# Isolation and molecular identification of endophytic bacteria from *Tylosema esculentum*

**Elisha Sinyinza[1] and Percy M Chimwamurombe[2]***

[1] Department of Biological Sciences, Faculty of Science, University of Namibia, Private Bag 13301, 340 Mandume Ndemufayo Avenue, Windhoek, Namibia

[2] Department of Natural and Applied Sciences, Namibia University of Science and Technology, Private Bag 13388, Windhoek, 13 Jackson Kaujeua Street, Namibia, 9000.



* Corresponding author

E-mail: pchimwa@gmail.com

Tel: +26461207236

Cell: +264812744678

## ABSTRACT

*Tylosema esculentum* is a perennial legume that is predominant in Namibia, Botswana, and South Africa. It is rich in nutrients, drought-tolerant, and a climate change contender for future agriculture. The objectives of this study were to isolate and identify culturable endophytic bacteria. Our study assessed the mean CFUs/mL among the tuberous roots, stems, and leaves to determine the density and diversity using ANOVA. It was a quantitatively controlled experiment in which seeds were grown in a greenhouse. The bacteria number varied from $1.29 \times 10^8$ to $3.5 \times 10^8$ CFU/ml of broth, with the highest in the tuberous roots and the lowest in the leaves. At a 5% significant level, we revealed that there is a difference among the bacterial densities of the plant parts. Shannon-Weiner diversity showed that there is a higher diversity of bacteria in the tuberous roots. Of the isolates that were obtained, 53.8% belonged to *Enterobacter*, 15.4% to *Bacillus*, 15.4% to *Pantoea*, and 7.67% to *Burkholderia*. The results will be helpful in future agriculture; bio-fertilizers will be used in place of chemical fertilizers and other pesticides. Further studies will determine the importance of the isolates in biological control, agriculture, and biotechnology for growth promotion.

**Keywords**: Bio-fertilisers; Nitrogen fixation; Plant Growth-Promoting Bacteria

## INTRODUCTION

*Tylosema esculentum*, also known as marama bean and gemsbok, is a long-lived, underutilized perennial legume native to the arid region of southern Africa. [4,10] It belongs to the *Leguminosae* family and has three subfamilies (*Caesalpinioideae*, *Mimosoideae*, and *Palpilionoideae*). [4] *T. esculentum* forms part of the indigenous people's diet, and it is known to have a high protein and oil content of about 29%-39% and 25%-28%, respectively. [10]

Endophytic bacteria colonize uninjured plant tissues without causing any harm to the plant host, the interaction is mutualistic.[1,21] Unfortunately, the relationship between endophytes and plants is not fully understood. The plant will provide nutrients for the bacteria and the bacteria will aid in the protection against pathogens, and secretion of gibberellins, phytopathogens, and cytokinins.[15] Almost all, if not all plants are colonized by endophytic bacteria, some of which include nitrogen fixation and plant growth-promoting bacteria (PGPB). Endophytes normally reside in the intracellular spaces that contain a high amount of inorganic minerals, carbohydrates, and amino acids.[3,8]

Advances in the molecular biology field have improved our understanding of microorganisms and have simplified the molecular identification of various bacterial species, which has led to the discovery of a huge number of bacteria.[20] 16S ribosomal RNA (rRNA), is used for bacterial identification proposes due to the following unique features: (i) It has a unique signature that is present in all bacterial species (ii) It contains about 1500 base pairs (bp), which is large enough for sufficient information (iii) Genes does not change over time, it is very accurate.[20] This would

enable us to identify the endophytic bacteria found in *T. esculentum.* In addition, universal primers such as 27F and 1492R are commonly used to amplify template bacterial DNA. [20]

*T. esculentum* tubers are used as traditional medicines in Southern Africa. [4] Rotaviruses are the major cause of diarrhea among infants and immunocompromised people. It has demonstrated that marama extracts are promising microbicides against rotavirus.[4,10]

The microbiome of endophytic bacteria promotes growth, yield, and acts as a bio-control agent. [24] These beneficial effects are characterized and brought about by metabolic interactions.[5] Micro-symbionts in plants produce a wide range of different types of metabolites, these substances have a role in communication, interaction, defense, and competition. [24] Endophytes induce the synthesis of essential metabolites.[5,9] The hosts benefit from the endophytic bacteria by producing a wide range of natural compounds, they have shown potential in eliminating soil contaminants by speeding up the phytoremediation process.[6] They are also known to act as natural fertilizers by solubilizing phosphates, synthesis of plant hormones such as auxin, and fixation of atmospheric nitrogen.[12]

There is insufficient knowledge about the endophytic bacteria inhabiting the tissues of *T. esculentum*. Therefore, the objective of this study was to isolate and identify the culturable endophytic bacteria from the leaves, tuberous roots, and stems of the plant. The outcomes will increase our awareness of the type of bacteria present, this will aid us in the development of beneficial strains for use as bio-inoculants.

