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Isolation and characterization of rhizospheric microorganisms from bacterial wilt endemic areas in Kenya

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Ralstonia solanacearum **is a soil-borne bacterium causing bacterial wilt, particularly in Solanaceae crops, including tomatoes. Due to pathogen's wide host plant range, pesticide resistance, and ability to overcome cultivars utilized for innate resistance, not even a single measure is effective against the pathogen. Screening the rhizosphere of bacterial wilt tolerant tomato plants from Bomet, Kiambu, Kajiado and Kirinyaga regions of Kenya for beneficial bacterial and fungal isolates was done using nutrient agar and potato dextrose agar, respectively. Morphological, biochemical, and microscopic characterization to identify the genus of fungal and microbial isolates revealed a total of 41 bacterial and 42 fungal isolates distributed as Bomet>Kiambu>Kajiado>kirinyaga while fungal isolates were as Bomet>Kiambu>Kirinyaga>Kajiado. Methyl Red (MR) and Voges-Proskauer (VP) tests, catalase reaction, citrate reaction, starch hydrolysis, and triple iron sugar (TSI) tests showed that the predominant bacteria were *Burkholderia*, *Bacillus* and *Micrococcus* species. *Pseudomonas, Streptomyces*, *Serratia*, and *Enterobacter* species were the least. *Aspergillus*, *Trichoderma,* and *Fusarium* species were the most dominant fungal isolates from the four counties. Hierarchical cluster analysis was done using DARwin software V6 to reveal different dissimilarity levels among the isolates based on biochemical and morphological characteristics.**

Key words: *Ralstonia solanacearum,* rhizosphere screening, fungal isolates, bacterial isolates, biological control.

INTRODUCTION

Ralstonia solanacearum is a complex pathogen with a wide host range (Salanoubat et al., 2002) causing bacterial wilt (Bocsanczy et al., 2012; Zuluaga et al., 2013), in over 200 plant species, including crops like potatoes, tomatoes, tobacco, pepper and bananas (Meng, 2013), by the colonizing the xylem vessels that transport water (Khokhani et al., 2017). The pathogen exhibits a very strong tropism towards specific tissues

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within the hosts, specifically invading the xylem where it multiplies (Salanoubat et al., 2002) and produces its costly virulence factors immediately it reahes a high population density (Khokhani et al., 2017). Host plant invasion first occurs at roots followed by colonization of root's intercellular spaces, then eventual entrance of the xylem vessels where high replication takes place, leading to wilt symptoms (Addy et al., 2012; Hikichi et al., 2017). Eventually, the plants die as a result of a lack of water and nutrients uptake. *R. solanacearum* survival capabilities are notable: for instance, its ability to scavenge for nutrients in water devoid of nutrients or carbon (Elsas et al., 2001), wide host range, wide geographical distribution (Genin, 2010) and diverse range of virulence factors which allow it to strive in the rhizosphere by breaking through the microbial shield and overcoming plant innate defense systems (Mendes et al., 2013). The regulation of virulence factors initiated in response to dynamic changes in the environment, interaction with host plants, and other microbial communities play a significant role in successful infection, multiplication, and survival under diverse environmental conditions throughout its life cycle (Ham, 2013). This allows its survival even when there are no host plants. Complex systems operated by the pathogen during signal perception, transduction, and exchange of resources at the cellular level ensures sound production of virulence factors to limit wastage and avoid the host plant from activating its defense system (Ham, 2013). The pathogen's sophisticated pathogenic machinery and its ability to change to resistant forms, that is, from fluidal to afluidal forms (Álvarez et al., 2010), help it overcome pesticides, physical and cultural barriers commonly utilized as control measures. Bactericides, like streptomycin, were initially regarded as effective in controlling *R. solanacearum,* but are no longer used because large dosages are required for it to be effective (Xue et al., 2009). This implies that the pesticides become costly to small scale farmers, and brings more harm to the environment due to high dosages. Though the use of latest technology of biological disinfection of soil using substrates that release volatile compounds (Nion and Toyota, 2015), and utilization of wilt-resistant cultivars could be the most appropriate strategy, there is a continuous problem of pathogen overcoming plant's resistance over time (Sudisha et al., 2013). More efforts are desirable to come up with living microorganisms that can effectively manage bacterial wilt disease. Studies indicate numerous biological controls as a promising alternative strategy for integrated control of bacterial plant diseases (Rado et al., 2015). Biological control methods, especially the use of microorganisms, present a huge potential in complementing other control strategies commonly used since they have the advantage of reaching underground plant organs where pesticides would not reach. Soil microorganisms have a significant impact on soil and plant processes because they are crucial in determining soil nutrient status, crop health, and productivity (Larkin, 2003). Biochar amendments to soils may alter soil function and fertility in various ways, including through induced changes in the microbial community (Lu et al., 2016). There has been an increased focus on the use of antagonistic microorganisms, which include non-virulent strains of *R. solanacearum*, some fungi, bacteriophages, and other bacterial species to control bacterial wilt. Strains of *Streptomyces,* for instance, have attracted attention due to their ability to inhabit similar ecological habitats as the pathogenic microorganism (Diallo et al., 2011) whereas isolates of *Trichoderma* have been found to have different mechanisms or combination which include direct parasitism, competition for nutrients, stimulators of plant health, or inducers of plant systemic resistance against various pathogens (Rai et al., 2015). The beneficial microorganisms limits activities of plant pathogens through parasitism, competition for space, and secretions of antibiotics (Shishido et al., 2007). Several rhizosphere bacteria, including species of *Pseudomonas, Azospirillum, Klebsiella, Enterobacter, Arthrobacter, Burkholderia, Bacillus, Rhizobium,* and *Serratia* have been found to enhance plant growth (Kumar et al., 2012). Bacterial wilt tolerant plants contain diverse microbes that play a critical role in enhancing and improving the growth and health of the plant. The presence of soil-borne pathogens and other deleterious organisms can result in high disease severity, reduced plant vigour, and low yield. In contrast, beneficial soil microorganisms can lead to the suppression of pathogens, increase plant growth, and often high yield (Larkin, 2003). The use of inoculants of rhizosphere bacteria to enhance plant growth in intensive agricultural production has gained positive attention in various parts of the world (Abbasi et al., 2015). In light of this, we hypothesized that those tomatoes that do not show symptoms of bacterial wilt contain beneficial microorganisms in its rhizosphere, which confers resistance and/or tolerance to *R. solanacearum*. In this study, sampling of rhizosphere soils from bacterial wilt tolerant tomatoes from Bomet, Kajiado, Kirinyaga, and Kiambu for isolation and characterization of potentially beneficial fungal and bacterial isolates was evaluated.

MATERIALS AND METHODS

Rhizosphere sample collection

Purposive rhizosphere sampling (Bernard, 2002) was done in Bomet, Kirinyaga, Kiambu, and Kajiado from Kenya, where bacterial wilt disease is known to be endemic. Rhizosphere soils were picked from bacterial wilt resistant tomato rhizospheres growing in the bacterial wilt infected soils when the plants were at the flowering stage. The following is the identification of infected tomato plants.

