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ISOLATION AND CHARACTERISATION OF *RALSTONIA SOLANACEARUM* STRAINS OF TOMATO WILT DISEASE FROM MASENO, KENYA

Buyela D. Khasabulli[1+]
David M. Musyimi[2]
David M. Miruka[3]
George T. Opande[4]
Pascaline Jeruto[5]

[1]*Sn. Technologist, SPBS, DEPT. of Botany, Maseno University Kenya*
[2,4]*Research scientist SPBS Dept. of Botany, Maseno University Kenya*
[3]*Research scientist SPBS Dept. of Zoology, Maseno University Kenya*
[5]*Research scientist Dept. of Bio Sciences, School of Science, University of Eldoret Kenya*

(+ Corresponding author)

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ABSTRACT

Ralstonia solanacearum (E.F. Smith) Yabuuchi, is the cause of bacterial wilt of tomato and one of the most severe pathogens of solanaceous crops with a very wide host range. There is little information on *R. solanacearum* strains in Maseno region in Kenya despite the fact that bacterial wilt of tomato is of economic importance. The objective of this study was to isolate the strains of R. solanacearum of tomato and characterize morphologically and biochemically *Ralstonia solanacearum* races and biovars from infected tomato plants in Maseno region. This study was conducted at Maseno University in the Department of Botany at Microbiology Laboratory. Ten diseased tomato plants from Maseno, Mariwa, Seme and Hollo were collected and cut into small sections (0.5–1cm). The plant sections were then plated onto Triphenyl tetrazolium chloride (TZC) media. Profiling of the pathogen was done morphologicaly using culture techniques; biochemical tests included Gram staining test, Potassium hydroxide test, Catalase oxidase test, Gas production test, Starch hydrolysis test and sugar utilization test. The races were then identified by pathogenicity test on wide host range. All the isolates had fluidal pinkish red centered colonies on TZC media, they were Gram negative, potassium hydroxide solubility positive, produced gas from glucose, ooze test positive and did not hydrolyse starch which is typical of *R. solanacerum. R. solanacearum* strains from infected tomato plants in Maseno region were race 3 biovar 1 and race 3 biovar 3. All isolates were pathogenic on tomato plants.

1. INTRODUCTION

Bacterial wilt is caused by *Ralstonia solanacearum*, which was formerly known as *Pseudomonas solanacearum* E.F. Smith, is one of the most devastating, important and wide-spread bacterial diseases of crops in tropical environments [1]; [2]. *R. solanacearum* is an aerobic non-sporing Gram negative plant pathogenic bacterium [3]. It is soil borne and motile with a polar flagellar tuft and sometimes 1 to 4 polar flagella. It colonizes the xylem, causing bacterial wilt in a wide range of potential host plants [4].

Ralstonia solanacearum is a complex species with exceptional diversity among strains regarding host range, geographical distribution, pathogenicity, epidemiological relationships and physiological properties [5]. This complex species has been subdivided into five Landraces on the basis of differences in host range [6] and six biovars on the basis of carbohydrate utilization [7]. There is no information on the races and biovars found in Maseno Region.

The landrace and biovar classification has gained wide acceptance for subdividing *R. solanacearum* [8]. The racial pattern system groups the strains of *R. solanacearum* according to their ability to infect different host plants, viz., landrace 1 the most widely distributed in the world and comprises of many strains having a wide host range and is pathogenic on different solanaceous plants and weed hosts [6] landrace 2 is restricted to triploid banana and Heliconia; landrace 3 usually occurs at higher altitudes in tropical areas and in those with temperate climate and affects mainly potato and tomato and to an extent, other hosts such as solanaceous weeds and geranium, landrace 4 infects ginger, and landrace 5 is pathogenic to mulberry [9] The biovar scheme divides the species into five groups on the ability of the strains to metabolise or oxidise specific hexose sugars and disaccharides [10]. At present, there is lot of controversy regarding the prevalence of strains in the various parts of the world. In Kenya however, limited information is available on the prevalence of biovars, landraces and strains in various parts of the country especially Western Kenya region. Therefore, the present investigations on isolates of *R. solanacearum* causing wilt on tomato plants in Maseno region was carried out.

2. MATERIALS AND METHODS

2.1. Study Site

This study was conducted at Maseno University in the Department of Botany Microbiology Laboratory. Maseno is situated in Western, Kenya, its geographical coordinates are 0° 10' 0" South, 34° 36' 0" East and the altitude is 1,503 metrers or 4,934 feet above sea level (KNBS, report, 2013). Maseno receives both short and long rains averaging 1750mm per annum with mean temperature of 28.7^{0}C.The study was carried out between October 2015 and April 2016.

2.2. *Ralstonia Solanacearum* Infected Plant Sample Collection

A total of 10 diseased tomato plants were collected from four sites i.e Maseno, Holo, Seme and Mariwa, where they were selected on the basis of tomato production and representative of Maseno division. From each plant, 5 samples were prepared. Field diagnosis of infected plant samples was done by critically observing the bacterial wilt symptoms which included wilting, yellowing of leaves, stunting of growth and observance of narrow dark stripes corresponding to the infected vascular bundles beneath the epidermis [7]. Simple random sampling technique was used for collection of samples so as to eliminate selection bias and for accuracy of representation.

Plate-1. *R.solanacearum* infected plant part showing leaf epinasty

2.3. Isolation of *Ralstonia Solanacearum*

Collected tomato plant materials were surface sterilized with 1% Sodium Hypochlorite (NaOCl) solution for 1 to 2 min, followed by three repeated washings with distilled water and blot dried according to procedure by Singh,

et al. [11]. The plant sections (0.5–1 cm) were then plated onto 2, 3, 5 triphenyl tetrazolium chloride (Kelman's TZC agar) medium (glucose 10 g, peptone 10 g, casein hydrolysate 1 g, agar 18 g, distilled water 1000 ml). 5 ml of TZC solution filter sterilized was added to the autoclaved medium to give final concentration of 0.005%) according to the procedure of Seleim, et al. [12]. The plates were incubated at 28°C ± 2°C for 24–48 hr. The virulent colonies in the medium characterized by dull white colour, fluidal, irregularly round with light pink centres were further streaked on TZC medium to get pure colonies of the bacterium (Plate 2).

