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# Comparative Study of Microflora Population on the Phylloplane of Common Okra [*Abelmoschus esculentus* l. (Moench.)]

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**Abstract**

**Microflora isolates on healthy green leaves of mature Okra were estimated. The leaves were categorized based on their point of harvest into old, new and middle with a week interval between each harvest. The diversity and frequency of occurrence was higher [18 (60.00 %)] at first sampling than at second sampling [9 (60.00 %)] for fungi, bacteria [12 (40.00 %)] and [6 (40.00 %)] respectively. Total microbial population in the second sampling was higher [177.5 cfu/ml] than the first [160 cfu/ml]. The total cumulative bacteria count was 390 cfu/ml and 366 cfu/ml for fungi during the studies. Ten genera of fungi and six genera of bacteria were examined. The predominant flora was identified to the genera *Rhodotorula, Saccharomyces, Mucor, Aspergillus* and *Penicilum,* for the fungi while *Micrococcus, Staphylococcus,* and *Serratia* for the bacteria. Further studies could help identify major players in the phylloplane microbial ecology.**

**Keywords: Okra** (*Abelmoschus esculentus),* Phylloplane, Microflora population, Bacteria and Fungi

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## Introduction

Common Okra [*Abelmoschus esculentus* L. (Moench] is thought to be native to West Africa (Tindall and Rice, 1983). It is cultivated throughout the tropical and warm climates of the world for its fibrous fruits or pods containing round, white seeds. It was formerly considered a species of *Hibiscus*, but is now elevated in the genus *Abelmoschus* and family Malvaceae (mallow family) (Santos *et al*., 2011). Angiosperm Phylogeny Group (2009) places Okra in the Eurosid II Malvales order. The taxonomical revision undertaken by van Borssum (1966) and its continuation by Bates (1968) constitutes the most fully documented studies of the genus *Abelmoschus* while the geographical distribution of *Abelmoschus* species is documented by Charrier (1984).

Okra is an annual, sometimes biannual crop, requiring warm growing condition and found in almost every market all over Africa (Schippers, 2000). Schippers (2000) observed a great diversification of Okra with the most important production regions localized in Ghana, Burkina Faso and Nigeria. Two main species, the dwarf and the tall type were also observed in these areas. It is an important vegetable suitable for cultivation in traditional agricultural systems as well as on large commercial farms. The fruits are harvested when immature and eaten or cooked in a variety of ways. It provides carbohydrates, proteins and vitamin C in large quantities and also essential and non-essential amino acids which are comparable to that of soybean (Adeboye and Oputa, 1996).

Okra fruit is mucilaginous, producing a slimy substance when cut and cooked but cooking them whole helps reduce the sliminess. Okra is an important source of vitamins, calcium, potassium and other mineral matters which are often lacking in the diet of developing countries. To retain most of these nutrients, the seed pods should be cooked as little as possible or eaten raw. Its medicinal value has also been reported by Siesmonsma and Hamon (2002). Jamala *et al.* (2001) observed that the production and economic importance of okra as a vegetable in Nigeria has rapidly increased in recent years with different varieties now used by farmers to meet the demand of okra by consumers.

According to Andrews (1991) and Lindow and Brandl (2003) the phylloplane, or leaf surface, represents an important terrestrial habitat that harbors a wide range of microorganisms. Phyllosphere which can be used in reference to entire above ground exposed surface of the plant, according to Muktar *et al.* (2012) is both scientifically and economically important habitat in which to study microbial ecology. Fungi, encompassing both filamentous and yeast taxa, are a major component of the phylloplane microbiota (Dickinson 1976, Andrews 1991, Bills and Polishook 1994, De Jager *et al.,* 2001). Filamentous fungi from the phylloplane may be either parasites, saprophytes, endophytes or epiphytes (Guimaraes *et al.,* 2011). Most of the endophytes colonize different compartments of the plant apoplast, including the intercellular spaces of the cell walls and xylem vessels (Malfanova *et al.,* 2013).