In the selected fields and greenhouses, tomato plants that showed yellowing, leaf epinasty, necrosis and growth of adventitious roots on stems were carefully uprooted and washed with distilled water before chopping the stems into 15 cm pieces. The pieces were suspended for 30 min on 500 mL glass beaker containing sterile distilled water, and observation was made to check for the white ooze emanating from the suspended stem. Confirmation of the presence of *R. solanacearum* on the oozed stem was done by checking the presence of brown discolouration of vascular tissues (Seleim et al., 2014) after longitudinally cutting the stem with a sterile knife (Doolotkeldieva and Bobusheva, 2016). Soil samples from the rhizosphere of such tomato plants were labelled as bacterial wilt infected samples and therefore were considered to be lacking effective potential bacterial and fungal isolates that can confer resistance to tomato against *R. solanacearum*. However, such rhizosphere soils were used to isolate *R. solanacearum* for subsequent studies. The identification of non-infected tomato plants was based on a lack of symptoms of wilting, yellowing, and leaf epinasty. Thus, the rhizosphere samples from such plants were labelled healthy and used to isolate potential bacterial and fungal isolates that can infer resistance to tomato against *R. solanacearum.* The intact root system was dug out, and the rhizosphere soils were carefully taken (Kumar et al., 2012) and placed in labelled khaki bags (Yanti et al., 2017) and transported to the lab where they were stored at 4°C (Kumar et al., 2012) to preserve the microbes until they were isolated.

Isolation of rhizosphere microorganisms

One gram of soil samples was placed in sterile test tubes, and 9 ml of sterile distilled water was added before vortexing for 5 min and allowing it to settle for another 5 min. An eight-fold serial dilution was made before plating 0.1 ml in nutrient agar (peptone 5 g/l, sodium chloride 5 g/l, beef extract 3 g/l from HiMedia laboratories) at pH of 7.4 and 25°C and left to solidify overnight. The plates were then sealed and placed in an incubator at 28°C for 48 h before observations were made. Purification was done by sub-culturing different colonies showing different morphology on fresh nutrient agar. Potato dextrose agar (Dextrose 20 g/l, potato extract 4 g/l and agar 15 g/l from HiMedia laboratories) amended with 25 mg/l of chloramphenicol at pH of 5.7 and 25°C was used to isolate potential beneficial fungi (Mwashasha et al., 2016). An aliquot of 0.1 ml was picked, plated, and placed in an incubator for 72 h at 28°C before the observation of fungal structures was done. Purification was done by sub-culturing different fungal structures showing different morphology on fresh potato dextrose agar and left for 72 h before observation, and microscopic descriptions were made.

Identification of fungal structures

Fungi were characterized according to Barnett and Hunter (1987) through microscopic and macroscopic observation. Shapes, spores, surface, peripheral and reverse colour, and hyphae septation, elevation, and margin were observed using a compound microscope. The *Genus* name of each fungal isolate was concluded after comparing spores against those found in Barnett and Hunter (1987) key.

Morphological characterization of bacterial isolates

The identification of different bacterial isolates was done, according to Bergey et al. (2010). Morphological characteristics included shape, elevation, colour, and margin (Swarupa et al., 2014), Gram status, and motility was studied for all isolated bacteria. Gram staining of the isolated bacteria was performed by placing a drop of sterile water on the sterile slide before aseptically picking bacterial organisms using a sterile wire loop, then placing it on a drop of water, and gently mixing it before flooding it with crystal violet and leaving it for 1 min. The slide was then held in a slanting position and gently washed with sterile water before flooding it with Gram's iodine and leaving it to stand for another 1 min before washing it with sterile water. The gently held slide was decolourized by adding 95% ethyl alcohol drop by drop until the alcohol turns clear. It was rinsed with sterile water and finally counterstained with safranin for 45 s, blot dried, and observed on a light microscope under oil immersion (Cappuccino and Sherman, 2014). Gram staining results were confirmed by adding a drop of 3% KOH test (Halebian et al., 1981). A loop full of the bacteria was introduced and mixed thoroughly for 1 min before gently lifting the wire loop immediately KOH solution turned viscous. Positive results were observed when a thread of slime followed the wire loop for 0.5 to 2 cm, and the isolates were considered Gram-negative, but the mixture that formed watery suspension without slime following the wire loop was considered as negative for KOH, and the bacteria were recorded as Gram-positive (Dida et al., 2018).

Biochemical characterization of isolated bacteria

Tests that were performed to investigate the biochemical characteristics of the bacterial isolates included motility, Methyl Red test (MR), Voges-Proskauer (VP) tests, catalase reaction, citrate reaction, starch hydrolysis and triple iron sugar tests (TSI) (Dinesh et al., 2015).

Motility tests

Motility tests for all bacterial isolates were done using sulfide indole motility (SIM) medium from HiMedia laboratories. Tubes containing SIM agar left to cool overnight were inoculated with 24 h old bacterial isolates. Inoculation was done by making a single stab down the centre of the tube using a sterile needle to about half the depth of the medium before placing them into the incubator at 37°C for 24 h. Motility was recognized when the medium turned turbid, and the bacteria were not restricted to the line of inoculation. On the other hand, bacterial isolates that did not show motility were confined to the line of inoculation.

Methyl Red-Voges-Proskauer (MR-VP) test

Each bacterial isolate was inoculated into MR-VP broth in duplicate, shaken, then incubated at 37°C for 72 h. For methyl red tests, drops of methyl red indicator were added to an aliquot of every bacterial culture tested while Barrit's reagent (4% KOH and 5% alpha naphthol in 95% ethanol) was added for VP tests. Bacterial isolates that turned red on methyl red tests were positive for methyl reaction, whereas pale yellow indicated a negative reaction. For VP tests, the production of deep red colouration indicated a positive VP reaction (Cappuccino and Sherman, 2002).

Catalase activity

Catalase tests detect catalase enzyme activity that is present in anaerobic bacteria that contain cytochrome complex. Catalase positive enzymes produce hydrogen peroxide while aerobically

Table 1. Bacterial and fungal isolates from bacterial wilt endemic regions in Kenya.

Isolate type	Bacterial wilt endemic regions				Total
	Kiambu	Kajiado	Kirinyaga	Bomet	
Fungi	12	10	8	13	43
Bacteria	11	7	3	19	40

Table 2. Colony and microscopic characteristics identification of fungal isolates.

Total No.	Surface	Reverse	Periphery	Margin	Hyphae	Elevation	Genus
8	Green	Yellow	Cream	Smooth	Septate	Raised	*Trichoderma* spp.
13	Black	Cream	White	Smooth	Septate	Raised	*Aspergillus* spp.
8	Pink	Red	Pink	Smooth	Non-Septate	Raised	*Fusarium* spp.
5	White	Cream	White	Smooth	Non-septate	Raised	*Phytophthora* spp.
2	Green	Yellow	White	Smooth	Septate	Raised	*Penicillium* spp.
2	White	Cream	White	Smooth	Septate	Raised	*Chladosporium* spp.
2	White	Cream	White	Smooth	Septate	Flat	*Sporidesmium* spp.
3	Cream	Yellow	White	Serrated	Septate	Raised	*Odecephalum* spp.

breaking sugars. Twenty-four-hour old bacterial colonies were aseptically picked from the plates and placed on glass slides before flooding with a 3% hydrogen peroxide solution (H_2O_2). When catalase breaks down hydrogen peroxide to water and oxygen, the formation of effervescence indicated that bacteria were catalase positive. The absence of effervescence indicated that bacteria were negative for catalase activity.

