Nig. J. Biotech. Vol. 37(2): 177-193 (Dec. 2020)
ISSN: 0189 1731
Available online at
http://www.ajol.info/index.php/njb/index
and www.biotechsocietynigeria.org
DOI: https://dx.doi.org/10.4314/njb.v37i2.18

NIGERIAN JOURNAL OF BIOTECHNOLOGY

In-Vitro Antagonistic Effect of *Bacillus thuringiensis* on *Ralstonia solanacearum,* the Causal Agent of Bacterial Wilt Disease of Tomato (*Lycopersicon esculentum* Mill*).*

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Abstract

Tomato (*Lycopersicon esculentum*, Mill) is a rich source of vitamins, minerals and lycopene, which has many health benefits. However, its production is hampered by bacterial wilt caused by *Ralstonia solanacearum* resulting in significant yield losses. Use of chemicals in the control of plant pathogens has detrimental effects on humans and the environment in terms of leaving residues in soil which later find their way into underground waters. Therefore, it is desirable to find an alternative to chemical control of this bacterial pathogen. This study investigates the potential of native *Bacillus thuringiensis* (Bt) for biological control of *Ralstonia solanacearum (Rs)* under laboratory conditions. *B. thuringiensis* was isolated from cultivated soil, non- cultivated soil, stagnant water, sawdust, horse dung, grain dust, dead leaves and poultry manure. *R. solanacearum* was isolated from stem exudates of bacterial wilt infected plants and its pathogenicity assay was carried out using 2-week-old seedlings of *Beske* tomato variety. The Bt and *R. solanacearum* isolates were then characterized phenotypically. Bt isolates were further identified using endospore and parasporal staining techniques. All the Bt isolates were tested for *in-vitro* antagonistic activity on *R. solanacearum using* agar well diffusion method. Isolates Bt2, Bt16, Bt17, Bt32 and Bt34 were confirmed as *Bacillus thuringiensis* while isolate Rs was confirmed as *R. solanacearum. Beske* showed wilting symptoms from the fourth day of inoculation and eventual death of seedlings. The zone of inhibition exhibited ranged from 0.0 mm to 20.0 mm.

Keywords: Bacillus thuringiensis, In-vitro, Bacterial wilt, Ralstonia solanacearum, Tomato.
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Introduction

Tomato *(Lycopersicon esculentum* Mill) belongs to the *Solanaceae* family (Khokar, 2013). It is widespread throughout the world and represents the most economically important vegetables even in Nigeria (Olaniyi *et al.,* 2010; Bergougnoux,2013).Tomatoes contribute to a healthy, well-balanced diet. They are rich in minerals, vitamins, essential amino acids, sugars and dietary fibers. Tomato contains vitamin B and C, iron and phosphorus; its fruits are consumed fresh in salads or cooked in sauces, soup and meat or fish dishes. They can be processed into purées, juices and ketchup. Yellow tomatoes have higher vitamin A content than red tomatoes, but red tomatoes contain lycopene, an antioxidant that may contribute to protection against carcinogenic substances (Khokar, 2013).

Recently, it has been estimated that huge proportions of vegetable crops get deteriorated annually during growth or post-harvest storage, owing to diseases caused by fungi, nematodes,

bacteria, and viruses. This is one of the major limiting factors influencing food production and human development over thousands of years (Dun-chun *et al.,* 2016).Bacterial wilt is a common bacterial disease in tropical, subtropical and some temperate regions of the world (Fegan and Prior, 2005). It is endemic in most tomato-growing areas of Nigeria, causing 60 to 100% yield losses (Popoola *et al.,* 2015).
For the past 50 years, application of chemical pesticides has been the prevailing control measure for disease management in vegetables and other crops production. The continuous exposure to chemical pesticides adversely affects productivity, soil texture, nutritional content of vegetables, as well as the health of human beings (Singh *et al*., 2017). Due to the hazards associated with chemically synthesized herbicides and pesticides, management of diseases via biological control method is the novel emerging technology gaining importance in better agricultural sustainability (Singh *et al* ., 2017).

Farmers are shifting towards eco-friendly technologies for the management of pests and diseases, i.e., Biological Control Agents (BCAs) or BCA - based formulations, commonly called "Biopesticides". Examples include *Trichoderma* spp., *Pseudomonas* spp., *Bacillus* spp., *Agrobacterium radiobacter*, non- pathogenic *Fusarium* spp., *Coniothyrium* spp., *Aspergillus niger, Bacillus thuringiensis* (Bt), *Metarthizium* spp., *Beauveria bassiana* and nuclear polyhedrosis virus (NPVs), which are popularly used in plant protection (Keswani *et al.,* 2015;Mishra *et al.,* 2015).