## Materials and Methods

### Study design and population

It was a quantitative controlled experimental study; the major variables were endophytic bacteria and plant parts. We focused on the leaves (L), stems (S), and tuberous roots (TR). We had twelve (12) plants and from each, three (3) parts were obtained producing 36 replicates.

### Study site and sample collection

Soil samples were randomly collected from the University of Namibia at coordinates 22.6122° S, and 17.0584° E. The soil was sterilized using an autoclave at 121ºC for 15 minutes. The soil samples were allowed to cool down for 3 hours prior to planting. The seeds were planted in 12 nursery pots and carefully placed on a flat surface in the greenhouse.

### Surface sterilisation

The fresh and healthy plant samples were washed thoroughly to remove sand particles. Surface sterilization was performed using 70% ethanol for 3 minutes, Sodium hypochlorite for 3 minutes, 70 % ethanol again for 30 seconds, and lastly washed them using distilled water.[9,11] The disinfection process was confirmed by plating the aliquots of the final rinse in Tryptone Soy agar (TSA) at 30ºC for 14 days.[8]

### Preparation of media

The modified SM-medium composed of 1g Malic acid, 1g Anhydrous glucose, and 20mg yeast extract in 1 liter (L) distilled water, was sterilized at 121ºC for 15 minutes. This was followed by the addition of 1 ml vitamin stock solution (D-biotin 0.2g, p-Amino benzoic acid 0.02g, pyridoxine hydroxide 0.04g, thiamine dichloride 0.004g, riboflavin 0.02, nicotinic acid 0.04 plus 0.676 ml of distilled water) and 90% ethanol through a sterile syringe and a WHATMAN 0.2µm PES w/GMF filter by filtration. VM-ethanol was composed of the following ingredients per liter: 0.6g $K_2HPO_4$,0.4g $KH_2PO_4$, 0.5g $NH_4Cl$, 0.2g $MgSO_4.7H_2O$, 1.1g NaCl; 0.026g $CaCl_2.2H_2O$; 0.01g

$MnSO_4.H_2O$; 0.002g $Na_2MoO_4.2H_2O$; 0.066g Fe (III)EDTA; 1g Yeast extract; 3g Tryptone; 12g bacteriological agar. The pH was adjusted to 7.56 using 0.5% sodium hydroxide and HCl. And then, the addition of 6ml of 90% ethanol and vitamin stock solution was done through filtration.[9,14]

## Endophytic bacteria isolation and culture

The plant samples of about 2 grams were sliced and macerated separately using a sterile mortar and pestle. The macerated samples were inoculated in nitrogen-free SM-broth and incubated at room temperature for 12 days. Room temperature incubation was followed by 2 weeks of incubation at 30ºC.[4,8,10]

## Bacterial enumeration and pure culture

The tissue extracts from the SM-broth were diluted by serial dilution on VM-ethanol plates from $10^{-1}$ to $10^{-6}$. The dilution tubes were mixed thoroughly and aliquots of 0.1ml dilution were inoculated in triplicates on the VM-ethanol plates followed by spreading using an L-shaped spreader.[6] The VM-ethanol plates were incubated. The plates between 30-300 colonies were selected, and the bacterial cell numbers were expressed as CFU per ml of liquid broth. It was done by calculating the number of colonies multiply by the dilution factor divided by the volume placed on the culture plate (0.1ml). The value CFU/ml was then converted to a logarithm value for easy processing and statistical analyses.[4,6,9]

Sub-culturing of single colonies using the four-quadrant streak method was performed prior to incubation for 7 days at 30°. The pure cultures were inoculated in nutrient broth for 48 hours. The plates were stored in the refrigerator at 40°C.[4]