Starch hydrolysis test

Isolated bacteria were streaked on starch agar plates and left in an incubator at 37°C for 48 h. After incubation, the plates were removed and flooded with Gram's solutions. Observations for clear zone around bacterial growth were done. Bacteria that showed clear zones around the growth were regarded as positive for starch hydrolysis. Those bacteria that did not show clear zones around the growth were regarded as negative for starch hydrolysis.

Triple sugar iron agar tests

Triple sugar iron tests were done to test the type of reaction and if the bacterial isolates produced hydrogen suphide (H_2S) and gas. Bacterial isolates were streaked on triple sugar iron agar slants and left to cool overnight. The streaked tubes were then incubated at 37°C for 24 h before taking any observation to determine the fermentative activities of bacterial isolates depending on the colour of slants and butt. The yellow colour indicated acid slants, whereas acid butt was indicated by yellow colour. Similarly, alkaline slants were indicated by red colour, while alkaline butt was indicated by red or orange-red colour. The blackening colour normally indicates production of H_2S within the medium, but under these tests, no blackening was observed, indicating that bacterial isolates were not able to produce H_2S gas. Formation of a transparent space between the slants and the butts indicated the presence of gas.

Citrate utilization

This test was performed to investigate the ability of bacterial isolates to utilize citrate as the only source of carbon. The bacterial isolates were tabbed using a sterile wire before tabbing the wire on Korsa citrate medium prepared and left to cool overnight in test tubes. The tabbed test tubes were then placed in the incubator at 37°C for 24 h. The change of colour from clear to turbid indicated that the bacteria were positive for citrate utilization, whereas those negative for citrate utilization remained clear.

RESULTS

Isolation of microorganisms

After purification, rhizosphere samples from Bomet accounted for 30 and 48% of total fungal and bacteria isolated, respectively, followed by Kiambu, which recorded 28 and 27% of total fungal and bacteria isolated, respectively (Table 1). Kajiado was rank third with 23% fungal isolates and 18% of bacterial isolates, while Kirinyaga was the least county with microbes. It accounted for 19% of total fungal isolates obtained, and only 8% of the total bacteria isolated. As presented in Table 2, the most dominant fungal isolates obtained were *Aspergillus* species that accounted for 30% of the fungal isolates characterized, followed by *Trichoderma* and *Fusarium* species, with each accounting for 19%. *Phytophthora* and *Oedecephalum* species accounted for about 12 and 7%, respectively, while *Chladosporium, Sporidesmium*, and *Penicillium* species accounted for 5% of the total count.

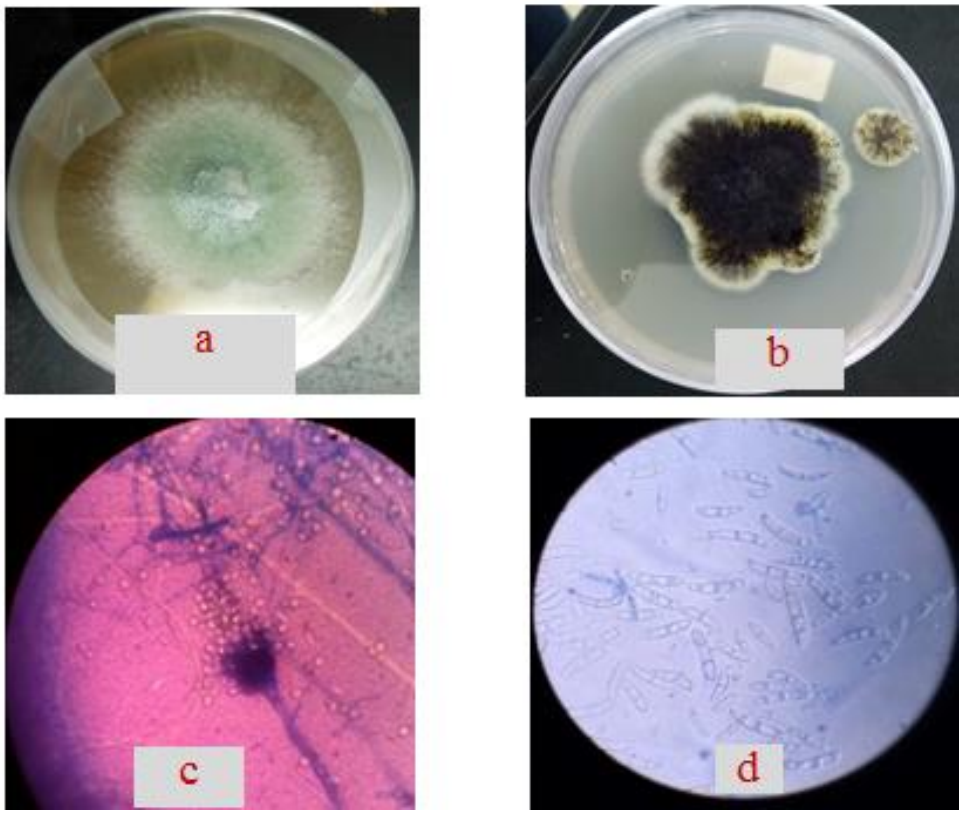

Plate 1. (a) Whitish green with smooth, hairy edge, (b) compact white-yellowish with dense of black colonies, (c) spherical vesicle with conidia arising from the vesicle, and (d) sickle shape microconidia.

Characterization of fungal isolates

Morphological characterization of fungal isolates was done based on the colour of the colony surface, reverse and periphery, and the shape of the margin and the isolates varied considerably in terms of surface, reverse, and periphery colour (Table 2 and Plate 1). The isolates were microscopically identified to genus level belonging to eight groups based on illustration provided in Barnet and Hunter (1987). A dendrogram (Figure 1) was drawn based on the colony and cell morphology characters giving 3 clusters. Hierarchical clustering using weighted pair group method with averaging (WPGMA) method and the distance between characters measured using Euclidean metric.

Characterization of bacterial isolates

Microscopic observations performed on bacterial isolates to investigate cell shape, motility, and Gram status revealed that the majority of the isolates were rod-shaped, and motile. One group was coccus in shape, and two were non-motile. In terms of Gram status, three were Gram-negative, and four turned out to be Gram-positive (Table 3). Based on biochemical tests performed (Table 4) and together with microscopic observations, bacterial isolates were identified to the *Genus* level belonging to seven groups. Plate 2 illustrates the changes in colour in some of the bacterial isolates on biochemical tests performed that included triple iron sugar test, citrate tests, and starch hydrolysis.

The identification of pure fungal colonies was done to genus level based on morphological characters, which included the elevation, margin, colour of the surface, reverse, and periphery. Other information included microscopic characteristics as shown in Table 2.

Plate 1 reveals the appearance of fungal isolates on PDA plates and fungal spores when observed under a light microscope.