Bacillus thuringiensis (*Bt*), a spore-forming bacterium is well known for its insecticidal properties, associated with its ability to produce crystal inclusions during sporulation. These inclusions are proteins encoded by *cry* genes and have shown to be toxic to a variety of insects and other organisms like nematodes and protozoa (Konecka*et al.,* 2007). Formerly, only the insecticidal properties of *B. thuringiensis* attracted extensive attention. However, in recent years, the roles of *B. thuringiensis* in plant disease control have been discovered. Apart from crystal protein and other insecticidal substances, B. *thuringiensis* also produces other active components with good prospects for application as the following: zwittermicin A, which is extremely efficient at preventing the damping-off of alfalfa caused by *Phytophthor amedicaginis* (Silo-Suh*et al.,* 1994), and acyl homoserine lactone (AHL) lactonases, which can quench bacterial pathogenicity (Dong *et al*.,2002). Therefore, there is a need to assess the *in-vitro* antagonistic effect of *Bacillus thuringiensis* on *Ralstonia solanacearum.*

Materials and methods

Study area and collection of soil samples
The study covered 3 locations within the village extension mandate of the Federal University of Agriculture, Abeokuta (FUNAAB), Ogun State, Nigeria. These are FUNAAB main campus, Owe village and Obantoko. Samples from cultivated soil, non-cultivated soil, dead leaves, dead insects, stagnant water, grain dust, saw dust, poultry manure and horse dung were collected aseptically. Diseased tomato plants showing symptoms of bacterial wilt were also collected. The samples were collected into Ziploc bags, kept on ice packs and transported to the Microbiology Laboratory, FUNAAB.

Isolation of Bacillus thuringiensis (Bt)

Isolation from soil

Bacillus thuringiensis was isolated using the method described by Palma (2015) with slight modifications. Vegetative cells from sporulated bacteria were isolated by homogenizing 3 g of each soil sample in 10 ml of sterile distilled water, intensely vortexed and mixed for 1 minute and incubated at 70±2°C for 15 minutes. After which the samples were vortexed and heated again. Each sample was then subjected to ten-fold dilutions and 20µl (from 10^{-3} to $10^{-5)}$ was dispensed on nutrient agar. Plates were incubated at 28±2°C for at least 72 hrs (Suguna *et al.,* 2011).

Isolation from stagnant water

Ten milliliter (10.0 ml) of each stagnant water sample was dispersed in 90 ml of saline solution. This was left to equilibrate for 20 minutes and then subjected to heating in a water bath at 80±2°C for 10 minutes (Saadeldin, 2007). Each sample was then subjected to ten-fold dilutions and 20µl (from 10^{-3} to $10^{-5)}$ was dispensed on nutrient agar. Plates were incubated at 28±2°C for at least 72 hrs (Suguna *et al.,* 2011).

Isolation from insects, grain dust, animal dung and dead leaves

Approximately 5 g of dead insects, saw dust, grain dust, animal dung and dead leaves were weighed

separately into 95 ml sterile distilled water in 250 ml conical flasks and incubated in an orbital shaker set at 25±°C for 5 hr. One milliliter (1 ml) aliquot was transferred into 5ml pre-warmed boiling tubes. The tubes were given heat-shock treatments at 80±°C for 15 minutes, in a water bath. Each sample was then subjected to ten-fold dilutions and 20µl (from 10^{-3} to $10^{-5)}$ was dispensed on nutrient agar. Plates were incubated at 28±2°C for at least 72 hrs (Suguna *et al.,* 2011).

Isolation of Ralstonia solanacearum

Ralstonia solanacearum was isolated as described by Shew and Lucas (1991) with slight modifications. A stem section was cut from diseased plants with vascular discoloration using a sterile sharp blade. The section was swabbed with 70% ethanol and subsequently placed against the inside wall of a water- filled clear test-tube so that the end of the section slightly touches the water surface. Milky white strands containing bacteria and extracellular polysaccharide streaming from the cut ends of the xylem were then cultured on nutrient agar plates incubated at 28±2°C for at least 24 hrs (Jayesh *et al.,* 2014). Smooth, circular, raised and dirty- white colonies were sub-cultured to obtain pure cultures. The isolates were kept on nutrient agar slants maintained at 4°C.

Characterization of Bacterial isolates

The bacterial isolates were subjected to standard microbiological methods such as morphological characteristics of the colony (shape, size, elevation, colour) and Gram staining to differentiate Gram negative and positive bacteria. Biochemical tests including catalase, oxidase, citrate utilization, Voges-Proskauer and methyl-red were carried out on the isolates (Fawole and Oso, 1998; Cheesbrough, 2006). The morphological and biochemical characteristics were examined according to the Bergey's Manual of Determinative Bacteriology (Holt *et al.,* 1994).

Pathogenicity test

Pathogenicity assay of the *Ralstonia solanacearum* isolates was carried out under screen house conditions by inoculating susceptible *Beske* tomato seedlings. Bacterial isolates were grown on nutrient agar medium for two days at 28±2°C. The cells were removed by centrifugation at 7,000 rpm for 10 minutes; pellets were re suspended in sterile distilled water and adjusted to a final density of 1×10^7 / ml (Elsharkawy *et al.,* 2015). Tomato seeds were nursed in a plastic tray and transplanted into disposable plastic cups 10 days after planting, while the inoculum was introduced into healthy seedlings 2 days after, by drenching the soil with 10ml each of the suspected pathogens (Hyakumachi *et al.,* 2013). Seedlings inoculated with sterile water served as negative control. Wilt intensity was calculated after 21 days of inoculation using the method described by Abeer and Hend (2013). $I\%=[\sum(nixv1/(VxN)] \times 100$, where I = wilt intensity, ni = no of plants with respective disease rating, v1= disease rating (following scale : 1= no symptoms, 2= 1 leaf wilted, 3=2 or 3 leaves wilted, 4 = four or more leaves wilted, 5= plant dead), V= the highest disease rating and N= the no of plants observed.

