ARTICLE

Variations in enzymatic activity and response to abiotic factors of endophytic bacteria isolated from Narra (*Pterocarpus indicus*) growing in a forest environment and urban arboretum

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Endophytic microorganisms are plant-colonizing microorganisms that cause no significant harm to the host. They are widely studied due to their production of secondary metabolites that are useful for the pharmaceutical, agricultural and food industries. The production of these metabolites also helps plants to better adapt to their environment. The species composition of endophytes can be greatly affected by environmental conditions and the location of their hosts. This study aimed to compare species and enzymatic production of endophytes isolated from Narra (*Pterocarpus indicus*) growing in two different locations in the Philippines i.e. the Mount Makiling Forest Reserve (MMFR) and the UP Arboretum in Quezon City. The results show that there was a higher number of bacterial genera isolated from MMFR compared to UP Arboretum. *Staphylococcus, Pseudomonas, Serratia, Pantoea*, and *Microbacterium* were uniquely found in the MMFR while *Lysinibacillus* was only found in the UP Arboretum. More isolates from MMFR were able to produce amylase, cellulase, chitinase and protease than those from the UP Arboretum. On the other hand, a greater percentage of endophytes from the UP Arboretum were able to produce laminarinase, L-asparaginase and xylanase. The results agree with previous studies that show that location affects the physiological activity of endophytes.

KEYWORDS

endophytes, enzymatic activity, Mt. Makiling Forest Reserve (MMFR), UP Arboretum

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INTRODUCTION

Endophytic microorganisms are endosymbionts that are widespread among plants, colonizing intercellularly and/or intracellularly without causing any significant infection to the

Table 1: Environmental variables measured for two sampling sites.

	UP Arboretum	MMFR
Climate	Wet season	Wet Season
Temperature (minimum)	24.590 ± 0.094	24.270 ± 0.100
Temperature (maximum)	31.620 ± 0.155	30.850 ± 0.239
Average Relative Humidity	79.090 ± 0.526	89.430 ± 0.4708
Average Rainfall	10.300 ± 1.824	10.660 ± 1.901
Common Vegetation in the Area	*Bauhinia* sp. *Swietenia* sp. *Leucaena leucocephala*	Bagtikan (*Parashorea malaanonan)*, Balete (*Ficus balete)*, Salisi *(Ficus benjamina),* and Balilang Uak (*Meliosma pinnata* Roxb*)*

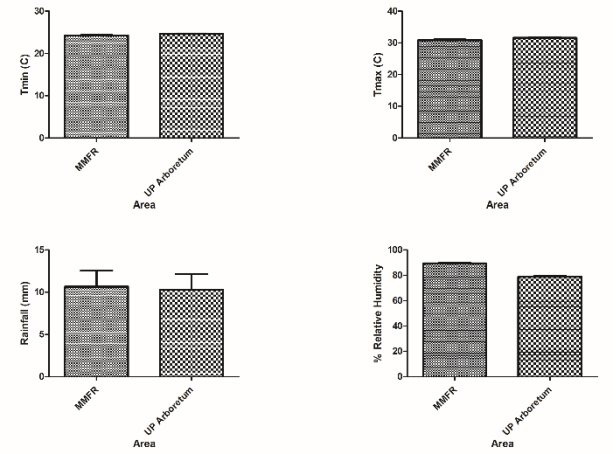

Figure 1: Environmental parameters in UP Arboretum and Mount Makiling Forest Reserve (MMFR) from August to December 2017. Data from DOST-PAG-ASA

host (Nair and Padmavathy 2014; Schulz and Boyle 2006; Holliday 1989). Several studies have emphasized their ability to influence plant growth, disease resistance, abiotic stress tolerance, water retention, and production of plant hormones (Wang et al. 2019). Interspecies interaction is exhibited by these endophytic microorganisms through the production of chemical signals that become essential to the hosts' survival fitness (Dhayanithy et al. 2019). Endophytic microorganisms also promote phosphate solubilization, indole acetic acid production and production of siderophore that help in plant growth promotion (Ryan et al. 2008). Furthermore, endophytes also contribute to plants' osmotic adjustment, stomatal regulation, modification of root morphology, and enhanced uptake of minerals (Compant et al. 2005). Secondary metabolites produced in large amounts by these endophytes can be useful for pharmaceutical, agricultural and food industries (Strobel et al. 2004; Strobel and Daisy 2003). Examples of these metabolic products are antibiotics, anticancer, antiviral and antifungal compounds, volatile organic compounds, immunosuppressants and insecticidal agents (Ryan et al. 2008). Endophytes are also considered good sources of extracellular enzymes such as hydrolases, lyases, oxidoreductases, and transferases targeting various macromolecules to break down into transportable products (Traving et al. 2015). From the study of Carrim et al. (2006) and Sturz et al. (2000), bacterial endophytes are known to produce aminocyclopropane-1-carboxylic acid (ACC) deaminase, cellulases, protease, amylase, pectinase, esterase, lipase, protease, asparaginase, phytase, etc.