Macroscopic description of fungal isolates indicated a wide variation in terms of surface colour that ranged from green to cream with the majority (13 out of 43) featuring black surface (*Aspergillus* spp.). Fungal isolates with

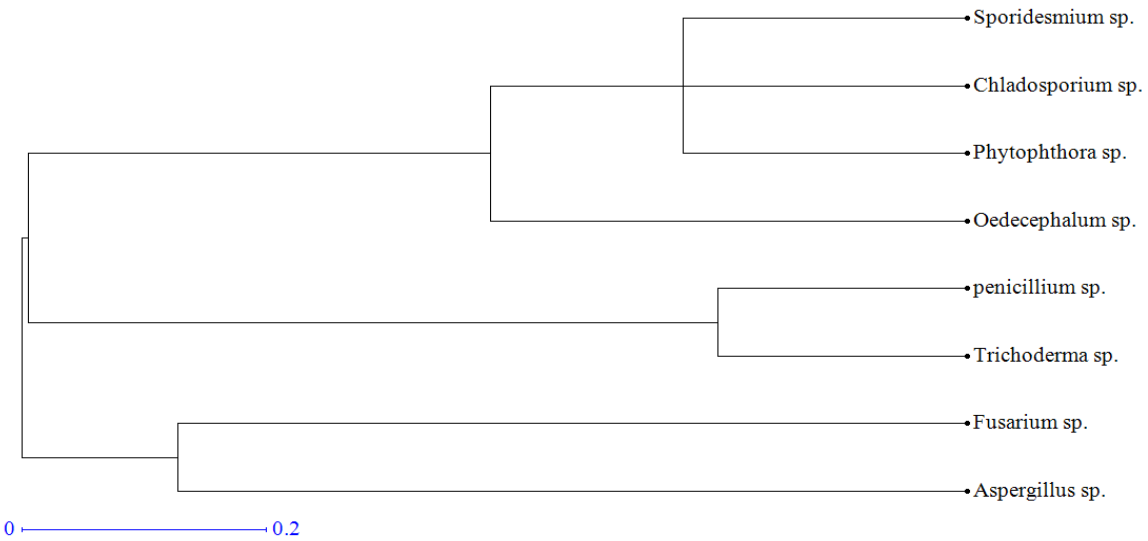

Figure 1. Dendrogram showing how the fungal isolates clustered morphologically.

Table 3. Microscopic observation of bacterial isolates.

No. of isolate	Cell shape	Motility	Gram reaction	Genus
11	Rod	Motile	Positive	*Bacillus* spp.
4	Rod	Motile	Negative	*Pseudomonas* spp.
2	Rod	Non-motile	Positive	*Chryseobacterium* spp.
11	Coccus	Non-motile	Positive	*Micrococcus* spp.
1	Rod	Motile	Negative	*Enterobacter* spp.
6	Rod	Motile	Negative	*Burkholderia* spp.
3	Rod	Motile	Positive	*Streptomyces* spp.
2	Rod	Motile	Negative	*Serratia* spp.

white surface colour (*Phytophthora*, *Chladosporium*, and *Sporidesmium* spp.) were the second majority followed by green (*Trichoderma* and *Penicillium* spp.) and pink (*Fusarium* spp.), then cream colour (*Oedecephalum* spp.). *Aspergillus, Phytophthora, Sporidesmium,* and *Chladosporium* spp. had cream reverse colour while *Trichoderma, Penicillium* and *Odecephalum* spp. were yellow. *Fusarium* spp. was the only fungal isolate that showed red reverse colour. All fungal isolates showed peripheral white colour except *Trichoderma* and *Fusarium* spp. that showed cream and pink colour, respectively. The margin of the fungal isolates was smooth except *Oedecephalum* spp., which were serrated. *Fusarium* spp. showed non-septated hyphae while the rest were all septated. *Trichoderma, Aspergillus, Fusarium, Phytophthora, Penicillium, Chladosporium*, and *Oedecephalum* showed raised elevation except for *Sporidesmium* species. The dissimilarity of the fungal isolates based on morphological characteristics is shown on the dendrogram drawn using the Euclidean metric in Figure 1 containing three clusters. *Trichoderma* and *Penicillium* spp. are more to each other than to *Phytophthora* and *Sporidemium*. However, *Phytophthora* and *Sporidesmium* spp. are more similar to each other than to *Chladosporium* and *Oedecphalum* spp., which are far closer to *Trichoderma* and *Penicillium*. *Chladosporium* and *Oedecephalum* are more close to one another than to *Fusarium* and *Aspergillus* spp., both of which are very different from the rest of the isolates.

Results in Table 3 reveal that cell shapes of bacterial isolates were rods except for *Micrococcus* that was Cocci. *Chryseobacterium* and *Micrococcus* species were non-motile while *Bacillus*, *Pseudomonas*, *Enterobacter, Burkholderia, Streptomyces*, and *Serratia* spp. showed positive motility. When bacteria were treated with KOH to test for Gram status, *Bacillus, Chryseobacterium, Micrococcus*, and *Streptomyces* spp. were positive while *Serratia, Burkholderia, Enterobacter* and *Pseudomonas*

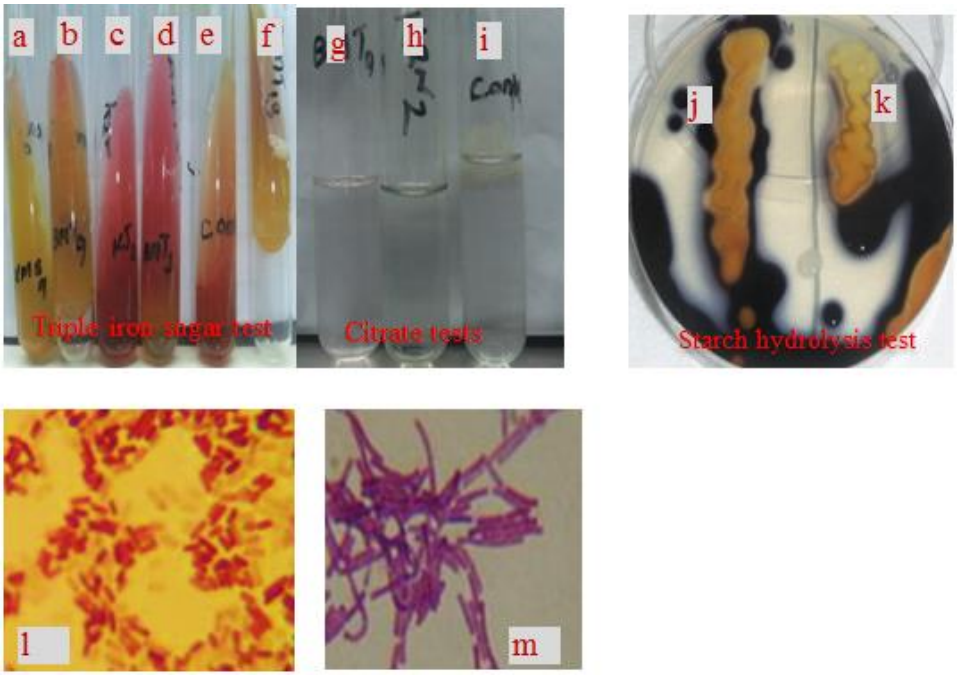

Plate 2. (a) Moderate acid slant, intense acid butt, no H_2S, and no gas (b) Alkaline slant, intense acid butt, positive for gas, no H_2S (c) Partial acid slant, partial acid butt, no gas, no H_2S (d) alkaline slant, moderate acid butt, no gas, no H_2S (e) Control, no reaction took place (f) moderate acid slant, intense acid, Butt, positive for gas, no H_2S (g) positive for citrate utilization (h) negative for citrate utilization (i) control (j) negative for starch hydrolysis (k) positive for starch hydrolysis. (l) gram-negative rod (m) gram-positive rod.

spp., were all negative for Gram status.