In-vitro activity

In-vitro antagonistic activity on the pathogen was determined using agar well diffusion method in which about 10µl of pathogen suspension (1×10^7 cfu/ml) was evenly spread on nutrient agar plates. Subsequently, with the aid of a sterile cork borer (6mm in size), wells of 20 mm apart and diameter of about 6mm were punched aseptically. Antagonist suspension (1.8×10^8 cfu/ml, about 100µl), was added into each well. Wells inoculated with sterile distilled water served as control (Elsharkawy *et al.,* 2015; Mounyr *et al.,*2016). This was carried out as follows: antagonist and pathogen were inoculated at the same time, pathogen was inoculated first followed by antagonist applied 48 hrs after, antagonist inoculated first while pathogen was applied 48 hrs after. The plates were then incubated for 24hrs at 30±2°C after which zones of inhibition were measured (Cleidson *et al,* 2007, Mounyr *et al.,*2016, Marissa *et al.,* 2016).

Molecular characterization of the Bacterial isolates

Genomic DNA of antagonistic Bt and Rs were extracted using Bacterial DNA isolation kit (Norgen BIOTEK, USA) followed by amplification of 16S rRNA gene in 10µl reaction mix (3.1µl of nuclease free H_2O,1.0 µl of 10x buffer,1.0µl of 25mM $MgCl_2$, 0.8 µl of 2.5mM DNTPs, 0.5 µl of 5pMol forward primer, 0.5 µl of 5pMol forward primer, 1.0 µl of DMSO, 2.0 µl of 10ng/ µl DNA and 0.1 µl of 5ng/ µl of Taq polymerase) using Forward primer 16SF (5′-

GTGCCAGCAGCCGCGCTAA-3′) and Reverse primer 16SR (5′-AGACCCGGGAACGTATTCAC-3′) (Taiwo *et al.,* 2017).

The conditions for Polymerase Chain Reaction (PCR) in the thermal cycler (GeneAmp PCR System 9600) were as follows: 94˚C for 5 minutes followed by 36 cycles of denaturation at 94˚C for 30 sec, annealing at 56˚C for 30 sec, extension at 72˚C for 45 sec, and final extension at 72˚C for 7 minutes. The amplified fragments were resolved by electrophoresis on a 1.5% agarose gel prepared in 0.5X TBE, stained with Gel red using a 50bp ladder. The gel ran for 50 minutes at 100V and was visualized under UV transilluminator. The PCR product of the amplified 16S rRNA amplified region was purified using ethanol precipitation method.

The PCR product was used for another PCR reaction (sequencing reaction), using the big Dye Terminator method with 3130xl genetic analyzer from Applied Biosystems. Amplification and sequencing were done at International Institute for Tropical Agriculture (IITA) Biosciences Laboratory, Ibadan, Oyo State.

Sequence editing and database matching

The sequences were edited, assembled and aligned using BioEditsoftware (version 7.1.9). Gene sequences were compared at the Genbank database of NCBI (National Centre for Biotechnology Information) using BLASTn search tool to identify the isolates. Phylogenetic analysis was done using Molecular Evolutionary Genetics Analysis (MEGA) version 6 (Tamura *et al.,* 2013).

Results

Morphological and Biochemical characteristics of bacterial isolates

All the bacterial isolates were rod-shaped and motile. Isolate T3 had dirty - white color while others were cream colored. Some were large while others were small in size. Some of the bacteria had raised elevation while others had flat elevation (Table 1). The bacterial isolates were Gram positive except T3 (Table 2).

Table 1: Morphological characteristics of the isolates

ID	Shape	Motility	Colour	Elevation	Size	Probable organism
1	Rod	Motile	Cream	Flat	Small	*Bacillus species*
2	Rod	Motile	Cream	Flat	Big	*Bacillus species*
3	Rod	Motile	Cream	Flat	Small	*Bacillus species*
4	Rod	Motile	Cream	Flat	Small	*Bacillus species*
5	Rod	Motile	Cream	Flat	Small	*Bacillus species*
6	Rod	Motile	Cream	Flat	Small	*Bacillus species*
7	Rod	Motile	Cream	Flat	Small	*Bacillus species*
8	Rod	Motile	Cream	Flat	Small	*Bacillus species*
9	Rod	Motile	Cream	Flat	Small	*Bacillus species*
10	Rod	Motile	Cream	Flat	Small	*Bacillus species*
11	Rod	Motile	Cream	Flat	Small	*Bacillus species*
12	Rod	Motile	Cream	Flat	Small	*Bacillus species*

