# Phylogenetic Diversity of Endophytic Bacteria Communities from marama bean *Tylosema esculentum* (Burchell.) A. Schreiber

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ABSTRACT

*Tylosema esculentum* is a nutritious drought-avoiding and climate change contender plant for future agriculture. It is endemic to the Kalahari Desert. This study assessed the density, diversity and distribution of endophytic microbial community structures associated with leaves, stems and tuberous roots of *T. esculentum* in Eastern Namibia using culture-dependent methods. Analysis of Variance with pairwise comparison revealed differences in bacterial density between below and above ground. Endophytic bacterial isolates were identified and grouped into 24 genera and three phyla. *Proteobacteria* were the most represented (67.4%) followed by *Firmicutes* (23.7%) and *Actinobacteria* (4.3%). Shannon diversity index revealed a significant difference between the tuberous roots and leaves ($p = 0.005$) and stems ($p = 0.006$) microbial communities. The PCA confirmed these findings. Our results suggested that the microbial community composition was mainly governed by the plant parts rather than the location or sampling time. The 16S rDNA based phylogenetic analysis showed that all these microbial communities fell into two clades distinct from known cultivated bacteria from NCBI. Our sequences have shown similarities with the ones occurring in water-stressed environments with plant growth promoting traits. In conclusion, *T. esculentum* bean lives in community with a large diversity of potentially plant growth-promoting bacteria.



## 1 Introduction

Bacterial endophytic communities are ubiquitously and symptomlessly contained within plant tissues at the time of the study (Reinhold-Hurek and Hurek, 2011). Bacterial endophytes are vital bio-stimulators committed to triggering host gene expression, bestowing several benefits upon the hosts including improved photosynthetic efficiency, nutrient and water use, tolerance to abiotic and biotic stress and defensive mechanisms (Aloo *et al.*, 2022; Bulgarelli *et al.*, 2013). Recent studies have attested to the physiological significance of endophytic bacterial-mediated drought avoidance and plant growth–promoting activities that are persevered even under critical drought conditions (Ahanger *et al.*, 2016; Bashir *et al.*, 2021; Zhang *et al.*, 2021). Considering that *T. esculentum* survives in harsh drought-prone environments, we hypothesized mechanism of its tolerance to drought to be partly due to endophytic bacteria.

It was recently advocated that significant economic and nutritional advantage can be gained by the increasing sustainable exploitation or even domestication of wild plants occurring on marginal, arid, and semi-arid lands (Alsharif *et al.*, 2020; Gupta *et al.*, 2015). Among prospective climate change-adapted crops, the “orphan crop"

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(Sprent *et al.*, 2010) and "Green gold of Africa" (National Research Council, 2006) *Tylosema esculentum* (Marama bean) stands out as a prominent contender for future agriculture. This drought-avoiding plant is endemic to the Kalahari Desert (Namibia, Botswana and to lesser extend to South Africa) (Castro *et al.*, 2005). Its seed protein and oil content (30-39% and 35-43% respectively) are comparable to that of soya and peanut (Holse *et al.*, 2010; Maruatona *et al.*, 2010). *T. esculentum* has great agricultural potential to produce harvestable material such as seeds and tubers (Holse *et al.*, 2010).

Further, *T. esculentum* can make up an informal self-insurance and an alternative source of income to the local communities since it grows in areas where animal husbandry is the sole source of income. Deciphering *T. esculentum's* endophytic microbial communities is essential for understanding and potentially improving plant-microbe interactions, and further enlightening its potential production sustainability.

The study was designed to isolates, compare their tissue specific (leaves, stems and tubers) density and phylogenetic diversity of bacterial endophytes communities from *T. esculentum*.

# 2 Materials and Methods

## 2.1 Site description and sample collection

The plant materials were collected once a year from 2011 to 2014, during the rainy season in Omitara (S$22°21.596'$, E$18°2.476'$), Harnas (S$21°47.705'$, E$19°19.921'$) and Otjinene (S$21°1333'$, E$18°7667'$) in Eastern part of Namibia. On each site, two healthy plants were collected annually. Tubers, stems, and leaves were separated and kept in different sterile plastic bags. Plant samples were kept in a cooler box for immediate laboratory experiments as described.

## 2.2 Endophytic bacteria isolations and growth conditions

Samples were separately washed using running tap water to dislodge any soil or dust particles. Plant leaves, stems and tuberous roots were then immersed for 30 sec in 70% ethanol solution and afterwards immersed in 100 ml of 2% sodium hypochlorite containing 0.1% Tween 20. To remove the disinfectant, the roots were rinsed five times in two washes of RNase free sterile water. Tubers, stems and leaves were dried on sterile paper towels. Aliquots of the final rinse (100 $\mu$l) were spread-plated on Tryptic Soy Agar (TSA) to check if the disinfection process was successful.

One gram of root, stem or leaf from each sampling site were macerated in a sterile mortar and pestle. Samples were incubated for a week at room temperature in a modified SM medium (Burbano *et al.*, 2015) followed by two-week incubation at $30°C$. The tissue extracts from SM medium were serially diluted in phosphate buffer (up to $10^{-7}$), 0. 1ml were spread-plated in triplicate on VM-ethanol plates and incubated at $30°C$ for 5–7 days (Burbano *et al.*, 2015; Grönemeyer *et al.*, 2011). The colony-forming units (CFU) were determined and were expressed as CFU $g^{-1}$ fresh tissue weight.