## Genomic DNA extraction and Polymerase Chain Reaction

The nutrient broth bacterial cells were washed with 100µl ringer solution and centrifuged for 10 minutes at 10,000rpm. DNA was extracted using ZR Fungal/Bacterial DNA MiniPrep™ according to the manufacturer's instructions. The presence of DNA was then confirmed by Nanodrop 2000

and onto 1% agarose stained with SYBR® Green I nucleic acid gel stain in 1X TBE buffer and then viewed under ultraviolet (UV) light to see if the quality and concentration is good enough for PCR.[4]

The amplification of 16S rDNA was performed in a reaction with a final volume of 25µl containing 5µl of template DNA, 0.8µM of each primer 27F and 1492R primers, 5µl of Nuclease-free water and 12.5µl of GoTaq® Green Master Mix: *Taq* DNA polymerase, dNTPs, $MgCl_2$ and buffers (Inqaba Biotechnology Industries, South Africa). A positive control (Bacteriophage lambda DNA with PCR mixture) and a negative control (PCR mixture without template DNA) were added to all the reactions.[7] The PCR profile on the thermal cycler was as follows: Pre-denaturation (1 cycle) at 95ºC for 4 minutes, 35 cycles of denaturation at 95ºC for 30 seconds, annealing at 50ºC for 1 minute, 72ºC extension for 1 minute, final extension (1 cycle) of 72ºC for 10 minutes and samples were stored at 4ºC.[7] The amplified DNA was observed on 1% agarose (100ml of 1XTBE, 1g of agarose, and 5µl of ethidium bromide) at 120 volts (v) for 35 minutes. The PCR products were sequenced and purified by Inqaba Biotechnical Industries (Pty) Ltd in Pretoria, South Africa using an ABI 3500XL sequencer.[1,7]

## Molecular identification

Nucleotide sequences were edited and cleaned manually using Bioedit. The forward and reverse sequences were aligned using the complements of their sequences. All the sequences that were overlapping were trimmed to a consensus sequence that was generated using the sequence alignment editor. These consensus sequences were pasted in the blast search on the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi).[9]

### Statistical Analysis

A normality test was performed on the mean CFU/ml results. ANOVA was performed, to determine if there is a difference in the bacterial density among the parts using SPSS version 23 at a 5% significance level.[10]

To compare the bacterial diversity among the plant parts, the Shannon-Weiner diversity index was employed on Microsoft Office 2013 (Excel) assuming that the species were repeated and randomly sampled.[22,33]

## Results and Discussion

### Bacterial enumeration

The following mean CFU per ml were obtained from the liquid broth of *T. esculentum*, the results (Table 1) indicate that the tuberous roots had the highest CFU/ml average value. Roots are rich in chemical compounds, they provide special environments for a variety of microorganisms.[3,6] It has been found that the bacterial niche in the root and the rhizosphere are distinct from each other, as well as those from the soil microbiome.[5,11]

Bacteria in roots affect the well-being of a plant and productivity.[6] It has been found that the microbes of the roots differ in how they behave depending on the association with different plant hosts.[9] They can also alter the interaction activity within a single host as environmental conditions or changes in the health of the host. [4,9,23]

Despite the importance of root microbes for plants and ecosystems, our understanding of how they are organized is still at its baseline. This is because of the poor advances in molecular biology.[6,8] They are difficult to study because of their complex diversity.[8] Evidence suggests both biotic and abiotic factors affect the composition of microbes.[5]

## Statistical Analyses

A Shapiro-Wilk's test ($p>0.05$), an inspection of the normal Q-Q plots, histograms, and box plot indicated that the CFU/ml of the bacterial samples were approximately normally distributed.[22] A skewness of 0.765 (standard error=0.637) and Kurtosis of-0.064 (standard error=1.232).[10,22,23]

### Analysis of Variances

The p-value (sig=0.013) in table 2 is less than $\alpha=0.05$. Hence, we reject the null hypothesis, and we conclude that there is sufficient evidence to claim that the bacterial densities among tuberous roots, stems, and leaves are different.

### Diversity indices

The tuberous roots had the highest diversity of endophytic bacteria, (H value= 2.95) followed by the stems (H value = 1.09) and the least was the leaves (H value= 0.563) (Fig.2). The results presented evidence that there is a difference in the bacterial densities among the plant parts. We can say that the bacterial density is based on the niche environment, the bacterial composition is based on the substances present at a specific plant location as well as exposure to environmental factors such as sunlight and soil.[15] The diversity of endophytic bacteria was the highest in the tuberous roots, this was proven by the Shannon-Weiner diversity index. We can say that the tuberous root is an ideal part of the plant for the isolation of the required bacteria due to the presence of nearly all strains of bacteria.[18]

## Genomic DNA isolation and Polymerase Chain Reaction

The DNA was successfully isolated, and the result was confirmed by Nanodrop and gel electrophoresis.

