ISSN 1996-0719

International Journal of Plant Pathology

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OPEN ACCESS International Journal of Plant Pathology

ISSN 1996-0719
DOI: 10.3923/ijpp.2023.21.28

Research Article
Diversity of *Ralstonia solanacearum* Causing Tomato Bacterial Wilt Disease in Tanzania

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Abstract

Background and Objective: *Ralstonia solanacearum* (Smith) is one of the most destructive bacterial plant pathogens in the world. It causes bacterial wilt disease (BWD) in several plant species including tomato. This research was carried out to understand the diversity of the causing bacterial isolates and develop management strategies based on the characteristics of the prevailing pathogens in Tanzania. **Materials and Methods:** Forty isolates were collected from the infected tomato plants preserved in the laboratory from the BWD field survey in 2018 in Tanzania. Isolates were grown on TTC medium and DNA was extracted from a single colony of each isolate using the bacterial DNA extraction kit for molecular analysis using PCR. The similarity coefficients were predicted by the Dice technique for all possible isolate pairs based on the fingerprint groups. Finally, the aligned sequences were contrasted with the standard strains in the National Center for Biotechnology Information (NCBI) database to determine resemblance using the Basic Local Alignment Tool (BLAST). **Results:** According to the results of the phylogenetic analysis, 80% of the samples belonged to phylotypes I and III of the *R. pseudosolanacearum* and 20% to phylotype II of the *R. solanacearum* genospecies, respectively. Findings have shown that Tanzania has a diversified population of the *Ralstonia solanacearum* species complex that causes tomato BWD. Two species and three phylotypes of infections that cause tomato BWD were found to predominate in various agro-ecological zones across the nation. Phylotypes I and III of *Ralstonia pseudosolanacearum* and phylotype II of *Ralstonia solanacearum* were the isolates that were found. **Conclusion:** Strong standard phytosanitary measures must be implemented worldwide by plant health authorities as a result of the first report of phylotype II of RSSC in Tanzania.

Key words: BLAST, BWD, bactericides, diversity, geno-species, phylotypes, tomato, *Ralstonia solanacearum* species complex

Citation: Aloyce, A. and E.R. Mbega, 2023. Diversity of *Ralstonia solanacearum* causing tomato bacterial wilt disease in Tanzania. Int. J. Plant Pathol., 14: 21-28.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Tomato (*Lycopersicon esculentum* Mill.) is one of the most important vegetable crops worldwide. It is one of the world's most eaten vegetables as a source of minerals, vitamins, essential amino and organic acids, lycopene, β-carotene and dietary fibres[1]. The β-carotene and lycopene contained in tomatoes have anti-cancer and antioxidant characteristics and are regarded as healthy food[2]. Economically, tomato is among the cash crops and source of employment especially for youth in peri-urban areas of developing countries[2]. Due to those benefits, the cultivation of tomatoes in terms of acreage, production and consumption has been on the increase globally[3].

Tomato ranks as the first most important vegetable crop produced to enhance nutrition and source of income for growers in Tanzania[4]. However, contrary to the global production of tomatoes which is substantially increasing, tomato production in Tanzania is comparatively lower by 48% than the global production[3]. Challenges that resulted in low production of tomatoes in Tanzania are several namely soil infertility, prolonged drought, poor quality inputs, unreliable markets and pests[4].

Bacterial wilt disease (BWD) caused by complex species of *Ralstonia solanacearum* (RSSC) has been categorized as one of the most significant plant diseases in the world. The BWD is considered a serious plant disease because of the biology of RSSC which composes several geno-species[5,6] or phylotypes[7-9]. Losses due to RSSC are known to be huge but cannot be estimated correctly due to differences in host plant species, geographical location, pathogen strains, farming environments and soil types. Tomato yield losses caused by BWD range from 10-100% contingent on cultivar, soils, climate, soils, cropping practices and strain of RSSC[10,11]. It is among serious tomato production challenges in different areas including the East African Region[12] including Tanzania where a total (100%) yield loss was reported in farmers fields[13].

