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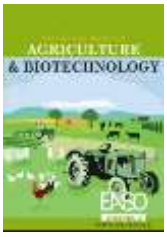

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Original Article

Diversity and Prevalence of Indigenous Soil Bacillus spp. in the Major Cabbage Growing Agroecological Zones of Uganda

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ABSTRACT

Different species of genus *Bacillus* have been reported from different environments of the world. They are reported to play a role of soil fertility improvement, plant growth promotion and disease and pest management. Most of these reports on Bacillus species are from studies conducted outside Uganda and therefore information on the prevalence and diversity of bacillus species in Ugandan soils is scanty. This study aimed at determining the prevalence and diversity of *Bacillus* spp. isolated from the cabbage rhizosphere in the four major cabbage-growing agroecological zones of Uganda. The experiment was conducted in a laboratory at the College of Veterinary Medicine, Animal Resources and Biosafety Makerere University for morphological and biochemical identification of the *Bacillus* bacteria. DNA extraction and PCR were conducted at the College of Natural Resources Makerere University while sequencing was done at Macrogen laboratories in Korea and Inqaba Biotec in South Africa. Morphological, biochemical and genomic analyses revealed five *Bacillus* spp. (22 Bacillus strains) grouped as *B. cereus, B. mycoides, B. thuringiensis, B. megaterium and B. bingmayongensis. B. cereus* and *B. megaterium* were the most dominant and widely spread *Bacillus* spp. A phylogenetic tree indicated three major clads, showing that *B. thuringiensis* was closely related to *B. cereus* while *B.bingmayongesis* was closely related *to B.megaterium.* The *B. mycoides* were closely related to some *B. cereus* strains and *B. bingmayongensis. The* phylogenetic tree further showed that some Bacillus strains of the same species were distantly related. It was therefore concluded that most abundant and prevalent *Bacillus* spp. in Ugandan soils were *B. cereus* and *B. megaterium*. The presence and abundance of these bacillus species in the Ugandan soil presents an opportunity for soil scientists to innovatively manipulate them for use as biofertilizers and biopesticides for crop production and management. Such innovations would reduce the reliance of farmers on synthetic fertilizers that are pollutants to the environment and unhealthy to the users and consumers.

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INTRODUCTION

Soil comprises of different bacterial genera including *Bacillus, Azospirillum, Pseudomonas, Azotobacter, Klebesiella, Enterobacter, Alcaligens, Arthrobacter, Burkholderia and Rhizobia* and these provide health benefits to the soil and plant relations (Bashan *et al*., 2014, Hirsch, 2018). The strains from genera *Bacillus*, *Pseudomonas* and *Agrobacte rium* play role in biological control of plant and soil pathogens (Hayat *et al*., 2010; Pérez-García *et al*., 2011; Sindhu *et al*., 2014; Mascarin and Jaronski, 2016). Most bacterial communities are dominant in the rhizosphere (Rousk, et al. 2010; Farina et al., 2012 and Tahir et al., 2015).

B*acillus* is the major bacterial genus that exists in soil, and produces many valuable antibiotics (Tabbene et al., 2009; Qiao et al.,2014; Cherif-Silini *et al,* 2016) due to production of antibacterial substances, enzymes and nutritional factors (Frikha-Gargouri *et al*., 2017 and Zhang *et al*., 2018). Majority of *Bacillus spp*. are non-pathogenic with the exception of *Bacillus anthracis* and *Bacillus cereus which cause anthrax and food poisoning respectively (*Spencer, 2003). *Bacillus spp*. are gram positive and ubiquitous in nature (Nicholson, 2002; Lyngwi and Joshi, 2014; Cote *et a*l., 2015) and *a*erobic or f*a*cult*a*tive *ana*erobes (Longan & Halket, 2011). They can form endospores within cells that provide high resistance to radiation, desiccation, UV light, heat and chemicals, which gives them the capacity to survive under adverse conditions (Mandic-Mulec et al., 2016)

