Molecular identification and characterization of *Ralstonia solanacearum* species complex causing bacterial wilt in solanaceae in Tanzania.

This project report entitled **Molecular identification and characterization of *Ralstonia solanacearum* species complex causing bacterial wilt in solanaceae in Tanzania.** Is the work of Mr. Mahesh babu Gandla, undergraduate student of BSc (Hons) Agriculture at (Acharya N.G.Ranga Agricultural University) submitted in partial fulfilment of the requirement for a 2 month (1 April 2023 to 30 May 2023) internship in Bacteriology unit under the Supervision of Dr. Ricardo olive and Jaw-rong chen, at World Vegetable Centre, Taiwan.

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ABSTRACT

Solanaceas crops infected by wide range of diseases one of the most important disease is bacterial wilt caused by *Ralstonia solanacearum* species complex one of the most destructive phytopathogenic bacteria. It enters into plant natural openings and wounds caused by implements& insect pest. *Ralstonia solanacearum* is a soil born pathogen having special features like wide host range, latent infection, survives in soil up to 20 years. So that the disease management is more difficult. Proper identification, distribution and mapping plays an important role in the development of effective management strategies. Wilted tomato, sweet pepper, hot pepper, African eggplant and African night shade plant samples are collected from different regions of Tanzania. The causal bacterial strain were isolated from the samples and all were identified as *Ralstonia solanacearum.* Multiplex PCR using phylotype specific primer pair identified the presence of Phylotype I strain is the most common then Phylotype III and Both Phylotype I & Phylotype III strains are present in same field /plant. Phylotype I is widely distributed in Tanzania fields whereas Phylotype III is confined to Kilimanjaro and Dodoma region both Phylotype I & Phylotype III are present in fields of Morogoro, Dodoma , and Arusha regions of Tanzania.

INTRODUCTION

Solanaceous crops play an important role in human diet and the economy of nations. They are also called as “Nightshades”. This family is distributed throughout the world in all continents except Antarctica. Solanaceae family consists of 98 genera and 2700 species, with greater habitat, morphological and ecological diversity. The family Solanaceae includes number of commonly cultivated species. Most important genus of the family Solanaceae is “Solanum”, which includes Potato, Tomato, Brinjal, Chilli and Capsicum, that are used as food. They can grow in several conditions ranging from tropics to subtropics. In this diverse climatic conditions they are infested by several diseases, one among them is bacterial wilt caused by bacterium Ralstonia solanacearum (most damaging plant pathogen). Ralstonia solanacearum has been reported to conquer 450+ plant species belonging to 54 different botanical families, the most susceptible hosts being solanaceous crops. Potato, tomato, brinjal and chilli are mostly affected by this bacterial wilt.[1]

Bacterial wilt is one of the main diseases of nightshades so called solanaceous crops such as potato, tomato and chilli. This disease occurs in wet tropics, sub-tropics and also in some temperate regions in different parts of the world. Bacterial wilt in solanaceous crops is caused by the bacterium Ralstonia solanacearum which was previously called as Pseudomonas solanacearu. Bacterial wilt is known as "green wilt" disease as the leaves of the infested plant remain green when the plant begins to show wilting symptoms. Ralstonia solanacearum, the causative agent of bacterial wilt, is one of the most devastating phytopathogenic bacter . Ralstonia solanacearum has been reported to conquer 450+ plant species belonging to 54 different botanical families, the most susceptible hosts being solanaceous crop. Bacterial wilt ends up with substantial losses in crops like tomato, eggplant, potato, tobacco and banan. Ralstonia solanacearum is a soil borne bacterium which penetrates the roots of the plant and invades the xylem vessels, then spreads rapidly to the aerial parts of the plant through the vascular system where its faster multiplication leads to wilting and ultimately to the death of the plant. In addition to

Ralstonia solanacearum lethal ability, its ability to remain in the soil for several years and form latent infection within native weeds contributes to the difficulty of eradicating this destructive bacteria. The spread of this bacterium is considered a threat to crops and this pathogen is considered a quarantine bacterium. Ralstonia solanacearum is an extraordinarily diverse and complex species. The pathogen is divided into five races (due to its ability to infect different plant species) and six biovars (due to its ability to oxidize hexoses, alcohols and sorbitol as well as disaccharides). These bacterial strains present a wide genetic diversity and are divided into four phylotypes that correspond roughly to the geographical origin of the strain: Asia (Phylotype I), America (Phylotype II), Africa (Phylotype III) and Indonesia (Phylotype IV). Phylotype II has two subgroups namely IIA and IIB. & only strains belonging to phylotype IIB are responsible for bacterial wilting of potatoes in cold and temperate regions.

