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MICROFLORA DIVERSITY ON THE PHYLOPLANE OF WILD OKRA (C*ORCHORUS OLITORIUS* L. JUTE)

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ABSTRACT
Indigenous people especially in southern Nigeria use Corchorus olitorius L. (Jute) as a staple vegetable. Population dynamics, richness and frequency of occurrence of microflora isolates on healthy green leaves of wild okra were estimated within two weeks at weekly intervals using the dilution technique. This study was conducted in the University of Benin intend to show the diversity of microorganisms on the leaves of wild okra. The leaves were categorized based on their period of harvest into old, new and middle with a week interval between each harvest. After serial dilution in distilled water, isolation was done using nutrient agar for bacteria and potato dextrose agar for fungi. After incubation colony forming units per millimeter were counted, isolated, identified and characterized using standard microbiological techniques. The fungal diversity and frequency of occurrence were higher in the first sampling (61.50% and 62.07% respectively) than those of the second sampling (38.50% and 37.93%). Total viable microbial population in the second sampling after two weeks was higher (11.23 X 10^2cfu/ml) than in the first sampling after one week (10.00 X 10^2cfu/ml). The total cumulative bacterial count was higher (15.69X 10^2cfu/ml) than those of fungi (55.40 X 10^2 cfu/ml) during the studies. Bacterial genera isolated included; Staphylococcus, Bacillus, Micrococcus, Serratia and Proteus. Rhodotorula, Mucor, Trichoderma, Aspergillus, Penicillium, and Helminthosporium were the genera of fungi isolated. Further studies could help to elucidate major players in wild okra phylloplane ecology.
Keywords: Wild Okra (Corchorus olitorius), Phylloplane, Microflora population, Bacteria, Fungi

INTRODUCTION

The aerial parts of living plants (leaves, stems, buds, flowers and fruits) offer a habitat for microorganisms, commonly called the phyllosphere and are generally colonized by a variety of bacteria, yeasts, and fungi (Mukhtar *et al.*, 2012). This study seeks to investigate the microflora diversity on the phylloplane of wild okra *Corchorus olitorius* L. The microorganisms will be isolated, identified and characterized as well as study their population density, the dynamics of the isolates and frequency of occurrence. The variation in phylloplane microbial isolates populations in the different leaf categories will also be studied.

Dynamics of microbial population on leaves in time and space is a function of immigration, emigration, growth and death (Kimkel*et al.*, 1997). The surface of leaves contain stimulatory and inhibitory substances that regulate the colonization of leaf surface by organisms (Fating and khare, 1978).Various seasonal studies have revealed seasonal fluctuations in microbial population on various plants, with maximum population sizes observed during the warmer months. Bacterial counts vary in fresh weights, depending upon the plant species and habitat examined (Crosse, 1965; Last and Deighton, 1965). Leben (1965) suggested that bacterial isolates occur in depression between epidermal cells and are distributed singly or in small groups. Microflora populations on the leaf surface vary in size and diversity depending on the influence of numerous biotic and abiotic factors which affect their growth and survival. These factors include leaf age, external nutrients, the interactions between populations of different microorganisms, temperature, relative humidity, duration of leaf wetness, light intensity, wind speed and the presence of air pollutants and pesticides. Pesticides in particular, have the potential to reduce phyloplane population diversity and to give advantages to some species (Bosshard *et al.*, 1987).

Wild okra*Corchorus olitorius*, L. belongs to the angiosperm family Tiliaceae. Angiosperm Phylogeny Group (2009) includes this family as part of Malvaceae in the Eurosid II Malvales order. It is widely consumed as a vegetable among rival communities in most part of Africa (Velempini *et al.*, 2004). In West Africa, it is commonly cultivated and very popular among people of all classes especially in Nigeria (Oyedele *et al.,* 2006). Leaves of *Corchorus* have been a staple food in Egypt since the time of the Pharaohs. The plant is also eaten in some parts of Asia (Furumuto *et al.*, 2002). According to Zakaria *et al.* (2006), wild okra is used in folklore medicine in the treatment of gonorrhea, chronic cystisis, pain, fever and tumors. *C. olitorius* is known to contain high levels of iron, which are useful for the prevention of anemia (Oyedele *et al.,* 2006). Ecologically, the crop grows easily in rural subsistence farming systems when compared to exotic species like cabbage and spinach (Modi *et al.*, 2006).