The leaf surface is a suitable environment for microbial growth because of a thin film of nutrients deposited on the leaf. The microbial communities are influenced by external and/or internal factors such as nutrient availability, humidity, temperature, leaf age and type, and presence of inhibitors (chemical compounds produced by the plant) (Andrews 1991, Kinkel 1997, De Jager *et al*., 2001, Santamaria and Bayman 2005, Evueh and Ogbebor 2008). This complex relationship can either be beneficial or harmful. They do not cause any disease symptoms, in contrast to phytopathogens (Malfanova *et al.,* 2013). The microflora of phylloplane is essential to understand microbial diversity because they provide information about their occurrence in the niche. It also suggests the role of such association to the health and wellbeing of the plant as well as on members of the food chain that consume them. Though microbial conservation has not gained much attention, studies of phylloplane microflora can play key roles.

The aim of the current study is to isolate, identify and characterize microbial isolates from the phylloplane of Okra leaves. This study also investigates the microbial population on different leaf positions on mature plant and considers its impact on microbial biodiversity.

## Materials and Methods

*Study Area:* The Experimental garden of Department of Plant Biology and Biotechnology and the 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 zone. The relief is characterized by lowland of less than 300 meters above sea level. The climate includes high rainfall up to 2000 mm – 3000 mm 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:* Common 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 plastic container and were later wetted with water. Four seeds were planted in each of the 15 plastic containers. The seeds were planted 2 cm deep into the soil and watered daily with 50 cl of water (Osawaru *et al.,* 2012).

*Sample Collection:* Healthy *Abelmoschus esculentus* 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 collected again after two weeks. 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 top is categorized as new. All the leaves were collected from the same plant. The leaf samples were put separately into sterile bags, taken back to microbiology 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 10 mm cork borer. Each leaf category was put in a 10 ml sterile distilled water and hand shaken for 20 minutes. A quantity (1 ml) of the stock suspension was diluted into 9.0 ml 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 microbiological 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, biochemical and morphological characteristics.
Frequency of individual microbial species was calculated in percentage as follows;
Microbial frequency (%) = number of colony of the species appeared ×100/ Total number of all colony isolated from each sample.

Sampling of the phylloplane was done weekly for two consecutive times. The frequency of occurrence of each isolates from each leaf categories was noted. The descriptive statistical analysis was done using Microsoft excel 2010.

## Results

Results are presented in Tables 1, 2, 3, 4 and 5. The frequency of occurrence of microbial isolates on the phylloplane of the different leave categories from the first sampling had higher fungi (60.0 %) isolates than bacteria (40.0 %). *Staphylococcus* sp. had the highest frequency [bacteria (10.0 %)]. *Rhodoturola, Saccharomyces, Aspergillus* sp. were also found to be high in their frequency [fungi (10.0 %)]

Table 1: Frequency of occurrence of phylloplane organism from common Okra (first sampling)

| **Isolates** | **Number (%)** | **Old** | **New** | **Middle** |
|---|---|---|---|---|
| **FUNGI** | | | | |
| *Rhodotorula* sp. | 3 (10.0) | √ | √ | √ |
| *Saccharomyces* sp. | 3 (10.0) | √ | √ | √ |
| *Mucor* sp. | 2 (6.7) | √ | √ | X |
| *Trichoderma* sp. | 2 (6.7) | √ | X | X |
| *Cladosporium* sp. | 2 (6.7) | √ | √ | √ |
| *Aspergillus* sp. | 3 (10.0) | √ | √ | √ |
| *Rhizopus* sp. | 2 (6.7) | X | √ | √ |
| *Penicillium* sp. | 1 (3.3) | X | √ | X |
| | | 6 | 7 | 5 |
| | | 18(60.0%) | | |
| **BACTERIA** | | | | |
| *Pseudomonas* sp. | 2 (6.7) | √ | X | √ |
| *Micrococcus* sp. | 2 (6.7) | √ | X | √ |
| *Proteus* sp. | 2 (6.7) | √ | √ | X |
| *Staphylococcus* sp. | 3 (10.0) | √ | √ | √ |
| *Serratia* sp. | 1 (3.3) | X | √ | X |
| *Bacillus* sp. | 1 (3.3) | X | √ | X |
| *Streptococcus* sp. | 1 (3.3) | X | X | √ |
| TOTAL | 30 | 4 | 4 | 4 |
| | | 12(40.0%) | | |
| OVERALL TOTAL | 30(100.2) | 10 | 11 | 09 |
| | | 30(100.0) | | |