13	Rod	Motile	Cream	Flat	Small	*Bacillus species*
14	Rod	Motile	Cream	Flat	Small	*Bacillus species*
15	Rod	Motile	Cream	Flat	Small	*Bacillus species*
16	Rod	Motile	Cream	Flat	Big	*Bacillus species*
17	Rod	Motile	Cream	Flat	Big	*Bacillus species*
18	Rod	Motile	Cream	Flat	Small	*Bacillus species*
19	Rod	Motile	Cream	Flat	Small	*Bacillus species*
20	Rod	Motile	Cream	Flat	Small	*Bacillus species*
21	Rod	Motile	Cream	Flat	Small	*Bacillus species*
22	Rod	Motile	Cream	Flat	Small	*Bacillus species*
23	Rod	Motile	Cream	Flat	Small	*Bacillus species*
24	Rod	Motile	Cream	Flat	Small	*Bacillus species*
25	Rod	Motile	Cream	Flat	Small	*Bacillus species*
26	Rod	Motile	Cream	Flat	Small	*Bacillus species*
27	Rod	Motile	Cream	Flat	Small	*Bacillus species*
28	Rod	Motile	Cream	Flat	Small	*Bacillus species*
29	Rod	Motile	Cream	Flat	Small	*Bacillus species*
30	Rod	Motile	Cream	Flat	Small	*Bacillus species*
31	Rod	Motile	Cream	Flat	Small	*Bacillus species*
32	Rod	Motile	Cream	Flat	Big	*Bacillus species*
33	Rod	Motile	Cream	Flat	Small	*Bacillus species*
34	Rod	Motile	Cream	Flat	Big	*Bacillus species*
35	Rod	Motile	Cream	Flat	Small	*Bacillus species*
36	Rod	Motile	Cream	Flat	Small	*Bacillus species*
37	Rod	Motile	Cream	Flat	Small	*Bacillus species*
38	Rod	Motile	Cream	Flat	Small	*Bacillus species*
39	Rod	Motile	Cream	Flat	Small	*Bacillus species*
40	Rod	Motile	Cream	Flat	Small	*Bacillus species*
41	Rod	Motile	Cream	Flat	Small	*Bacillus species*

42	Rod	Motile	Cream	Flat	Small	*Bacillus species*
43	Rod	Motile	Cream	Flat	Small	*Bacillus species*
44	Rod	Motile	Cream	Flat	Small	*Bacillus species*
45	Rod	Motile	Cream	Flat	Small	*Bacillus species*
46	Rod	Motile	Cream	Flat	Small	*Bacillus species*
47	Rod	Motile	Cream	Flat	Small	*Bacillus species*
48	Rod	Motile	Cream	Flat	Small	*Bacillus species*
49	Rod	Motile	Cream	Flat	Small	*Bacillus species*
50	Rod	Motile	Cream	Flat	Small	*Bacillus species*
51	Rod	Motile	dirty- white	Raised	Small	*Ralstonia solanacearum*
52	Rod	Motile	dirty- white	Raised	Small	*Ralstonia solanacearum*

Table 2: Biochemical characteristics of the bacterial isolates

s/no	Gram reaction	Cat	Cit	MR	VP	St	Glu	Man	Mal	Xy	Lac	Ox	Ur	Ind	Suc	Gal	Fru	Suspected organism
1	+	+	+	+	-	+	-	+	-	+	+	+	-	-				*Bacillus lentus*
2	+	+	+	-	+	+	+	+	-	-	-	-	+	-				*Bacillus thuringiensis (BT2)*
3	+	+	+	+	-	-	+	+	-	+	+	-	-	-				*Bacillus niaciani*
4	+	+	+	+	-	+	-	+	-	+	+	-	-	-				*Paenibacillus timonensis*
5	+	+	+	+	-	-	-	+	-	+	+	-	-	-				*Lysinibacillus fusiformis*
6	+	+	+	+	-	-	-	+	-	+	+	-	-	-				*Lysinibacillus fusiformis*
7	+	+	-	+	-	-	-	+	-	+	+	-	-	-				*Aneuribacillus aneurinilyticus*
8	+	+	+	+	-	-	-	+	-	+	+	-	-	-				*Lysinibacillus fusiformis*
9	+	+	+	+	-	+	-	+	-	+	+	-	-	-				*Paenibacillus timonensis*
10	+	+	+	+	-	+	-	+	-	+	+	-	-	-				*Paenibacillus timonensis*
11	+	+	+	+	-	+	-	+	-	+	+	-	-	-				*Paenibacillus timonensis*
12	+	+	+	+	-	+	-	+	-	+	+	-	-	-				*Paenibacillus timonensis*
13	+	+	-	+	-	-	-	+	-	+	+	-	-	-				*Aneuribacillus aneurinilyticus*
14	+	+	-	+	-	+	+	+	-	+	+	+	-	-				*Paenibacillus glucanolyticus*
15	+	+	-	+	-	+	+	+	-	+	+	+	-	-				*Paenibacillus glucanolyticus*
16	+	+	+	-	+	+	+	+	-	-	-	-	+	-				*Bacillus thuringiensis (Bt16)*
17	+	+	+	-	+	+	+	+	-	-	-	-	+	-				*Bacillus thuringiensis (Bt17)*
18	+	+	-	+	-	-	+	+	-	+	+	+	-	-				*Paenibacillus mendelii*
19	+	+	+	-	+	+	+	+	-	-	+	+	-	-				*Bacillus amyloliquefaciens*