Bacillus spp. exhibit functional differences due to different compounds produced and some show a narrow spectrum of activity, while others have a broad spectrum (Abriouel et al., 2011). The beneficial *Bacillus* spp. such as *B. megaterium*, *B. circulans*, *B. coagulans*, *B. subtilis*, *B. azotofixans*, *B. macerans*, *B. velezensis*, *B. pumilus*, *B. thuringiensis*, and *B. mycoides* are reported as *Plant growth-promoting rhizobacteria* (PGPR) (Zhang, *et al*., 2009; Basurto-Caden, *et al*., 2012; Fan *et al., 2018)* due to their capacity to fix nitrogen, mineralization of phosphorus and other nutrients, phytohormone production, production of antimicrobial compounds and hydrolytic enzymes (Senthilkumar et al., 2009; Goswami *et al., 2016).* The beneficial *Bacillus spp*. associate with plant roots in the rhizophere and develop biofilms to increase plant growth as biofertilizers and biopesticides (Beauregard *et al., 2013*; Kang *et al*., 2015b, Goswami *et al*., 2016). Whereas *Bacillus* spp. are reported essential in the soil, information on their prevalence and diversity in Ugandan soils is scanty. There is no study that has

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been done in Uganda on the *Bacillus* spp. in the soil. The objective of this study was to determine the prevalence and diversity of indigenous soil *Bacillus* spp. in the four agro-ecological zones of Uganda. The study hypothesis was "the genetic diversity of indigenous soil *Bacillus* spp. strains differs across the major cabbage growing agro-ecological zones of Uganda".

MATERIALS AND METHODS

The soil samples were collected from four agro-ecological zones of Uganda, West Nile, Mid Northern, Lake Victoria crescent and Southern highlands (Fig. 1). From each zone, five cabbage fields were randomly selected making a total of twenty cabbage fields for soil sampling. The names of farmers growing cabbage were obtained from the agricultural extension officers at the sub counties of the sampled districts. Soil samples were obtained from the rhizosphere of cabbage plants at the time of head formation to ensure enough time for the microorganisms to perform their activities. Cabbage rhizosphere was preferred because being a commercial green vegetable, the presence of *Bacillus* spp. could be useful biofertilizers and biopesticides for enhancing its growth. The rhizosphere was selected as a source of soil samples because most active soil microorganisms are found in zone (Rodrigo et al., 2013; Bulgarelli et al., 2013; Zhang et al., 2017). Sampling was done diagonally in a traverse at a distance of 5 meters apart using clean sterile spatula and a dry polythene bag. Ten spots were sampled for a composite sample (Motsara, 2008). From each cabbage field, 500cm^3 of soil were collected. The soil samples were transferred to the laboratory in an ice box at 4^{0}C to prevent damage to bacterial DNA (Chen et al., 2021; Pavlovska et al., 2021)

Figure 1. Soil Sampling Sites

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The experiment was conducted in a laboratory at the college of Veterinary Medicine, Animal Resources and Biosafety Makerere University to determine the morphological and biochemical identification of the *Bacillus* bacteria. *Bacillus spp*. were isolated by the serial dilution and spread plate method (Filippi *et al.*, 2011). The 1g of each soil sample was dissolved in 10mls of phosphate buffered saline (PBS, pH 7.2) followed by serial dilution (Chilcott et al., 1993). After dilution, the solution was heated at 80^0C for 15 minutes to kill vegetative organisms and leave only spore-forming bacteria. Then 100 µL of each dilution was inoculated into nutrient agar plates containing LB broth (El-Gayar, *et al*., 2020) supplemented with 50 µg ml–1cycloheximide (Sigma) to prevent fungal growth (Wattiau *et al.*, 2001) and incubated at 37 °C for 24 hours (Toulouse, 2016). The inoculated agar plates were laid in a completely randomized design (CRD) in the laboratory. This design was preferred because the experimental units have homogenous conditions (Salkind, 2010; Festing, 2014).

The purified colonies were identified according to Berge's manual of determinative bacteriology (Bergey, 2004). A microscope of 100 magnification was used to observe the colonies. The Gram-positive, rod-shaped, spore-forming *bacilli* were then selected for biochemical tests. Catalase test, starch hydrolysis, sucrose fermentation, nitrate reduction, methyl red test, citrate test and urease test were all performed for the bacterial species following standard procedures (Chauhan & Jindal, 2020). *Bacillus* spp. *were* stored in tryptic soy broth containing 6% yeast extract and glycerol (Cagri-Mehmetoglu, 2012) at -20°C, in an airtight container, away from light and moisture (Adama, 2018).