Plate-2. Appearance of *R.solanacearum* on TZC media.

2.4. Preservation of *Ralstonia Solanacearum*

Two loopfuls of bacterium from 48hr old colonies grown on Kelman's TZC Agar was transferred to 5 mL of sterile double distilled water in screw capped vials according to procedure by Grover, et al. [13]. They were stored under refrigeration at 20°C for maintenance of virulence. To revive an isolate, the stored bacteria were streaked on TZC Agar medium and well separated fluidal colonies were selected.

2.5. Preparation of *Ralstonia Solanacearum*

A bacterial suspension prepared by pouring sterile distilled water over 24hr old bacterial growths on Nutrient agar slants, the suspension was then poured into a test tube and adjusted to optical density (O.D) 0.5 in Spectrophotometer (Novaspec II) in blue filter (425nm) to obtain a bacterial population of 1 x 10^8 colony forming unit per millilter of the suspension according to the procedure by Mushore and Matuvhunye [14].

2.6. Simple Staining of *Ralstonia Solanacearum*

Fresh pure culture of the bacterium were stained by Ziehl's carbolfuchsin according to the procedure by Weller, et al. [15]. Bacteria cells were spread over the central area of the slide by use of an inoculating loop. The slides were placed on a dryer with smeared surface upwards, and air dried for 30 minutes. The dried smear was then heat fixed. The smear was then covered with carbol fuchsin stain and heated until vapour just began to rise (about 60 degree Celsius). The heated stain was allowed to remain on the slide for 5 minutes and then washed off with clean water. The smear was then covered with 3% v/v acid alcohol for 2-5 minutes (or 20% sulfuric acid). It was then washed well with clean water. The cells were then stained with methylene blue and viewed on a microscope (Nikon) under oil immersion for determination of size, shape and arrangement of cells.

2.7. Cultural Studies of the Bacterium *(Ralstonia Solanacearum)*

Cultural characteristics of the bacterium such as size and shape of colonies, colour of colonies, surface margin of colonies, and growth on nutrient agar (NA) and in nutrient broth were studied according to the procedure by Pawaskar, et al. [16].

2.8. Gram Staining Test of *Ralstonia Solanacearum*

Gram staining of *R.solanacearum* was done according to the procedure by Chaudhry and Rashid [17]. A loop full of the bacterium was spread on a glass slide and fixed by heating on a very low flame. Aqueous crystal violet solution (0.5%) was spread over the smear for 30 seconds and then washed with running tap water for one minute. It was then flooded with iodine for one minute, rinsed in tap water and decolorized with 95% ethanol until colorless runoff. After washing, the specimen was counter-stained with safranin for approximately 10 seconds, washed with water, dried and observed microscopically at Mg X10, Mg X 40 and MgX 100 using oil.

2.9. Potassium Hydroxide Test for *Ralstonia Solanacearum*

Bacteria was aseptically removed from petri plates with an inoculating wire loop, placed on glass slide in a drop of 3% Potassium hydroxide (KOH) solution, stirred for 10 seconds and observed for the formation of slime threads according to the procedure by Senthilkumar, et al. [18] 18. The colony's solubility in 3% KOH solution eliminated any possible confusion of the organism with other wilt causing pathogen of tomato.

2.10. Catalase Oxidase Test of *Ralstonia Solanacearum*

Young agar cultures (18-24 hrs) and 3% hydrogen peroxide (H_2O_2) were used to observe production of gas bubbles according to the procedure by Chaudhry and Rashid [17]. A loop full of bacterial culture was mixed with a drop of H_2O_2 on a glass slide and observed for the production of gas bubbles with unaided eye and under a dissecting magnification of 25X.

2.11. Gas Production Test for *Ralstonia Solanacearum*

The ability of the culture to produce gas was tested by growing the organism in nutrient broth containing 2 per cent glucose, according to the procedure by Pawaskar, et al. [16]. The medium was distributed in test tube containing inverted Durham's tube. These were sterilized by autoclaving at 15 lbs psi for 20 minutes. The tubes were inoculated with 0.5 ml of bacterial suspension and incubated at room temperature (28 ± 1 ^{0}C) for seven days. Gas production was indicated by air bubbles in the inverted Durham's tube.

2.12. Starch Hydrolysis Test for *Ralstonia Solanacearum*

The ability of bacterium to hydrolyze starch was studied by growing on nutrient agar containing one per cent soluble starch according to the procedure by Kumar and Khare [19]. The sterilized liquefied nutrient agar was poured to sterilized Petri plates and allowed to solidify. The culture was inoculated in the center of the plates and incubated for seven days at room temperature (28 ± 1 ^{0}C). The plates were then flooded with Lugol's iodine (Iodine 1g, potassium iodide 2gm and distilled water 300 ml). Clear zone around bacterial culture was an indication of positive test.

2.13. Profiling of *R. Solanacearum* Isolates into Biovars

Biovars of *R. solanacearum* strains were determined by standard procedure according to Grover, et al. [13]. The following basal medium was used for biovar identification: $NH_4H_2PO_4$, 1.0 g; KCl, 0.2 g; $MgSO_4.7H_2O$, 0.2 g; Peptone, 1.0 g; 1% (wv^{-1}) aqueous solution of bromothymol blue, 0.3 ml; agar, 1.5 g; distilled water, 1litre. The pH of the medium was adjusted to 7.1 with 40% (wv^{-1}) NaOH solution before addition of the agar. 5ml of a 10% (wv^{-1})

pre-sterilized solution of the sugars (lactose, maltose and cellobiose) and sugar alcohols (mannitol, sorbitol and dulcitol) was added to 45 ml of molten cooled basal medium separately. 200µl of these media was then dispensed into each test tube. Hayward's medium without a carbon source and un-inoculated test tubes served as control. Each test tube was inoculated with 3 µl of a 2 x 10^9 CFU ml^{-1} cell suspension prepared from overnight Kelman's TZC broth culture. The cultures were incubated at 28±1°C and examined at 3, 7 and 14 days for change of pH (yellow colour). Positive cultures changed the culture medium from green to yellow. Each test was replicated three times.