The pathogen has been accounted for to cause serious yield losses in numerous solanaceous crops, with 88% loss of tomatoes detailed in Uganda, and 70% loss of potato in India and different nations in changing degrees. Bacterial wilt was accounted for to influence 50–100% of potatoes crop yield loss in Kenya. In Ethiopia, bacterial wilt frequency is practically 100% on pepper, 63% on potato and 55% on tomato. On account of potato, since most wilted potato plants don't produce attractive or marketable tuber, crop yield losses from the bacterial wilt disease could be extremely high.

The race, biovar and phylotype classification has gained wide acceptance for subdividing R. solanacearum species complex. The racial pattern system groups the strains of R. solanacearum according to their ability to infect different host plants, viz., race 1 comprised of many strains having a wide host range and pathogenic on different solanaceous plants and weed hosts, race 2 is restricted to triploid banana and Heliconia, race 3 (potato race) affects potato, race 4 infects ginger, and race 5 is pathogenic on mulberry (Buddenhagen et al., 1962; Aragaki and Quinon, 1965; He et al., 1983; Begum, 2005; Rahman et al., 2010; Chandrashekara et al., 2012, Popoola et al., 2015). Five races are also different in geographical distribution and ability to survive under different environmental conditions (French, 1986). R. solanacearum was grouped into four biovars on the basis of utilizing and/or oxidizing three hexoses (mannitol, dulcitol and sorbitol) and three disaccharides (lactose, maltose and cellobiose) (Hayward, 1954; He et al., 1983; Begum, 2005; Rahman et al., 2010; Chandrashekara et al., 2012, Popoola et al., 2015). Fegan and Prior (2005) developed a new hierarchical classification scheme by analysis of the internal transcribed spacer (ITS) region, the endoglucanase (egl) gene and the hrpB gene. A phylotype-specific multiplex PCR (Pmx-PCR) was employed in this scheme to distinguish four phylotypes (I, II, III, IV). A number of different phenotypic and genotypic methods are presently being employed for the identification and classification of bacteria, including plant pathogenic bacteria like Ralstonia. Each of these methods permits a certain level of phylogenetic classification from the genus, species, subspecies, biovar to the strain level. Moreover, each method has its advantages and disadvantages with regard to ease of application, reproducibility, requirement for equipment and level of resolution. Modern phylogenetic classification is based on 16S rRNA sequence analysis (Cook et al., 1989; Gillings et al., 1993; Seal et al., 1992; Seal et al., 1993; Poussier et al., 2000; Popoola et al., 2015) There exists a lot of controversy regarding the prevalence of strains in the various parts of the world.[2]

MATERIALS AND METHODS

Survey and sample collection

A field survey was conducted in the horticultural regions of Tanzania covering six regions with two districts each namely Arusha (Arumeru, Arusha), Kilimanjaro (Moshi,Hai), Tanga (Lushoto, Korogwe), Morogoro (Kilosa, Mvomero), Dodoma (Chamwino,Bahi), Manyara (Babati).

A multistage random sampling procedure was used which involved selection of the wards within the district, then the villages and the fields. In each locality, five counts were taken diagonally per field and 3 to 5 fields per location were assessed. Within the field; five (four at each corner and one at the center of the field) plots measuring 50 m 2 were assessed by critically observing symptoms of BWD.
A bacterial ooze test was performed to confirm the presence of R. solanacearum bacteria in the solanaceous crops. A cut stem from a fresh infected plant was placed in a transparent glass beaker containing water to observe streaming from the cut end of the stem within 15 min in which threads of a viscous white slime occurred. Samples showed threads (bacterial ooze exuding from the infected xylem vascular bundles) were taken for isolation. Total 596 samples are collected from various regions of Tanzania.

Isolation of R.solanacearum species complex bacteria and transfer to FTA cards

The infected tomato stem that signified the presence of bacterial wilt disease-causing pathogen was divided further into smaller portions and washed in running tap water. The tissue was surface disinfected in a 70 % Ethanol for 3 minutes and then rinsed with sterile distilled water two times. The plant material was air-dried under a laminar floor chamber onto sterile Petri dishes containing Whatman filter paper for 5 minutes and macerated using sterile mortar and pestle. Two milliliters of sterile sterile distilled water were added to a mortar prior to sample homogenization in order to obtain bacterial suspension. Using a sterile loop, samples of suspension were streaked on a modified and autoclaved semi-selective medium for R. solanacearum, triphenyl tetrazolium chloride (TTC) (10 g of peptone, 10 g of dextrose, 18 g of agar agar, 5 g of skimmed milk, 956 ml of distilled water and 1 mL of filtered and autoclaved 1% aqueous stock solution of 2,3,5-triphenyl tetrazolium chloride) at 121°C for 15 min and incubated for 48 h at 28°C. The isolated cultures showed characteristic morphological traits of a pathogenic R. solanacearum strain with a thick, fast-growing biofilm that was cream-white with pink pigments were transferred to new Petri dishes containing TTC media. One loopful sterilized by flaming and cooled of the purified R. solanacearum culture was transferred into an eppendorf tube containing 500 µl of sterile distilled water and vortexed for 30 s. 100 µl of bacterial suspension was removed and placed onto a FTA card and allowed to air dry under the laminar flow chamber for 2 hours. The dried FTA cards were kept into a desiccator containing silica-gel crystals until shipped to Taiwan HQ.