Several management options for BWD have been proposed varying from the use of physical, biological and chemical approaches[14]. Nevertheless, the use of such methods has not successfully addressed the BWD. The biology of the causative pathogen has been associated with difficulties that exist in managing the BWD[12,15]. For instance, the unusual genetic diversity of the RSSC, its ability to inhabit the hidden plant parts such as the xylem vessels and survive in a diverse environment with an extremely wide host range[16,17]. The characteristics of RSSC in the coastal agro-ecological zone of the country were reported by Aloyce *et al.*[17] but no study has been conducted to determine species and phylotypes of RSSC affecting tomato production in Tanzania.

The development of effective and sustainable management strategies is based on the characteristics of the targeted pathogen. Accurate diagnosis of the pathogen to detect its presence in the environment is believed to be the strongest foundation for its effective management[18]. Understanding the composition and characteristics of the prevailing *Ralstonia* species and phylotypes will help to tailor management measures towards the particular traits of strains prevailing in a specific geographical location, thus designing a pathogen-targeted management strategy. Therefore, the current study was conducted to establish the phylogenetic relationships of the BWD-causing pathogen(s) isolated from infected tomato plants collected from the main agro-ecological zones of Tanzania to generate information on the diversity of the BWD-causing pathogens as a key step to guide the development of effective and sustainable management measures of the disease. The study was important and urgently needed since there was little or no information on RSSC diversity in Tanzania.

MATERIALS AND METHODS

Study area: This research was conducted in the laboratory at The Nelson Mandela African Institution of Science and Technology (NM-AIST), Arusha, Tanzania from November, 2022 to January, 2023.

Bacterial isolates: Forty isolates of RSSC were collected from infected tomato plants preserved in the laboratory after the BWD field survey conducted in 2018 in the main agro-ecological zones of Tanzania. Twenty isolates i.e., 19 most pathogenic[5,6] of the 40 isolates and one outgroup labelled XYZ were used for the phylogenetic study (Table 1). The XYZ, an out-group isolated from the infected potato stems was included for comparison. All isolates were stored at 20°C and revived in triphenyl tetrazolium chloride (TTC) medium[5]. After growing bacterial isolates on TTC medium, DNA was extracted from a single colony of each isolate using the bacterial DNA extraction kit as per the manufacturer's instructions. The extracted DNA was used as a PCR template while a non-DNA template reaction mixture was included to serve as a negative control.

Table 1: Description of isolates used during the phylogenetic study of *Ralstonia solanacearum* species complex in Tanzania

Agro-ecological zone	District	Code of isolate	Agro-ecological zone	District	Code of isolate
Northern	Arumeru	NAA1	Central	Kongwa	CDK1
Northern	Arumeru	NAA4	Central	Kongwa	CDK4
Northern	Babati	NMB1	Lake	Nyamagana	LMN1
Northern	Babati	NMB2	Lake	Nyamagana	LMN3
Southern	Kilolo	SIK1	Lake	Kibondo	LKK1
Southern	Kilolo	SIK3	Lake	Kibondo	LKK4
Southern	Kilolo	SIK4	Coastal	Chake-Chake	CZC1
Southern	Mbeya	SMM2	Coastal	Chake-Chake	CZC3
Central	Manyoni	CSM2	Coastal	Temeke	CDT4
Central	Manyoni	CSM4	Northern	Arumeru	XYZ

Letter in the isolate code represents zone, region and district where the respective isolate was collected

Table 2: Reaction mixture for the Rep-PCR experiment for phylogenetic analysis

Component	Reaction (1 µL)	Reaction (20+1 µL)	Procedure
OneTaq® Quick-Load® 2X Master Mix with standard buffer	12.50	262.50	Primers and One Taq® Quick-Load® 2X Master Mix were thawed with standard buffer
10 µM forward primer	0.50	10.50	Primers were vortexed for 3-5 sec
10 µM forward primer	0.50	10.50	All tubes were pulse spined and held onto the ice
Template DNA	0.50		One Taq® Quick-Load® 2X Master was mixed by gently pipetting it up and down, then pulse spined and hold on ice
Nuclease free-water	11.00	231.00	Components 2 and 4 were combined in appropriate tube
Total volume	25		

Table 1 describes the geographical location of isolates used for the phylogenetic research in this study.