## Molecular identification of endophytic bacteria

We obtained nineteen isolates of endophytic bacteria from *T. esculentum*, the isolates were selected based on the morphological features of the colonies on the plates. A total of thirteen isolates were

successfully amplified. Analysis by bioinformatics grouped the endophytic bacteria into the following genera: *Bacillus*, *Enterobacter*, *Pantoea,* and *Burkholderia*.

*Enterobacter* was the predominant genus, representing 53.8% of the total, *Bacillus,* and *Pantoea* (15.4%), and lastly *Burkholderia* (7.67%). Our study demonstrated that the 3 taxa we recovered are also living in association with *Curcuma longa* L, sugarcane, and Rambutan fruits (*Nephelium lappaceum*).[9,12,14]

### *Enterobacter cloacae*

It has been found in the roots, stems, and leaves of sugarcane.[12] At present the bacterium is used as a post-harvest bio-control for fungal diseases affecting fruits and vegetables and as a supplement of seeds before plantation.[13] Their results confirmed that *E. cloacae* exhibited traits for plant growth-promoting bacteria (PGPB).[6,8,17] It can be concluded that the strain *E. cloacae*, could be used to promote the growth of *T. esculentum* and other potential crops.

### *Enterobacter aerogenes*

It had been found to induce resistance against phytopathogens, by the production of acetoin.[13,20] The process is mediated through metabolites that are synthesized by *rhizobacteria.*[13,15,16] Acetoin produced by *Enterobacter aerogenes* has contributed to the effective resistance of maize plants against phytopathogens known as *Setosphaeria turcica.*[13,17] We isolated *Enterobacter aerogenes* from the tuberous roots, the bacterium has been found to be living in association with the root tissues. The results are consistent with other studies.[6,14,26] We can conclude that *Enterobacter aerogenes* can confer resistance against phytopathogens and can fix atmospheric nitrogen.

### *Enterobacter asburiae*

*Enterobacter asburiae* is a facultatively anaerobic, gram-negative non-spore-forming rod-shaped bacterium belonging to the family of *Enterobacteriaceae*.[24] It inhabits plants, soil, and water surfaces.[15] Most strains of *E. asburiae* have the ability to suppress a number of plant diseases by

protecting roots, stems, leaves, and seeds from fungal infection.[15,22] It has been found that *E. asburiae* has the capability to improve plant growth, production of auxins, and fixation of atmospheric nitrogen.[15,16]

### *Enterobacter xiangfangensis*

*Enterobacter xiangfangensis* occurs in a wide range of environments, which include, plants, water, and soil. It grows optimally at 30°C.[17] It has been discovered that *E. xiangfangensis* can synthesize IAA, (indole-3-acetic acid) essential for cell growth, elongation, and solubilization of phosphates.[5,16,17]

### *Enterobacter spp*.

*Enterobacter* spp. is a gram-negative, non-spore-forming endophytic plant growth-promoting bacteria. It resides in the soil but mostly in plant tissues living in a mutualistic relationship.[21,25] It has been described as nitrogen fixation and nodule-forming bacteria.[8] Recent studies have shown that the strain *Enterobacter* spp exhibits plant growth-promoting traits such as ACC (1-Aminocyclopropane-1-Carboxylatedeaminase), siderophores, IAA, acetoin, and 2,3-butanediol production.[12] The traits are commonly found in bacteria that confer plant growth-promoting characteristics.[18]

### *Pantoea agglomerans*

*Pantoea agglomerans* is a diazotrophic, non-spore-forming, rod-shaped endophytic bacteria, it was formerly known as *Enterobacter agglomerans* or *Erwinia herbicola.*[3,14] It has been commonly isolated from plants, fruits (e.g., mandarin oranges), seeds, and animal or human faeces.[19] The bacterium grows well at temperatures between 25 to 30°C. It is also known to grow in environments with high salt concentrations or low water levels.[15] In addition, *P. agglomerans* has also been found to be a plant growth-promoting bacteria.[4]