2.14. Landrace Identification

The landraces of *Ralstonia solanacearum* were identified by pathogenicity test on wide host range [20]. Seedlings of eggplant, tomato, potato, tobacco, peanut and pepper were raised in tray. One month (30 days) old seedlings were inoculated by soil inoculation method. The incubated plants were then kept in the greenhouse until symptoms development.

3. RESULTS

3.1. Isolation of *Ralstonia Solanacearum*

Isolation of the bacterium was done from tomato (*S. lycopersicum*), plants showing typical symptoms of bacterial wilt. Such signs were: lower leaves turning pale yellow, loss of leaf turgidity followed by drooping of leaves and sudden wilt of the plants (plate 3). The vascular bundles of the infected plants showed brown discoloration (plate 4). Infected plants showed milky white bacterial streaming from diseased stem and thus were ooze test positive (plate 5).

Plate-3. Tomato plant showing symptoms of *R.solanacearum* infection

Plate-4. Longitudinal section of *R.solanacearum* infected tomato stem with vascular bundles showing brown discoloration.

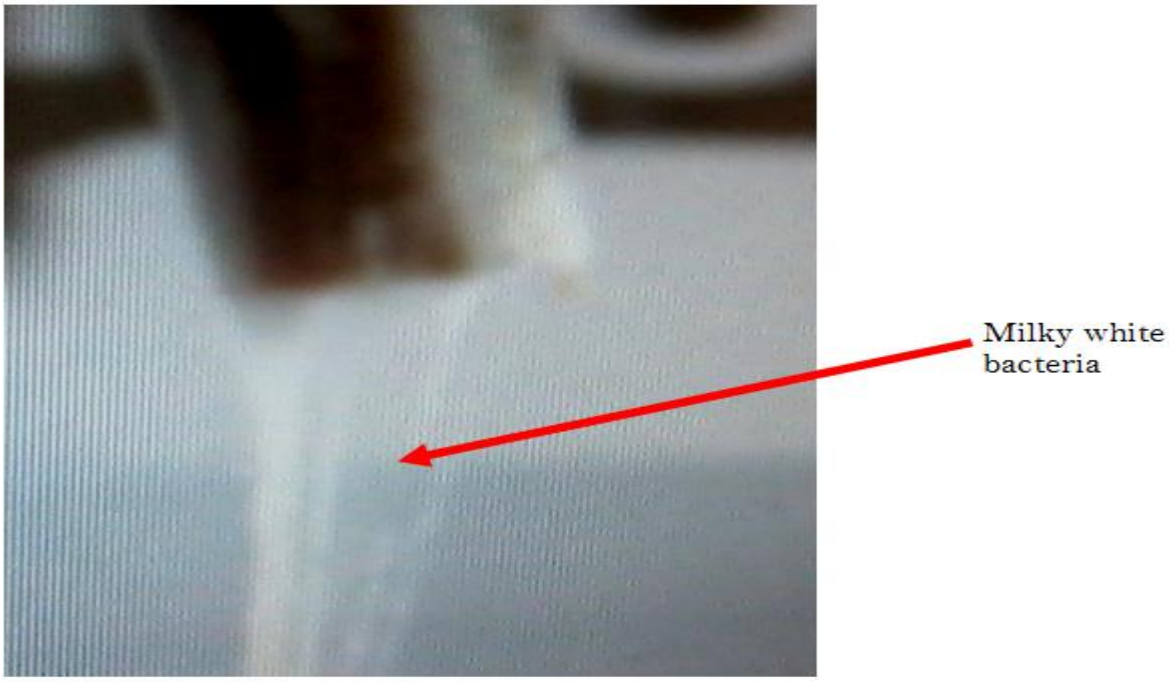

Plate-5. Milky white bacteria streaming from diseased stem suspended in water.

3.2. Morphological Profiling of *Ralstonia Solanacearum*

Fluidal pinkish red centered colonies of typical *R. solanacerum* were observed on TZC media (Plate 6, 7 and 8). Virulent colonies appeard white with pink colour at the centre. The different colony morphology was observed, colonies were irregular, white and fluidal (plate 9 and 10) on nutrient agar media.