The diversity and population of these endophytic microorganisms are greatly affected by climatic condition and location where the host plant grows (Nair and Padmavathy 2014; Dhayanithy et al. 2019). Plants and trees in different forest areas, such as natural and urban forests, are often subjected to different climactic and environmental factors. Urban forests, as opposed to natural forests, are often more vulnerable as they are affected by various stressors and disturbances (Steenberg et al. 2017). These disturbances in the ecosystem may, therefore, affect the diversity of the microecosystem of a specific plant/tree. The Mount Makiling Forest Reserve (MMFR) located in UP Los Baños is considered an ASEAN Heritage Park located at 14°8' north and 121°12' east and lies 65km south of Metro Manila. The forest reserve covers about 4,224 hectares across 80% of the mountain. On the other hand, the University of the Philippines Arboretum is a 16-hectare man-made forest located beside the UP Diliman main campus and is dubbed as the last remaining rainforest in Quezon City (Galauran and Hidalgo 2015). In this study, variation of bacterial endophytes isolated from *Pterocarpus indicus* leaf samples growing within the Mount Makiling Forest Reserve (MMFR) and those growing in an urban space, UP Arboretum were compared. It is hypothesized that the differences in the environmental conditions where their host plants live affects their enzymatic activities and tolerance to different abiotic stresses.

MATERIALS AND METHODS

Sample Collection. Ten grams of healthy mature leaf samples were collected from three randomly selected Narra trees (*Pterocarpus indicus*) growing in each of the two sites, Mt.

Table 2: Identities of endophytic bacteria isolated from Narra (*Pterocarpus indicus*) growing in MMFR and UP Diliman Arboretum.

Bacterial Genera	MMFR	UP Arboretum
Bacillus	*B. pumilus* (NLSB1), *B. subtilis* (NLSB2), *B.aerius* (NLSB3), *B. toyonensis, B. altitudinis* (5 Bacillus species)	*Bacillus sp.* (NL1B1), *B. weidmannii* (NL1B2), *B. subtilis* (NL1B3), *B. marisflavi* (NL2B2), *B. cereus* (NB1B1), *B. circulans* (NB1B3), *B.amyloliquefaciens* (NB2B1, NB2B2)
Staphylococcus	*Staphylococcus saprophyticus* (NLCB1), *S. sciuri* (NLSB8)	
Pseudomonas	*Pseudomonas azotoformans* (NLSB4)	
Serratia	*Serratia marcescens* (NLCB2, NLCB3)	
Pantoea	*Pantoea dispersa* (NLCB4)	
Microbacterium	*Microbacterium sp.* (NLSB10)	
Lysinibacillus		*Lysinibacillus macroides* (NB1B6)
	6 Bacterial Genera	**2 Bacterial Genera**

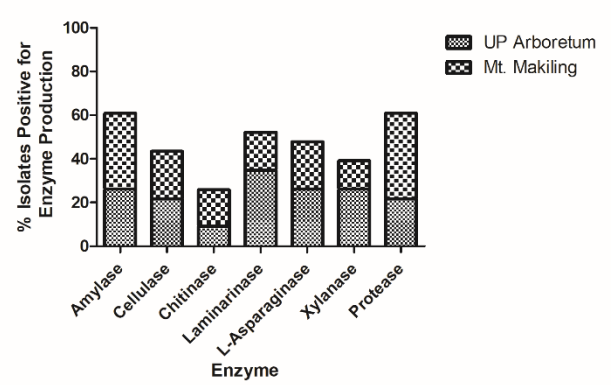

Figure 2: Percentage of isolates from MMFR and UP Arboretum capable of producing enzymes responsible for biomass degradation.