DNA extraction and PCR were conducted at the College of Natural Resources Makerere University Kampala. DNA was extracted from the bacteria using Quick-DNA™ Fungal/Bacterial Miniprep kit using manufacturer instructions (ZymoResearch Corporation, USA, CA) and was quantified using a Nanodrop to amplify the 16S rRNA gene. Use of 16S rRNA gene sequencing is ideal for identification of bacteria (Chauhan & Jindal, 2020). Universal 16S rRNA gene primers 27F(5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) were used. Thermo cycling was done with an initial heating at 94 ◦C for 3 min, denaturation at 94 ◦C for 25 s, annealing at 58^0C for 30 s, elongation at 72 ◦C for 50 s, and a final extension step at 72 ◦C for 3 min. The PCR products were separated and visualized on a 1.5% agarose gel along with the Gene Ruler BH 1kbp DNA Ladder RTU（100-1,500 bps). PCR products were cleaned and sequenced using sanger at Macrogen laboratories in Korea and Inqaba Biotec in South Africa.

A phylogenetic tree was constructed to reveal the genetic closeness of the different bacillus strains. The forward and reverse ABI files generated from the Sanger sequencer were first assembled into a single counting file and the merged AB1 files converted into fasta format using Tracy Version 0.7.5. The fasta files for each sample were merged into a single multifasta file. Multiple sequence alignment was then performed on the multifasta file using MAFFT Version 7.310 and the phylogenetic tree was constructed using the Maximum likelihood method in MEGA version 11 using the default parameters with 1000 bootstrap replications. The resultant tree generated was then imported into *R* version 4.2.1 and manipulated using the *ggtree* package.

RESULTS

Morphological and Biochemical Identification of *Bacillus spp*.

Morphological and biochemical tests, revealed 40 *Bacillus* spp. from soil samples obtained from the four-cabbage-growing agroecological zones of Uganda. The 40 isolates were rod shaped with different sizes of colonies; small, medium and large. Gram staining and microscopy work revealed that all isolates were Gram positive while Biochemical

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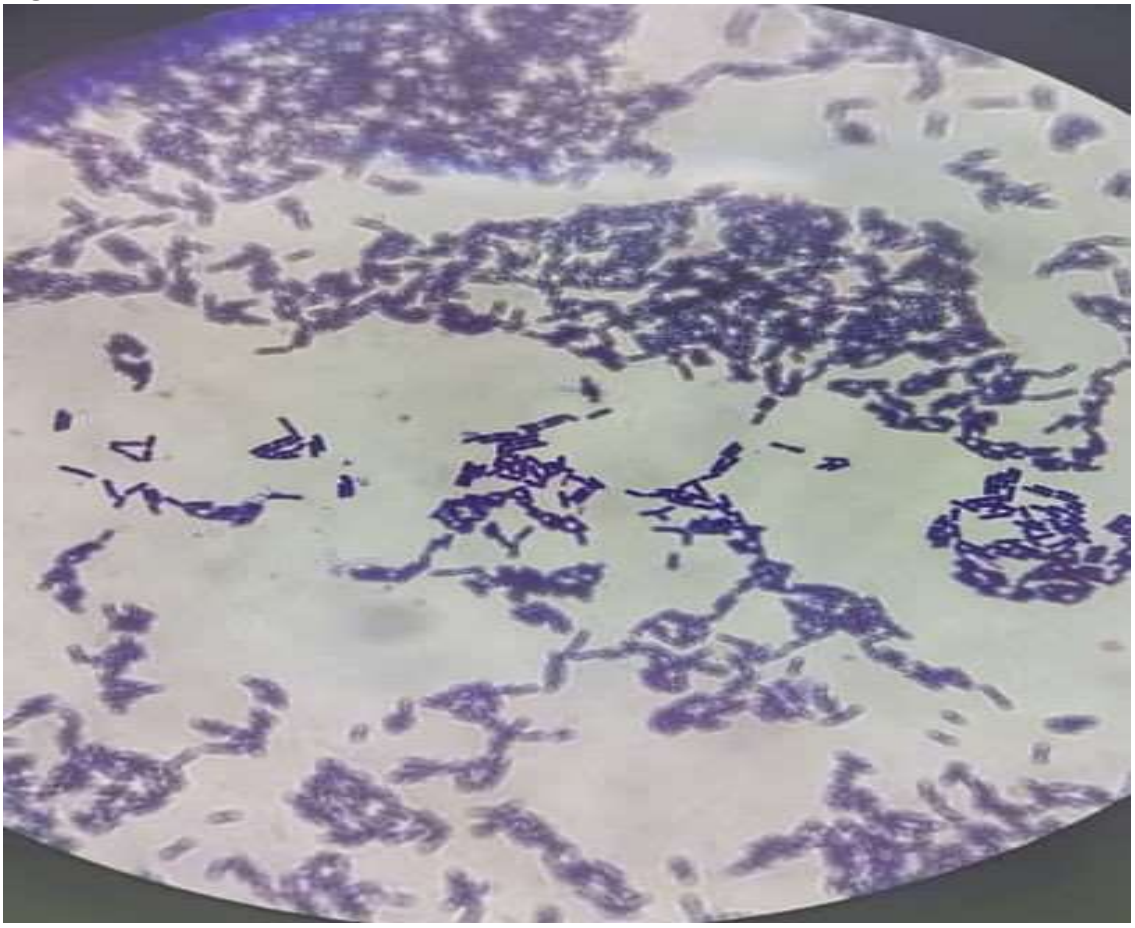
tests showed positive results for Catalase, Urease, Oxidase, starch hydrolysis, glucose utilization and nitrate reduction tests (table 1).