C. olitorius develops a characteristics slimy texture when exposed to water and moist heat, is sun dried after picking. Due to contact with soil during growing and drying, this dry vegetable is usually soaked in water and should be washed thoroughly prior to cooking, otherwise the relish becomes gritty.

The nutritive value, as well as micro nutrient content of *C. olitorius* has not been well researched in parts of Africa and Asia. Indigenous vegetables such as Okra (*Abelmoschus* spp) like wild okra play important roles in human diets especially in Nigeria. They can supply the body with minerals, vitamins and certain hormones precursors in addition to protein and energy (Antia *et al*., 2006).

MATERIALS AND METHODS

Study Area

The Experimental garden of Department of Plant Biology and Biotechnology and Laboratory of Department of Science Laboratory Technology, University of Benin, Benin City (6.20^{0}N, 5.37^{0}E) were used. It lies within the Tropical Rainforest (TRF) zone. The relief is characterized by lowland of less than 300 meters above sea level. The climate includes high rainfall up to 2000mm – 3000mm of bimodal pattern with peaks at July and September respectively, high temperature ranging between 20^{0}C-40^{0}C and high atmospheric humidity (Omuta, 1980). Radiation is fairly high and varies according to different period of the year. Above 1,600 hours per year have been reported in surrounding areas, NIFOR (Onwueme and Sigh, 1991). The soils are slightly ferrallitic.

Source of Seeds

Wild Okra seeds were collected from Nigerian Institute of Horticulture (NIHORT), Ibadan. The seeds were stored in stapled paper and stored in a drawer in the laboratory at ambient temperature for five days before sowing.

Sowing of Seeds

Ten (10) kilograms of top soil was collected and transferred into 15 medium sized polythene bags. A small portion was cleared from weeds, transferred into small polythene bags and the bags stored for 24 hours before cultivation. The bags were later wet with water. Four seeds were planted in each 15 polythene bags containing the top loamy soil. The seeds were planted 2cm deep into the soil and watered (Osawaru *et al.,* 2012).

Sample Collection

Healthy *Corchorus olitorius* leaves used in this study were harvested from the mature plants and categorized as old, new and middle leaves based on the point of collection. The leaves were collected and labelled initial sampling and again after two weeks interval resulting in two samples. The leaves collected from the point closest to the soil is categorized as old and those collected midpoint as middle while those collected close to the topis categorized as new. All the leaves were collected from the same plant.The leaf samples were put separately into sterile bags, taken back to microbial laboratory in less than 2 hours for isolation of phyllospheric microorganisms (Mukhtar *et al*., 2010 and 2012).

Isolation of Phylloplane Microorganisms

Twenty discs each of 10 mm in diameter was cut from each of the leaf categories using a 10mm corkborer. Each leaf category was put in a 10ml sterile distilled water and hand shaken for 20 minutes. A quantity (1ml) of the stock suspension was diluted into 9.0ml of diluent for up to five times. This was repeated for the two other leaf categories, each time shaking for uniform distribution of the cells (conidia). One mililitre of the aliquots from 10^{-1} and 10^{-5} dilutions of each leaf wash, were transferred to sterile Petri plates, two replicates for each dilution were made for each of bacteria and fungi isolates. Cheek cool molten agar (Nutrient agar) for bacteria and Potato Dextrose agar (PDA) for fungi were poured into Petri dishes (pour plate method). Plates were incubated at room temperature (28±2^{o}C) for 24 hours (for bacterial isolates) in inverted position and 3-5 days for fungal isolates under fluorescent day light. Colony forming units per millilitres (cfu/ml)were counted as described by Codina *et al.,*(2008); Mukhtar *et al*., (2010) and (2012).