20	+	+	-	+	-	-	-	+	-	+	+	-	-	-	*Aneuribacillus aneurinilyticus*
21	+	+	-	+	+	-	+	+	-	+	+	-	-	-	*Bacillus pumilus*
22	+	+	-	+	+	-	+	+	-	+	+	-	-	-	*Bacillus pumilus*
23	+	+	+	-	+	+	+	+	-	-	+	+	-	-	*Bacillus amyloliquefaciens*
24	+	+	-	+	-	+	+	+	-	+	+	+	-	-	*Paenibacillus glucanolyticus*
25	+	+	+	+	-	-	+	+	-	+	+	-	-	-	*Bacillus niaciani*
26	+	+	-	+	-	-	-	+	-	+	+	-	-	-	*Aneuribacillus aneurinilyticus*
27	+	+	+	+	-	-	+	+	-	+	+	-	-	-	*Bacillus niaciani*
28	+	+	-	+	-	+	+	+	-	+	-	+	-	-	*Bacillus smithii*
29	+	+	-	+	+	-	+	+	-	+	+	-	-	-	*Bacillus pumilus*
30	+	+	-	+	-	+	-	+	-	+	+	+	-	-	*Bacillus megaterium*
31	+	+	-	+	-	+	-	+	-	+	+	+	-	-	*Bacillus megaterium*
32	+	+	+	-	+	+	+	+	-	-	-	-	+	-	*Bacillus thuringiensis (Bt32)*
33	+	+	-	+	-	+	+	+	-	+	+	+	-	-	*Paenibacillusglucanolyticus*
34	+	+	+	-	+	+	+	+	-	-	-	-	+	-	*Bacillus thuringiensis (Bt34)*
35	+	+	-	+	+	+	+	+	-	-	-	+	-	-	*Bacillus assamensis*
36	+	+	-	+	+	+	+	+	-	-	-	+	-	-	*Bacillus muralis*
37	+	+	+	+	-	-	-	+	-	+	+	-	-	-	*Lysinibacillus fusiformis*
38	+	+	+	+	-	+	+	+	-	-	+	-	-	-	*Paenibacillus massilliensis*
39	+	+	+	+	-	+	-	+	-	+	+	-	-	-	*Paenibacillus timonensis*
40	+	+	+	+	-	+	-	+	-	+	+	-	-	-	*Paenibacillus timonensis*
41	+	+	-	+	+	-	+	+	-	+	+	-	-	-	*Bacillus pumilus*

42	+	+	-	+	+	-	+	+	-	+	+	-	-	-				*Bacillus pumilus*
43	+	+	-	+	-	+	+	+	-	+	-	+	-	-				*Bacillus smithii*
44	+	+	-	+	+	-	+	+	-	+	+	-	-	-				*Bacillus pumilus*
45	+	+	-	+	+	-	+	+	-	+	+	-	-	-				*Bacillus pumilus*
46	+	-	-	+	+	+	+	+	+	+	+	+	-	-				*Bacillus siamensis*
47	+	+	-	+	+	-	+	+	-	+	+	-	-	-				*Bacillus pumilus*
48	+	-	-	+	+	+	+	+	+	+	+	+	-	-				*Bacillus siamensis*
49	+	+	-	+	+	+	+	+	+	+	+	-	-	-				*Bacillus lichenfomis*
50	+	+	-	+	+	-	+	+	-	+	+	-	-	-				*Bacillus pumilus*
51	-	+	+	-	-	-	+	-	+	+	+	+	+	-	+	+	+	*Ralstonia solanacearum (T1)*
52	-	+	+	+	+	-	+	-	+	+	+	+	+	-	+	+	+	*Ralstonia solanacearum (T3)*

Cat-catalase; Cit-citrate; MR-methyl-Red; VP-Voge-Proskauer; St-starch; Glu-glucose; Man-mannitol; Mal-maltose; Xy-xylose; Lac-lactose; Ox-oxidase; Ur-urease; Ind- indole; Suc-sucrose; Gal-galactose; Fru-fructose.

+ Positive reaction; - Negative reaction

Endospore and parasporal crystal staining for Bacillus thuringiensis identification

Isolates Bt2, Bt16, Bt17, Bt32, and Bt34 were positive for endospore and crystal staining (Table 3).

Table 3: Endospore and parasporal crystal staining of the bacterial isolates

Bacterial isolate	Endospore staining	Parasporal body staining
Bt2	+	+
Bt16	+	+
Bt17	+	+
Bt32	+	+
Bt34	+	+

Percentage occurrence of Bacteria species isolated from different sources

Bacillus pumilus had the highest percentage occurrence (53%) followed by *Paenibacillus timonensis* (41%) while the least occurrence was observed in *Paenibacillus mendelis, Bacillus assamensis, Bacillus muralis, Paenibacillus massilliensis and Bacillus licheniformis*(6%) as shown in Figure 1.

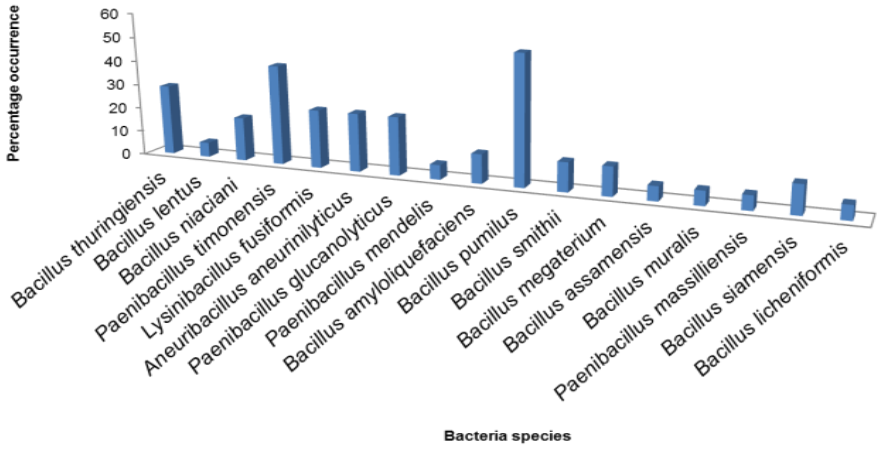


Fig. 1: Percentage occurrence of bacteria species isolated from different sources

Percentage occurrence of Bacillus thuringiensis isolated from different sources

Cultivated soil had the highest percentage occurrence of *Bacillus thuringiensis* (60%) while the least occurrence was observed in stagnant water and dead insects (20%) (Figure 2).