### *Pantoea ananatis*

*Pantoea ananatis*, a member of the family *Enterobacteriaceae*, the bacterium is characterized by its ubiquitous nature and it is associated with both plant and animal hosts.[20] *P. ananatis* has been isolated from roots, leaves, and stems of a broad range of plants and it exists as part of endophytic and epiphytic flora.[15] It has been found as an endophyte in rice seeds and banana leaves.[10, 22] Other studies have found pathogenic strains of *P. ananatis* that infect plants.[6] Secondary metabolites have been obtained from *P. ananatis* and it has been found that they have anti-fungal activities against *Mycosphaerella musicola*.[22]

### *Burkholderia spp*.

*Burkholderia* spp. is a versatile bacterium that occupies a wide range of niches. The bacterium is a slow grower. [5,6,24] *Burkholderia* spp is mainly used for to prevent plant infections caused by bacteria or fungi, as a biocontrol, bioremediation, and plant growth promoting purposes.[23] In a recent study, *Burkholderia* spp was isolated from the tobacco rhizosphere in China. [23] It has been reported that it produces antimicrobial products which can therefore provide important ideas in the synthesis of bio-control chemicals and eradication of plant diseases.[16,23] In addition, *Burkholderia* spp. isolated from rice confers some plant growth-promoting characteristics such as ACC deaminase synthesis, the ability to fix nitrogen, and antifungal activities.[7]

### *Bacillus safensis*

*Bacillus safensis* is mostly found as an endophyte in plants, it is also found in other habitats such as water and soil.[23] *B. safensis* relies on its genotypic and physiological characteristics to withstand extreme environments.[23] Studies have found that *B. safensis* exhibits plant growth-promoting traits and has promising applications in biotechnology.[7,21]*B. safensis* acts as a bio-control and therefore could stimulate the growth of *T. esculentum* and essential plants.

### *Bacillus pumilus*

*Bacillus pumilus* is an endophytic bacterium that has been used as a bio-control product against soil-borne and other plant pathogens by means of resistance and direct antagonism.[17] *B. pumilus* was isolated from the stem in this study. It has also been found in the stem of a diseased cucumber plant.[16] The bacterium has been known to induce resistance against multiple plant pathogens.[21]

We successfully isolated 12 different bacterial endophytes from the internal tissues of the tuberous roots, stems, and leaves of *T. esculentum*. The isolated endophytes identified had a good homology of between 97% to 99%, we can confidently say that the isolates we obtained are closely identical to those in the database. Our results agree with other studies, the endophytic bacteria recovered are well-known endophytes in sugarcane, grasses as well as other plants. The endophytic bacteria were suspected to have an anti-pathogenicity action and belong to a group of plant growth-promoting bacteria, which produce gibberellin and auxin growth hormones.

Furthermore, these bacteria not only directly promote plant growth, but also protect plants against flooding, drought, salt, flower wilting, metals, organic contaminants, and both bacterial and fungal pathogens. The potential of the endophytic bacteria to promote the marama bean plant growth and their bio-control potential in fungal and bacterial diseases that affect this important legume for the human diet will be addressed in future studies.

**References**

1. Aman M, V RR. Antifungal activity of novel indole derivative from endophytic bacteria Pantoea ananatis4G-9 against *Mycosphaerella musicola*. Biocontrol Science and Technology. 2016;26(4):476-491. doi:10.1080/09583157.2015.1126223

2. Ft C, Adachi K. Isolation of endophytic diazotroph Pantoea agglomerans and nondiazotroph Enterobacter asburiae from sweet potatoes stem in Japan. Letters in Applied Microbiology. 2004;38(1):19-23. doi:10.1046/j.1472-765x.2003. 01434.x

3. Aswani R, Jishma P, Radhakrishnan E. Endophytic bacteria from the medicinal plants and their potential applications. Microbial Endophytes. 2020:15-36. doi:10.1016/b978-0-12-818734-0.00002-4

4. Chingwaru W, Majinda RT, Yeboah SO, et al. *Tylosema esculentum* (Marama) Tuber and Bean Extracts Are Strong Antiviral Agents against Rotavirus Infection. Evidence-Based Complementary and Alternative Medicine. 2011; 2011:1-11. doi:10.1155/2011/284795

5. Chowdhury SP, Hartmann A, Gao X, Borriss R. Biocontrol mechanism by root-associated *Bacillus amyloliquefaciens* FZB42 – a review. Frontiers in Microbiology. 2015;6. doi:10.3389/fmicb.2015.00780