## 2.3 DNA extraction and amplification of 16S ribosomal RNA gene

Genomic DNA was extracted from cultures grown in Luria Bertani (LB) using the DNeasy Plant Mini Kit (QIAGEN, GmbH, Hidlen, Germany) as per manufacturer's instructions. Primer pair 27F and 1492R (Weisburg *et al.*, 1991) was used to amplify the 16S rRNA in the thermo-cycler (Bio-Rad, Hercules, CA). The thermal cycle consisted of 1 cycle of pre-denaturation at $94°C$ for 4 minutes, 30 cycles of denaturation at $94°C$ for 1 minute, annealing at $50°C$ for 30 seconds, Extension at $72°C$ for 1 minute, and with a final extension at $72°C$ for 10 minutes. The amplification was performed in 50 $\mu$l containing 0.5 $\mu$M of each primer, 23.5 $\mu$l of nuclease free

water, 25 $\mu$l master mix (Inqaba Biotechnology Industries, South Africa) and 1 $\mu$l of DNA template. PCR products were visualized on 1% agarose gel and sequenced at Inqaba Biotechnology Industries, SA to identify the particular isolates. The 16S rRNA Sanger sequencing was commercially performed at Inqaba Biotechnical Industrie (Pretoria, South Africa). Sequence were aligned and edited using Chromas lite (`http://technelysium.com.au/wp/chromas/`) and Bioedit (`https://bioedit.software.informer.com/7.2/`, version 7.2). Putative bacterial taxonomic affiliation of each library was assigned based on the closest match to sequence using the Classifier program of the Ribosomal Database Project (RDP) release 11 with confidence level of 80%. Phylogenetic trees were constructed using partial rRNA gene sequences of bacterial isolates obtained in this study. The resulting sequences were matched against those available in the GenBank database using BlastN algorithm (Altschul *et al.*, 1997). They were then aligned using the multiple sequence alignement program CLUSTAL X (Thompson *et al.*, 1997) with default parameters. The evolutionary history was inferred using the Maximum Parsimony method.

### 2.4 Statistical analysis

Analysis of Variance (ANOVA) was performed using PAST (Hammer *et al.*, 2001) and SPSS 22.0 Software to detect difference among means of colony-forming units (CFU) of the entire sample collected from different locations and different tissues. The statistical significance of the differences between bacterial densities in samples from the different sampling location over 4 years were tested by two-way ANOVA followed by Tukey's significant difference test (Tukey's HSD, $p < 0.005$). For ecological association, the Principal Component Analysis was performed using PAST software (Hammer *et al.*, 2001).

## 3 Results

### 3.1 Enumeration of *T. esculentum* endophytic bacterial population

Over a period of 4 years 6 samples (leaves, stems and tubers) were collected from each site. Six hundred and fifty endophytic bacterial isolates were obtained. In leaves, the maximum microbial density was recorded in Otjinene, $(3.73 \pm 0.24)10^5$ CFU $g^{-1}$ fresh leaf tissue weight in 2013. The minimum was $(6.3 \pm 0.97)10^3$ CFU $g^{-1}$ fresh leaf tissue weight in 2011 in Harnas (Table 1). In stem tissues, the microbial density ranged $(1.38 \pm 0.13)10^3 - (5.50 \pm 1.2)10^5\, g^{-1}$ CFU fresh stem weight in Harnas in 2013 and 2014 respectively (Table 1). In tuberous root tissue, our results showed that CFU were constantly higher than in stems and leaves. Both the highest root bacteria population density $(5.09 \pm 0.13)10^6\, g^{-1}$ fresh root tissue weight and the lowest $(1.08 \pm 022)10^6\, g^{-1}$ fresh root tissue weight originated from Omitara in 2012.

The total microbial density Two-way ANOVA analysis indicated that the time of sampling ($p > 0.05$), the location of plant samples ($p > 0.05$) and their respective interactions had no effect on the microbial density. It was indicated that at least one of the three plant parts had a mean CFU significantly different from others ($p - \text{value} = 0.000 < 0.05$). Therefore, a pairwise comparison was required. It was revealed that there is a significant difference microbial density between the above ground (stems and leaves) and belowground (tuberous roots).

### 3.2 Identification and Bacterial community structure

In *T. esculentum's* leaves, stems and tubers bacterial endophytes were affiliated to 3 phyla: *Proteobacteria* (class alpha, beta and gamma), *Firmicutes* and *Actinobacteria*. However, of the 605 isolates, one was consisted of unclassified bacteria at the domain level (0.7%), while 4 *Bacillaceae* and 3 *Bacillale* were not classified. In the *Streptomycetacea* family 3 were unclassified. Four and one isolates from the *Xanthomonaceae* and *Pseudomonadaceae*, respectively were also unclassified. The most abundant isolates grouped as unclassified belonged to the *Enterobacteriaceae* family where out of 291 isolates, 126 (43%) were unclassified.

The analysis of all sequences amplification products of the 16S rRNA genes led to the identification of 24 different bacterial genera namely: *Streptococcus, Lactococcus, Brevibacillus, Lysinibacillus, Paenibacillus, Bacillus, Arthrobacter, Curtobacterium, Streptomyces, Ochrobactrum, Rhizobium, Achromobacter, Burkholderia, Acinetobacter, Azomonas, Cosenzaea, Enterobacter, Escherichia/Shigella Klebsiella, Kosakonia, Pantoea, Stenotrophomonas, Pseudomonas, Trabulsiella*, Unclassified *Enterobacteriaceae* Unclassified *Pseudomonadaceae*, Unclassified *Xanthomonadaceae*, Unclassified *Actinomycetales* and Unclassified *Bacilli*.