The total microbial count obtained from the old, new and middle leaves of the plant shows that the frequency of occurrence was higher in the first sampling than what was obtained from the second sampling The old leaf shows higher bacteria count during the first and second sampling than the new and middle leaf, whereas the bacteria population from the second count were higher than those of the new leaf. The fungi count for the second counting where higher than those of the second counting with the old leaves showing higher colony forming units per mililitre (cfu/ml) of leaf wash than the new and the middle (Table 3).

Table 2: Frequency of occurrence of microbial isolates from the phylloplane of Okra after second sampling

| | | Treatment/frequency | | |
|---|---|---|---|---|
| **ISOLATES** | **Number (%)** | **Old leaves** | **New leaves** | **Middle leaves** |
| **FUNGI** | | | | |
| *Saccharomyces* sp. | 2(13.33) | × | √ | √ |
| *Mucor* sp. | 2(13.33) | √ | √ | × |
| *Rhodotorula* sp. | 1(6.67) | × | √ | × |
| *Penicillium* sp. | 1(6.67) | × | × | √ |
| *Aspergillus* sp. | 2(13.33) | √ | × | √ |
| *Botydiplodia* sp. | 1(6.67) | √ | × | × |
| | | 3 | 3 | 3 |
| | | 9(60.0%) | | |
| **BACTERIA** | | | | |
| *Serratia* sp. | 1(6.67) | × | × | √ |
| *Staphylococcus* sp. | 2(13.33) | √ | × | √ |
| *Micrococcus* sp. | 3(20.00) | √ | √ | √ |
| | | 2 | 1 | 3 |
| | | 6(40.0%) | | |
| **TOTAL** | 15 | 5 | 4 | 6 |
| **OVERALL TOTAL** | **15(100.00)** | **15(100.0%)** | | |

Table 3: Total microbial counts from Phylloplane of Okra [counts in colony forming units per millitre/leaf (cfu/ml)]

| **Sampling time (weeks)** | **Bacteria** | **Fungi** | **Bacteria** | **Fungi** | **Bacteria** | **Fungi** |
|---|---|---|---|---|---|---|
| | Old | | New | | Middle | |
| 1 | 152.0 ± 68.0 | 44.0 ± 4.0 | 114 ± 2.0 | 54.0±60 | 100.0±20.0 | 62.0±10.0 |
| 2 | 160.0 ± 70.0 | 57.0 ± 3.0 | 112.0± 2.0 | 55.0±5.0 | 118.0±6.0 | 65.5±5.5 |

See appendix 1

**Discussion**

Microorganisms can establish their niche on plant surface or internal tissues. Thus, plant surface have been recognized as an important habitat for microorganisms based on either a transient (unspecific or ephemeral epiphytic saprophytes) or permanent (epiphytic residents, endophytes or pathogens) association for more than a century (Forcesa and Inaceo, 2006) In this study, considerable biodiversity of microflora were observed during isolation of phylloplane organism on Okra leaves. The fungal diversity and frequency of occurrence were higher during the first sampling than in the second sampling. These could be as a result age (time difference), nature of the leaf