NOTE: SUREVEY, SAMPLE COLLECTION, ISOLATION OF *R. solanacearum species complex* AND TRANSFER TO FTA CARD. BY JUDITH HUBERT – PLANT PATHOLOGIST CONSULTANT, WORLDVEG-ESA (IMPLEMENTOR OF WORK)

METHOD 1: Recovery of DNA from FTA card (IRRI METHOD)

1. Six discs (<2mm) from the center of the FTA card with the sample using a Harris Uni-Core™ - 2.00,
2. Transfer the discs into a 1.5 ml eppendrof microtube,

3. Wash the section with 200µl of 80% ethanol,
4. Vortex at maximum setting for 10 s,
5. Remove the ethanol using a pippete,
6. Add 200µl of 1x TE buffer,
7. Gently rotate and invert the tubes 10-20 times to allow the TE to flow over all the inner tube surface,
8. Spin down briefly with the help of centreifuge,
9. Remove the TE using a pipette,
10. Repeat steps 6-8,
11. Resuspend in 30 µl of 1x TE buffer,
12. Let stand for a minimum of 1 hr prior to use in PCR.

METHOD 2: PURIFICATION OF DNA SAMPLES FROM FTA CARD (PURIFICATION REAGENT METHOD)

Proceed as follows of purified Bacterial DNA samples from FTA card.

1. A small disc (<2 mm) from the center of the dried sample area was cut using a Harris Uni-CoreTM - 2.00 and put into 1.5 ml centrifuge tube. (Max. 2 discs in a tube)

2. Add 100 µl of purification Reagent (WhatmanTM) into 1.5 ml centrifuge tube then keep in room temperature for 5 minutes

3. Centrifuge for 1 minute by 10,000 r.p.m.

4. Remove the purification Reagent, add 100 µl of fresh purification Reagent, then direct centrifuge for 1 minute by 10,000 r.p.m..

5. Remove the purification Reagent, add 200 µl of 1x TE buffer, then direct centrifuge for 1 minute by 10,000 r.p.m..

6. Remove the TE buffer, add 200 µl of fresh 1x TE buffer, then direct centrifuge for 1 minute by 10,000 r.p.m..

7. Remove the TE buffer and move this small dot to the tube wall, keep in oven at 60 °C for 20 minutes to dry the small dot.

8. Transfer the disc into a PCR tube for PCR reaction.

Species and phylotype identification

Species and phylotype identification were performed on DNA from the samples captured on FTA™ cards. Two-millimetre diameter disks cut from the sample-loaded area of the FTA card using a Harris Uni-

Core™ -2.00 punch were placed into separate 1.5 ml microfuge tubes and washed using 80% EtOH and 1x TE buffer following the PCR-ready DNA from bacterial cells and plant tissue stored on FTA card developed by MARIAN HANNA R.NGUYEN, Associate scientist and CASIANA M. VERA CRUZ, Senior Scientist modified by Jaw –rong chen Principal Research Assistant Bacteriology at WVC. The Bacterial suspension volume of 30µl transferred into new PCR tubes and species were detected using RSSC specific primer pairs AU759f and AU760r (Table 1). PCR was performed in a total volume of 12.5 µl mixture reaction comprising 2.75µl of NF water, 0.5µl of P759(10µM), 0.5 µl of P760(µM),6.25 µl of Q-Amp 2x screening fire taq master mix and 2.5 µl of bacterial suspension. DNA amplifications were conducted in a Bio-Rad DNAEngine ® Peltier Thermal Cycler with an initial cycle of 94 °C for 3 min, 53 °C for 1 min and 72 °C for 1.5 min, then followed by 30 cycles of 94 °C for 18 s, 60 °C for 18 s and 72 °C for 18 s, with a final extension step of 72 °C for 5 min and holding at 4 °C.