*Proteobacteria* dominated the collection of sequences, comprising 67.4% of the total isolates with 0.7% of unclassified bacteria (Table 2). The phyla P*roteobacteria* included 3 classes *Alphaproteobacteria* (0.7%), *Betaproteobacteria* (2%) and *Gammaproteobacteria* (97.3%). In the *Gammaproteobacteria*, the most abundant group of sequences was affiliated with the family of *Enterobacteriaceae* (73.4%) followed by the *Moraxellaceae* (15.8%), *Xanthomonadaceae* (7.3%) and *Pseudomonaceaea* (3.5%).

*Firmicutes* (23.7%) included the *Bacillaceae* family (83.9%), the *Planococcaceae* (11.2%) and one genera of the *Paenibacillaceae*. The genera *Bacillus* accounted for 97% of the *Bacillaceae*. *Actinobacteria* (4.3%), *Cyanobaccteria* / *Chloroplast* (0.3%). All three phyla were found across the three sites and all samples.

The highest number of sequences associated with leaves were isolated from members of the *Gammaproteobacteria* (13.22%) and *Firmicutes* (4.1%). The *Firmicutes* were well represented in leaves, stems and tuberous root microbial communities, 4.1%, 5.61% and 17.5%, respectively. A decrease in abundance was observed from tuberous root to the leaves. In contrast, genera *Lactobacillus* and *Streptococcus* were only twice isolated in leaves. This high number was additionally found in stems with 12.33% and 5.61% for *Gammaproteobacteria* and *Firmicutes* respectively.

The *Proteobacteria* were prominent in tuberous roots and mainly restricted to the class *Gammaproteobacteria* with a high abundance of the genus *Enterobacter* (13.74%) followed by the *Acinetobacter* (10.51%). *Alpha* and *Beta proteobacteria* were poorly represented. The phylum *Actinobacteria* had the lowest diversity with only 3 genera represented namely *Arthrobacter, Curtobacterium, Streptomyces* and unclassified *Actinomycetales*. Half of them were found in the tuberous root (2.14% of the total microbial population recorded in this study). The second highest (1.48%) populated plant organ was the stem followed by the leaves (0.66%) (Table 2).

The number of phyla in the three plant organs (leaves, stems and tuberous roots) though the same is characterized by a net dominance of the $\gamma-$*Proteobacteria* class in all plant parts. The four most frequent bacterial taxa (*Bacillus, Acinetobacter, Anterobacter* and the Unclassified *Enterobacteriaceae*) had a widespread presence in leaves, stems and tuberous root. Of the 24 genera isolated from *T. esculentum*, more than 50% of them did not occur in the leaves (*Streptococcus, Lactococcus, Brevibacillus, Paenibacillus, Arthrobacter, Curtobacterium, Ochrobactrum, Rhizobium, Achromobacter, Burkholderia, Azomonas, Cosenzaea, Escherichia/Shigella* and *Trabulsiella*). Similarly, the stems harboured fewer genera than the leaves. For example, isolates of *Brevibacillus, Arthrobacter, Rhizobium, Escherichia/Shigella* were not found in stems while, they were present in leaves. The isolate of *Trabulsiella* was detected in stems, while it was absent in leaves. All recorded genera were found in the tuber with some, such as *Paenibacillus, Curtobacterium, Achromobacter, Azomonas, Cosenzaea* and *Trabulsiella* that were only recovered once in the tuberous root during the whole study period. Each pair of test suggested that distinctly different tuber bacterial communities were present in the different locations and different part of the plants ($p < 0.001$).

## 3.3 Bacterial richness analyses

The Shannon's index ranged from the lowest 1.22 in leaves from Otjinene in 2013 (sample LOTJ2013) to 2.69 found in a tuber in Omitara in 2011 (TOM2011) (Data not shown). In addition, based the one-way analysis of variance (ANOVA) and Tukey's post hoc tests, there is a significant difference between the tuberous root and leaves ($p = 0.005$) and stems' ($p = 0.006$) microbial communities. Simpson's diversity dominance index values

were very low and ranged between 0.72 (sample OM2012) and 1.0 in a tuberous root in Harnas 2012 (Data not shown). It is higher in tuberous root compared to the leaves and stems. This would indicate that the microbial endophytic diversity is more in tubers than leaves and stems. The lowest Simpson dominance value was recorded in stems (0.72) in Omitara 2012, while the highest Simpson dominance index (0.95) was in a tuberous root in Omitara in 2011.

## 3.4 Microbial community analysis of the three sites and plant parts using Principal Component Analysis (PCA)

The original 29 Principal Component (PC) were reduced to 4 main PCs (PC1, PC2, PC3 and PC4) that encompassed 93.2% of the total variance present in the original data. The positive loadings on the first component (PC1) (explaining approximatively 59% of total variation) were moderate for *Bacillus* (0.74) and Unclassified *Enterobacteriaceae* (0.56) which reflects a moderate prevalence with the *T. esculentum* microbial community and weak for *Acinetobacter* (0.31). The second PC (PC2) contributing to variation among the observed taxonomic groups represented approximatively 15% of the variation. Weak and negative loadings for this PC are represented by the taxa *Bacillus* ($-0.33$) and for *Acinetobacter* ($-0.44$) while the Unclassified *Enterobacteriaceae* and the genus *Enterobacter* have moderate loadings of 0.50 and 0.53 respectively. The third component, PC3, explained 12.38% of the variance with loadings ranging from moderate (0.74) for *Enterobacter* to weak and negative ($-0.46$) for the unclassified *Enterobacteriaceae*. While the fourth component explained 6.5853% of the total variance and its loadings only for *Pantoea* (0.76) had a moderate one and others were weak and negative (*Lysinibacillus* ($-0.39$) and unclassified *Enterobacteriaceae* ($-036$).