surface, climatic conditions and availability of an alternate host. More so, the bacteria diversity and frequency was higher in the first sampling than those of the second sampling. Generally, most phylloplane microorganisms show little specificity to their plant host; hence changes during their life cycle and in the environment may affect their number. There is need for investigation of the complete phylloplane microflora of a particular host, its development and the factors affecting its size and composition (Nicholsen, 1972). In a study by Andrews *et al.* (1980) height and lateral position were significant factors for the observed variations in density of filamentous fungi and yeast. These groups of organisms were among the predominant flora identified in the present study alongside members of these genera *Rhodotorula*, *Mucor*, *Aspergillus* and *Penicilum* for the fungi while *Micrococcus*, *Staphylococcus* and *Serratia* for the bacteria. The following microbial isolates were more frequent and found at all the three sites of the leaves sampled: *Rhodotorula* sp, *Saccharomyces* sp, and *Aspergillus* sp for the fungi, *Staphylococcus* sp., and *Micrococcus* sp, which was identified at only two sites. In a similar study on wheat (*Triticum vulgare)* by Abdel-Hafez (1981), more than ninety species were isolated, some of which were also found in the present study.

Table 5: characterization of bacteria isolates from the phylloplane of okra leaves (common okra)

| **Cultural characterization** | **B1** | **B2** | **B3** | **B4** | **B5** | **B6** |
|---|---|---|---|---|---|---|
| Shape | Rod | Cocci in chain | Cocci in cluster | Straight rods | Straight in chains | Straight in chains |
| Elevation | Raised | Raised | Raised | Raised | Raised | Flat |
| Surface appearance | Opaque | Whitish Opaque | | Opaque | Opaque | Opaque |
| Margin | Entire | Entire | Entire | Irregular | Irregular | Entire |
| Colour | Pink (NA) | Whitish (NA) | Pink (MCC) | Greenish (NA) | Cream (NA) | Cream (NA) |
| **Colonial Morphology** | | | | | | |
| **Gram stain** | **–ve** | **–ve** | **–ve** | **–ve** | **–ve** | **–ve** |
| Cell type | Rods | Cocci | Cocci | Rods | Rods | Rods |
| Cell arrangement | Single | Chains | Cluster | Single | Single | Chains |
| **Biochemical Test** | | | | | | |
| Methyl red | + | + | – | – | – | + |
| Vogesproskauser | + | – | + | – | – | + |
| Indole | – | – | + | – | + | + |
| Oxidase | – | + | + | + | – | + |
| Catalase | + | + | + | + | – | + |
| Coagulase | – | – | + | – | + | – |
| Hydrogen sulphide | – | – | – | + | + | – |
| Motility | + | – | – | + | + | + |
| Gelatin liquefaction | + | – | – | – | – | + |
| Citrate | – | + | + | – | + | – |
| **Sugar tests** | | | | | | |
| **Sugar** | B1 | B2 | B3 | B4 | B5 | B6 |
| Glucose | A/G | A/G | + | A | A | A |
| Mannitol | A/G | A | + | – | – | A |
| Sucrose | + | – | – | + | + | – |
| Lactose | + | – | – | + | – | + |
| Galactose | A | – | – | – | – | – |
| Tentative identity | *Serratiasp* | *Mcrococcus sp.* | *Staphylococcus sp* | *Pseudomonas sp* | *Proteus sp* | *Bacillus sp* |

The composition and quantity of nutrients, including carbohydrates, organic acids, and amino acids that support the growth of epiphytic and endophytic microorganisms, are affected by the plant species, leaf age, leaf physiological status, and the presence of tissue damage (Hallmann *et al.,* 1997, Annapurna and Rao, 1982). Similarly, host plants, leaf age, leaf position, physical environmental

Table 4: Characterization of Fungi Isolates from the Phylloplane of Common Okra Leaf