Table 1: Morphological and biochemical characteristics of *Bacillus* strains isolated from cabbage rhizosphere.

Colony shape	Rod shaped
Gram reaction	+ve
Voges proskauer's	+ve
Citrate utilization	+ve
Catalase activity	+ve
Glucose utilization	+ve
Nitrate reduction	+ve
Starch hydrolysis	+ve

Figure. 2: Gram Positive Isolates



Molecular Characterization of the Bacillus spp.

Molecular analysis identified 22 bacillus strains from the four major cabbage growing agroecological zones of Uganda. The PCR products were separated and visualized on a 1.5% agarose gel along with the Gene Ruler BH 1kbp DNA Ladder RTU（100-1,500 bps) (Fig 3).

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Figure. 3. Gel electrophoresis

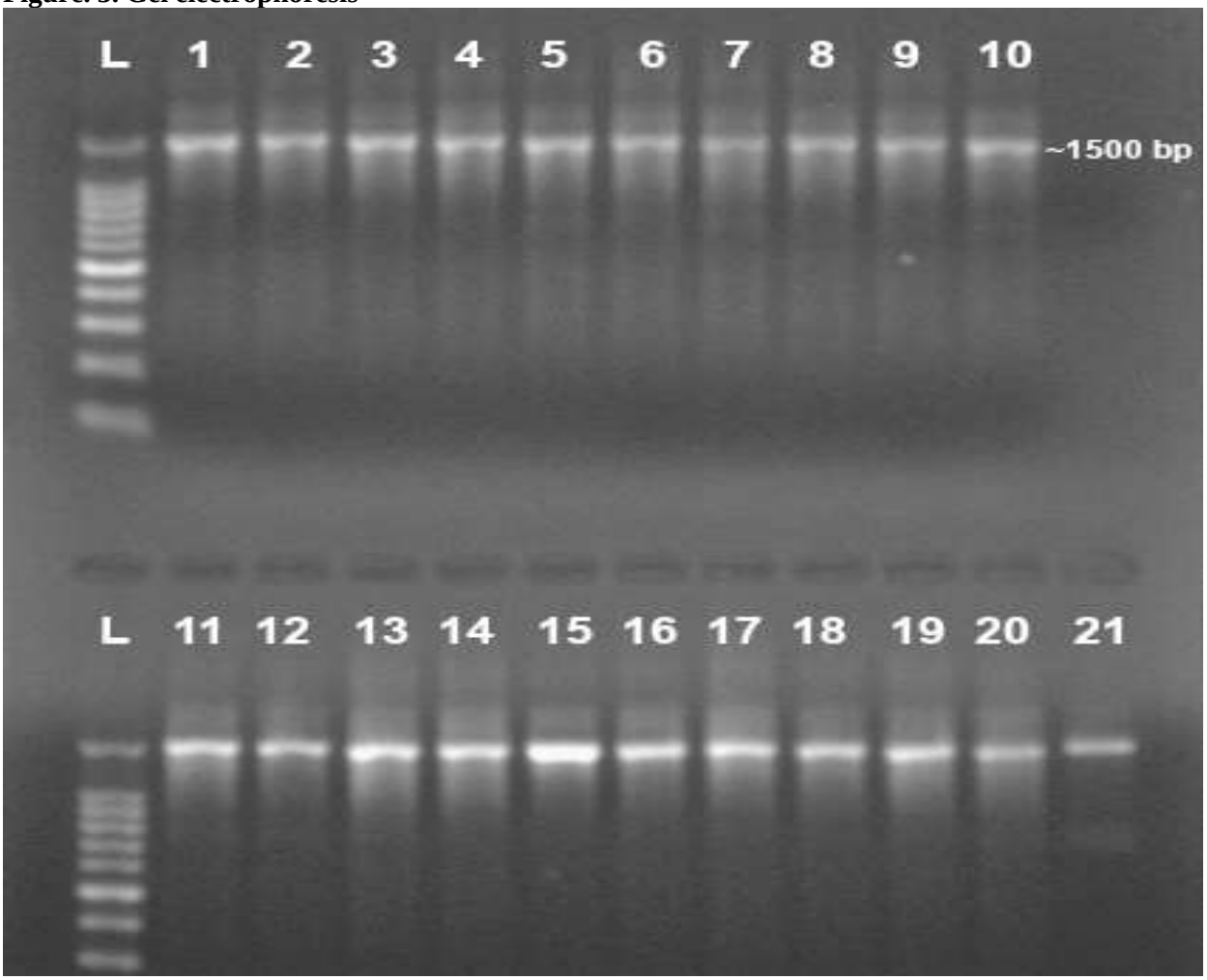

Figure. 3. Gel electrophoresis of the PCR amplicons obtained from 24F and 1492R primer pairs from the 16S rRNA gene. Lane 1, Bsub; Lane 2, K1; Lane 2, K2; Lane 4, K11; Lane 5, K12; Lane 6, L1; Lane 7, L4; Lane 8, L5; Lane 9, L6; Lane 10, L8; Lane 11, L10; Lane 12, LU35; Lane 13, LU36; Lane 13, LU36; Lane 14, LU38; Lane 15, LU39; Lane 16, LU40; Lane 17, N1; Lane 18, N2-1; Lane 19, N5; Lane 20, N7; Lane 21, N9; L, BH 100bp DNA Ladder RTU（100-1,500 bps)~