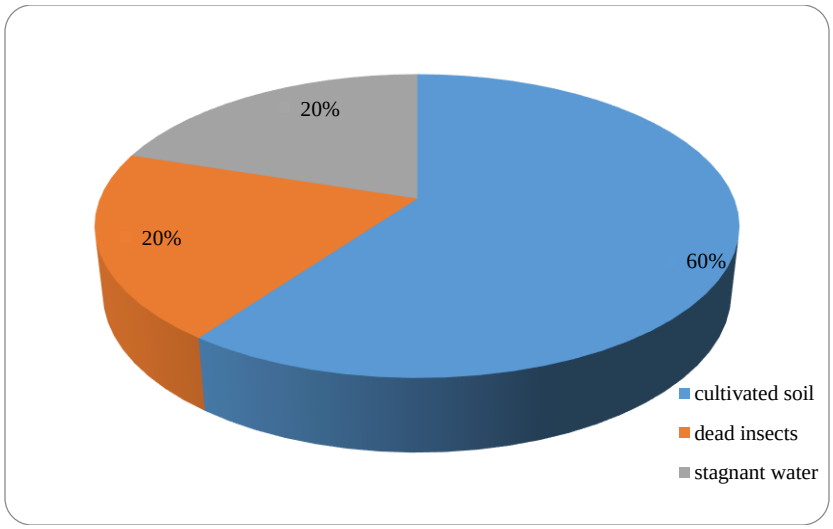


Figure 2: Percentage occurrence of *Bacillus thuringiensis* isolated from different sources

Pathogenicity

Wilting of varying degrees were observed on seedlings inoculated with pathogens while seedlings inoculated with sterile water showed no sign of wilting as shown in Table 3.

In-vitro activity

There were no zones of inhibition when either antagonist or pathogen was applied first. When pathogen and antagonist were applied at the same time, isolate Bt34 had the highest zone of inhibition (20 mm) while isolate Bt17 had the least zone of inhibition (0 mm) (Table 4).

Table 3: Pathogenicity Test for *Ralstonia solanacearum* on healthy tomato seedlings

ID	No of wilted leaves
R1	All (dead)
R2	4
R3	All (dead)
R4	All (dead)
R5	5
R6	All (dead)
C1	None
C2	None
C3	None
C4	None

C5	None
C6	None

KEY: R1-R6- seedlings with pathogen; C1-C6- seedlings with sterile water

Table 4: *In-vitro* antagonistic effect of *Bacillus thuringiensis* against *Ralstonia solanacearum*

ID	Zone of inhibition (mm)
Bt2	4
Bt16	2
Bt17	0
Bt32	1
Bt34	20

Molecular Characterization of the Bacterial Isolates

Purity and concentration of DNA extracted from the bacterial isolates ranged from 1.74 to 1.92 and 32 ng/µl to 160.50 ngµl, respectively, as shown in Table 5. Similarity of sequences of the bacterial isolates with sequences obtained from NCBI is shown in Table 6.

Gel images of genomic DNA are shown in Plate 1 while Plate 2 shows the gel image of amplified 16S rRNA gene of the isolates. Phylogenetic relationship of the bacterial isolates is shown in Figure 3.

Table 5: Purity and Concentration of the DNA extracted from the bacterial isolates

Sample	Purity	Concentration (ng/µl)
RsT1	1.74	130.00
RsT3	1.86	32.40
Bt2	1.82	145.40
Bt34	1.92	160.50

KEY: RsT1 – *Ralstonia solanacearum;* RsT3 – *Ralstonia solanacearum;* Bt2 - *Bacillus thuringiensis;* Bt34 - *Bacillus thuringiensis*

Table 6: Similarity of the sequences from the Bacterial strains with sequences obtained from NCBI genbank database.

Bacterial isolate	Closest related taxa	% Similarity	Accession Number

Ralstonia solanacearum(T1)	*Ralstonia solanacearum* APK76	95%	MF973211.1
Ralstonia solanacearum (T3)	*Ralstonia solanacearum* Rs8	95%	HRG425352.1
Bacillus thuringiensis (Bt2)	*Bacillus thuringiensis* strain INF-71	85%	KP813739.1
Bacillus thuringiensis (Bt34)	*Bacillus thuringiensis* strain PKN 3	81%	KF922484.1