Identification and Characterization of Isolates

Microbial isolates were identified and characterized using standard microbial techniques. Fungal isolates were Identified using non-culturable or culturable analysis as a surrogate measure of exposure to fungi and the spores identified at the genus level or classified into groups following general taxonomic guidelines currently accepted by the scientific community (Codina *et al.,*2008). Fungal colonies were counted after 3 - 5 days. Each fungal colony was purified and identified on the basis of morphological characteristics to meet relevant taxonomic requirements. Characteristics features of bacterial strains/colonies were also identified based upon standard physiological test, biochemical tests and morphological characters including:

Shape: circular, irregular or rhizoid; Size: small, medium, large(or in millimetres); Elevation: elevated, convex, concave, umbonate/umbilicate; Surface: Smooth, wavy, rough, granular, papillate or glistening; Edges: entire, undulate, crenated, fimbriate or curled; Colour of the colony: Yelow, green etc.; Structure: opaque, translucent or transparent and Degree of growth: scanty, moderate or profuse.

Nature: discrete or confluent, filiform, spreading or rhizoid. This were in addition to Bergey's manual.

Frequency of individual microbial species was calculated in percentage as follows;

Microbial frequency (%) = number of colony of the species appeared ×100/ Total number of allcolony isolated from each sample. Sampling of the phylloplane was done once a week for two weeks. The frequency of occurrence of each isolates from each leaf categories was noted. All statistical analysis was done using Microsoft excel 2010.

RESULTS

The total fungal diversity and frequency of occurrence was highest in the initial sampling but less than bacteria after two weeks. The microbial count was highest after two weeks. The middle leaves and old leaves categories showed high diversity and frequency of occurrence than the new leaves in both sampling periods and microbial isolates. The viable microbial counts were studied by weekly sampling of leaves categories and the result presented in Table 1. Results show that bacteria load decrease from old, middle and the new leaves having the lowest.

Fungal load decrease from the new, old to the middle leaves within the first week, whereas, bacteria load decreased from middle, new to old leaves in the second week. Fungi load decreased from old, new to the middle leaves showing the least all during the first sampling.

Table 1: Viable microbial counts from leaf surface of *Corchorus olitorius*in colony forming units per milliliter/ leaf category (cfu/ml/leaf category)

Sampling period (weeks)	BACTERIA	FUNGI	BACTERIA	FUNGI	BACTERIA	FUNGI
	OLD		NEW		MIDDLE	
	120.00	56.00	32.00	56.00	40.00	60.00
1	96.00	64.00	60.00	68.00	108.00	40.00
X± SX	180.00 ±12.00	60.00 ±4.00	46.00 ±14.00	62.00 ±6.00	74.00 ± 34.00	50.00 ±10.00
2	120.00	52.00	112.00	48.00	220.00	40.00
	80.00	72 .00	116.00	60.00	84.00	80.00
	100.00 ± 20.00	62.0±10.0	114.00 ± 2.00	54.00 ± 6.00	154.00 ± 68.00	44.00 ±4.00

X± SX= Mean ± Standard error

During the second sampling period the result of viable microbial counts are presented in table 2. The first sampling show decrease was observed from new, old and middle (first week, bacteria) to new, middle, old leaves (fungi) whereas, in the second week decreased from new, old to the middle leaves (bacteria) and new, old and middle (fungi). The viable microbial count during the first sampling was lower 10.00 x 10^2cfu/ml whereas, total cumulative bacteria count was higher (15.69 x 10^2cfu/ml) than the total cumulative fungi (5.54 x 10^2cfu/ml) during the sampling period of study.

Table 2: Viable microbial counts from leaf surface of *Corchorus olitorius* in colony forming units per millilitre/ leaf position on plants (cfu/ml/leaf position)

Sampling period (weeks)	BACTERIA			FUNGI		
	OLD	**NEW**	**MIDDLE**	**OLD**	**NEW**	**MIDDLE**
	115.00	40.00	130.00	48.00	160.00	52.00
1	180.00	48.00	190.00	60.00	80.00	48.00
X± SX	147.50 ±32.50	44.00±4.00	160.00±30.00	54.00 ±6.00	120.00 ± 40.00	50.00±2.00
	120.00	56.00	152	56.00	180.00	60.00
2	196.00	64.00	192	68.00	108.00	52.00
X± SX	158.00± 38.00	60.00±4.00	172.00± 20.00	62.00± 6.00	144.00± 36.00	6.00±4.00

X± SX= Mean ± Standard error

Generally, microbial count during the first sampling was lower (1.0 X 10^3cfu/ml) whereas, total cumulative bacteria count was higher (1.57 X 10^3 cfu/ml) than the total cumulative fungi (5.54 X 10^2 cfu/ml) during the sampling period of study.