Makiling Forest Reserve, Los Baños Laguna and UP Arboretum, Quezon City. Parameters such as climate, average temperature, average relative humidity and average rainfall were acquired from the Philippine Atmospheric, Geophysical and Astronomical Services Administration (PAGASA). Samples from the field were transported in a cooler and immediately processed in the laboratory. Surface sterilization of leaves was done by washing the leaf samples with distilled water to remove attached materials. The leaves were subsequently immersed in 70% ethanol, washed with hypochlorite solution, rinsed with 70% ethanol, and finally washed with sterile distilled water.

Isolation of Endophytes. Surface sterilized leaves were cut into 5-mm^2 segments and were placed into petri plates containing Nutrient Agar, NA, (5 g peptone, 3 g beef extract, 5 g NaCl, 15 g agar per 1 liter of water) for bacterial growth. Five replicated plates with 20 5-mm^2 segments were prepared for each sample. Plates were incubated at 37°C for 48 h. Following incubation, morphologically distinct bacterial colonies were isolated and purified on NA. Pure cultures were then qualitatively screened for enzyme activities. The water used in the last wash of the leaves was also plated in NA to test for effectivity of leaf sterilization.

Screening for Xylanase, Cellulase and Amylase Activity. Qualitative analysis for xylanase, cellulase and amylase activity was based on Kim et al. (2014) with minor modifications for amylase and xylanase activities ie. instead of carboxymethylcellulose (CMC), 1% soluble starch and 1% xylan was used as sole carbon source, respectively. Pure isolates were point-inoculated on minimal salts agar plates (containing 0.5% CMC, 0.1% $NaNO_3$, 0.1% K_2HPO_4, 0.05% $MgSO_4$, 0.1% KCl, 0.05% yeast extract, 1.5% agar for cellulose agar plates; 1.0% xylan, 0.1% yeast extract, 0.5% NaCl, 0.1% K_2HPO_4, 0.02% $MgSO_4$, 0.01% $CaCl_2$, Na_2CO_3, and 1.5% agar for xylan agar plates; and 0.3% beef extract, 1% soluble starch and 1.2% agar for starch agar plates) and incubated at 37°C for 24 h. Following incubation, plates for the detection of xylanase and cellulase activity were flooded with 1% Congo red for 15 min. The Congo red solution was then poured off and plates were further flooded with 1 M NaCl for 15 min. Colonies exhibiting zones of clearing were taken as positive cellulose-degrading bacterial colonies. Plates for the detection of amylase activity were flooded with Gram's iodine for three to five min and zones of clearing were observed. Quantitative assays for cellulase and amylase were conducted as described by Kim et al. (2014) using xylan from Birchwood, CMC and soluble starch as 1% substrate solutions. Standardized bacterial isolates, using a 0.5 McFarland standard, were grown on minimal salts broth for 48 h at 37°C. After incubation, cultures were centrifuge at 12,000 rpm for 3 min, and the supernatants were collected. The reaction mixture of 200 µl of crude enzyme solution, 200 µl of appropriate 1 % substrate solutions were incubated at 37°C for 60 min. After incubation, 1600 µl of 3,5-Dinitrosalicylic acid (DNS) solution was added to the reaction mixture and boiled in a water bath for 20 min. From the mixture, 200 µl was transferred to a 96-well microplate and analyzed at 570 nm with a microplate reader (BMG LABTECH Spectrostar® Nano) to determine the amount of reducing sugar liberated. Series of xylose, glucose and maltose standards were independently prepared for DNS standard curve development of xylanase, cellulase and amylase activities, respectively.

Screening for Protease Activity. Skim milk agar plate containing 1% peptone, 0.5% NaCl, 2% agar and 10% skimmed milk was used in screening for protease activity. Pure isolates were point inoculated and incubated at 37°C for 24 h. After incubation, the diameter of the colonies was measured as well as the zones of clearing. Quantitative protease assay was performed by inoculating standardized inoculum, using a 0.5 MacFarland standard, in protease production medium (0.5% peptone, 1% glucose, 0.05% NaCl, 0.01% $CaCl_2$. $2H_2O$, 0.03% K_2HPO_4, 0.04% KH_2PO_4, 0.01% $MgSO_4$.7H2O, and 1.0% skim milk powder) and incubating at room temperature for 48 h (Gaur et al. 2010). Following incubation, protease activity was determined