Identification of phylotypes was performed using a multiplex PCR, combining four phylotype-specific primer pairs. Amplification was performed in a 25 µl total reaction volume with 1 µl of Nmult21:1F(10µM), 1 µl of Nmult21:2F(10µM), 2 µl of Nmult23:AF(10µM), 1 µl of Nmult22:InF(10µM), 1 µl of Nmult22:RR(10µM), 1 µl of AU759f(10µM),1 µl of AU760r(10µM), 12.5 µl of Q-Amp 2x screening fire taq master mix, 4.5 µl of bacterial suspension. The PCRs were performed in a Bio-Rad DNAEngine ® Peltier Thermal Cycler with an initial cycle of 94°C for 3min, 53°C for 1.0min, 72°C for 1.5 min, then followed by 30 cycles at 94°C for 0.3 min, 60°C for 0.3 min,72°C for 0.3min, 72 °C for 5 min and holding of 4 °C. PCR products were separated alongside a 100 bp DNA ladder for size comparison by electrophoresis in 1.5% agarose (aMResco®) gels. The gels were stained with ethidium bromide and amplicons were visualized and scored under UV illumination.

RESULTS

Collection of samples

A total of 596 diseased samples from different regions of TANZANIA covering six regions with two districts each namely Arusha (Arumeru, Arusha), Kilimanjaro (Moshi,Hai), Tanga (Lushoto, Korogwe),Morogoro (Kilosa, Mvomero), Dodoma (Chamwino,Bahi), Manyara (Babati). Multistage random sampling was used for collecting samples from bacterial wilt infected plants from various regions of Tanzania for further isolation and preservation of DNA samples on FTA card. A total of 596 samples are sent to Headquarters of WVC for MOLECULAR identification.

Out of 596 samples 200 samples are selected for molecular identification at bacteriology unit WVC. The samples includes 32 from Kilimanjaro, 36 from Tanga, 74 from Morogoro, 30 from Dodoma, 15 from Arusha, 13 from Manyara, a total of 200 samples.

Species identification with species primers AU759&AU760 as follows.

10 % (20) samples identified as positive of species identification with Multiplex PCR.

It includes 6.2% (2) from Kilimanjaro, 8.1% (6) from Morogoro, 3.3% (1) from Dodoma, 73.3% (11) from Arusha, no positive result from Tanga and Manyara. The positivity rate is very high in Arusha region then Mrogoro region.

Phylotype analysis:

The multiplex PCR which distinguishes between the four geographically linked monophyletic phylotypes within the members of RSSC (Fegan and Prior 2005) was performed on DNA from 20 samples which positive in species identification. The multiplex PCR revealed that 10 (50%) of the strains are Phylotype I, 2(10%) of the strains are Phylotype III and 8(40%) were both intermix of Phylotype I & III. As Safni et al. (2014) placed both phylotype I and phylotype III strains as *R. pseudosolanacearum,* this means that only strains of *R. pseudosolanacearum,* were detected in TANZANIA samples. None of the strains from TANZANIA belonged to either the phylotype II (*R. solanacearum*; American origin) or phylotype IV (*R. syzygii*; Indonesian- Australian origin).

CONCLUSION:

PHYLOTYPE I IS MORE COMMON IN TANZANIA THEN PHYLOTYPE II

THE IMPORTANT THING WAS IN SAMPLES WE OBSERVED BOTH PHYLOTYPE I & III SO THAT WE CAN CONCLUDE THAT IN SOME SOILS OF TANZANIA ESPICIALLY MOROGORO AND ARUSHA BOTH STRAINS PHYLOTYPE I & III ARE PRESENT.

THE CONCLUSION WAS MADE BASED ON RESULTS UPTO MY KNOWLEDGE.

DISCUSSION:

This study confirmed that bacterial members of the RSSC cause bacterial wilt in tomato, sweet and hot pepper, African night shade and African eggplant in Tanzania. Testing using specific primers revealed that in Tanzania *R. pseudosolanacearum* (both phylotype I and III strains) could be detected.

In Arusha region of Tanzania phylotype I strain is most common, in Kilimanjaro and Morogoro region Phylotype III strain was identified, in some fields of morogoro and Arusha region both Phylotype I and III strains are identified in same field or plant sample.

The study reveals that in some fields of Tanzania reveals that both phylotype I and III strains are present in same field. But we the conformation should be made by placing Phylotype III using a Check.

One of the most important point should be noted will preparation of FTA card plays an important role in Molecular identification of RSSC because we observed that positive percentage was very in Tanzania samples out 200 samples only 20 samples are showed positive in species identification even though some sample the DNA concentration was high but they showed negative in species identification because of wrong handling while preparing FTA card and identification of virulent strain when the FTA card was prepared from FTA card the virulent strain of RSSC forms cream white colonies with red or pink center. The research study may helpful for breeders for the development of resistant varieties and DNA sequencing for future work.

Table and Figure Legends

Table 1 Details of PCR primers used for species, phylotype identification.

Table 2 Proportion of samples in each collection Region of species identification positive and Phylotype.