Microscopic descriptions of the bacterial isolates together with the biochemical characteristics, were used for the identification of the organism to the genus level (Krieg et al., 2010) as shown in Table 4.

Plate 2 illustrates the appearance of the individuals isolates on biochemical tests that included the triple iron sugar test, citrate tests, and starch hydrolysis and the Gram reaction of the bacterial isolates observed under a light microscope under oil immersion at X100.

Table 4 reveals that *Bacillus* and *Micrococcus* spp. were both positive and negative for citrate utilization while *Pseudomonas, Enterobacter, Streptomyc*es, and *Burkholderia* were all positive for citrate utilization. The majority of bacterial isolates (58%) were positive for starch hydrolysis, while the remaining 42% were negative. Some of the *Bacillus* spp. were either negative or positive for starch hydrolysis. *Micrococcus* spp. were all negative for starch hydrolysis, except two (KJ9) and (BMT 10), which hydrolyzed starch. All bacterial isolates were negative for hydrogen sulphide and gas, except *Enterobacter* (BMT9), which was positive for gas production. All bacterial isolates positive for acid butts and acid slants, except some *Micrococcus* spp. BMT10, BMT11, BMT1, and BMT13, which were negative for acid slants and acid butts. The intensity of acidity in the butt ranged from intense, moderate, and finally, slight acidity, as demonstrated by a few species of *Micrococcus*, *Bacillus*, and *Chryseobacterium*. Some isolates of *Bacillus*, *Pseudomonas*, *Burkholderia*, and *Micrococcus* were negative for slant acidity while the rest were all positive. The majority of bacterial isolates except for some isolates of *Bacillus* and *Burkholderia* spp. were all negative for MR tests. Finally, the results indicated that the majority of bacterial isolates were negative for VP tests except for some few isolates of *Bacillus, Pseudomonas, Micrococcus*, and *Burkholderia* were positive for VP tests. Those tests that turned red were positive for MR, whereas those that turned pale yellow to yellow were negative for MR tests. Based on the morphological characters and biochemical tests performed, a dendrogram was drawn using the WPMGA method, and the distance between characters was measured using the Euclidean metric. From Figure 2, BMT10 and BMT11 are closer to one another than they are to BMT15, BMT12, BMT13, KRN2, BMT20, KJ2, KMB6, KJ8, and KMB10. Similarly, KJ7 and KMB7 are closer to one another than they are to KMB11, while BMT16 and BMT4 are closer to one another than they are to BMT1. BMT 9 is dissimilar than BMT3, and

Table 4. Biochemical characterization of individual bacterial isolates.

Isolate no.	MR	VP	Catalase	Starch	Citrate	TSI				Organism ID
						Slant	Butt	Gas	H_2S	
BMT1	+	+	+	+	+	-	++	-	-	*Bacillus* spp.
BMT2	-	-	+	-	+	-	+	-	-	*Bacillus* spp.
BMT3	-	-	+	+	+	-	++	-	-	*Pseudomonas* spp.
BMT4	+	+	-	+	-	-	++	-	-	*Bacillus* spp.
BMT5	-	-	+	+	-	++	++	-	-	*Chryseobacterium* spp.
BMT6	-	+	+	+	-	+	++	-	-	*Bacillus* spp.
BMT7	+	+	-	+	+	+	+++	-	-	*Bacillus* spp.
BMT8	-	-	+	-	+	++	+++	-	-	*Micrococcus* spp.
BMT9	-	+	-	+	+	-	+++	+	-	*Enterobacter* spp.
BMT10	-	-	-	+	+	-	-	-	-	*Micrococcus* spp.
BMT11	-	-	-	-	+	-	-	-	-	*Micrococcus* spp.
BMT12	-	+	+	-	+	-	-	-	-	*Micrococcus* spp.
BMT13	-	+	+	-	+	-	-	-	-	*Micrococcus* spp.
BMT14	+	-	+	+	+	+	++	-	-	*Burkholderia* spp.
BMT15	-	+	+	-	+	+	+	-	-	*Micrococcus* spp.
BMT16	+	+	+	+	-	-	++	-	-	*Bacillus* spp.
BMT17	+	+	-	+	+	+	++	-	-	*Burkholderia* spp.
BMT20	-	-	+	+	+	-	+	-	-	*Burkholderia* spp.
BMT21	+	-	+	+	+	-	+++	-	-	*Burkholderia* spp.
KRN1	+	+	+	-	-	+	++	-	-	*Bacillus* spp.
KRN2	+	-	+	+	+	+	+	-	-	*Burkholderia* spp.
KRN3	-	-	-	+	-	+	+++	-	-	*Bacillus* spp.
KMB1	-	-	+	-	+	+	+++	-	-	*Streptomyces* spp.
KMB2	-	-	-	-	-	++	++	-	-	*Micrococcus* spp.
KMB3	-	-	+	-	-	+	++	-	-	*Streptomyces* spp.
KMB4	-	-	-	-	+	+	++	-	-	*Micrococcus* spp.
KMB6	-	+	+	-	+	+	+	-	-	*Serratia* spp.
KMB7	-	+	+	-	+	+	+++	-	-	*Serratia* spp.
KMB8	-	-	-	-	+	+	+++	-	-	*Micrococcus* spp.
KMB9	-	-	-	+	+	++	+++	-	-	*Bacillus* spp.
KMB10	-	-	+	+	+	+	+	-	-	*Streptomyces* spp.
KMB11	-	+	+	+	-	+	+++	-	-	*Pseudomonas* spp.
KMB12	-	-	-	-	+	+	++	-	-	*Micrococcus* spp.
KJ2	-	-	+	-	+	+	+	-	-	*Pseudomonas* spp.
KJ4	-	-	+	+	+	+++	+++	-	-	*Bacillus* spp.
KJ5	+	-	+	+	+	+	++	-	-	*Burkholderia* spp.
KJ6	-	-	-	+	-	+	++	-	-	*Bacillus* spp.
KJ7	-	+	+	-	+	+	++	-	-	*Pseudomonas* spp.
KJ8	-	-	+	+	+	+	+	-	-	*Chryseobacterium* spp.
KJ9	-	-	-	+	-	+	++	-	-	*Micrococcus* spp.

+: Positive; -: Negative; MR: Methyl Red tests; VP: Voges-Proskauer test; and H_2S: Hydrogen Sulphide gas tests; Slants: Acidity level of the slant. Butt: Acidity level of the butt.