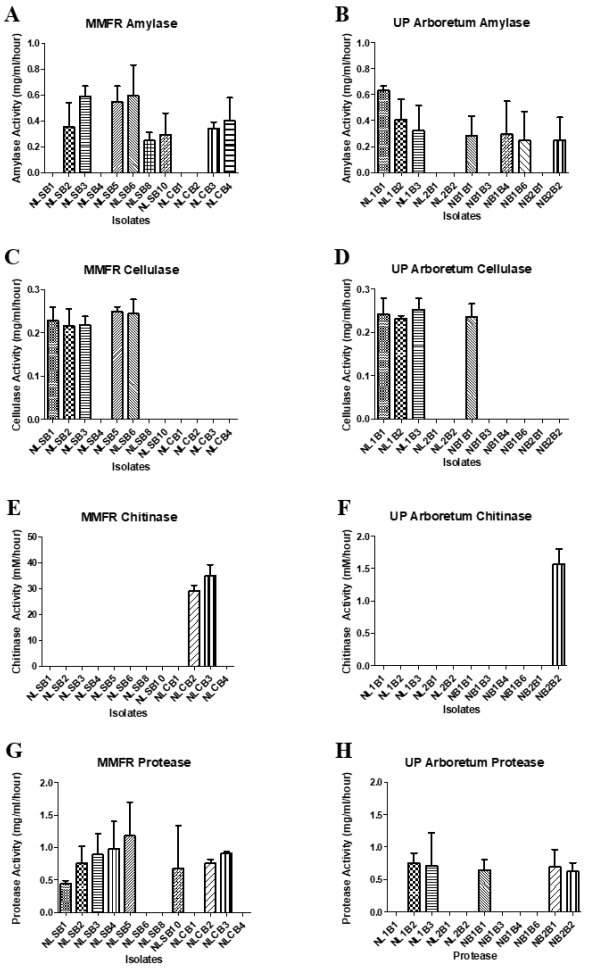

Figure 3: Quantitative screening of the different isolates for their enzymatic activities.

using the method described by Cupp-Enyard (2008) with few modifications. Casein was used as substrate and the amount of liberated tyrosine was measured at 660 nm using a UV-Vis Spectrophotometer (BMG LABTECH Spectrostar® Nano).

Screening for Chitinase Activity. Chitinase activity was determined using the method of Murthy and Bleakley (2012). Colloidal chitin (CC) medium (pH 7.0±0.2), composed of the following ingredients: 0.07% K_2HPO_4, 0.03% KH_2PO_4, 0.05% $MgSO_4.5H_2O$, 0.001% $FeSO_4.7H_2O$, 0.0001% $ZnSO_4$, and 0.0001% $MnCl_2$) was **s**upplemented with 2.0% moist colloidal chitin. One hundred microliters of pure isolates with 0.1 OD_{600} were inoculated in CC broth and incubated at 37°C with shaking (150 rpm) for 3-5 days. After incubation, the amount of *N*-acetyl-*D*-glucosamine produced was measured as quantitative chitinase activity following the protocol of Gómez Ramírez et al. (2004).

Screening for L-asparaginase Activity. Rapid plate assay method was used in screening for L- asparaginase producing microorganisms. A modified M9 medium was used (1% L-asparagine, 0.152% K_2HPO_4, 0.052% $MgSO_4.7H_2O$, 0.003% $CuNO_3.3H2O$, 0.005% $ZnSO_4.7H_2O$, 0.2% glucose, 0.003% $FeSO_4.7H_2O$, 0.052% KCl, 1.8% agar supplemented with 0.009% phenol red (prepared in ethanol and pH adjusted to 6.2 using 1 M HCl) as an indicator. Isolates were streaked on the medium and incubated at 28°C for 24-48 h. Isolates producing pink haloes were selected for further screening. *Escherichia coli* 25922 strain was used as positive control.

Screening for Laminarinase Activity. Laminarinase screening was based on the procedure of Kim et al. (2014) with minor modifications. A concentration of 0.1% of laminarin from *Laminaria digitata* (Sigma ®) was used as sole carbon source in 2% Bacto agar to selectively grow laminarin-degrading microorganisms. Isolates were point-inoculated onto the agar plates and incubated at 28°C for 48 h.