Table 3 Details of bacterial wilt strain about region, crop, disease severity and Phylotype.

Fig. 1 Amplicon of Phylotype identification from Tanzania samples. Lanes M 100bp ladder, lanes 1&10 strains of Phylotype III, lanes 2 to 5 and 12 to 17 strains of Phylotype I, lanes 6 to 9 ,11, 18 to 20 strains of both Phylotype I and Phylotype III. Lane B1 is positive for Phylotype I, lane B2 is positive check for Phylotype II. Lane D1&D2 strains of phylotype I which cololected from experimental fields at WVC. Species identification was done at 282 bp.

Fig. 2 Distribution of *R. psedusolanacearum* (Phylotype I represented by blue 7-point star Phylotype III represented by light green diamond Phylotype I&III represented by red oval) in different regions of TANZANIA.

Fig. 3 Graphical representation of Phylotype distribution in various regions of TANZANIA

Fig. 4 Preparation of FTA card from diseased smaple.

Fig. 5 Preparation of FTA card from Bacterial pure culture.

Table 1 Details of PCR primers used for species, phylotype identification.

Gene	Primer name	Sequence	Specificity	Amplicon size(bp)	Authors
16S-23S ITS region	Nmult21:1F	CGTTGATGAGGCGCGCAATTT	Phylotype I	144	Fegan and Prior (2005)
	Nmult21:2F	AAGTTATGGACGGTGGAAGTC	Phylotype II	372	
	Nmult23:AF	ATTACGAGAGCAATCGAAAGA	Phylotype III	91	
	Nmult22:InF	ATTGCCAAGACGAGAGAAGTA	Phylotype IV	213	
	Nmult22: RR	TCGCTTGACCCTATAACGAGT	(reverse)		
Spel	AU759F	GTCGCCGTCAACTCACTTTCC	RSSC*	282	Opina et al.1997
	AU760R	GTCGCCGTCAGCAATGCGGAATCG			

**Ralstonia solanacearum species complex*

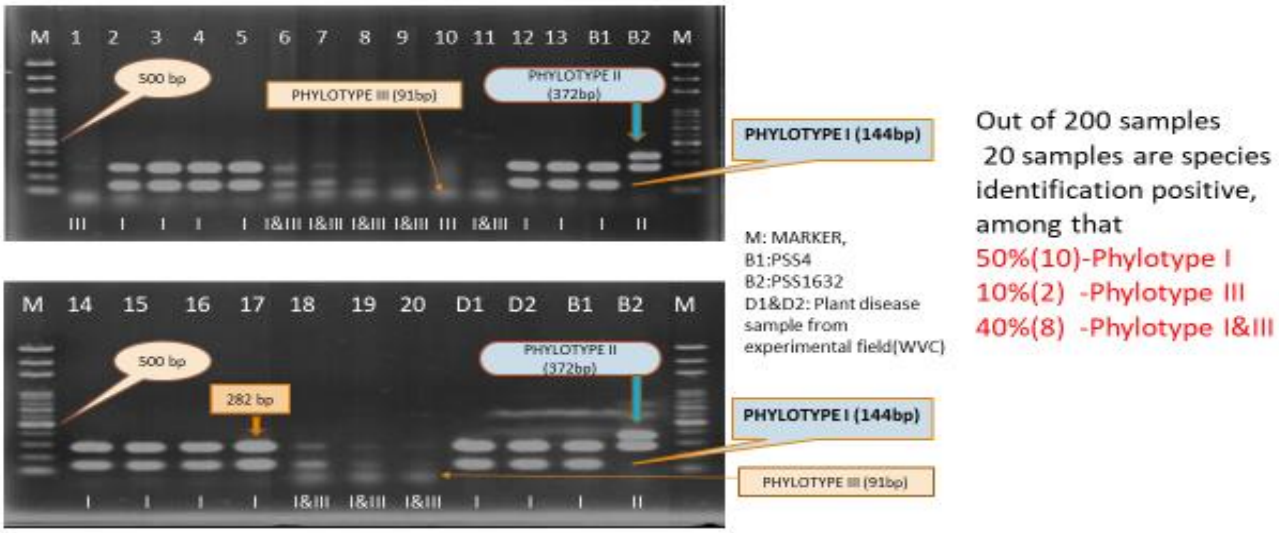
Table 2 Proportion of samples in each collection Region of species identification positive and Phylotype.

S.NO	REGION	Number of samples collected	Species identification positive	% positive	PHYLOTYPE I	PHYLOTYPE III	PHYLOTYPE I&III
1	Kilimanjaro	32	2	6.2	1	1	0
2	Tanga	36	0	0	0	0	0
3	Morogoro	74	6	8.1	1	1	4
4	Dodoma	30	1	3.3	**0**	0	1
5	Arusha	15	11	73.3	8	0	3
6	Manyara	13	0	0	0	0	0
	Total	200	20	10	10	2	8

Table 3 Details of bacterial wilt strain about region, crop, disease severity and Phylotype.