BMT21, both of which are closer to one another. However, all the microbial isolates in clusters are dissimilar to BMT14 and KJ5, both of which appear to be closer to one another. KMB3 and KMB1 are closer to one another but very dissimilar to other microorganisms found in cluster 1. BMT6 and KRN1, which fall under the fourth cluster, are closer to one another but significantly dissimilar to the rest of the bacterial isolates in the first

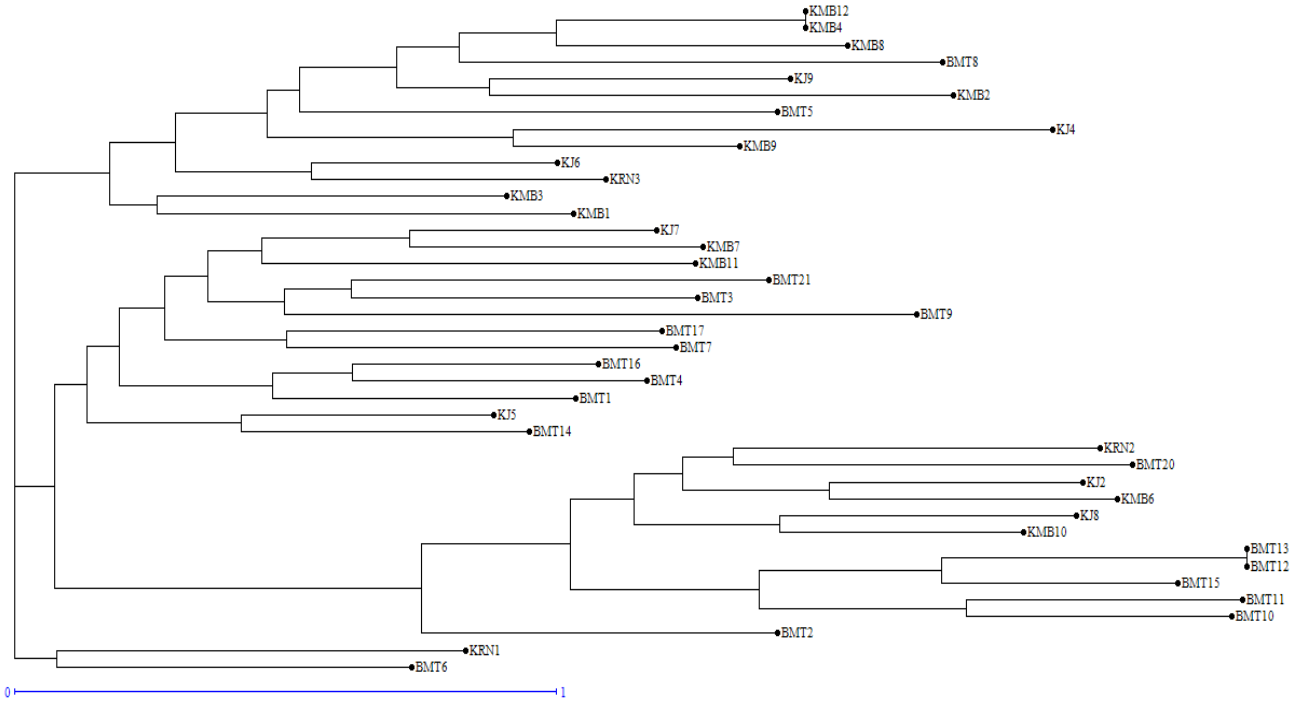

Figure 2. Dendrogram showing bacterial isolates clustered biochemically.

three clusters. Figure 2 shows how the isolates were clustered, and the dissimilarity of one isolate from the other.

DISCUSSION

Soil bacterial communities and the soil processes mediated by bacteria are critical for ecosystem functioning and productivity. Rhizosphere samples from bacterial wilt tolerant tomatoes from bacterial endemic areas Bomet, Kiambu, Kajiado, and Kirinyaga were screened for the isolation fungal and bacterial isolates considered to confer resistance to plants against bacterial wilt caused by *R. solanacearum*. After purification, rhizosphere samples from Bomet had the leading number of fungal and bacterial isolates followed by Kiambu. Kajiado was ranked third whereas Kirinyaga was fourth least count of fungal and bacterial isolates (Table 1). Most rhizospheric samples obtained from Kirinyaga and Kajiado were from the greenhouse where farmers do soil amendments like steaming, high use of fertilizers, and constant use of pesticides. Some of these synthetic substances cause a shift in the population of microbes in the soil due to altered rhizosphere environment. The least number of microorganisms obtained from the regions can be attributed to the intensive use of pesticides to control pests and diseases. The distribution of microorganisms in the soil is affected by factors that include farming systems employed by farmers, weather, and crop species distribution. According to Naylor et al. (2017), plant root microbiome can exhibit significant shifts during drought, for instance, increasing the *Actinobacteria*, and the shifts are host specific due to diversity in hosts' response mechanisms to drought. The rhizosphere harbours a wide variety of bacteria species, and the compositions of bacterial communities differ according to the root zone, plant species, plant phenological phase, stress, and disease events (Marschner et al., 2011). The results obtained confirm that soil characteristics, environmental conditions, and crop management strategies are important factors that drive the changes in the soil microbiology (Larkin, 2003). The dynamic parameters in the soil affect microbial growth and survival (Timmusk et al., 2011). Results revealed that the majority of organisms obtained from screening included fungi like *Penicillium, Trichoderma*, some species of *Fusarium* and *Aspergillus,* while bacteria included *Bacillus*, *Pseudomonas*, *Burkholderia* and *Enterobacter* spp. The dominance of *Bacillus* spp. was similar to that found by Pedrinho (2009), who reported predominance of *Bacillus* spp. in the rhizosphere. The dissimilarity of bacterial isolates in the four clusters dependent on shared morphology and biochemical characteristics. Organism closer to one another could be belonging to the same genera, for instance, BMT10 and BMT11 are both *Micrococcus* spp., but they could be of different species. Similarly, the relationship could be based on their ability

to utilize different substrates for their survival in the soil. KMB6 and KJ2 are *Serratia* and *Pseudomonas* spp., respectively, but hierarchical analysis some closer relationship between the two organisms yet to belong to the different species name. The similarity of the two organisms lies in their ability to both utilize citrate and catalase as well as demonstrate acidic butt and slants. However, both did not utilize starch and were negative on MR tests, gas, and hydrogen sulphide production. On the other hand, microorganisms of the same genus, for instance, KJ5 and KRN2 both belong to *Burkholderia* spp. and clustered together, but are not closer to one another. This is based on the ability of KRN2 to form a moderate acidic slant in relation to KJ5, which demonstrates slightly acidic slant. This can be concluded to be bacterial isolates that belong to similar genus but different species. Similarly, Gutiérrez et al. (2003) also found similar results revealing *Bacillus* spp. as the most dominant rhizosphere bacteria that aggressively colonize plant roots and rhizosphere, and promote plant growth through various mechanisms. Most rhizobacteria must colonize the roots to establish itself at population densities sufficient enough to produce beneficial effects on plants (Manivannan et al., 2012). The rhizosphere harbours a wide variety of bacteria species, and the compositions of bacterial communities differ according to the root zone, plant species, plant phenological phase, stress, and disease events (Naylor et al., 2017). The population of *Trichoderma*, *Aspergillus*, and *Fusarium* spp. were consistent with that of Thormann and Rice (2007), which pointed out *Trichoderma* spp. as one of the most frequently occurring fungi in the rhizosphere. The population of the fungal and bacterial isolates can be their interaction in the rhizosphere under a diverse environment. According to Frey-Klett et al. (2011), both fungi and bacteria have a significant role in enhancing the survival of interacting partners. However, Brulé et al. (2001) found out that the effect can be reciprocal sometimes. For instance, *Pseudomonas fluorescens BBc6R8* can promote the viability of *Laccaria bicolor*, a kind of mycorrhizal fungus in adverse soil conditions, but according to Deveau et al. (2010), the fungus, on the other hand, can also promote the survival of the bacterium. MR-VP tests are conducted to identify mixed acid fermenting bacteria through the detection of isolates' ability to utilize glucose and produce sufficient acid end products (Harold, 2002). Mixed acids, which are the characteristics end products of specific pathway fermentation that make the medium to be acidic (pH≤4.5) is detected by MR tests upon the addition of an indicator (Cappuccino and Sherman, 2002). The VP test, on the other hand, identifies bacteria that can produce non-acidic/neutral end products from organic acids that result from fermentation of glucose by detecting the intermediate products of fermentation pathway that yields 2, 3-butanediol (acetoin) when Barret's reagent was added. The ability of microorganisms to produce and take up various types of siderophores, including those from other microbial organisms often leads to a scenario where microorganisms benefit from others as a source of carbon while failing to utilize their own (Marschner et al., 2011) implies that those microorganisms that can utilize different types of siderophores have the competitive ability (Mirleau et al., 2000) than the others. This, therefore, explains the reason why some bacterial microorganisms like *Bacillus*, *Micrococcus*, and *Burkholderia* spp. were dominant in the sampled soils. Citrate utilization helps the bacteria maintain and competitive colonization of the roots (Weisskopf et al., 2011). For citrate utilization, organisms that are positive for citrate can use citrate as their source of carbon (Harold, 2002). Citrate utilization tests are indicated by the growth of bacteria, followed by an alkaline pH (Cappuccino and Sherman, 2002). For VP tests, deep red colouration indicated a positive VP test (Cappuccino and Sherman, 2002). The dendrograms (Figures 1 and 2) show how isolates were clustered based on morphological and biochemical characteristics. Relying on biochemical and morphological characteristics in determining the diversity of rhizospheric microorganisms may be reliable because two organisms that may belong to different species may show similar characteristics, yet they are not related. Application of biocontrol agents in the field requires a proper understanding of their diversity, but their accurate species identification based on morphology is challenging as a result of scantiness and similarity of morphological characters, which can further be made more challenging by the cryptic species (Kullnig et al., 2001). Molecular techniques that rely on DNA analyses to evaluate bacterial community composition and identifying the abundance of genes that are involved in the rhizosphere processes are therefore necessary for a better understanding of these communities (Lagos et al., 2015). Molecular identification is therefore necessary for accurate identification of species to gauge their diversity and similarity.