The PCR products were sequenced and revealed that the identified *Bacillus spp*. are grouped in four species; *B. megaterium, B.cereus, B. thuringiensis, B. mycoides and B. bingmayongensis* with accession numbers and percentage identities (table 2).

Table 2: Shows *Bacillus* spp. identities based on the 16S rRNA gene

Isolate code	Query Coverage (%)	Identity (%)	Identity
K11	100	100	*B. cereus*
K1	100	100	*B. cereus*
K2	100	100	*B. cereus*
L5	100	100	*B. cereus*
L6	100	100	*B. cereus*
L10	100	100	*B. cereus*

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Isolate code	Query Coverage (%)	Identity (%)	Identity
LU36	100	100	*B. cereus*
LU40	100	99.7	*B. cereus*
N1	100	100	*B. cereus*
N3	100	100	*B. cereus*
N7	100	100	*B. cereus*
N9	100	100	*B. cereus*
L1	88	98.73	*B. mycoides*
LU35	87	98.2	*B. mycoides*
L4	68	97.46	*B. megaterium*
L8	79	97.37	*B. megaterium*
LU38	100	100	*B. megaterium*
N5	78	95.74	*B. megaterium*
K12	83	98.06	*B. megaterium*
LU39	90	99.36	*B. bingmayongensis*
K14	90	96.51	*B. thuringiensis*
LU6	95	92.86	*B. thuringiensis*

Key: N; West Nile, L; Mid Northern, LU; Lake Victoria crescent K; Southern highlands

The results in Table 2 reveal the percentage identity for the different *Bacillus* spp.; *B. cereus* above 99%, *B. mycoides* above 98%, *B. megaterium* above 95% and *B. thuringiensis* above 92% identity.