S.NO	Card no	Card code	Village	City	Region	Crop	Severity	PHYLOTYPE
1	9	TBWK34	KIMARORONI	HAI	KILIMANJARO	TOMATO	4	III
2	60	TBWMr234	SANGASANGA	KILOSA	MOROGORO	TOMATO	3	I
3	144	TBWAr571	KISANGAI	ARUMERU	ARUSHA	TOMATO	4	I
4	146	TBWAr581	MAWENI	ARUSHA	ARUSHA	TOMATO	5	I
5	3	TBWK10	MAWELLA	MOSHI	KILIMANJARO	TOMATO	2	I
6	86	TBWMr339	RUDEWA	KILOSA	MOROGORO	TOMATO	5	I&III
7	88	TBWMr345	RUDEWA	KILOSA	MOROGORO	TOMATO	5	I&III
8	88	TBWMr346	RUDEWA	KILOSA	MOROGORO	TOMATO	5	I&III
9	90	TBWMr353	RUDEWA	KILOSA	MOROGORO	HOT PEPPER	4	I&III
10	95	TBWMr376	MELELA	MVOMERO	MOROGORO	TOMATO	2	III
11	104	TBWD409	BUIGIRI	CHAMWINO	DODOMA	SWEET PEPPER	2	I&III
12	142	TBWAr564	NDURUMA	ARUMERU	ARUSHA	TOMATO	5	I
13	142	TBWAr565	NDURUMA	ARUMERU	ARUSHA	TOMATO	5	I
14	144	TBWAr570	NDURUMA	ARUMERU	ARUSHA	TOMATO	5	I
15	144	TBWAr572	KISANGAI	ARUMERU	ARUSHA	TOMATO	4	I
16	144	TBWAr573	KISANGAI	ARUMERU	ARUSHA	TOMATO	4	I
17	146	TBWAr580	KISANGAI	ARUMERU	ARUSHA	TOMATO	4	I
18	148	TBWAr586	KIWOWO	ARUSHA	ARUSHA	TOMATO	5	I&III
19	148	TBWAr587	KIWOWO	ARUSHA	ARUSHA	TOMATO	4	I&III
20	148	TBWAr588	KIWOWO	ARUSHA	ARUSHA	TOMATO	5	I&III

Fig. 1 Amplicon of Phylotype identification from Tanzania samples. Lanes M 100bp ladder, lanes 1&10 strains of Phylotype III, lanes 2 to 5 and 12 to 17 strains of Phylotype I, lanes 6 to 9 ,11, 18 to 20 strains of both Phylotype I and Phylotype III. Lane B1 is positive for Phylotype I, lane B2 is positive check for Phylotype II. Lane D1&D2 strains of phylotype I which cololected from experimental fields at WVC. Species identification was done at 282 bp.