The characterization of bacteria isolates are shown in table 3. The shapes range from rods, cocci and straight while the margins were either entire or irregular. Thecolors are pink, white, cream and greenish. Some bacteria isolates were negative to the Gram stain while others were positive. The characterization implicated the following bacterial genera: *Serratia*, *Micrococcus*, *Staphylococcus*, *Pseudomonas*, *Proteus* and *Bacillus*.

Table 3: Cultural, morphological and biochemical characteristics of bacteria isolates from wild okra leaf (*Corchorus olitorius*)

CULTURAL CHARACTERISATION	**B1**	**B2**	**B3**	**B4**	**B5**	**B6**
Shape	Rods	Cocci in chain	Cocci in cluster	Straight rods	Straight rods in chains	Straight rods in chains
Elevation	Raised	Convex (raised)	Raised	Raised	Raised	Flat
Surface Appearance	Opaque	Whitish (opaque)	Opaque	Opaque	Opaque	Opaque
Margin	Entire	Entire	Entire	Irregular	Irregular	Entire
Colour	Pink (NA)	Whitish (NA)	Pink (Mcc)	Greenish (NA)	Cream (NA)	Cream (NA)
COLONAL MORPHOLOGY						
Gram stain	-ve	+ve	+ve	-ve	-ve	+ve
Cell type	Rod	Cocci	Cocci	Rods	Rods	Rods
Cell arrangement	Single	Chains	Cluster	Single	Single	Chains
BIOCHEMICAL TESTS						
Methyl red	+	+	-	-	-	
Voges proskauser	+	-	+	-	-	+
Indole	-	-	+	-	+	+
Oxidase	-	+	+	+	-	+
Catalase	+	+	+	+	-	+
Coagulase	-	-	+	-	+	+
Hydrogen sulphide	-	-	-	+	+	-
Motility	+	-	-	+	+	-
Gelatin liquefaction	+	-	-	-	-	+
Citrate	+	+	+	-	+	+
SUGAR TESTS						
Glucose	AG	AG	+	A	A	A
Mannitol	AG	A	+	-	-	A
Sucrose	+	A	-	+	+	-
Lactose	+	-	-	+	-	+
Galactose	A	-	-	-	-	-
Tentative Identity	*Serratia* sp.	*Micrococcus* sp.	*Staphylococcus* sp.	*Pseudomonas* sp.	*Proteus* sp.	*Bacillus* sp.

Table 4 shows result for fungal characterization on the wild okra surface. The result implicate varying growth forms and colour of reverse plates. Some isolates had no hyphae whereas others were septate and non septate. Rhizoids were present in some and absent in others while the spore colour varied from pink, white, colourless and green. Based on the result of characterization the fungal isolates were identified as from the following genera: *Rhodotorula*, *Saccharomyces*, *Mucor*, *Aspergillus*, *Penicilium*, *Rhizopus*, *Trichoderma* and *Fusarium*.

Table 5 show the result of frequency of occurrence of microbial isolates on the leaf of wild okra after one week. Among the fungal isolates, *Saccharomyces* and *Mucor* were found present in all leaf categories. *Rhodotorula* was only absent in new leaves while *Rhizopus* and *Trichoderma* were only present in old leaves. *Aspergillus* and *Penicillium* were only present in new and middle leaves. *Fusarium* was present only in new leaves while *Helminthosporium* was present only in middle leaves. Among the bacterial isolates, Staphylococcus and Bacillus are present only on the old leaves. Micrococcus and Serratia were present in all leaf categories while Proteus was only absent on middle leaf. The result suggest that fungi had higher frequency than bacteria.