## 3.5 Phylogenetic analysis of 16S-rRNA sequences of cultured endophytic bacteria

Clade one (red colour) in Figure 1 was made of 9 distinctive classes. Clade one consisted of 51 sequences accounting for 36% of all endophytes isolated. Blast matches for these sequences were close to members of the *Gammaproteobacteria* with 100% similarity index. A close examination of these results indicated that they are all members of the *Enterobacteriaceae* family. Of the 51 sequences, 33 sequences (JD10, JD63, JD28, JD33, JD30, JD8, JD27, JD24, JD9, JD49, JD47, JD14, JD56, JD16, JD4, JD12, JD46, JD48, JD19, JD21, B20, B14, B11, B2, R7, B5, B3, B4, B13, R1, B16, JD39B and B7) formed the majority and polytomic section of this clade. Only 30% of this clade's endophyte sequences had significant identities ($\geq 99\%$) to recorded species in the RDP database. But the use of the 16S rRNA library could not clearly resolve the identity of these species.

This clade included the genera *Enterobacter* (percentage similarity 54-100%), *Pantoea* (percentage similarity 87 to 100%), *Klebsiella* (67 – 80% percentage similarity) and *Citrobacter* with the percentage similarity ranging between 42 and 62%. *Trabulsiella* and *Escherichia/Shigella* had both 38% percentage similarity to the closest strain from the RDP Database while *Raoultella's* was a bit higher (48%). There was a weak phylogenetic support that would lead to a conclusion that 70% of these endophytes were not affiliated to a given genus. The remaining 30% (JD18, JD26 and JD13) were nested with *Citrobacter* with a percentage similarity ranging from 87 to 100% and a bootstrap value of 94. *Pantoea* nested with JD52, JD55, JD31, JD35, JD53, JD22, JD42, JD36, JD3, JD17, JD44, JD20 and JD32 with a moderate bootstrap value (52%) and 100% sequence similarity.

Four sequences derived from the deeply branched clade two (blue) (B6, B17, B18, and B19) and accounting only for 3% of all sequences isolated from *T. esculentum*. They have been identified as *Acinetobacter* based on 16S rRNA sequence similarity (100%) of known taxa present in RDP database with a 99% bootstrap value. This attested that B6, B17, B18 and B19 all belong to the genus *Acinetobacter*.

Clade three (green) had five clusters (R8, JD5, JD34, JD61, and B10) which accounted for 4%. Their sequences showed 100% similarity with their best BLAST hits. This resulted in these clades being strongly supported with good bootstrap values ranging from 65 to 99%. R8 (*Burkholderia*) and JD6 (*Bacillus*) sequence

formed a distinct and moderately-supported clades with a boostrap value of 65% and 57% respectively. Their sequence similarity (100%) clades within *Betaproteobacteria* and *Firmicutes*. JD8, JD34, JD61, B10, JD37and JD23 were all supported by a strong bootstrap value (99%) and a 100% sequence similarity clearly show they were respectively related to *Pseudomonas, Bacillus, Rhizobium, Paenibacillus* and *Lysinbacillus*.

The taxonomic placement of endophytes belonging to Clade 4 (maroon) showed similarity among the six sequences ranged between 67 and 100%. Sequences JD15, R6 and B12 were virtually identical displaying 100% sequence similarity. These 3 sequences belonged to 2 classes with JD14 and B12 sharing the upper class with a 99% bootstrap while R6 though sharing the same sequence similarity, showed a strong but lower bootstrap value (94%) suggesting all these sequences belong to the same genus *Bacillus*. JD25 sequence, however, though clustering with B12 and JD15 showed 67% sequence similarity with its best RDP database hit which resulted in phylogenetic placements with moderate bootstrap support. Accordingly, JD25 sequence is likely represented a new genus given its low sequence similarities (67%) with Bacillus strains. Finally, JD23 sequence was the only *Lysinibacillus* belonging to clade 4 with 100% sequence similarity and 99% bootstrap support and JD 37 was the only *Paenibacillus* of this clade. However, it had 91% similarity sequence with a strong bootsrap value suggesting the latter might represent a new genus. JD23 uniquevocally represent the genus *Lysinibacillus*. This was supported by the high sequence similarity and a strong bootstrap value.

In clade 5 (purple), samples sequences belonged to 3 classes (D4, NN1, UZ4, UZ5 D2; D14, D15 NN10 and UZ3). With the exception of UZ3 sequences which belongs to *Xylella* genus with 47% sequence similarity, other sequences (D4, NN1, UZ4, UZ5 D2; D14, D15 and NN10) had 100% sequence similarity to the RDP database and fell into *Xanthomonadaceae* family and *Stenotrophomonas* genus. However, D4, NN1, UZ4, UZ5 and D2 sequences were supported with a strong bootstrap value (83%) while D14, D15 and NN10 showed a lower bootstrap value of 61%. With the exception of UZ3 despite its strong bootstrap value (94%) which is most likely a new genus, other sequences probably belonged to the *Stenotrophomonas* genus.

The 18 sequences derived from clade 6 (black) were divided into 6 classes that represented 18% of all selected sequences. Of all sequences of this clade, 72% exhibited $\geq 99\%$ similarity to its closest relatives. Despite their subdivision in different classes, all members of this clade belong to the *Bacillus* genus. They are all well supported with bootstrap values ranging from 57% for XY3 to 99% (D6 and UZ6). However, some sequences (XY4, b1, 25, UZ6) showed at least 87% sequence similarity with their best BLAST hit in RDP database which resulted in phylogenetic placements with good bootstrap support. They most likely represent a new genus given their low sequence similarities (87% at least and 98% at the most) with *Bacillaceae* type strains.