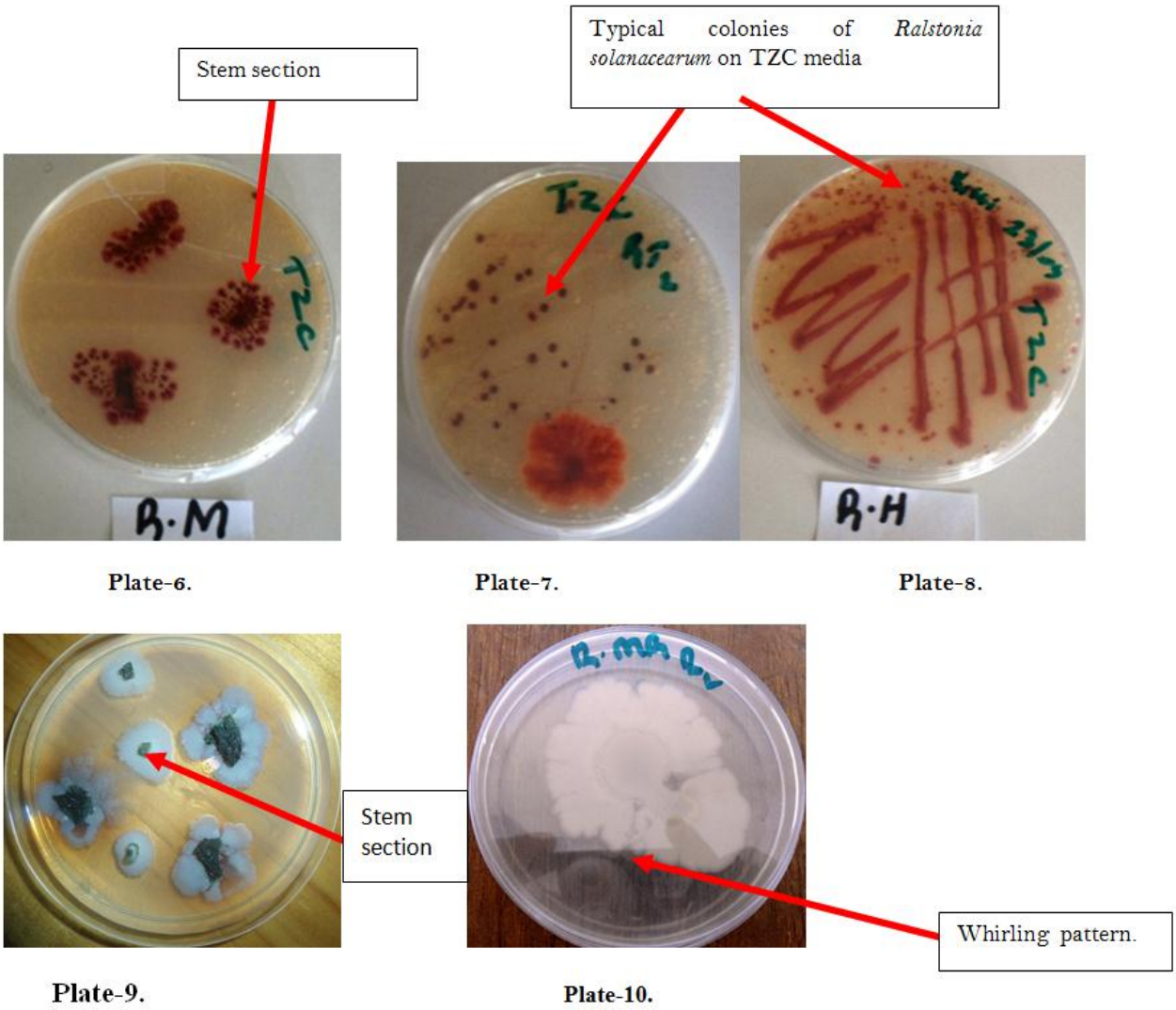

Plate-6. Plate-7. Plate-8.

Plate-9. Plate-10.

Plate-6. Tomato stem sections isolation on TZC medium.

Plate-7. Typical colonies of *R.solanacearum* on TZC medium.

Plate-8. Confirmation of *Ralstonia solanacearum* on TZC medium.

Plate-9. Tomato stem sections isolation on Nutrient Agar medium.

Plate-10. Appearance of of *Ralstonia solanacearum* on Nutrient Agar medium showing whirling pattern.

Table-1. Morphological profiling of four isolates of *R. Solanacearum* on TZC media

Sno.	Isolate	Morphology on TZC medium
1	Maseno	irregular with smooth margin, slimy dull white colonies with pink to red center
2	Mariwa	moderately fluidal, irregularly shaped, convex, dull white colonies with pink colored center and bluish margin
3	Seme	irregular with smooth margin, slimy dull white colonies with pink to red center
4	Hollo	irregular with smooth margin, slimy dull white colonies with pink to red center

3.3. Gram's Stain of *R.Solanacearum*

The microscopic results showed that all the isolates of *R.solanacearum* did not retain violet colour i.e. the isolates retained counter stain (pink colour) (plate 10). This was an indication that all isolates of *R. solanacearum* representing each group are gram negative and straight or curved rod shaped.

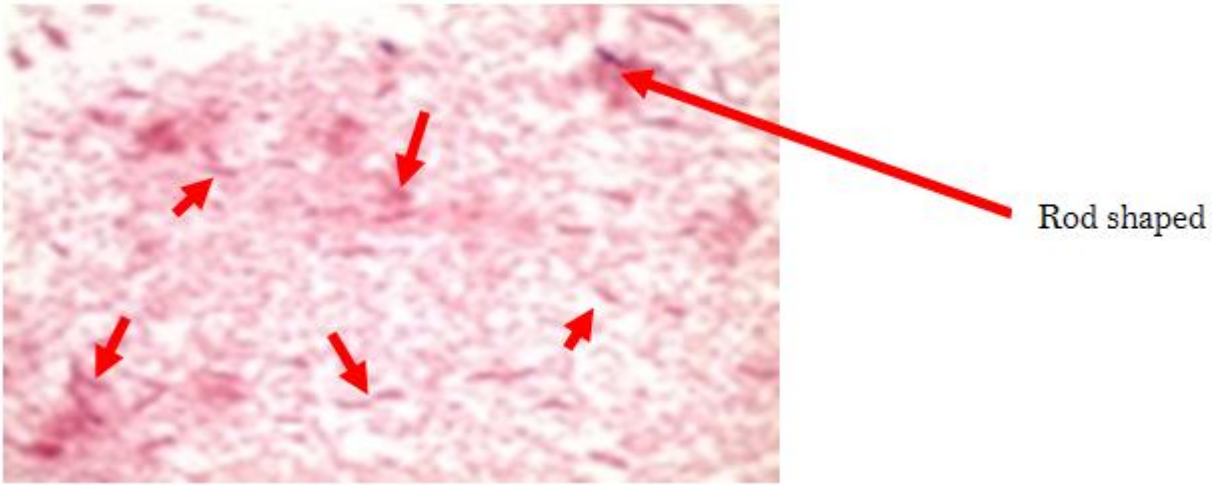

Plate-10. Appearance of *Ralstonia solanacearum* cells after gram staining (Mgx100)

3.4. Potassium Hydroxide Solubility Test

The Gram negative test of *R.solanacearum* was also confirmed by Potassium hydroxide solubility test. An elastic thread or viscous thread was observed when the loop was raised from the bacterial solution by tooth pick, a few centimeters from glass slides for all the isolates from the four sites indicating that all groups of *R.solanacearum* isolates were Gram negative (Table 2).

3.5. Catalase Oxidase Test

All the isolates tested produced gas bubbles during these tests (Table 2), indicating that these might be *Ralstonia solanacearum.*

3.6. Gas Production

All the isolates tested produced gas from glucose within eighteen hours of incubation (Plate 11, Table 2).