Prevalence of Bacillus Species

The prevalence of the different *Bacillus* spp. was calculated using percentages and the results are presented in table 3.

Table 3: The prevalence of Different *Bacillus* spp

Isolate	Sampling site				Percentage
	K	LU	L	N	
B. cereus	03	02	03	04	54.5%
B. megaterium	01	01	02	01	22.7%
B. mycoides	-	01	01	-	9.1%
B. thuringiensis	01	01	-	-	9.1%
B. bingmayongensis	-	01	-	-	4.6%

N-West Nile, K-Southern Highlands, LU-Lake Victoria Crescent and L-Mid northern

The results in Table 3 reveal that *B. cereus* and *B. megaterium* are distributed in all the four agroecological zones studied. Further, *B. cereus* was the most prevalent (54.5%) followed by *B. megaterium* (22.7%). On the other hand, *B. bingmayongensis* was only identified in the Lake Victoria Crescent while *B. thuringiensis* was found in southern highlands and Lake Victoria Crescent. *Bacillus mycoides* were identified in Lake Victoria Crescent and Mind Northern agroecological zones. This means, some *Bacillus* spp. are common to certain ecological conditions.

Phylogenetic Tree

Phylogenetic tree of 22 *Bacillus* species based on 16S rRNA gene sequences constructed using the neighbor-joining method and genetic distances were generated using the Kimura 2-parameter method was constructed (fig. 4) to present the genetic relationship between the Bacillus strains.

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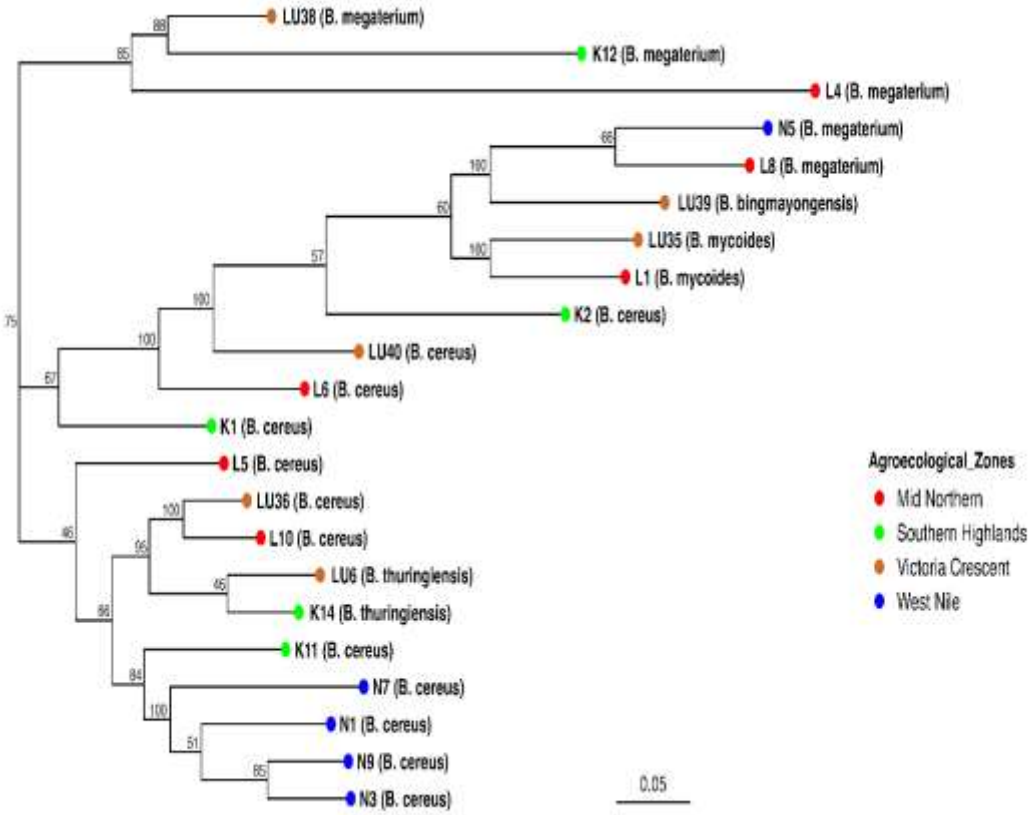
Figure 4: Phylogenetic Tree for *Bacillus* strains from Cabbage Rhizosphere