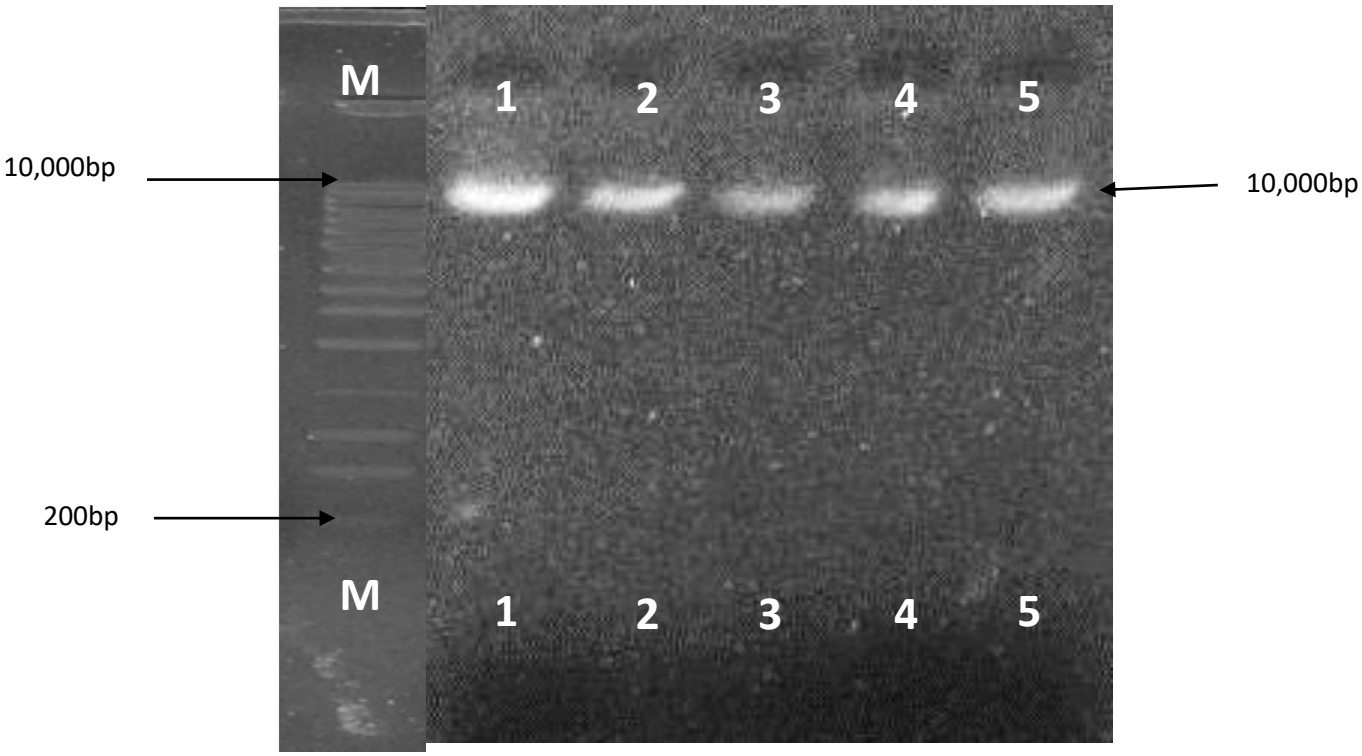


Plate 1: Agarose gel electrophoresis of genomic DNA
M- Molecular ladder; 1-*Ralstonia solanacearum* (T1); 2-*Ralstonia solanacearum* (T3) 4-*Bacillus thuringiensis*

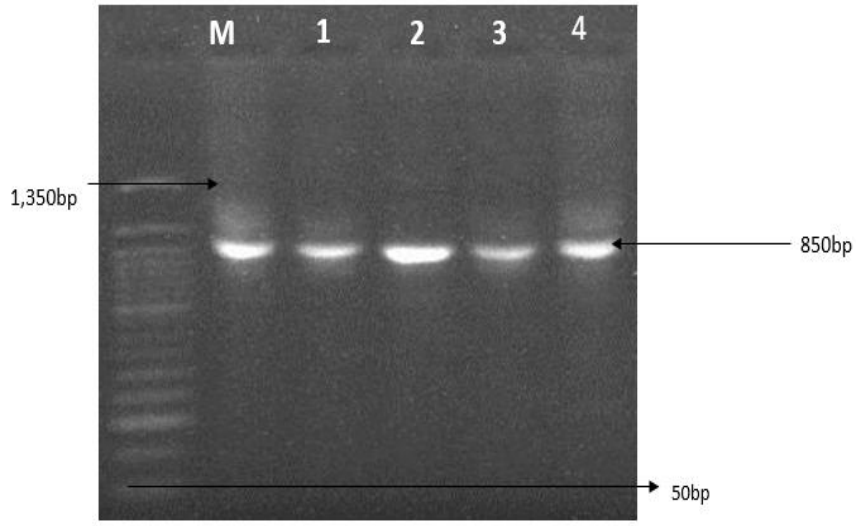
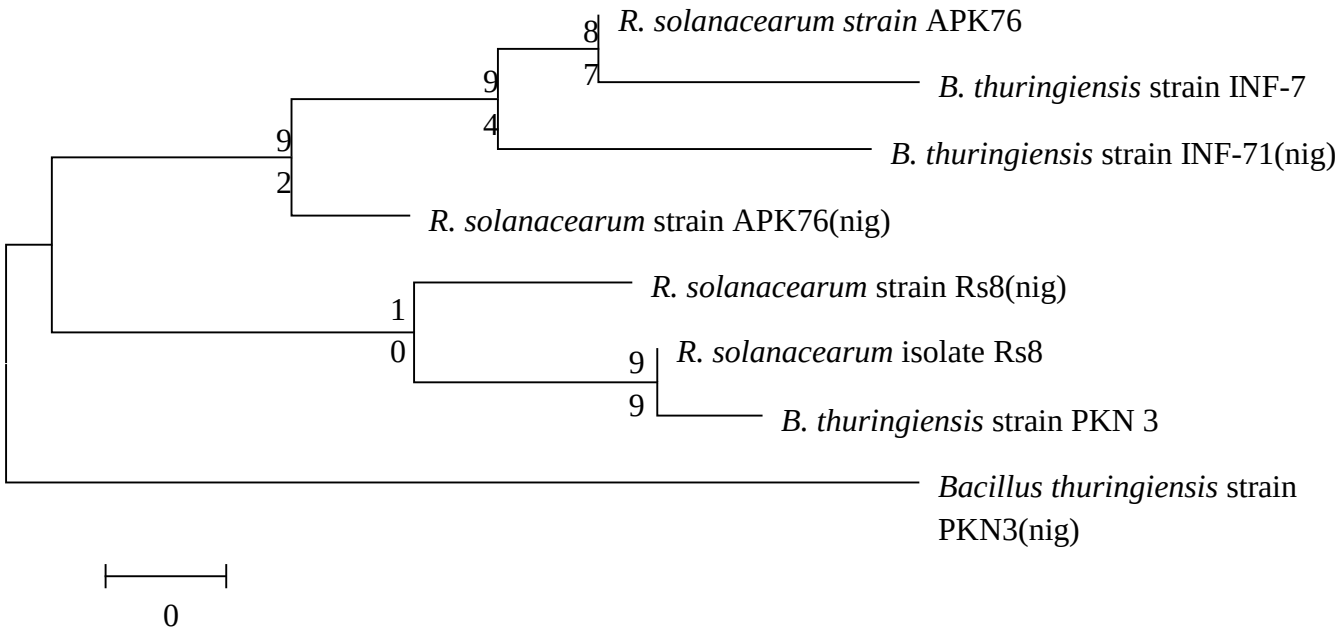