The 27 sequences that comprised the clade 7 (dark green) were supported by a moderate bootstrap value (66%). They account for 21% of all sequences of which only 14% exhibit $\geq 99\%$ similarity to its closest relative. The current clade comprised 3 classes where the third clade is made of 2 deep branching parts (4, 12, 16, 14, and 19) and (XY1, B2, R3, 22, 17, 20 JD31). These 2 classes are supported by a high to moderate bootstrap values, 86% and 65% respectively. All these sequences (4, 12, 16, 14, and 19) have sequence similarity below the accepted threshold. Like the previous group, these sequences (XY1, B2, R3, 22, 17, 20 JD31) did not cluster with any reference strain from the database. However, our phylogeny had established a close relationship of these species to the members of the genus *Enterobacter*, but due to their weak sequence similarity to the known database strains, their identity cannot clearly be resolved to any particular *Enterobacteriaceae*. Between the two deep branching classes, there was a more polytomic section of this class (Figure 1). The bootstrap value is moderate (65%) and their sequence similarity are far below the recommended threshold (Figure 1). As a result, there were no strong phylogenetic signals to conclude on their genus affiliation.

In phylogenetic studies, a 16S rRNA sequence identity between 98.5% and 99% is currently considered to be the absolute boundary for species restriction. Sequences (D7, NN6, NN4, D19, UZ14, D18, NN12, D22) and (13, 18, 26) constituted class one and two respectively. Despite their very close similarity in DNA sequence and phylogenetic affiliation to the *Enterobacter* and *Citrobacter* genus, there was no reference strain from the

database that clustered with them. Taking all this into consideration, there is a weak support to confirm that these sequences might be belonging to these two genera of *Enterobacter* and *Citrobacter*.

Cluster 8 (dark green) (Figure 1) contained sequences B8 and B15 that clustered with significant support. The bootstrap value was 98%, while the sequence similarity was 100%. These high values would indicate that these isolates belonged to the *Acinetobacter* genus. However, these isolates never clustered with any reference strain from the database. In this case more data is needed to confirm the reliability of the isolates identification. Given the sequences obtained in this work were only mostly 700 - 800bp and were not perfect matches to the known type strains, it can be assumed that the bacterial isolates obtained in this study had unique strains Stackebrandt and Ebers, (2006) in (Burbano *et al.*, 2015).

# 4 Discussion

The numerical average of endophytes associated with *T. esculentum* in leaves, stems and tuberous roots were in the range of 106 CFU $g^{-1}$. This average was higher than figures found in other studies (Mercado-Blanco and Lugtenberg, 2014). However, higher density ($10^9$ to $10^{13}$) were accounted in 20 days old maize plant (Cavaglieri *et al.*, 2009). Plate counts were constantly significantly higher in tuberous roots as compared to leaves and stems. Root exudates are important constituent of the plant micro-ecological system. They control mutual plant-microbial communities and nutrient exchange in the rhizosphere (Sessitsch *et al.*, 2012) de facto increasing their density. This agreed with our results where microbial density was higher in tubers compared to the above ground parts.

Plant leaf tissues are subjected to abiotic factors that can act as selective components to the leaf niche for the formation of an adapted bacterial endophytic communities. Additionally, while maturing, the accumulated carbohydrates in leaves are channeled into the grains (Ferrando *et al.*, 2012; Méndez-Espinoza *et al.*, 2021). Consequently, the mature leaf nutrient quality declines. Similarly, the stem key specific tissue could provide a homeostatic sucrose rich environment and yet be poor in secondary metabolites (Emiliani *et al.*, 2014). These conditions may select for bacterial communities that would have adapted and developed a strong interaction with the *T. esculentum* communities in above ground niche. The wide distribution of genera accounted in *T. esculentum* has been documented as endophytes with plant growth promotion traits (Emiliani *et al.*, 2014). Additionally, most sequences obtained in this study, have shown similarities with those from stressed microbes occurring in environmental samples from arid, semi-arid, cold desert and contaminated soils (Chowdhury *et al.*, 2009).

All the three above-ground phyla *Proteobacteria, Firmicutes* and *Actinobacteria* comprise 38% of the total *T. esculentum* above ground microbial community. Almost 42% of all isolated taxa recorded were confined in leaves (*Lysinibacillus, Bacillus, Streptomyces, Acinetobacter, Enterobacter, Klebsiella, Kosakonia, Pantoea, Stenotrophomonas*, and *Pseudomonas*). A lower diversity was found in stems. However, several taxa recorded in leaves were absent in stems. Members of these three phyla were the most abundant in the endophytic communities of *Crocus albiflorus* (Reiter and Sessitsch, 2006); radish leaf (Seo *et al.*, 2010); Arabidopsis and Citrus leaves (Bodenhausen *et al.*, 2013) and olive oil leaf (Muller *et al.*, 2015).

*T. esculentum* bacterial endophytic communities shared several similar adaptation traits with endophytic communities of other plant species. The *Gammaproteobacteria* dominance has been observed in *Catalystegia soldanella* and *Elymus mollis*, two sand dune plant species (Kim, 2005), poplar trees (Ulrich *et al.*, 2008) and seagrass (Jensen *et al.*, 2005) growing in marginal soils, and pearl millet, sorghum and maize grown in Kavango East (Namibia) (Grönemeyer *et al.*, 2011). In the tuber, the class *Alphaproteobacteria* was the most prevalent. Similar findings were observed in *Lavandula angustifolia* (Emiliani *et al.*, 2014) and desert soils (Makhalanyane *et al.*, 2013, 2015). Organ plant-specific microbial distribution might attest that *T. esculentum* microbial community colonization was not passive, at least at a certain developmental stage (Bulgarelli *et al.*, 2013). This assumption

was later confirmed in root-associated rice microbiomes (Edwards *et al.*, 2015).