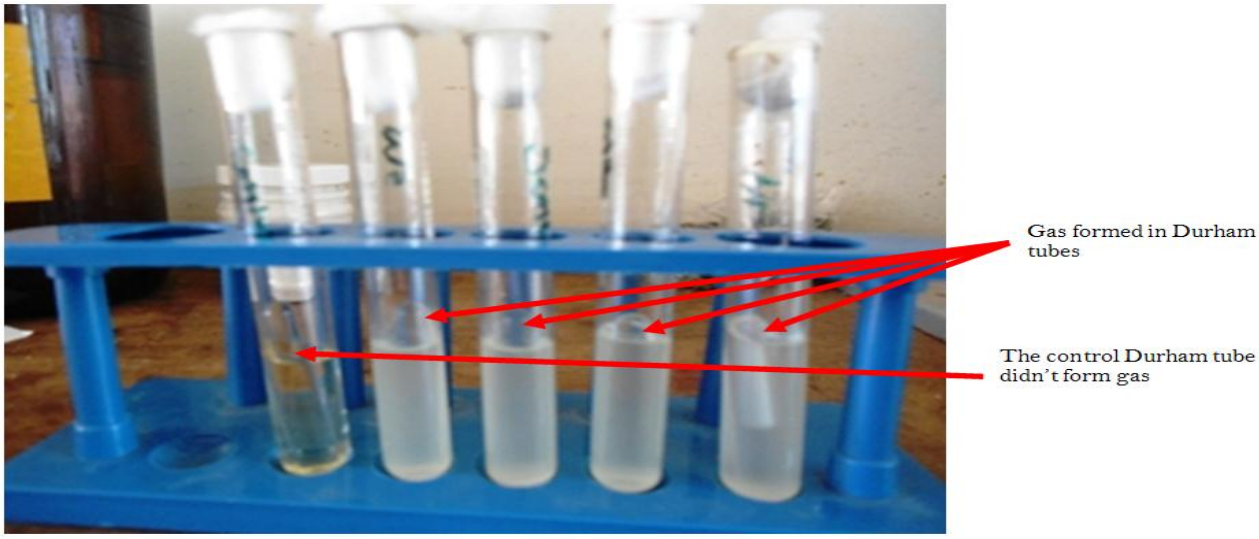

Plate 11 .Gas production by *R.solanacearum* isolates.

3.7. Starch Hydrolysis

Starch hydrolysis test of the bacterium showed that all the isolates from the four sites were unable to hydrolyse starch (Plate 12, Table 2); no clear zone surrounding the bacteria growth was observed when plates were flooded with Lugol's iodine (IKI) solution.

Plate-12. Starch hydrolysis

Table-2. Profiling of *Ralstonia solanacearum* strains isolated from bacterial wilt infected tomato plants in Maseno region.

TEST	Maseno isolate	Mariwa isolate	Seme isolate	Holo isolate
Gram's Stain	_	_	_	_
Potassium Hydroxide Solubility Test	+	+	+	+
Catalase oxidase test	+	+	+	+
Gas production	+	+	+	+
Starch hydrolysis	_	_	_	_
Ooze test	+	+	+	+
Growth on Tzc	+	+	+	+

Legend: += Positive reaction; - =Negative reaction

3.8. Biochemical Profiling of *R. Solanacearum*

The oxidation reaction was indicated by the change of the color of Hayward's medium. The results revealed a change of color from green to yellow color indicating the oxidization of sugars by bacterial isolates (plate 13). All tested strains utilized glucose and fructose invariably. However marked differences were observed in the ability of the strains to oxidize three dissacharides (Lactose, Celiobiose and Maltose) and three sugar alcohols (Mannitol, Sorbitol and Ducitol). Three strains were classified as biovar III and one strain as biovar I based on Haywards classification scheme [21]. Biovar III strains (Maseno, Seme and Holo) oxidized all of the sugar and sugar alcohols while biovar I strain (Mariwa) didn't oxidize any of the disaccharides or sugar alcohols even after five weeks of incubation (Table3). No reaction was produced in inoculated media without carbohydrate source.

Table-3. Biochemical differentiation of *Ralstonia solanacearum* into biovars

ISOLATE	Glucose	Fructose	Lactose	Celiobiose	Maltose	Mannitol	Sorbitol	Ducitol	Biovar
Distilled water(control)	-	-	-	-	-	-	-	-	-
Maseno	+	+	+	+	+	+	+	+	III
Mariwa	+	+	-	-	-	-	-	-	I
Seme	+	+	+	+	+	+	+	+	III
Holo	+	+	+	+	+	+	+	+	III

Legend: += Positive reaction or change of colour;
-= Negative reaction or no change of colour