Repetitive-Polymerase Chain Reaction (Rep-PCR): The phylogenetic relationship of RSSC infecting tomatoes in Tanzania was determined by using the Repetitive order-based Polymerase Chain Reaction (Rep-PCR) method. The random amplification of polymorphic DNA (RAPD) marker type known as the entero-bacterial repetitive intergenic consensus (ERIC) was selected due to its robust, reproducible and highly discriminatory fingerprint characteristic[19-25].

The forward (5'-AAGTAAGTGGGG GTGGGG-3') and reverse (5'-ATGTAAGCTCCTGGGGGATTCAC-3') pairs of primers, synthesized by the Inqaba Biotec East Africa Ltd. (IBE002), Nairobi, Kenya were used. The PCR amplification was carried out in a final 25 µL volume comprising a PCR response buffer (Table 2).

The Rep-PCR amplification was performed with automated C 1000 Touch® Thermal Cycler (BIO-RAD) programmed as an initial denaturation of 95°C for 2 min, followed by 30 cycles of 94°C designed for 30 sec, 50°C for one min and 65°C for 4 min with a final extension of 65°C for 5 min, followed by a holding time of 4°C until specimens were collected[26].

Rep-PCR product analysis: The Rep-PCR products were visualized in 1.5% agarose gels stained with µg mL^{-1} of blue vision DNA dye solution in a TBE buffer using a 100 bp DNA ladder at 100 V. The results were viewed under ultraviolet light and the base pair length of each isolate was measured and recorded. Genetic similarity between isolates was assessed using the fingerprint profile of Rep-PCR in which a position number was allocated to each group with distinct electrophoretic mobility and scored as either one (presence of band) or zero (lack of band).

The similarity coefficients were predicted by the dice technique for all possible pairs of isolates based on the fingerprint groups[27]. The dendrogram was produced by the Unweighted Pair Group Method with arithmetic means (UPGMA) clustering from the similarity coefficient information[28] and was facilitated by the Molecular Evolutionary Genetics Analysis (MEGA 7.0.14). Finally, the aligned sequences were contrasted with the standard strains in the National Center for Biotechnology Information (NCBI) database of taxonomic browsers to determine resemblance using the basic local alignment tool.

RESULTS

Fingerprint profile of the RSSC isolates: The ERIC primer pair generated genomic PCR profiles for all 20 isolates tested. The polymorphic bands that were observed at different base pair were used to group isolates and at about 150 bp monomorphic single band was noted (Fig. 1).

The 20 isolates of RSSC investigated were classified into 3 main groups at a 55% similarity level (Fig. 2). At a similarity point of 90%, group 1 isolates were categorized into