The smooth plant–*Enterobacteriaceae* (*Klebsiella, Enterobacter, Citrobacter, Pseudomonas* and several others yet to be unidentified) interaction was facilitated by the animal–soil–plant rhizosphere nutritional cycle and their motility (Kennedy *et al.*, 2004). They are renowned for their cellulase and pectinase enzyme production, capacity to metabolize a widespread variety of sugars and plant growth promoting bacteria (Kennedy *et al.*, 2004).

*Firmicutes* accounted for 23.7% of the total isolates in *T. esculentum* and were represented by the *Bacilli* and *Clostridiales*. Member of the *Firmicutes* phyla have been reported in desert soils and plants mainly the *Clostridia* class (Makhalanyane *et al.*, 2013). This class was dominant in rhizospheric soils associated with Antarctic vascular plants (Makhalanyane *et al.*, 2013). This emphasized Firmicutes importance in arid environments (Makhalanyane *et al.*, 2013). In *T. esculentum*, the genus *Bacillus* was the most dominant. According to (Malfanova *et al.*, 2011) evidence suggest that the genera Bacillus have been shown to be able to produce siderophores, IAA and antibiotic compounds which can be effective against deleterious effects of phytopathogen agents. Berendsen *et al.* (2012) have attested that plant genotypes can affect the accumulation of microorganisms that help the plant to defend itself against pathogen attacks. Finally, Oa *et al.* (2008) also demonstrated that the excretion of malic acid via plant exudates was responsible for *Bacillus* recruitment. Moreover, some members of the genus Bacillus can produce the $\alpha-$amylase enzyme responsible for starch hydrolysis (Makhalanyane *et al.*, 2013). Conversely, it would be premature to correlate the strong presence of *Bacillus* in *T. esclentum* tuber with the starch hydrolysis trait of some Bacillus genera. There is no data to support *Bacillus*' presence with the amount of starch present in the tuber. The relative abundance of the genus *Bacillus* in *T. esculentum* and particularly in the tuber could be due to the plant tolerance of this specific genus and its recruiting abilities for specific functions.

*Actinobacteria* were poorly represented and were mostly found in tubers (2.14%) with three genera namely *Arthrobacter, Curtobacterium, Streptomyces* and unclassified *Actinomycetales*. *Endophytic Actinobacteria* isolated from wheat were able to suppress fungal pathogens both in vitro and in vivo. They induced systemic acquired resistance (SAR) in *Arabidopsis thaliana* (Jiang *et al.*, 2013). *Endophytic Arthrobacter* have been previously found in wheat (Conn *et al.*, 2008), root and stems of black pepper (*Piper nigrum* L.) (Aravind *et al.*, 2009). *Endophytic Streptomyces* strains with the ability to produce IAA in vitro have been isolated from plants occurring in poor sandy soil and arid climatic conditions of the Algerian Sahara (Benhadj *et al.*, 2019; Messaoudi *et al.*, 2015).

*Proteobacteria, Firmicutes* and *Actinobacteria* phyla that were constantly found in *T. esculentum* specific organs across samples can be predicted to have a critical function in microbial communities. The dominance of *Proteobacteria* and *Firmicutes* reflect the ability to survive desert conditions that are known for their nitrogen and carbon deficiency. Hence, describing the interaction of these bacteria with the host plant would be of great interest with regards to our knowledge about the plant–microbe interactions and thereafter their economic potential and environmental benefits in agriculture. Very little is known about *T. esculentum* interactions with the microbial community in natural ecosystems.

Principal component analysis was performed using PAST software (Hammer *et al.*, 2001). Considering the composition of the first component (PC1), the taxa present seemed to be fairly representative of the plant growth promoter in the most represented phyla *Firmicutes* and *Proteobacteria*. It can be suggested that *Bacillus, Acinetobacter* and unclassified *Enterobactericeae* were selected for their plants growth promoting traits to compensate for the lack of nutrients in the Kalahari soils (Lü *et al.*, 2009; Romanens *et al.*, 2019). These microbes are adapted to elastic environments as their cycle can survive between wet and dry season.

The microbial dominance in the second component differs from the PC1's. In PC2, we had *Klebsiella* which is phylogenetically related to *Acinetobacter* and *Enterobacter*. This strong differentiation of endophytic communities belonging to the *Enterobacteriaceae* family suggested that their selection is strongly influenced by the recruitment of the rhizosphere microbiome and the genotype of the host (*T. esculentum*).

In this study, the change in Shannon–Wiener index ranged from 1.22 in Otjinene. After one way ANOVA analysis and Tukey test, it was concluded that there is a significant difference between the tuber and leaves ($p = 0.005$) and stems ($p = 0.006$) microbial communities. This trend reflects that bacterial diversity became abundant presumably because of the combined effects of root exudates and environmental conditions especially pedo-climatic conditions (Marasco *et al.*, 2012; Soussi *et al.*, 2016). The reduced microbial diversity in the above ground plant tissues may reflect specific physiological requirements to enter the interior of the leaf and stem to establish endophytic populations.

In the tuber, the higher microbial diversity might be the result of the rhizosphere effect. It has been demonstrated that it is pronounced especially in nutrient-poor soils and under severe abiotic stresses, as previously observed for herbaceous and arboreal plants grown in arid lands (Marasco *et al.*, 2013). This might be the case in the Kalahari Desert known for its poor sandy soils with very little water retention capacity coupled with its extreme environmental conditions. The higher values of the Simpson dominance in the tubers (0.84 in OM2013, 0.87 in OM2012, 0.85 in HARN2014, 0.90 in HARN2011 and 0.80 in OTJ2011) could be explained by the tuber's species specificity with genus such as *Streptococcus, Lactococcus, Curtobacterium, Ochrobactrum, Burkholderia, Rhizobium, Achromobacter, Azomonas, Consenzaea, Trabulshiella* and the unclassified *Pseudomonadaceae* that were at least recorded once. However, a comparison of the dominance values for microbial communities in different plant parts of *T. esculentum* using ANOVA, it was revealed there was no significant difference in microbial community dominance between leaves, stems and tubers ($p = 0.20$).