Abiotic Stress Tolerance. Temperature, salinity, and pH tolerance assays were determined qualitatively. Isolates were streak-inoculated to different culture media. For the temperature stress assay, NA plates were incubated at 28°C, 37°C and 42°C. The tolerance to salinity of the isolates was determined by point inoculation to NA supplemented with 0%, 3%, 6%, 9%, 12% and 15% NaCl. For the pH tolerance assay, the ability of the isolates to grow on NA adjusted to pH 2, 4, 6, 10 and 12 was determined. All plates were incubated at room temperature and assessed for growth after 24 h (Meléndez et al. 2017). All tests were carried out in triplicates.

Genomic DNA Extraction. Boil lysis method (Ahmed et al. 2014) was used for the extraction of bacterial genome. One ml of 24-h culture was centrifuged at 10,000 rpm for 5 min. The supernatant was discarded, and the pellet were resuspended in 1 ml of sterile double distilled water. The suspension was vortexed and boiled at 100°C for 15 min using a dry block heater (Premier®) and then centrifuged at 10,000 rpm for 5 min. The supernatant was collected and kept at -20°C for further use.

Polymerase Chain Reaction. Identification of the isolates through PCR amplification of the 16S rDNA gene using the universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1541R (5′-AAGGAGGTGATCCAGCCGCA-3′) (Weisburg et al. 1991) was performed. The reaction mixture consisted of 1.0 µL of each primer, 2 µL of 100 ng template DNA, 25 µL of master mix (MyTaqTM Mix, BIOLINE) and 21 µL of sterile water for a total of 50 µL. The thermocycling conditions were initial denaturation of 94° C for 4 min, 30 cycles of denaturation at 94° C for 30 s, annealing at 52.9° C for 30 s, extension at 72° C for 1 min and final extension at 72° C for 4 minutes.

Sequence Data Analysis. Sequencing of 16S rDNA of the isolates was done by Macrogen Inc., South Korea. Partial 16S rDNA sequences were imported into, checked for sequencing quality, and trimmed using the Staden Package software (Staden 1996) after which the consensus sequences were compiled and matched to the reference strains found in the National Center of Biotechnology Information (NCBI) GenBank database through BLASTn (Basic Local Alignment Search Tool) (NCB1, 2013).

RESULTS AND DISCUSSION

Sampling from UP Arboretum and Mount Makiling Forest Reserve

Samples were collected during the wet season of August to December 2017 in the UP Arboretum and Mt Makiling Forest Reserve (MMFR). The different environmental parameters of the sites are given in Table 1. Based on the unpaired *t*-test analysis, the two locations had significant differences in terms of minimum temperature, maximum temperature and relative humidity, while no significant differences were observed in average rainfall (Figure 1). The differences could be due to their location, wherein the MMFR is located at the upper slope of Mount Makiling, 1090 meters above sea level, while UP Arboretum is situated in the urban area of Quezon City, 70

meters above sea level. The MMFR sampling site is comprised of a large of number of endemic flora surrounding subwatersheds of Mt. Makiling. Other trees growing around the sampling site were Bagtikan (*Parashorea malaanonan)*, Balete (*Ficus balete)*, Salisi *(Ficus benjamina),* and Balilang Uak (*Meliosma pinnata* Roxb*)* (Terminal Report Forest-CANOPI Program, 2018). The UP Arboretum, on the other hand, houses 77 plant species, with the most common trees being *Bauhinia* sp., *Leucaena leucocephala*, and *Swietenia* sp. (UPDate Diliman Online n. d., Gubalane unpublished thesis, 2018).

Isolation and Enzymatic Activities of Endophytic Microorganisms

A total of 23 distinct bacterial isolates were purified and identified from the two sites. Twelve bacterial isolates were obtained from Narra growing at the MMFR site and 11 isolates were obtained from the UP Arboretum. Each isolate was screened for its ability to produce enzymes responsible for biomass degradation. Results showed that 14 out of the 23 isolates exhibited amylase activity, 10 had cellulase activity, 6 had chitinase activity, 12 had laminarinase activity, 11 had L-asparaginase activity, 9 had xylanase activity and 14 possessed protease activity. More isolates from MMFR were able to produce amylase, cellulase, chitinase and protease than those from the UP Arboretum (Figure 2). On the other hand, a greater percentage of UP Arboretum isolates were able to produce laminarinase, L-asparaginase and xylanase. Fig. 3 shows that chitinase was produced by only two isolates from MMFR, and one isolate from the UP Arboretum. For the amylase and cellulase activities, isolates NL1B1 and NL1B3 of UP Arboretum were observed to have the highest enzymatic activity, with an average of 0.631mg/ml/h and 0.253mg/ml/h respectively. Isolate NLSB5 isolated from MMFR showed the highest protease activity with an average of 1.179mg/mL/h. The production of these extracellular enzymes targets various macromolecules such as carbohydrates, lignin, organic phosphate, proteins and sugars polymers needed for growth and development (Strong and Claus 2011). These enzymes are also needed for the establishment of host symbiosis and for counteracting plant pathogenic infections (Leo et al. 2016; Tan and Zou 2001).