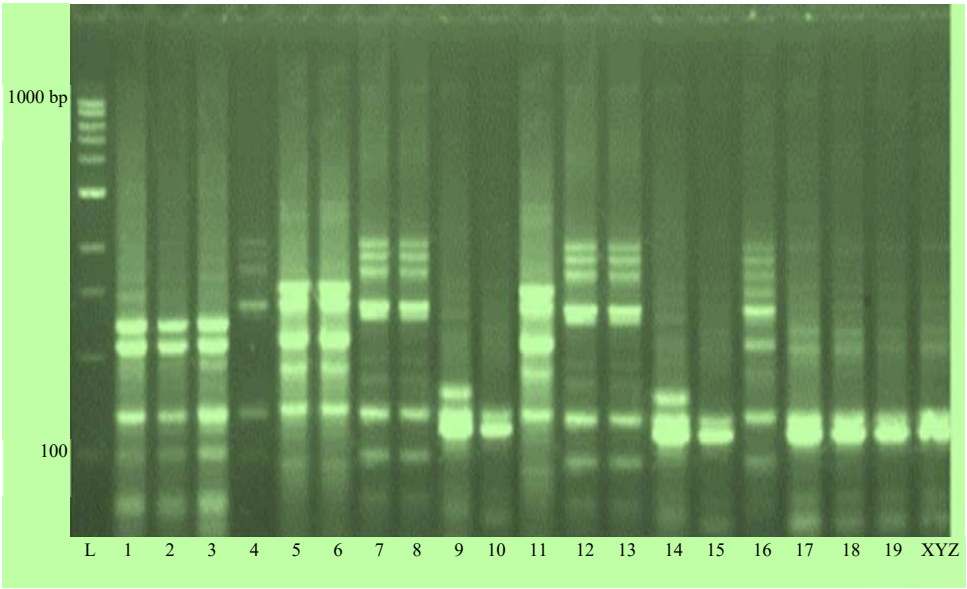

Fig. 1: Band patterns of *Ralstonia* isolates collected in Tanzania

L: DNA ladder (100 bp), 1-19: Isolates and XYZ: Out group potato isolate

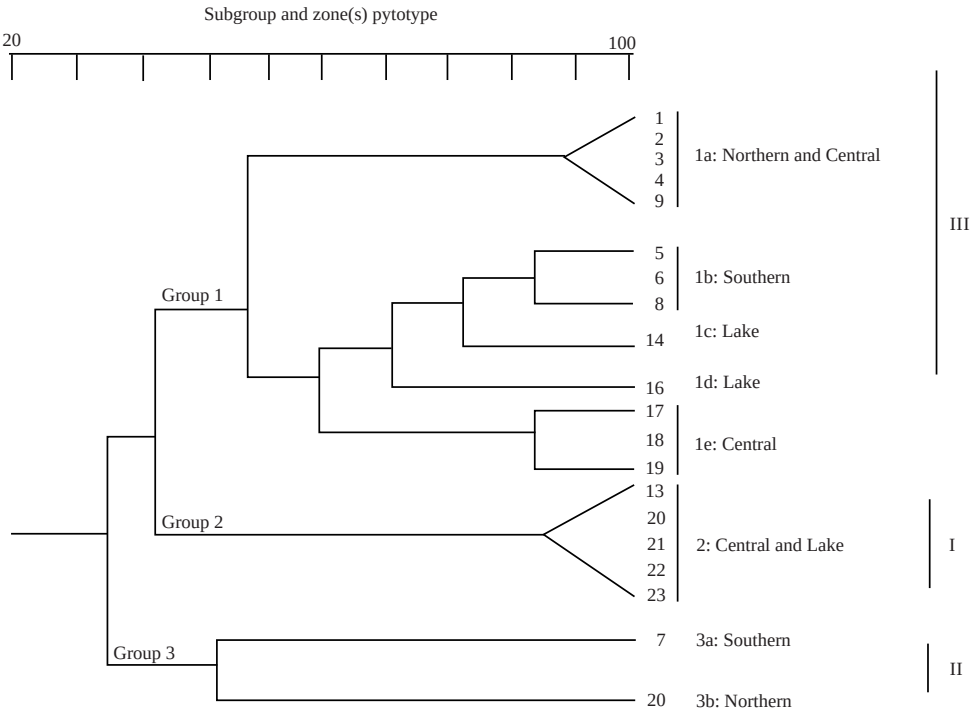

Fig. 2: Phylogenetic relationship of *Ralstonia solanacearum* species complex in Tanzania

5 sub-groups (1a-e) with the majority (38%) in subgroup 1a followed by 1b and 1e (23%) and 1c and 1d, (1%). Group 2 isolates consisted of 5 isolates and group 3 isolates were separated into 2 subgroups (3a-b) (Fig. 2).

Comparison of isolates with standard strains in the National Center for Biotechnology Information (NCBI) database: The identity of RSSC isolates collected in Tanzania was compared with standard strains in the NCBI database through BLAST (Fig. 3).