Conclusions

This study reveals that the presence of both bacteria and fungi will benefit plants' health and promotes growth. Biochemical and morphological characterization of these organisms revealed *Trichoderma*, *Aspergillus*, *Fusarium*, and *Phytophthora* spp. as the most abundant fungi in the rhizoplane of bacterial wilt tolerant tomatoes just like *Bacillus*, *Burkholderia*, *Micrococcus* and *Pseudomonas* spp. Though the biochemical and morphological characteristics can be used to show the dissimilarity between rhizospheric microorganisms, additional methods that rely on DNA are important to show their phylogenetic relationships.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

REFERENCES

Abbasi M, Majeed A, Hameed S, Imran A, Rahim N (2015). Isolation and characterization of plant growth-promoting rhizobacteria from wheat rhizosphere and their effect on plant growth promotion. Frontiers In Microbiology 6(198):2.

Addy HS, Askora A, Kawasaki T, Fujie M, Yamada T (2012). Loss of virulence of the phytopathogen Ralstonia solanacearum through infection by φ RSM filamentous phages. Phytopathology 102(5):469-477.

Álvarez B, Biosca EG, López MM (2010). On the life of *Ralstonia solanacearum*, a destructive bacterial plant pathogen. Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology 1:267-279.

Barnett HL, Hunter BB (eds) (1987) Illustrated genera of imperfect fungi, 4th edn. Macmillan Inc, USA

Bernard H (2002). Research methods in anthropology: Qualitative and quantitative approaches. (C. Creek, Ed.) (3rd ed). CA: Alta Mira Press.

Bergey D, Garrity G, Staley J, Boone D (2010). Bergey's manual of systematic bacteriology. New York: Springer.

Krieg N, Staley J, Brown D, Hedlund B, Paster B, Ward N, Ludwig W, Whitman W (2010). Bergey's Manual Of Systematic Bacteriology. 2nd ed. New York: Springer.

Brulé C, Frey-Klett P, Pierrat J, Courrier S, Gérard F, Lemoine M, Rousselet JL, Sommer G, Garbaye J (2001). Survival in the soil of the ectomycorrhizal fungus Laccaria bicolor and the effects of a mycorrhiza helper *Pseudomonas fluorescens*. Soil Biology and Biochemistry 33(12-13):1683-1694.

Bocsanczy AM, Achenbach UCM, Mangravita-Novo A, Yuen JMF, Norman DJ (2012). Comparative effect of low temperature on virulence and twitching motility of *Ralstonia solanacearum* strains present in Florida. Phytopathology 102(2):185-194.

Cappuccino J, Sherman N (2014). Microbiology (p. 77). San Francisco: Benjamin-Cummings.

Cappuccino JG, Sherman N (2002). Microbiology. A laboratory manual. 6th edition. Pearson education inc. San Francisco, California pp. 215-224.

Deveau A, Brulé C, Palin B, Champmartin D, Rubini P, Garbaye J, Sarniguet A, Frey-Klett P (2010). Role of fungal trehalose and bacterial thiamine in the improved survival and growth of the ectomycorrhizal fungus Laccaria bicolor S238N and the helper bacterium *Pseudomonas fluorescens* BBc6R8. Environmental Microbiology Reports *2*(4):560-568.

Diallo S, Crépin A, Barbey C, Orange N, Burini JF, Latour X (2011). Mechanisms and recent advances in biological control mediated through the potato rhizosphere. FEMS Microbiology Ecology *75*(3):351-364.

Dida G, Etisa D, Gizawu B (2018). Isolation and Characterization of Starch Degrading Rhizobacteria. Open Access Journal Of Microbiology and Biotechnology *3*(2).

Dinesh R, Anandaraj M, Kumar A, Bini YK, Subila KP, Aravind R (2015). Isolation, characterization, and evaluation of multi-trait plant growth promoting rhizobacteria for their growth promoting and disease suppressing effects on ginger. Microbiological Research 173:34-43.

Doolotkeldieva T, Bobusheva S (2016). Identification of *Ralstonia solanacearum* in Kyrgyzstan 's Potato Fields and the Possibility of Using Biocontrol Agents Against this Pathogen. International Journal of Environmental and Agriculture Research 5:146-155.

Elsas J, Kastelein P, de Vries P, van Overbeek L (2001). Effects of ecological factors on the survival and physiology of *Ralstonia solanacearum* bv. 2 in irrigation water. Canadian Journal Of Microbiology 47(9):842-854.

Frey-Klett P, Burlinson P, Deveau A, Barret M, Tarkka M, Sarniguet A (2011). Bacterial-Fungal Interactions: Hyphens between Agricultural, Clinical, Environmental, and Food Microbiologists. Microbiology And Molecular Biology Reviews 75(4):583-609.

Genin S (2010). Molecular traits controlling host range and adaptation to plants in Ralstonia solanacearum. New Phytologist 187(4):920-928.

Gutiérrez Mañero FJ, Probanza A, Ramos B, Colón Flores JJ, Lucas García JA (2003). Ecology, Genetic Diversity and Screening Strategies of Plant Growth Promoting Rhizobacteria (PGPR). Journal of Plant Nutrition 26(Suppl 5):1101-1115

Halebian S, Harris B, Finegold S, Rolfe R (1981). Rapid method that aids in distinguishing Gram-positive from Gram-negative anaerobic bacteria. Journal of Clinical Microbiology 13(3):444-448.