Tolerance of Endophytes to Different Abiotic Conditions

The endophytes were also tested for their ability to grow under different conditions of pH, temperature and salt concentration. Figure 4 shows the percentage of isolates growing best in different temperature conditions. Six of the UPArboretum isolates, NL1B1, NL1B2, NL1B3, NL2B1, NB1B3, and NB1B4, and 3 of MMFR isolates, NLSB1, NLSB6 and NLSB8, were capable of growing at 42°C. It was also noted that isolate NLSB5 of MMFR grew best at 28°C, while no endophytes from UP Arboretum grew best at this temperature. There was a higher number of endophytes in UP Arboretum capable of growing at higher temperature (42°C). From the environmental data, UP Arboretum experiences a higher maximum temperature than MMFR. Furthermore, MMFR exhibits higher relative humidity than the UP Arboretum. It is possible that these conditions might have selected for more organisms capable of tolerating higher temperature conditions. High and low temperatures could destroy photosynthetic apparatus and cell membranes, and endophytes could help protect host plants from these extreme damages (Mei and Flinn 2010).

Isolates were also grown in pH 4, 6, 8, 10, and 12. All isolates preferred relatively neutral conditions, from pH 6 to pH 8, although isolates NLSB2, NLSB4, NLSB5, NLSB8, NLCB1, NLCB2, NLCB3, and NLSB4, from MMFR, and isolates NL1B2, NL2B1, NL2B2, NB1B1, NB1B3, NB1B4 and NB1B6, from UP Arboretum were also capable of growing at pH 10. These results show that the endophytes were capable of growing

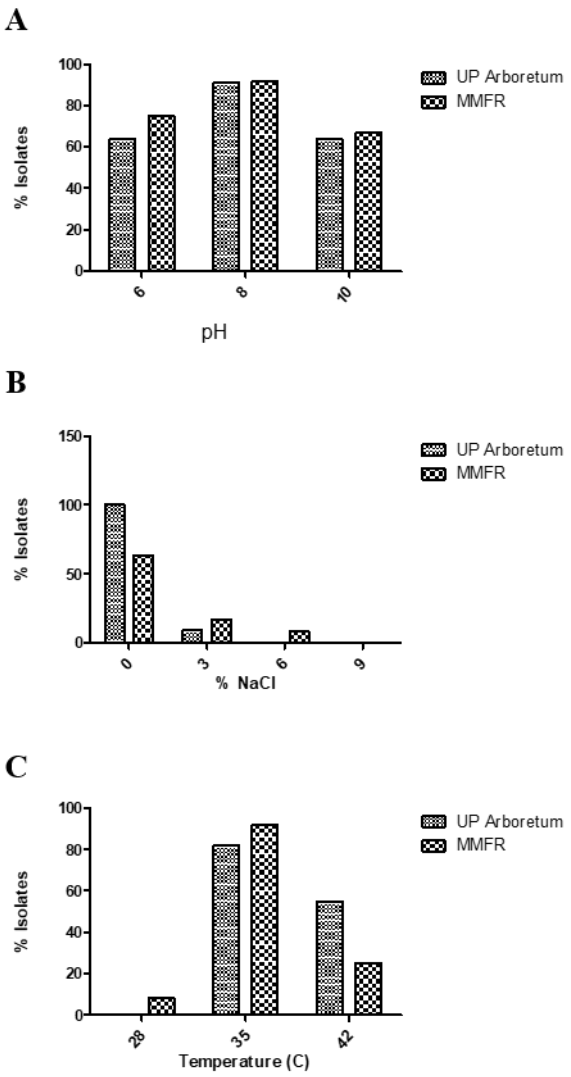

Figure 4: Percentage of isolates growing at their optimum under different abiotic conditions.

at a wide range of pH. According to Adejumo and Olugbenga (2010), some bacteria have a broad pH range, and their populations can be affected by the pH of the habitat. Increase in pH usually stresses and eliminates fungi, that makes bacteria and actinomycetes dominate (Yamanaka 2003).