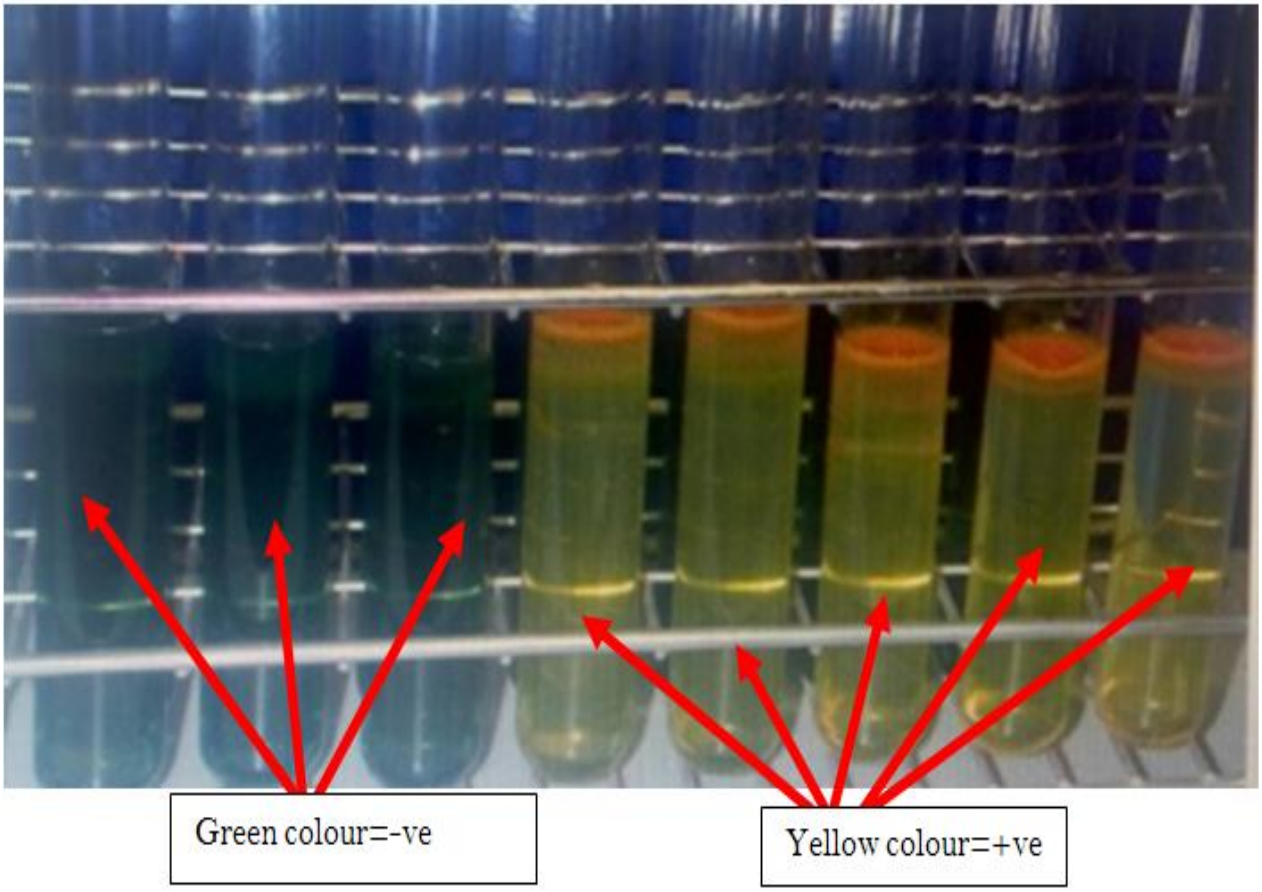

Plate-13. Carbohydrate utilization test for strains of *R.solanacearum.*

3.9. Designation of Landraces of *R. Solanacearum* in Maseno Region

The landraces of *R. solanacearum* were identified by pathogenicity tests in wide host which included; tomato, Potato, Pepper and eggplant. Initial symptoms of wilting in susceptible hosts appeared 3-4 days after stem inoculation. The symptoms consisted of wilting of the inoculated leaf and eventually the whole plant wilted. The result of the pathogenicity test showed that all of the groups of *R.solanacearum* isolates tested in the study were able to cause wilt symptom in inoculated tomato, Potato, Pepper and eggplant plants. The four strains (Maseno, Mariwa, Seme and Holo) were highly virulent on potato and tomato plants but were moderate to slight virulent on eggplant and Pepper after four weeks of inoculation (Table 4).

The four strains (Maseno, Mariwa, Seme and Holo) were highly virulent on potato and tomato plants but were moderate to slight virulent on pepper and low virulent on eggplant after four weeks of inoculation. But other hosts such as tobacco and peanut did not show wilting symptoms. Therefore, all the four strains from Maseno region had characteristic of race 3 with a limited host range on potato, tomato and a few other hosts (Table 4). The limited host range is the characteristic of landrace 3 of *R.solanacearum* [22].

Table-4. Pathogenicity test on potato, tomato, pepper and egg plant and classification of *Ralstonia solanacearum* strains in Maseno region

ISOLATE	TOMATO	POTATO	PEPPER	EGGPLANT	TOBACCO	PEANUT
Distilled water	0	0	0	0	0	0
MASENO	H	H	M	L	0	0
MARIWA	H	H	M	L	0	0
SEME	H	H	M	L	0	0
HOLO	H	H	M	L	0	0

Legend: Average disease indices of 9 plants at 28 days after inoculation and rating scales [20] were as followed: H, High(disease index 4.1 to 5.0); M, Moderate (2.6 to 4.0); L, Low (1.1 to 2.5); and, 0, None (1.0)

3.9. Response of Tomato to Infection by *Ralstonia Solanacearum*

Typical bacterial wilt symptoms were observed one week after transplanting. The cause of wilt was confirmed to be *R. solanacearum* by looking for bacterial ooze (Plate 14) and isolating the bacterium from wilted plants followed by culture on TZC medium. The tomato cultivar used, Rio Grande, was very susceptible to bacterial wilt. Disease incidence progressed rapidly beginning week one after transplanting.

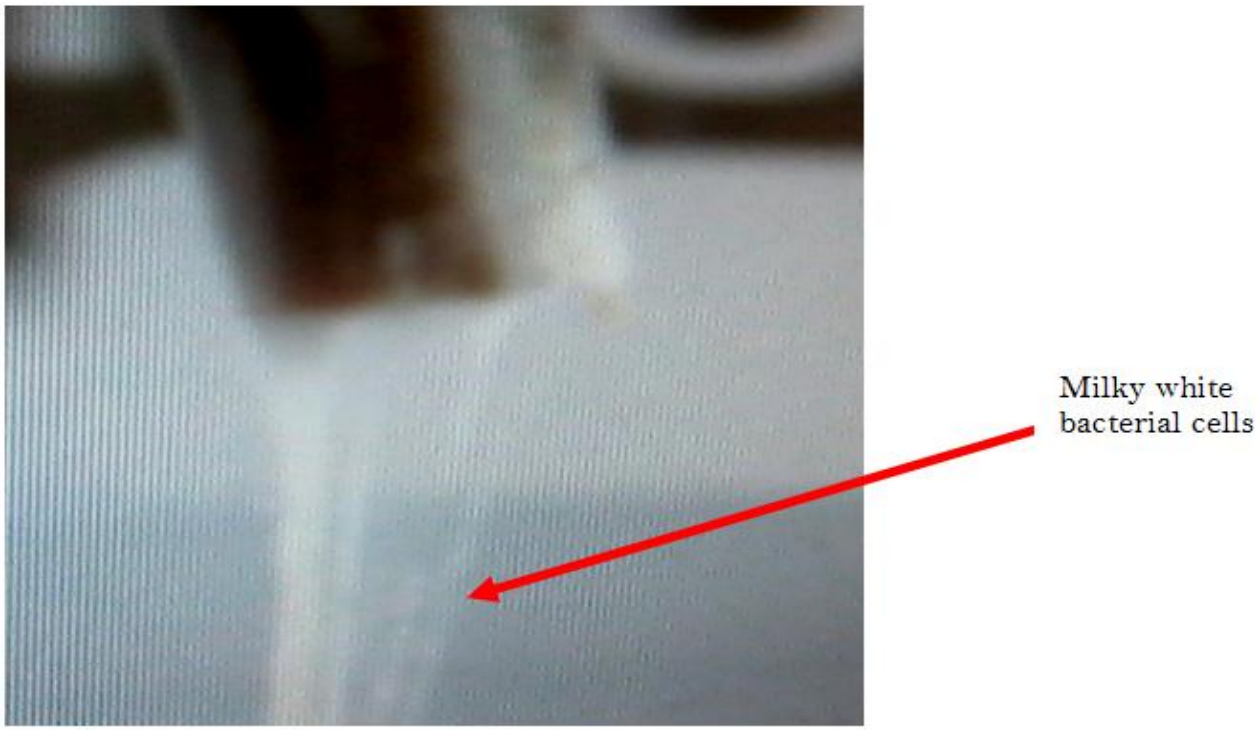

Plate-14. Milky white bacteria streaming from diseased stem suspended in water.