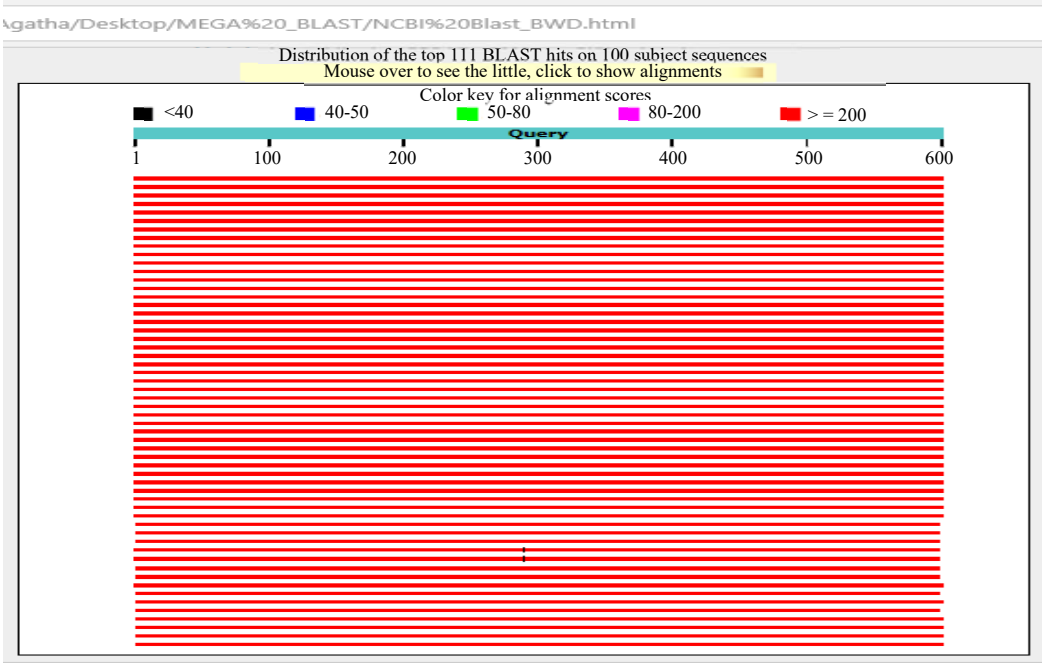

Fig. 3: Graphical summary of identity of *Ralstonia solanacearum* species complex isolates in Tanzania with standard strains in the National Center for Biotechnology Information (NCBI) database

Table 3: Similarity index of the Tanzanian isolates of *Ralstonia* with standard strains in the National Center for Biotechnology Information (NCBI)

Species	Strain	Max score	Total score	Query cover (%)	E-value	Identity (%)	Accession
Ralstonia solanacearum	FJAT 1458	1110	2220	100	0.0	100	CP016554.1
Ralstonia solanacearum	SL2330	1103	2205	100	0.0	99.83	CP022794.1
Ralstonia solanacearum	SL3755	1103	2205	100	0.0	99.83	CP022782.1
Ralstonia solanacearum	T25	1103	2205	100	0.0	99.83	CP023014.1
Ralstonia solanacearum	T110	1103	2205	100	0.0	99.93	CP023012.1
Ralstonia solanacearum	SL2729	1064	2127	100	0.0	98.67	CP022792.1
Ralstonia solanacearum	SL3300	1064	2127	100	0.0	98.67	CP022786.1
Ralstonia solanacearum	SL3730	1064	2127	100	0.0	98.67	CP022784.1
Ralstonia solanacearum	SL3822	1064	2127	100	0.0	98.67	CP022780.1
Ralstonia solanacearum	SL3882	1064	2127	100	0.0	98.67	CP022778.1
Ralstonia solanacearum	T42	1064	2127	100	0.0	98.67	CP022772.1
Ralstonia solanacearum	T60	1064	2127	100	0.0	98.67	CP022768.1
Ralstonia solanacearum	T78	1064	2127	100	0.0	98.67	CP022765.1

E-value is the number of expected hits of similar quality (score) found by chance[29]

The similarity index of the tested isolates obtained maximum (100%) query cover with a degree of identity from 98.67 to 100% (Table 3).