Isolate NLCB1 from MMFR was capable of growing at up to 12% salt concentration, and isolate NL2B1 from UP Arboretum up to 9% salt concentration. All isolated bacterial endophytes grew best without salt. The salt tolerance of these two endophytes is much greater than the salinity present in their actual habitats. Endophytic microorganisms have been shown to induce salt tolerance in host plants by producing antioxidants (Baltruschat et al. 2008) and are of great biotechnological value to agriculture. According to Mei and Flinn (2010), the presence of endophytic microorganisms contribute to enhanced plant tolerance against biotic and abiotic stresses in order to attain better growth and development and biomass yield. Endophytic microorganisms producing phytohormones, solubilizing phosphate and inhibiting ethylene biosynthesis contribute to plant positive response to biotic and abiotic stresses (Singh et al., 2017). Other environmental factors such as carbon dioxide and nitrogen could also have an effect on the endophyte-host plant interaction, wherein the higher the carbon dioxide and nitrogen content the higher the biomass of endophyte present (Hunt et al. 2005).

Identification of Endophytic Microorganisms

The identities of the isolates were determined through 16S rDNA sequencing and are presented in Table 2. Six bacterial species were observed in MMFR while two genera were observed for the UP Arboretum. *Staphylococcus, Pseudomonas, Serratia, Pantoea,* and *Microbacterium* were unique to MMFR while *Lysinibacillus* was only found in the UP Arboretum.

Members of the *Bacillus* genera are the most prevalent species in samples obtained from both locations. *B.* species such as *B. subtilis* and *B. amyloliquefaciens* are known plant growth-promoting rhizobacteria that could suppress plant pathogen and accelerate host plant growth (Kloepper et al. 1999). The presence of *Bacillus* spp. in the leaf could indicate the passive colonization of endophytes from the roots as its main gateway to other plant parts. From the study of Hardoim et al. (2008), colonization of endophytes of their host plant could occur through the mechanism of bacterial motility, the growth of roots or by mechanisms that could allow the colonization passively.

The presence of *Pseudomonas* species as endophytes also contributes to the ability of plants to degrade organic compounds that can be used as energy source (Porteous-Moore et al. 2006). Differences in isolated microbial community could vary from one location to another and could also differ due to environmental conditions. Salinity, chlorophyll concentration, wind, precipitation, solar radiation, conductivity and dissolved organic carbon are just some of the environmental and spatial factors that could also affect microbial community composition (Pagaling et al. 2009; Kirschner et al. 2002). Botella et al. (2010) also demonstrated that water availability, shade, light exposure, age, and elevation influenced endophytic communities. From this study, isolates with the highest chitinase activity belonged to the genus of *Serratia.* Isolates with highest enzymatic activities in terms of amylase, cellulase and protease belonged to the genus *Bacillus*. From the study of Ryan et al. (2008), bacterial genera, such as *Pseudomonas, Burkholderia* and *Bacillus*, are well-known producers of a diverse range of secondary metabolic products which include antibiotics, anticancer compounds, volatile organic compounds, antifungal, antiviral, insecticidal, and immunosuppressant agents.

Different genera of culturable endophytic bacteria colonizing the leaf of Narra were found to be specific for the location where the host plant thrived. The ability of these endophytes to produce extracellular enzymes was also demonstrated to vary between the two locations. Effects of other biotic and abiotic stresses that were not measured in this study could have also affected the activity of bacterial endophytes.

CONCLUSION

The variation of species and enzymatic activities of endophytes associated with Narra trees of UP Arboretum and MMFR were studied. Endophytes isolated from MMFR belonged to more bacterial genera. Furthermore, more isolates were able to produce amylase, cellulase, chitinase and protease than those from the UP Arboretum. On the other hand, a greater percentage of endophytes from the UP Arboretum were able to produce laminarinase, L-asparaginase and xylanase. *Bacillus* species were noted to be the most prevalent on both locations and are well-known for their plant growth-promoting activities. Temperature maximum and minimum as well as relative humidity were found to be significantly different between the two locations. Other biotic and abiotic factors not measured in this study could also play a significant role in the observed differences.

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