Ham J (2013). Intercellular and intracellular signalling systems that globally control the expression of virulence genes in plant pathogenic bacteria. Molecular Plant Pathology 14(3):308-322.

Harold JB (2002). Microbiological applications: A laboratory manual in general Microbiology. 8th Edition, pp. 1-478.

Hikichi Y, Mori Y, Ishikawa S, Hayashi K, Ohnishi K, Kiba A, Kai K (2017). Regulation Involved in Colonization of Intercellular Spaces of Host Plants in *Ralstonia solanacearum*. Frontiers in Plant Science *8*(June):967.

Khokhani D, Lowe-Power T, Tran T, Allen C (2017). A Single Regulator Mediates Strategic Switching between Attachment/Spread and Growth/Virulence in the Plant Pathogen *Ralstonia solanacearum*. Mbio 8(5).

Kullnig C, Krupica T, Woo S, Mach R, Rey M, Benítez T, Lorito M, Kubicek CP (2001). Confusion Abounds Over Identities of Trichoderma Biocontrol Isolates. Mycological Research 105(7):770-772.

Kumar A, Kumar A, Devi S, Patil S, Payal C, Negi S (2012). Isolation, screening and characterization of bacteria from Rhizospheric soils for different plant growth promotion (PGP) activities: An in vitro study. Recent Research in Science and Technology *4*(1):1-5.

Larkin R (2003). Characterization of soil microbial communities under different potato cropping systems by microbial population dynamics, substrate utilization, and fatty acid profiles. Soil Biology And Biochemistry 35(11):1451-1466.

Lagos L, Maruyama F, Nannipieri P, Mora M, Ogram A, Jorquera M (2015). Current overview on the study of bacteria in the rhizosphere by modern molecular techniques: A mini review. Journal of Soil Science and Plant Nutrition 15(2):504-523. doi: 10.4067/s0718-95162015005000042

Lu Y, Rao S, Huang F, Cai Y, Wang G, Cai K (2016). Effects of Biochar Amendment on Tomato Bacterial Wilt Resistance and Soil Microbial Amount and Activity. International Journal of Agronomy, pp. 1-10.

Manivannan M, Ganesh P, Kumar RS, Tharmaraj K, Ramya BS (2012). Isolation, Screening, Characterization and Antagonism Assay of PGPR Isolates from Rhizosphere of Rice Plants in Cuddalore District. International Journal of Pharmaceutical and Biological Archive *3*(1):179-185.

Marschner P, Crowley D, Rengel Z (2011). Rhizosphere interactions between microorganisms and plants govern iron and phosphorus acquisition along the root axis – model and research methods. Soil Biology and Biochemistry *43*(5):883-894.

Mendes R, Garbeva P, Raaijmakers JM (2013). The rhizosphere microbiome: Significance of plant beneficial, plant pathogenic, and human pathogenic microorganisms. FEMS Microbiology Reviews *37*(5):634-663.

Meng F (2013). The Virulence Factors of the Bacterial Wilt Pathogen *Ralstonia solanacearum*. Journal of Plant Pathology and Microbiology 04(03):3-5.

Mirleau P, Delorme S, Philippot L, Meyer J, Mazurier S, Lemanceau P (2000). Fitness in soil and rhizosphere of *Pseudomonas fluorescens* C7R12 compared with a C7R12 mutant affected in pyoverdine synthesis and uptake. FEMS Microbiology Ecology 34(1):35-44.

Mwashasha RM, Hunja M, Kahangi EM (2016). The effect of inoculating plant growth promoting microorganisms on rice production. International Journal of Agronomy and Agricultural Research 9(3):34-44.

Naylor D, DeGraaf S, Purdom E, Coleman-Derr D (2017). Drought and host selection influence bacterial community dynamics in the grass root microbiome. The ISME Journal *11*(12):2691-2704.
Nion Y, Toyota K (2015). Recent Trends in Control Methods for Bacterial Wilt Diseases Caused by Ralstonia solanacearum. Microbes and Environments 30(1):1-11.
Pedrinho EAN (2009). Isolation and Characterization of Growing Bacteria in Corn (*Zea mays* L.). Mr. Thesis. University of São Paulo, Brazil.
Rado R, Andrianarisoa B, Ravelomanantsoa S, Rakotoarimanga N, Rahetlah V, Fienena FR, Andriambeloson O (2015). Biocontrol of potato wilt by selective rhizospheric and endophytic bacteria associated with potato plant. African Journal of Food, Agriculture, Nutrition and Development 15(1):9762–9776.
Rai S, Kashyap P, Kumar S, Srivastava A, Ramteke P (2015). Comparative analysis of microsatellites in five different antagonistic Trichoderma species for diversity assessment. World Journal Of Microbiology and Biotechnology *32*(1).
Salanoubat M, Genin S, Artiguenave F, Gouzy J, Mangenot S, Arlat M, Boucher CA (2002). Genome sequence of the plant pathogen *Ralstonia solanacearum*. Nature 415(6871):497–502.
Seleim M, Abo-Elyousr K, Abd-El-Moneem K, Saead F (2014). First Report of Bacterial Wilt Caused by *Ralstonia solanacearum* Biovar 2 Race 1 on Tomato in Egypt. The Plant Pathology Journal 30(3):299-303.
Shishido M, Miwa C, Usami T, Amemiya Y, Johnson KB (2007). Biological Control Efficiency of Fusarium Wilt of Tomato by Nonpathogenic *Fusarium oxysporum* Fo-B2 in Different Environments. Phytopathology 95(9):1072-1080.
Sudisha J, Abdelrahman M, Tran LP, Shin-ichi I (2013). Characterization of rhizosphere fungi that mediate resistance in tomato against bacterial wilt disease 64(12):3829-42. https://doi.org/10.1093/jxb/ert212
Swarupa V, Ravishankar KV, Rekha A (2014). Plant defense response against *Fusarium oxysporum* and strategies to develop tolerant genotypes in banana. Planta 239(4):735-751.
Thormann MN, Rice AV (2007). Fungi from peatlands. Fungal Diversity 24:241-299.
Timmusk S, Paalme V, Pavlicek T, Bergquist J, Vangala A, Danilas T, Nevo E (2011). Bacterial Distribution in the Rhizosphere of Wild Barley under Contrasting Microclimates. Plos ONE 6(3):e17968.
Weisskopf L, Heller S, Eberl L (2011). Burkholderia Species Are Major Inhabitants of White Lupin Cluster Roots. Applied And Environmental Microbiology *77*(21):7715-7720.
Xue QY, Chen Y, Li SM, Chen LF, Ding GC, Guo DW, Guo JH (2009). Evaluation of the strains of Acinetobacter and Enterobacter as potential biocontrol agents against Ralstonia wilt of tomato. Biological Control *48*(3):252–258.
Yanti Y, Astuti FF, Habazar T, Nasution CR (2017). Screening Of Rhizobacteria From The Rhizosphere Of Healthy Chili To Control Bacterial Wilt Disease and to Promote Growth and Yield of Chili. Biodiversitas 18(1):1-9.
Zuluaga A, Puigvert M, Valls M (2013). Novel plant inputs influencing *Ralstonia solanacearum* during infection. Frontiers In Microbiology 4:349. https://doi.org/10.3389/fmicb.2013.00349.