Using the 16S rRNA, 49% the threshold percentage (98.7-99%) at 16S rRNA loci has shown to correspond to phylogenetically delimited species in the domain Bacteria. Thus, showing that in this study; there might be a high proportion of putatively new species in endophytes associated with *T. esculentum*. Furthermore, there was no clustering by geographic origin or plant organs observed in our samples. This was in line with Grönemeyer *et al.* (2011) who considered this as consistent boundary in species delimitation. The reliability of the alignment was confirmed using the E-value that indicated that the probability of obtaining an alignment a great number of reported taxa (49%) showed the identity similarity values below 98.7-99%.

# 5 Conclusion

This study presented evidence that time and sampling sites had no impact on the endophytic numerical microbial community recruitment of *T. esculentum's* in its native environment. However, there is a clear niche plant partitioning microbial density between below (tuberous roots) and above (leaves and stems) ground parts. The tuberous roots harbored the highest microbial density. Additionally, sequencing and BLAST analysis of 16S rRNA gene revealed that *T. esculentum* isolates species formed a tight sub-cluster, strongly supported by bootstrap analysis distinct from their nearest match from the NCBI database. It was shown that *T. esculentum* plants contain a reservoir of undiscovered bacterial diversity. Generally, plants growing in unique environments or interesting endemic locations possess novel endophytic micro-organisms which can supply new leads. Hence, it can be suggested that members of these endophytic phyla described above that were constantly found in plant specific organs across host plant species, have a critical function in microbial communities.

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**Conflict of Interest**

The authors declare that no conflict of interest exists.

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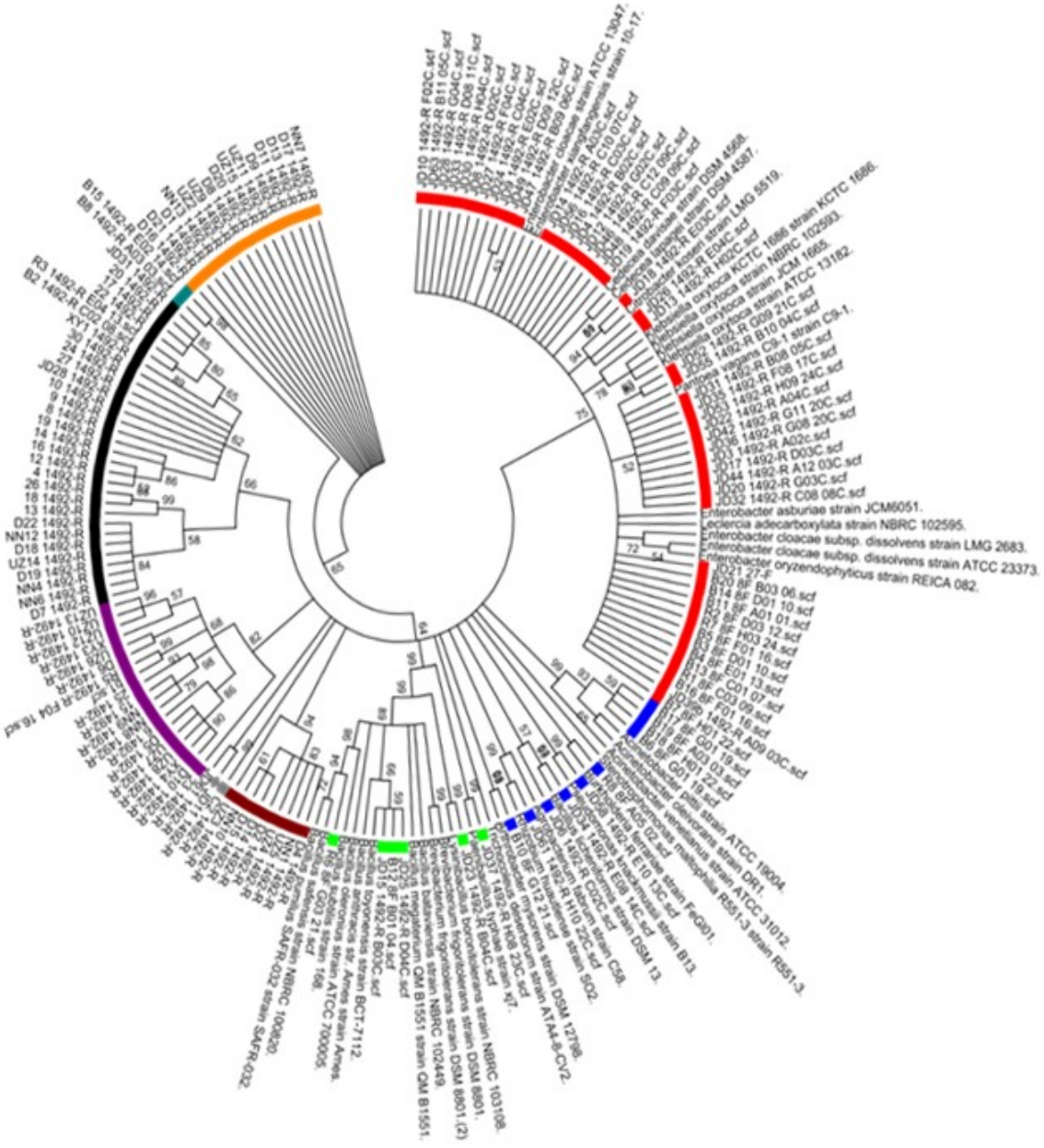


**Figure 1:** Strict consensus of the most parsimonious tree inferred using 16S rRNA sequences associated with *T. esculentum* based on 16S rRNA sequences. The phylogenetic tree represents a maximum parsimony analysis of 143 taxa with their reference strain. Bootstrap values higher than or equal to 50% (500 replicates) are shown at each branches.