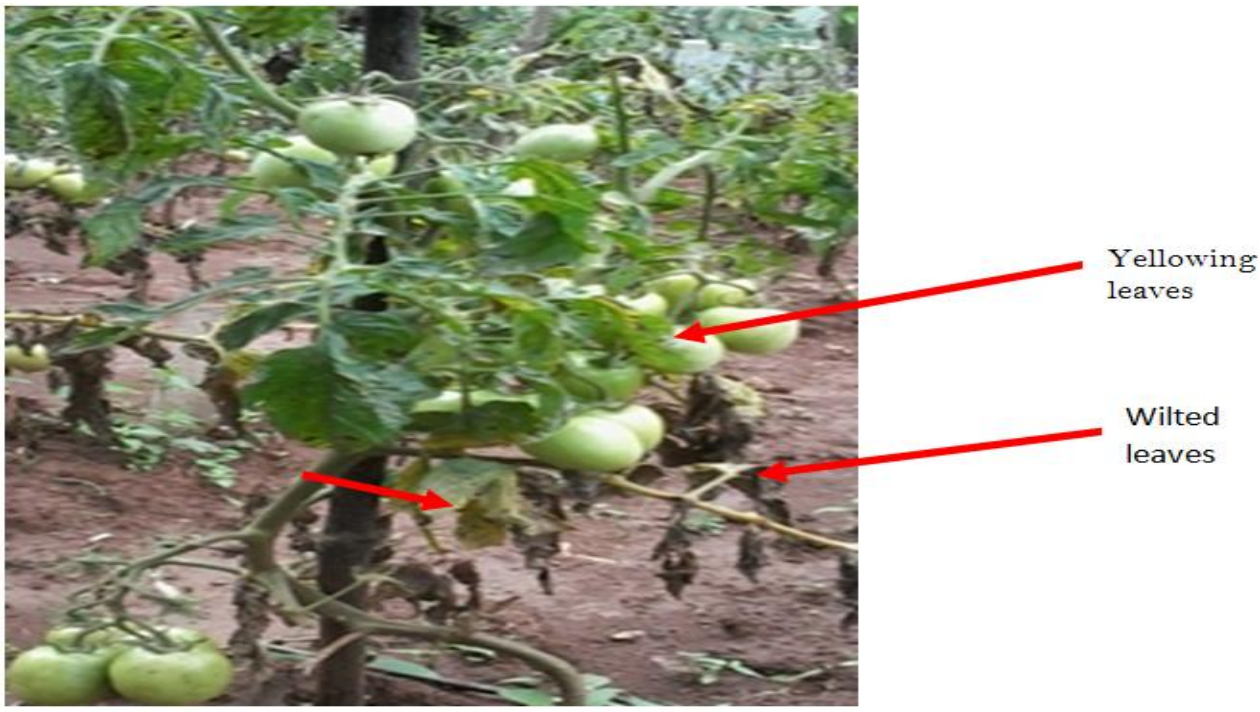

Plate-15. A mature tomato plant grown under greenhouse conditions showing symptoms of bacteria wilt.

4. DISCUSSION

4.1. Isolation of *Ralstonia Solanacearum*

The results described herein are similar to those reported by Zubeda and Hamid [23]. Virulence of an isolate can be determined on the basis of colony colour on TZC media. Virulent wild type colonies are usually large, elevated, fluidal and either entirely white or with a pale red center; avirulent mutant colonies were butyrous, deep-red often with a bluish border.

4.2. Morphological Profiling of *Ralstonia Solanacearum*

Cultural traits on different media are important tools for identification of *Ralstonia solanacearum*. Present results agreed with those reported by [24-26] and Maji and Chakrabartty [27]. This is the characteristic feature of *R. solanacearum*. According to Rohini, et al. [28] triphenyl tetrazolium chloride (TZC) medium is used to distinguish *R. solanacearum* among other bacteria during isolation. Also when TZC medium is used with *R. solanacearum*, it shows the difference between avirulent colonies that look dark red from the fluidal virulent that are white with pink center [25, 29]. In the present study, the colonies were fluidal whitish with a pink center, indicating virulent species of *R.* solanacearum.

The results of cultural studies are in close conformity with Mahdy, et al. [30] who described the colonies of *R. solanacearum* as white, wet, shining, circular, raised and smooth. Pawaskar, et al. [16] and Tahat and Sijam [31] also recorded similar observations regarding colony characters of *R. solanacearum* . The above results showed that these isolates were *R. solanacearum*.

4.3. Gram Stain

All of the plant pathogenic bacteria are usually Gram negative except Clavibacter and Streptomyces [32] ;[33]. All four tomato strains of *R. solanacearum* (Maseno, Mariwa, Seme and Holo) from Maseno region were Gram-negative. The results of this study agreed with Wang, et al. [34] who reported that *R. solanacearum* are Gram-negative. Gram reaction by staining is a necessary initial step for the identification and classification of bacteria from any source. The outcome of this test is literally fragile cell walls, which are bound by an outer membrane. Gram-positive bacteria have a thick mesh-like cell wall made of peptidoglycan (50-90% of cell wall), which stain purple and Gram-negative bacteria have a thinner layer (10% of cell wall), which stain pink. Gram-negative bacteria also have an additional outer membrane which contains lipids, and is separated from the cell wall by the periplasmic space.

