spore formation	negative	negative	negative	negative	Positive	positive	negative
Motility	positive	positive	positive	negative	Positive	positive	negative
gram stain reaction	negative	negative	negative	positive	Negative	positive	negative
nitrate reduction	positive	positive	positive	negative	Positive	positive	positive
Catalase	positive	positive	positive	positive	Positive	positive	positive

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Oxidase	positive	negative	negative	positive	Positive	negative	negative
Urease	positive	negative	negative	negative	Positive	positive	positive
utilisation of carbohydrates							
Glucose	positive	positive	positive	positive	Positive	positive	positive
Lactose	positive	positive	positive	negative	Positive	negative	positive
Mannitol	negative	positive	positive	negative	Positive	negative	positive
salt tolerance	positive	negative		positive	Positive	positive	positive
H_2S production	negative	negative	negative	negative	Positive	positive	negative
Indole	negative	positive	negative	Negative	Positive	negative	negative
voges proskaeur	negative	negative	positive	Positive	Positive	positive	negative
Citrate	positive	negative	positive	Negative	Negative	positive	positive
methyl red	negative	positive	positive	Negative	Positive	positive	negative

Table 2: Plant growth promoting (PGP) characteristics of the selected isolates

PGP characteristics	P. flourescens	E.coli	E. areogenes	Micrococcus sp	Agrobacterium sp	Bacillus sp	Klebsiella sp
Hydrogen cyanide production	positive	positive	negative	negative	negative	positive	positive
Ammonia production	positive	positive	positive	positive	positive	positive	positive
Phosphate solubilisation	positive	positive	positive	positive	positive	positive	positive
Indole acetic acid production	positive	positive	positive	positive	positive	positive	positive
Amylase production	5	2.6	3.2	4.2	5.2	7.1	4.5
Protease production	5	4.2	4.1	3.3	4.1	6.2	5.1

Note: Amylase and Protease production were measured in millimeter (mm).

Table 3: Action of the isolates against the phytopatogenic bacteria

Isolates	Bacteriostatic	Bactericidal
P. flourescens a		positive
P. flourescens b		positive
E.coli a	positive	
E.coli b	positive	
E. areogenes a		positive
E. areogenes b	positive	
Micrococcus sp a	positive	
Micrococcus sp b	positive	
Agrobacterium sp a	positive	
Agrobacterium sp b	positive	
Bacillus sp a		positive
Bacillus sp b		positive
Klebsiella sp a	positive	
Klebsiella sp b	positive	

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Note: Ascribed a and b assigned to each isolates showed the phytopathogenic bacteria tested against it. Where a is Xanthomonas campestris and b is Pseudomonas syringae.

IV. DISCUSSION:

Trees chosen for this study were trees with edible fruits because parts from these trees have always be cut to treat infections and diseases in southwest Nigeria since times past up till now. This claim has been established in several journal articles, thus the choice of edible fruit bearing trees for this research. The results from the cultivation of the soil from the root area of different selected trees showed that the highest number of colonies in cfu/g was recorded in Hog plum (6.03 x10^8) while the least was recorded in Indian almond (2.07 x10^8). Production Hydrogen cyanide (HCN) which is a secondary metabolite might be responsible for strong antagonistic activity seen in P. flourescens (20 and 18mm), E.coli, Bacillus (22 and 21mm) and Klebsiella sp (21 and 23 mm) against Xanthomonas campestris and Pseudomonas syringae respectively while Enterobacter, Micrococus and Klebsiella sp gave negative results. Hydrogen cyanide production has been linked to biocontrol ability in microorganisms due to the ascribed toxicity against pathogenic microorganisms as positive correlation between HCN production and suppression of pathogenic activities has been reported since 1990 by Defago. Although the mode of biocontrol action of HCN was not understood but some researches had suggested that this might be due to the ability of the hydrogen cyanide to bind iron and as such deprived the phyto-pathogen of available iron (Dzombak et al., 2009). Ammonia was produced by all and indole acetic acid, a main auxin in plants which controls many necessary physiological processes was produced by all isolates studied. All isolates studied were positive for phosphate solubilising ability which is useful in phyto remediation of heavy metal impacted soil (Ahemad, 2015). Bacillus sp are one of the most useful sources of bacterial proteases as they are capable of yielding neutral and alkaline proteolytic enzymes with great properties. This was also established in this result as the highest amylase production recorded was seen in Bacillus sp (7.1mm) while the least was recorded in E.coli (2.6mm). The high protease production of 6.2mm recorded in Bacillus sp showed that protease which is a hydrolytic enzyme capable of breaking the peptide bond in proteins was in abundant in Bacillus sp studied. The test of bacteriostatic and bactericidal action of the isolates against the two phyto-pathogens revealed that Pseudomonas sp and Bacillus sp exerted purely bactericidal action against two phyto-pathogens tested while E.coli, Micrococcus and Agrobacterium sp exerted a bacteriostatic action. The action of enterobacter sp differs as it was bactericidal against Xanthomonas campestris and bacteriostatic against Pseudomonas syringae.

In conclusion, nature has varieties of all that is needed to treat infections caused by pathogenic microorganisms are present in the environment.Using rhizobacteria as a substitute for promoting resistance to pathogenic Xanthomonas campestris and Pseudomonas syringae which are the causative organisms of black rot in vegetables and bacterial speck in monocot respectively. The success recorded in this study might be attributed to the fact that the rhizobacteria used were from the root area of trees that have parts implicated in treatment of infections in humans and as such the isolates are adapted to the root environmental conditions.

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