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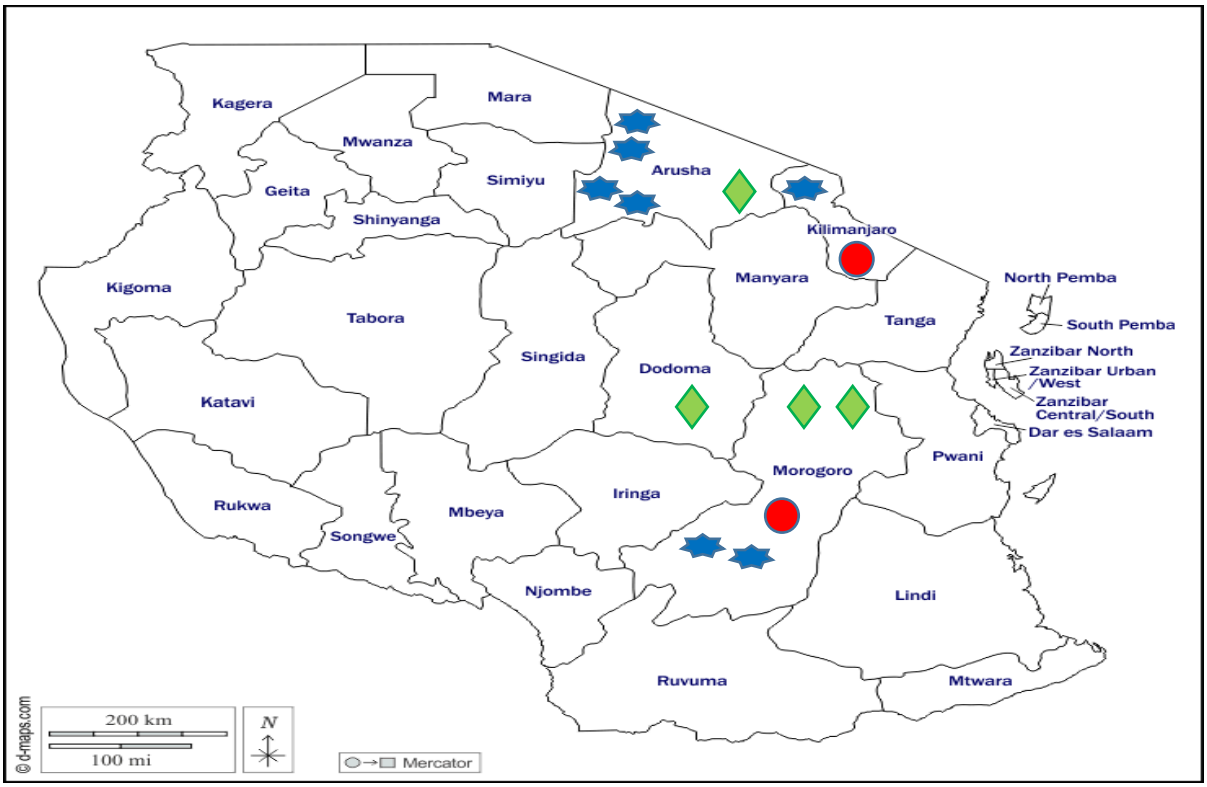
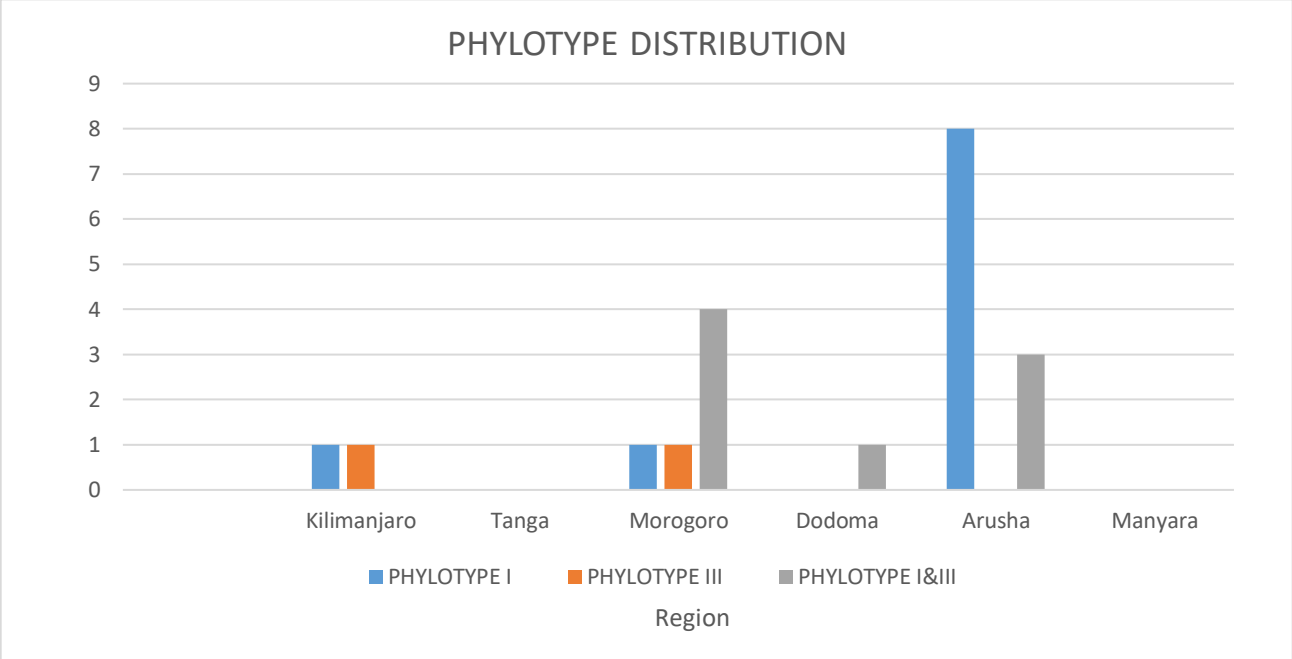


Fig. 3 Graphical representation of Phylotype distribution in various regions of TANZANIA