The frequency of occurrence of microbial isolates at second sampling is presented in table 6. Result show that bacteria had higher frequency than fungi. *Penicillium*, *Mucor*, *Helminthosporium*, *Trichoderma* and *Aspergillus* are present in all leaf categories. *Rhodotorula* was only absent in middle leaves while *Botrydiplodia*for the fungal isolates. Bacterial isolates like *Serratia* and *Micrococcus* are present in all leaf categories. *Staphylococcus*, *Pseudomonas* and *Bacillus* are present only in old leaves while *Proteus* is absent only in middle leaves.

Table 4: Cultural and morphological characteristics of fungi isolate for wild okra leaf (*Corchorus olitorius*)

Growth form	Pinkish splashes, mucoid edges entire flat.	Whitish mucoid, edges entire raise.	Whitish extensive woolly cottony with genolytic hyphae	Black, woolly with profuse growth.	Green, non-luxuriant with concentric ring.	Whitish , luxuriant with profuse growth, fluffy.	Green patches or cushion, luxuriant growth.	Extensive and cottony in culture , creamy in colour.
Colour of reverse plate	Pinkish	Creamy	Whitish	Dark	orange	Creamy	green	Whitish
Hyphae	No hyphae	No hyphae	Non-septate (young) septate (ocd)	Septate	septate	Non- Septate	Septate	Septate
Conidiophore	No conidiophore	No conidiophore	Non-septate, long erect usually unbranched.	Non-septate terminating in globose swelling.	Septate arise from a mycelium single, branched near apex.	Non septate, upright terminating in globose swelling.	Hyaline, upright much branched.	Simple and slender, branched irregular.
Conidia	Ellipsoid cells	Ellipsoid cells with buds on the sides.	Present,hyaline one-celled, globose non-motile.	Present, one celled globose in dry basipetal chains.	Present one-celled hyaline globose brightly coloured basipetal	Present , one celled globose in dry basipetal chain	Hyaline, one-celled ovoid borne in small terminal clusters.	Large sickle or cane shaped multi-septate hyaline, macro-Conidia
Stolon	Absent	Absent	Absent, presence of genocytic hyphae	Absent	absent	present	absent	Absent
Rhizoid	Absent	absent	Absent	Absent	absent	Present multi branched short rooted	absent	Absent
Spore colour	Pinkish	whitish	White	Dark	colourless	dark	green	Colourless
Spore attachment	Single cell	Buds growing on the side.	Tips of sporangiophore in the sporangia.	Bear phialides at the apex with conidia at the tip.	Phialides which pinch off conidia in dry chains at the tip.	Consist of terminal swelling of multi nucleated hyphal branches with Conidia at the tip.	Phialids single with small terminal clusters at the tip.	At the tip some intermediate..
Tentative identity	*Rhodotorula* sp	*Saccharomyces* sp.	*Mucor* sp.	*Aspergillus niger*	*Penicillium* sp.	*Rhizopus* sp.	*Trichoderma* sp.	*Fusarium* sp.

Table 5: Frequency of occurrence of microbial isolates from the phylloplane of wild okra leaf(*Corchorus olitorius*) from the initial sampling (after one week)

		TREATMENTS/FREQUENCY		
ISOLATES	**NO.(%)**	**OLD LEAVES**	**NEW LEAVES**	**MIDDLE LEAVES**
Fungi				
Rhodotorula sp.	2 (7.7)	+	-	+
Saccharomyces sp.	3(11.5)	+	+	+
Mucor sp.	3(11.5)	+	+	+
Rhizopus sp.	1(3.8)	+	-	-
Trichoderma sp.	1(3.8)	+	-	-
Aspergillus sp.	2(7.7)	-	+	+
Penicillium sp.	2(7.7)	-	+	+
Fusarium sp.	1(3.8)	-	+	-
Helminthosporium sp.	1(3.8)	-	-	+
Sub total	16 (61.5%)Fungi	5	5	6
Bacteria				
Staphylococcus sp.	1(3.8)	+	-	-
Bacillus sp.	1(3.8)	+	-	-
Micrococcus sp.	3(11.5)	+	+	+
Seratia sp.	3(11.5)	+	+	+
Proteus sp.	2(7.7)	+	+	-
Sub total	10 (38.5%)Bacteria	5	3	2
Total	26	10	8	8
% Total	26 (99.6%)	26 (100.0)Microbial		