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Cultural characteristics Growth form** | White fluffy growth, small compact colony. | Whitish, mucoid edges, entire raised | Pinkish splashes mucoid edges entire flat | Whitish extension woolly cottony with loenocytic hyphae. | Black woolly with profuse growth | Green non-luxuriant with concentric rings with white margin | Green patches or cushion luxuriant growth | Grayish black green woolly profuse growth | Whitish, luxuriant with profuse growth fluffy. | Pycnidia black crumpent and confluent |
| **Colour of reverse plate** | Creamy | Creamy | Pinkish | Whitish | Dark | Creamy | Green | Grayish | Creamy | Darkish |
| **Microscopy hyphae** | Non-septate | No hyphae | No hyphae | Non-septate (young) septate (old). | Septate | Septate | Septate | Septate | Non-septate | Non-septate |
| **Conidiophore** | Branched, slender segmented to produce conidia | No conidiophores | No conidiophores | No septate upright usually unbranched, coenocytic | Non-septatefer minating in glucose swelling | Septate arise from a mycelium singly branched near apex | Hyaline upright much branched | Tall, upright branches near apex bearing conidia | Non-septate, upright ferminating in globose swelling | Non-septate simple short upright and globose |
| **Conidia** | Minute, produced in chain by segmentation of hyphae | Ellipsoid cells with buds on the sides | Ellipsoid cells | Present hyaline one-celled, globose non-motile | Present, one-celled globose in dry basipetal chain | Present, one-celled hyaline globose brightly colouredbosi petal | Hyaline one-celled ovoid born in small terminal cluster. | Present cluster or single (1 or 2 celled) | Present one-celled globose in dry basipetal chain | Dark, two celled at maturity, ovoid to elongate |
| **Stolon** | Absent | Absent | Absent | Absent, present of coenocytic hyphae | Absent | Absent | Absent | Absent | Present | Absent |
| **Rhizoid** | Absent | Absent | Absent | Absent | Absent | Absent | Absent | Absent | Present, multi-branched short rooted | Absent |
| **Spore colour** | Whitish | Whitish | Pinkish | Whitish | Dark | Greenish | Green | Grayish black | Dark | Dark |
| **Spore attachment** | Produced at the tip of the hyphae | Buds growing on the side | Single cells | Tips of sporangiophore | Bear phialide at the apex with conidia at the tip | Phialids which pinch off conidia in dry chain at the tip | Phialids single with small terminal cluster | Terminal bear phialides at the apex terminating in conidia | Consist of terminal swelling of multi-nucleated hyphal branches with conidia at the tip. | No phialids, single with small dark erumpent terminal conidia at the tip of conidiophores |
| **Tentative identity** | *Streptomyces* sp | *Saccharomyces* sp | *Rhodotorula* sp. | *Mucor* sp. | *Aspergillus sp.* | *Penicillium* sp. | *Trichoderma* sp | *Cladosporium* sp. | *Rhizopus* sp. | *Botryodiplodia* sp. |

condition, and availability of immigrant inoculum have also been suggested to be involved in determining population size and diversity of microbes in the phyllosphere (Andrews *et al*., 1980; Cabral, 1985; Wilson and Lindow, 1994; Hata *et al.,* 1998; Yadav *et al*., 2011). One or more of these conditions may be implicated for the Okra phylloplane microflora in this study. Bacterial endophytes are ubiquitous colonizers of the inner plant tissues where they do not normally cause any substantial morphological changes and disease symptoms as some endophytes can promote plant growth and/or protect their host against phytopathogens (Malfanova *et al.,* 2013). Although fungi and bacteria diversity and populations vary with leaves site, it suggest their relative present. Phylloplane microorganisms have also been implicated as biocontrol agents in plants (Shahjahan *et al.,* 2001, Kawamata *et al.,* 2004). Application of fertilizers containing substantial amounts of nitrogen have also been found to affect colonization of certain phylloplane microorganism (Giorgio *et al.*, 1997) likewise treatment with cement dust during pre and post inoculation process (Singh and Rai, 1997).

In conclusion, microflora populations are present on the phylloplane of Okra and may pose public health concerns especially when cultivated for market and/or for consumption. There is need for further research to enhance our knowledge of microbial interaction and ecology on the phylloplane. This may be of help in understanding some plant disease(s) control mechanisms, prevent unpredictable consequences especially when these vegetables are consumed and a resultant assessment of the microflora effects on the nutrient status of their host.

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