6. Coenye T, Vandamme P. Diversity, and significance of *Burkholderia* species occupying diverse ecological niches. Environmental Microbiology. 2003;5(9):719-729. doi:10.1046/j.1462-2920.2003. 00471.x

7. Costa LEDO, Queiroz MVD, Borges AC, Moraes CAD, Araújo EFD. Isolation and characterization of endophytic bacteria isolated from the leaves of the common bean (*Phaseolus vulgaris*). Brazilian Journal of Microbiology. 2012;43(4):1562-1575. doi:10.1590/s1517-83822012000400041

8. Enterobacter cloacae. Definitions. 2020. doi:10.32388/ywfut4

9. Gu CT, Li CY, Yang LJ, Huo GC. *Enterobacter xiangfangensis* sp. Nov., isolated from Chinese traditional sourdough, and reclassification of *Enterobacter sacchari* Zhu et al. 2013 as Kosakonia sacchari comb. Nov. International Journal of Systematic and Evolutionary Microbiology. 2014;64(Pt_8):2650-2656. doi:10.1099/ijs.0.064709-0

10. Holse M, Husted S, Hansen Å. Chemical composition of marama bean (*Tylosema esculentum*)—A wild African bean with unexploited potential. Journal of Food Composition and Analysis. 2010;23(6):648-657. Doi: 10.1016/j.jfca.2010.03.006

11. Isolation and Characterization of Bacterial Endophytes from Ramie (*Boehmeria nivea*). International Journal of Agriculture and Biology. 2021;25(02):436-440. doi:10.17957/ijab/15.1685

12. Isolation and Identification of Soil Bacteria Collected from Dibru-Saikhowa, the National Park and Biosphere Reserve Forest of Assam, India. International Journal of Science and Research (IJSR). 2016;5(1):1937-1940. doi:10.21275/v5i1.nov153208

13. Jeong H, Choi S-K, Kloepper JW, Ryu C-M. Genome Sequence of the Plant Endophyte *Bacillus pumilus* INR7, Triggering Induced Systemic Resistance in Field Crops. Genome Announcements. 2014;2(5). doi:10.1128/genomea.01093-14

14. Kaga H, Mano H, Tanaka F, Watanabe A, Kaneko S, Morisaki H. Rice Seeds as Sources of Endophytic Bacteria. Microbes and Environments. 2009;24(2):154-162. doi:10.1264/jsme2.me09113

15. Khalifa AY, Alsyeeh A-M, Almalki MA, Saleh FA. Characterization of the plant growth promoting bacterium, *Enterobacter cloacae* MSR1, isolated from roots of non-nodulating

Medicago sativa. Saudi Journal of Biological Sciences. 2016;23(1):79-86. doi: 10.1016/j.sjbs.2015.06.008

16. Leme A, Bevilaqua M, Rhoden S, Mangolin C, Machado M, Pamphile J. Molecular characterization of endophytes isolated from *Saccharum* spp based on esterase and ribosomal DNA (ITS1-5.8S-ITS2) analyses. Genetics and Molecular Research. 2013;12(3):4095-4105. doi: 10.4238/2013.september.2fmic

17. Maayer PD, Chan W, Rubagotti E, et al. Analysis of the *Pantoea ananatis* pan-genome reveals factors underlying its ability to colonize and interact with plant, insect and vertebrate hosts. BMC Genomics. 2014;15(1):404. doi:10.1186/1471-2164-15-404

18. Mayer E. 1758 - Identification of plant growth-promoting bacterial endophytes. 2018. doi:10.26226/morressier.5b5199c4b1b87b000ecee995

19. Pedrosa F, Elmerich C. Regulation of Nitrogen Fixation and Ammonium Assimilation in Associative and Endophytic Nitrogen Fixing Bacteria. Associative and Endophytic Nitrogen-fixing Bacteria and Cyanobacterial Associations Nitrogen Fixation: Origins, Applications, and Research Progress.:41-71. doi:10.1007/1-4020-3546-2_3

20. Poonawala H, Peaper D. Bacterial Identification using 16S rRNA Gene Sequencing in a University Teaching Hospital. Open Forum Infectious Diseases. 2017;4(suppl_1). doi:10.1093/ofid/ofx163.1576