4.4. Potassium Hydroxide Solubility Test

The results reported herein are similar to those reported by Rahman, et al. [25] who reported that Gram negative bacteria had viscous thread when lifted glass slide when mixed with 3% KOH. These results could be due to the outer membrane of Gram-negative bacteria being readily disrupted on exposure to 3% KOH releasing the viscous DNA according to Ahmed, et al. [7] who performed a similar test on *R. solanacearum* causing bacterial wilt disease of potato in Bangladesh. Solubility test confimed that *R.solanacearum* isolates were Gram negative.

4.5. Catalase Oxidase Test

All Gram-negative bacteria produced gas bubbles when they were mixed with a drop of Hydrogen peroxide on glass slide thus they were catalase oxidase positive. Present results agreed with those reported by Mwankemwa [35] and Teng, et al. [36]. These results could be attributed to the presence of catalase enzyme in *R. solanacearum*. Slesak, et al. [37] working with Gram-negative concluded that Hydrogen peroxide (H_2O_2) is a byproduct of aerobic respiratory metabolism in aerobic bacteria. Production of gas bubbles gives a clue for presence of aerobic bacteria. Catalase is an enzyme, which is produced by microorganisms that live in oxygenated environments to neutralize

toxic forms of oxygen metabolites; H_2O_2. The catalase enzyme neutralizes the bactericidal effects of hydrogen peroxide and protects them against the toxic effects of H_2O_2. Anaerobes generally lack the catalase enzyme. Catalase mediates the breakdown of hydrogen peroxide H_2O_2 into oxygen and water. Only aerobic bacteria produce catalase enzyme hence the test bacteria (*Ralstonia solanacearum*) was conrnfirmed to be aerobic in nature.

4.6. Gas Production Test

Gas production test indicated that the bacterium (*R. solanacearum*) produced gas from glucose within eighteen hours of incubation. The result are in conformation with Pawaskar, et al. [16]; Zhou, et al. [38] and Houfani, et al. [39] who reported that *R. solanacearum* produce gas from dextrose, glucose and salicilin. Production of gas may be attributed to the presence of enzyme systems in bacteria which allow them to oxidize environmental nutrient sources. Many bacteria possess the enzymes system required for the oxidation and utilization of the simple sugar, glucose. Bacterial utilization of different energy sources in the medium depends on the specific enzymes of each bacterium. The characteristics feature of the enzyme production in the bacteria enables them to use diverse carbohydrates and this in turn aid in the identification of unknown bacteria.

4.7. Starch Hydrolysis Test

Starch hydrolysis test of the bacterium showed that the bacterium were unable to hydrolyse starch. This results are similar to those of Pawaskar, et al. [16] who reported *R. solanacearum* as negative in starch hydrolysis. Similarly Zhang, et al. [40]; Nouri, et al. [41] and Vaneechoutte, et al. [42] also reported *R. solanacearum* to be negative in starch hydrolysis. These results can be attributed to the absence of some exoenzymes (amylases) by *R.solanacearum* [43]. Starch agar is a differential medium that tests the ability of an organism to produce certain exoenzymes including amylase and oligo-1, 6-glucosidase that hydrolyze starch. Starch molecules are too large to enter the bacterial cell, so some bacteria secrete exoenzymes to degrade starch into subunits that can then be utilized by the organism. It is on this basis that *R.solanacearum* strains were classified as negative in starch hydrolysis.

4.8. Profiling of *R. Solanacearum* Isolates into Biovars

Ralstonia solanacearum isolates varied in utilization pattern of different sugars but Biovar 1 didn't utilize any sugar from sugar test Biovar 3 isolates, utilized Mannitol, Sorbitol, Ducitol, Maltose, Lactose and Cellubiose. Biovar 3 seems more prevalent in Maseno region; it was present in Maseno, Seme and Holo sites. Biovar 1 is restricted to Mariwa only. These results concurred with those obtained by Hassan, et al. [44] who performed similar biochemical test on all bacterial wilt strains from Rawalpindi and Islamabad whose results of the biovar test showed that all seven groups of *R.solanacearum* isolates oxidized disaccharides (Sucrose, lactose, maltose) and sugar alcohols (manitol, sorbitol and dulcitol) within 3-5days.

In different utilization pattern of various sugars; pathogenic variability and virulence pattern of *R. solanacearum* belonging to Biovar 1 and Biovar 3 could be due to genetic variability among different strains of pathogen [13]. Genetic variation represents an alternative adaptive strategy, which by providing some degree of diversity ensures survival in specific niches and adaptation to sudden changes in the environment. By responding to specific environmental signals, regulatory genes allow bacteria to adapt to changes in their habitat. High virulence of Biovar 3 strains could be due to their wide host range and compatibility with number of environmental factors favorable for disease appearance such as temperature, rainfall, soil type, inoculum potential, and other soil biological factors such as wilt complexes formed among nematodes (Meloioigyne spp), Fungi (Fusarium spp.) and *R. solanacearum.*

4.9. Designation of Landraces of *R. Solanacearum* in Maseno Region

All groups of *R.solanacearum* isolates causing bacterial wilt of tomato collected from four (Maseno, Mariwa, Seme and Holo) tomato growing areas in Maseno region belong to landrace 3. The strains showed high to moderate virulence on potato, tomato and eggplant. Therefore, all of them had characteristic of landrace 3 with a limited host range on potato, tomato and pepper (Table 4). In a host range study, all strains were pathogenic (moderate to high) on potato, tomato and eggplant. But other hosts such as pepper showed low level of pathogenicity but tobacco and peanut did not show any wilt symptoms. The limited host range is the characteristic of landrace 3 of *R.solanacearum*. The findings of this study were in line with those obtained by Tjou-Tam-Sin, et al. [45]; Manasa, et al. [46] and Ahmed, et al. [7] who classified *R. solanacearum* into five landraces. Five landraces have been described so far, but they differ in host range, geographical distribution and ability to survive under different environmental conditions. Landace 1 infects many solanaceous plants such as brinjal, tomato, tobacco, pepper and other plants including some weeds. Landace 2 causes wilt of triploid banana (Musa spp.) and Heliconia spp., while landrace 3 infects potato and tomato but it is weakly virulent on other solanaceous crops, landrace 4 infects ginger and landrace 5 infects mulberry. The results of this study primarily indicated that bacterial wilt pathogen of tomato; *R. solanacearum* belongs to landrace 3.

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