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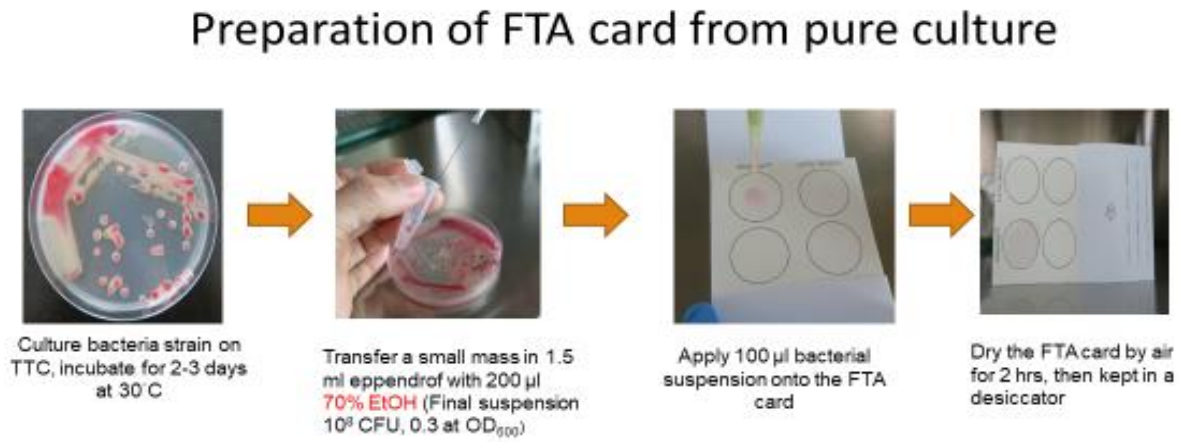

References

[1]Manda, R. R., Addanki, V. A., & Srivastava, S. (2020). Bacterial wilt of solanaceous crops. *Int. J. Chem. Stud*, *8*(6), 1048-1057.

[2]Sujeet Kumar, Kedarnath, N. Hamsaveni, I.B. Rohini, K.T. Rangaswamy, P.H. Ramanjini Gowda and Raghavendra Achari. 2017. Isolation and Characterization of Ralstonia solanacearum Causing Bacterial Wilt of Solanaceae Crops. Int.J.Curr.Microbiol.App.Sci. 6(5): 1173-1190. doi: https://doi.org/10.20546/ijcmas.2017.605.128

Fegan M, Taghavi M, Sly LI, Hayward AC (1998) Phylogeny, diversity and molecular diagnositcs of *Ralstonia solanacearum*. Pages 19-33 In: Bacterial wilt disease: molecular and ecological aspects. Prior P, Allen C, Elphinstone J, eds. Springer, Berlin.
Fegan M, Prior P (2005) How complex is the *Ralstonia solanacearum* species complex? Pages 449-461 In:Bacterial wilt disease and the *Ralstonia solanacearum* species complex. Allen C, Prior P and Hayward AC eds. Aps press. St. Paul, MN.

Opina N, Tavner F, Hollway G, Wang J, Li T, Maghirang R, Fegan M, Hayward AC, Krishnapillai V, Hong WF, Holloway BW, Timmis JN (1997) A novel method for development of species and strainspecific DNA probes and PCR primers for identifying *Burkholderia solanacearum* (formerly *Pseudomonas solanacearum*). Asia Pac J Mol Biol Biotechnol 5:19–30.
Peeters N, Guidot A, Vailleau F, Valls M (2013) *Ralstonia solanacearum*, a widespread bacterial plant pathogen in the post-genomic era. Mol Plant Pathol 14(7):651-662 http://dx.doi.org/10.1111/mpp.12038

Prior P, Fegan M (2005) Recent development in the phylogeny and classification of *Ralstonia solanacearum*. Acta Hortic 695: 127-136 http://dx.doi.org/10.17660/ActaHortic.2005.695.14

Safni I, Cleenwerck I, De Vos P, Fegan M, Sly L, Kappler U (2014) Polyphasic taxonomic revision of the *Ralstonia solanacearum* species complex: proposal to emend the descriptions of *Ralstonia solanacearum* and *Ralstonia syzygii* and reclassify current *R. syzygii* strains as *Ralstonia syzygii* subsp. *syzygii* subsp. nov., *R. solanacearum* phylotype IV strains as *Ralstonia syzygii* subsp. *indonesiensis* subsp. nov., banana blood disease bacterium strains as *Ralstonia syzygii* subsp. *celebesensis* subsp. nov. and *R. solanacearum* phylotype I and III strains as *Ralstonia pseudosolanacearum* sp. nov. Int J Syst Evol Micr 64(9):3087-3103.

Bihon, W., Chen, J. R., & Kenyon, L. (2020). Identification and characterization of Ralstonia spp. causing bacsterial wilt disease of vegetables in Mali. *Journal of Plant Pathology*, *102*(4), 1029-1039.