21. Sorani, Nil sari P. Isolation and molecular identification of Endophytic bacteria from Noni fruits (*Orinda vitriolic* l.) and their antibacterial activity. IOP Conference Series: Earth and Environmental Science. 2019; 299:012020. doi:10.1088/1755-1315/299/1/012020

22. SYSTAT, Inc. announces new data transfer package for SYSTAT software on IBM-compatible computers. Computational Statistics & Data Analysis. 1990;9(1):162-163. doi:10.1016/0167-9473(90)90080-2

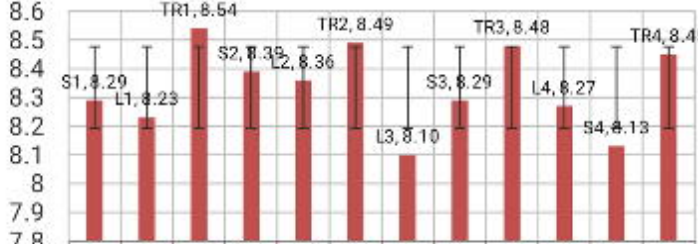
23. The role of statistics and statisticians. Statistical Thinking for Non-Statisticians in Drug Regulation. 2014:316-330. doi:10.1002/9781118470961.ch21

24. Ulla I. Improvement of Cadmium Detoxification Potential and Plant Growth Promotion by Bacterial Endophytes. 2019. doi:10.20944/preprints201908. 0235.v1

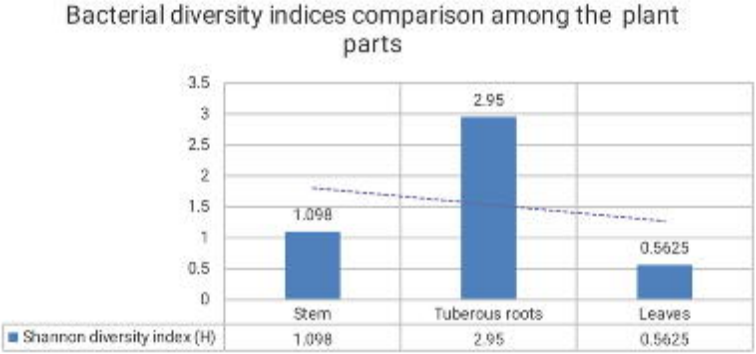
| | Sum of squares | DF | Mean Squares | F | Sig. |
|---|---|---|---|---|---|
| **Between groups** | 14.000 | 10 | 1.400 | 2.000 | 0.018 |
| **Within groups** | 0.000 | 1 | 0.000 | | |
| **Total** | 15.000 | 11 | | | |

DF (Degrees of freedom); F (F-distribution value); Sig (Significance probability)

Mean CFU/ml of the roots, stem & leaves of *Tylosema esculentum*




| | S1 | L1 | TR1 | S2 | L2 | TR2 | L3 | S3 | TR3 | L4 | S4 | TR4 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| log (Mean CFU/ml) | 8.29 | 8.23 | 8.54 | 8.39 | 8.36 | 8.49 | 8.10 | 8.29 | 8.48 | 8.27 | 8.13 | 8.45 |


Bacterial diversity indices comparison among the plant parts
3.5
3
2.5
2
1.5
1
0.5
0
1.098
2.95
0.5625
Stem
Tuberous roots
Leaves
Shannon diversity index (H)
1.098
2.95
0.5625

| Plant part | Mean CFU/ml | Log (mean CFU/ml) |
|---|---|---|
| S1 | $1.04 \times 10^{7}$ | 8.19 |
| L1 | $1.63 \times 10^{7}$ | 8.21 |
| TR1 | $9.50 \times 10^{7}$ | 8.24 |
| S2 | $2.51 \times 10^{7}$ | 8.39 |
| L2 | $2.30 \times 10^{7}$ | 8.36 |
| TR2 | $3.14 \times 10^{7}$ | 8.49 |
| L3 | $1.25 \times 10^{7}$ | 8.10 |
| S3 | $1.03 \times 10^{7}$ | 8.29 |
| TR3 | $2.21 \times 10^{7}$ | 8.48 |
| L4 | $1.40 \times 10^{7}$ | 8.27 |
| S4 | $1.34 \times 10^{7}$ | 8.13 |
| TR4 | $2.72 \times 10^{7}$ | 8.45 |

S (Stem); L (Leaves); TR (Tuberous root); CFU/ml (Colony Forming Units per millilitre)