The phylogenetic tree shows that the 22 isolates were divided into three major clads. At 46%, clad 1 comprised of 10 *Bacillus* strains, at 67%, clad 2 had 9 strains while at 85%, and clad 3 had 3 strains closely related. The phylogenetic tree indicates that *B. thuringiensis* are closely related to *B. cereus* while *B. bingmayongensis* is closely related to *B. mycoides* and *B. megaterium* L8 and N5. *B. megaterium* L8 and N2 are distantly related to *B. megaterium* LU38, K12 and L4. *B. cereus* K1, K2, L6 and LU40 are distantly related to other six strains of *B. cereus.* The genetic distance in relationship means that members of the same species may exhibit functional differences. The differences may be due to mutations or genetic combinations.

DISCUSSIONS OF RESULTS

This study aimed at determining the composition and prevalence of bacillus species in the four selected agro-ecological zones of Uganda. Literature shows that there are about 260 *Bacillus* spp. widely distributed. In this study, only five *Bacillus* spp. (22 strains) were confirmed using genomic tests based on 16S rRNA gene sequencing. This means, not all the *Bacillus* spp. may be found in the same environment. Whereas Saxena et al., (2019) found that bacillus was a predominant bacterial genera in the soil and widely distributed in different environments (Nicholson, 2002; Ge et al., 2016), the findings show that some species were more prevalent and abundant than others (Table 3). *B. cereus* was most abundant compared with the other *Bacillus* spp. The abundance and prevalence of *B. cereus* had been reported by Marwa (2023) and Majed et al., (2016). The abundance and prevalence difference could be due to the type of climate and soil composition which affects species distribution. For example, Southern Highlands and Lake Victoria Crescent agroecological zones have humid tropical climate because they receive more rains. West Nile

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and Mid Northern agro-ecological zones have hot tropical climate because they have higher temperatures. This climate difference influences soil characteristics differently (DEFRA., 2005). The distribution of *B. cereus* in all the agro-ecological zones studied was in agreement with Ehling-Schulz et al., (2004) who reported that *B. cereus* survived wide range of stress conditions in the environment. The phylogeny of *Bacillus* spp. indicates that some strains of bacillus strains of the same species both from the same and different agroecological zones genetically differed. For example, *B. cereus* strains appeared in different clads, showing evolutionary diversity. The findings of this study agree with the study hypothesis that the genetic diversity of indigenous soil *Bacillus* spp. strains differs across the major cabbage growing agro-ecological zones of Uganda".

CONCLUSION

The most abundant and prevalent *Bacillus* spp. in Ugandan soils are *B. cereus* and *B. megaterium*. The presence and abundance of these bacillus species in the Ugandan soil present an opportunity for soil scientists to innovatively manipulate these bacteria for use as biofertilizers and biopesticides for farmers to improve crop production and management and reduce their reliance on synthetic fertilizers that are pollutants to the environment and unhealthy to the users and consumers.

Recommendation

Further studies to develop innovations for isolation and conversion of soil bacillus species into well packaged usable biopesticides and biofertilizers in organic crop production and management is recommended.

Ethical statement

This study was approved by the Ethics Committee of St. Francis Hospital Nsambya. All methods were carried out in accordance with the relevant guidelines. Reference number SFHN-2020-10.

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Authors' Contributions

Silver Baryakabona (Planning for the study, data collection and preparation, data analysis, presentation and discussion of findings).

Joseph Ssekandi (quality control in data collection and analysis and manuscript review).

Laban Frank Turyagyenda (quality control in data collection and analysis and manuscript review).

Conflict of interest

The authors declare no conflict of interest.

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