Plate 2: Agarose gel electrophoresis showing amplified 16S rRNA gene of the isolates
M- Molecular ladder; 1: *Ralstonia solanacearum*(T1); 2: *Ralstonia solanacearum*(T3)
4- *Bacillus thuringiensis* (Bt2); 5- *Bacillus thuringiensis* (Bt34)

Figure 3: Phylogenetic relationship of the bacterial isolates



Discussion

Biocontrol is particularly desirable because it is sustainable, environment friendly, cost-effective and could be used in integrated pest management programs (Elshakawy *et al,* 2015). In this study, 17 bacterial isolates were isolated from cultivated and non-cultivated soils, stagnant waters, dead leaves and dead insects. This corroborates the findings of Argôlo-Filho and Loguercio, (2013), El-kersh *et al.,*(2016), and Denane *et al.,* (2017), that were able to isolate Bt from different sources. One bacterial isolate was also isolated from diseased tomato plants showing symptoms of bacterial wilt. Based on their morphological and biochemical characteristics, 5 out of the 17 bacterial isolates (Bt2, Bt16, Bt17, Bt32 and Bt34) recovered from the different sources (cultivated soils, dead insects and stagnant waters) were tentatively identified as *Bacillus thuringiensis* (Bt). Endospore and crystal staining further confirmed the Bt isolates. This corroborates the works of Mohsina *et al.,* (2013), Palma (2015) and Meihiar*et al., (*2015). They were able to establish Bt as crystal protein and endospore producers. Ammons *et al.,*(2016) and Neethu *et al.,*(2015) also reported that crystal proteins account for their pesticidal and insecticidal activities. Out of the 17 different *Bacillus* species isolated from the different sources, *Bacillus pumilus* occurred most, this could be as a result of their spores' extreme resistance to radiation, desiccation, and hydrogen peroxide treatment as reported by Link *et al.,* (2004) and Kempf*, et al.,* (2005). As a result, *B. pumilus* has been classified as an extreme microorganism according to the planetary protection standards (Vaishampayan *et al.,* 2012). *Bacillus thuringiensis* had the highest percentage occurrence in cultivated (agricultural) soil samples; this contradicts the findings of Kassougue *et al.,* (2015) but agrees with that of Ralte *et al*., (2016). Kassogue found low Bt strains in cultivated soil in Mali while Ralte found higher frequency of *Bacillus thuringiensis* in agricultural soils than non-agricultural soils. This could be as a result of plants serving as a form of protection to the soil from the harsh ultraviolet rays of the sun and as such the soil organisms remain intact. The bacterial isolate from diseased tomato plants was also tentatively identified as *Ralstonia solanacearum*. Pathogenicity assay of *Ralstonia solanacearum* isolate on *Beske* tomato variety showed wilting of varying degrees while the control showed no sign of wilting. This is in agreement with Popoola *et al.,* (2015) who reported *Beske* variety as susceptible to bacterial wilt of tomato. *In-vitro* activity of the Bt isolates (Bt2, Bt16, Bt17, Bt32 and Bt34) against *Ralstonia solanacearum* revealed isolate Bt34 as having the highest zone of inhibition with whole cell (20.00 cm) and supernatant (18.00 cm) while isolate Bt17 had the least zone of inhibition and whole cell. Similarly, isolates Bt 16, Bt 17 and Bt 32 had the least zones of inhibition (0.00 cm) only. This corroborates the work of Abeer and Hend (2013), reporting that *Bacillus thuringiensis* was able to significantly reduce the growth of *Ralstonia solanacearum* under laboratory conditions with a zone of inhibition of 8.2 mm. This result shows that native *Bacillus thuringiensis* may be used to control bacterial wilt of tomato under laboratory conditions.

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