**Table 1:** Enumeration of putative endophytic diazotroph bacteria isolated from aseptic fresh *T. esculentum* tissue leaves (L), stems (S) and tuberous roots (T) (means of three replicates, ± standard deviation) from different locations (Omitara, Harnas and Otjinene) over a period of 4 years (2011-2014).

| | | 2011 | 2012 | 2013 | 2014 |
|---|---|---|---|---|---|
| | | CFU $g^{-1}$ fresh plant weight | | | |
| $Omitara_1$ | L | $(3.2 \pm 0.7)10^5$ | $(2.9 \pm 0.42)10^5$ | $(2.7 \pm 0.22)10^5$ | $(2.40 \pm 0.55)10^5$ |
| | S | $(2.2 \pm 0.6)10^5$ | $(1.6 \pm 0.52)10^5$ | $(2.4 \pm 0.54)10^5$ | $(1.04 \pm 0.3)10^5$ |
| | T | $(2.24 \pm 0.32)10^6$ | $(1.08 \pm 0.22)10^6$ | $(1.2 \pm 0.45)10^6$ | $(1.5 \pm 1.3)10^6$ |
| $Omitara_2$ | L | $(2.4 \pm 0.08)10^5$ | $(2.72 \pm 0.26)10^5$ | $(1.66 \pm 0.35)10^5$ | $(2.20 \pm 0.25)10^5$ |
| | S | $(1.51 \pm 0.18)10^5$ | $(2.11 \pm 0.15)10^5$ | $(2.26 \pm 0.43)10^5$ | $(0.75 \pm 0.3)10^5$ |
| | T | $(3.57 \pm 0.05)10^6$ | $(5.09 \pm 0.13)10^6$ | $(1.87 \pm 0.12)10^6$ | $(1.19 \pm 2.7)10^6$ |
| $Harnas_1$ | L | $(6.3 \pm 0.97)10^3$ | $(2.54 \pm 0.12)10^4$ | $(1.97 \pm 0.92)10^5$ | $(1.65 \pm 0.16)10^5$ |
| | S | $(1.39 \pm 0.11)10^5$ | $(2.28 \pm 0.98)10^5$ | $(1.58 \pm 0.17)10^5$ | $(5.50 \pm 1.2)10^5$ |
| | T | $(1.24 \pm 0.07)10^6$ | $(1.73 \pm 0.05)10^6$ | $(1.17 \pm 0.11)10^6$ | $(1.88 \pm 0.47)10^6$ |
| $Harnas_2$ | L | $(2.04 \pm 0.52)10^5$ | $(1.09 \pm 0.13)10^5$ | $(2.49 \pm 0.41)10^5$ | $(1.62 \pm 0.35)10^5$ |
| | S | $(2.51 \pm 0.31)10^5$ | $(1.99 \pm 0.11)10^5$ | $(1.38 \pm 0.13)10^4$ | $(2.06 \pm 0.72)10^5$ |
| | T | $(2.02 \pm 0.07)10^6$ | $(1.16 \pm 0.04)10^6$ | $(3.02 \pm 0.15)10^6$ | $(2.23 \pm 0.42)10^6$ |
| $Otjinene_1$ | L | $(1.29 \pm 0.49)10^5$ | $(5.47 \pm 0.14)10^4$ | $(3.73 \pm 0.24)10^5$ | $(0.5 \pm 0.11)10^5$ |
| | S | $(1.22 \pm 0.89)10^5$ | $(3.17 \pm 0.18)10^4$ | $(2.34 \pm 0.03)10^4$ | $3.2 \times 10^5$ |
| | T | $(1.26 \pm 0.07)10^6$ | $(2.22 \pm 0.14)10^6$ | $(1.97 \pm 0.92)10^6$ | $(1.45 \pm 0.42)10^6$ |
| $Otjinene_2$ | L | $(2.37 \pm 0.77)10^3$ | $(1.84 \pm 0.12)10^4$ | $(1.57 \pm 0.62)10^5$ | $(0.39 \pm 0.03) \times 10^5$ |
| | S | $(1.19 \pm 0.16)10^5$ | $(2.78 \pm 0.68)10^5$ | $(1.28 \pm 0.37)10^5$ | $64 \times 10^4$ |
| | T | $(1.94 \pm 0.27)10^6$ | $(1.43 \pm 0.15)10^6$ | $(1.37 \pm 0.21)10^6$ | $(1.85 \pm 0.25)10^6$ |

**Table 2:** Phylum and class distribution frequencies (%) of endophytic bacteria associated with *T. esculentum's* tissues (leaves, stems and tuberous roots).

| | Firmicutes | Actinobacteria | Proteobacteria | | |
|---|---|---|---|---|---|
| | | | $\alpha-$Proteobacteria | $\beta-$Proteobacteria | $\gamma-$Proteobacteria |
| Leaves | 25 (4.1%) | 4 (0.66%) | 0 | 0 | 80 (13.22%) |
| Stems | 34 (5.61%) | 9 (1.48%) | 2 (0.33%) | 0 | 74 (12.23%) |
| Tuberous roots | 106 (17.52%) | 13 (2.14%) | 6 (0.99%) | 3 (0.49%) | 243 (40.16%) |