Key: + Present, - Absent

Table 6: Frequency of occurrence of microbial isolates from the phylloplane of wild okra leaves(*Corchorus olitorius*)after two weeks

		TREATMENT/FREQUENCY		
ISOLATES	**NO.(%)**	**OLD LEAVES**	**NEW LEAVES**	**MIDDLE LEAVES**
Fungi				
Penicillium sp.	3(10.34)	+	+	+
Mucor sp.	3(10.34)	+	+	+
Helminthosporium sp.	3(10.34)	+	+	+
Rhodotorula sp.	2(6.89)	+	+	-
Trichoderma sp.	3(10.34)	+	+	+
Aspergillus sp.	3(10.34)	+	+	+
Botrydiplodia sp.	1(3.45)	-	-	+
		6	6	6
Sub total18(62.07) Fungi				
Bacteria				
Serratia sp.	3(10.34)	+	+	+
Micrococcus sp.	3(10.34)	+	+	+
Staphylococcus sp.	1(3.45)	+	-	-
Pseudomonas sp.	1(3.45)	+	-	-
Proteus sp.	2(6.89)	+	+	-
Bacillus sp.	1(3.45)	+	-	-
		6	3	2
		12	09	08
Sub total	11(37.93) Bacteria			
Total	29			
% Total	29(99.96)	29(100.0)		

Key: + Present, - Absent

DISCUSSION AND CONCLUSION

In this study, results obtained revealed that during the first sampling, fifteen microbial isolates were observed, while thirteen were isolated one week after (during the second sampling), the difference being *Saccharomyces sp., Botrydiplodia* sp (fungi) and *Pseudomonas* sp. (bacteria). Wild okra play significant role in the provision of cheap and affordable protein for rural population (Ndlovu *et al.*2008). Thus, the presence of these microbes is important when considering public health.

In this study, the frequency of occurrence and population of microbial isolates and dynamics of microbial isolates on the phylloplane of the three leaf categories were investigated. A fluctuating relationship was observed between the frequency of occurrence, population in colony forming units per disc Area (78.6mm^2), which reflect a complex variations in weight per unit area of leaf categories, throughout the growing period of the experimental plant. This was in part, due to the continuous growth of the leaf lamina(Dickinson and Wallace,1975). Some of the organisms obtained in this work were pathogens or antagonists including, *Micrococcus sp, Pseudomonas* sp*. and Bacillus* sp*.* Bacterialspecies include *Trichoderma* sp.*, Aspergillus*sp.*, Penicillium* sp.*, Helminthosporium* sp*.* and *Fusarium* sp. which are responsible for the population and species diversity and dynamics. Leaf infected with a pathogen modifies the surface microflora which may partially or completely protect the leaf against subsequent infection by the other pathogens (Sinha, 1965).

An overall higher abundance and diversity of microorganisms was observed in old leaves than the new and middle leaves of the second sampling periods compared with that observed in the first sampling periods, whereas the diversity of the first sampling was higher (14 species) than the first (13 species). The bacteria population decreased from old, middle and the new leaves having the lowest. Generally, total microbial counts were lower in the first sampling 10.00 x 10^{2}cfu/ml than in the second sampling (11.23 x 10^2 cfu/ml). As similar population dynamics and diversity were observed at all three sites, some components of the phylloplane microflora may be less sensitive to regional and climatic variation in time of harvest, age of leaf. This is in agreement with Waipara *et al.* (2001) hence epiphytic phylloplane microorganisms can be exploited to act as bio indicators of different plant production practices. However, further taxonomic investigation is required to identify the exact components of the microflora that are the most sensitive and useful bio indicators.

The progressive increase observed in the populations during sampling may have reflected alterations in the characteristic of the leaves and sources and activity of the phylloplane microorganisms, particularly in the consistent differences between the first and second sampling periods and different leaf categories. With the results of this study, it could be suggested that meteorological data may be employed to help explain major fluctuations in the phylloplane populations, but minor changes will require studies of the micro-environment at the leaf surfaces.

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