DISCUSSION

The findings of this study have shown that the causing pathogens of tomato BWD are diverse in Tanzania. The pathogen population differs with geographical locations based on the phylogenetic analysis. Results indicated a considerable genetic variation among the isolates according to the agro-ecological zones. For instance, isolates belonging to phylotype III were found from all the agro-ecological zones except the central agro-ecological zone. Phylotype I isolates were found in the central and lake zones while phylotype II was found to prevail in the southern and northern zones. Based on the present study and literature, two major species and three phylotypes of RSSC namely pylotypes I and III of *R. pseudosolanacearum* and phylotype II of *R. solanacearum* prevail in Tanzania[8,9,30]. The diversity of pathogen isolates could be among the causes of variations of disease incidence and severity in the study area as previously reported by Yuliar *et al*[13].

To the best of the acquaintance of this study, this is the first report of the prevalence of phylotype II of RSSC of tomato

BWD in Tanzania and hence, it is an alarm to plant health regulators to design and implement stronger standard phytosanitary procedures to avert the new and/or spread of the disease to other geographical locations. The RSSC phylotype II infects both tomatoes and potatoes and is thus considered of relatively more economic importance than phylotype III which infects tomatoes only. The prevalence and survival of phylotype II in the southern agro-ecological zone of Tanzania could be associated with the continuous cultivation of potatoes[31] and thus farmers should be guided on plant protection practices such as crop rotation to discourage survival of RSSC persistence in the environment.

The findings indicated that groups 1 and 2 could be clustered together while group three formed another cluster at about 30% level of similarity which agreed with previous research[32,33] which identified two major genetically distinctive divisions of RSSC strains namely group one comprising all isolates of phylotypes III-V and suggested to be Asian in origin (Asiaticum) and group two included all isolates of phylotypes I-II and proposed to American in origin (Americanum).

Therefore, phylotypes II and III isolates reported in this study could be classified in the Americanum and Asiaticum divisions respectively. Relating the *Ralstonia* isolates collected from the infected tomato sample in Tanzania with the standard strains in the NCBI database revealed that the isolates of RSSC found in Tanzania are closely aligned with those available in the NCBI database. Therefore, it is a collaboration avenue for scientists across the world in designing effective management strategies against BWD.

The pathogenic and non-pathogenic[5,34,35] groups of RSSC were encountered in different surveyed main agro-ecological zones. The non-pathogenic isolates can be classified as saprophytic despite comparable colony appearance with RSSC on TTC medium. Non-pathogenic strains of RSSC have a potential antagonist effect against the pathogenic strains[14] and hence can be explored as microbial-based bactericides for BWD management. In this study, several non-pathogenic isolates have been found. Such findings open a window for further research towards exploring these isolates for the development of microbial-based bactericides.

CONCLUSION

The present study has generated knowledge on the diversity of *Ralstonia solanacearum* species complex, causing tomato BWD based on the geographical location which has major implications in developing effective and sustainable BWD management measures and is thus considered valuable. It is a key step in developing effective and sustainable management method(s) of BWD based on isolated characteristics prevailing in the specific geographical location. The detection of a new pathogen, phylotype II of *Ralstonia solanacearum* species complex in Tanzania alerts plant health regulators globally to implement strong standard phytosanitary measures to prevent the further introduction or spread of the disease into the uninfected areas. There should be a joint effort among the scientific community globally to combat BWD through efficient collaboration, research and communication. Follow-up research is thus suggested to develop effective and sustainable management measures by considering specific pathogen diversity in geographical locations. Further research is recommended to explore the potential of avirulent strains of *Ralstonia solanacearum* species complex to develop a microbial-based bactericide(s) against the virulent strains.

SIGNIFICANCE STATEMENT

This research was conducted to understand the diversity of tomato bacterial wilt disease (BWD) isolates so as to develop management strategies based on prevailing pathogens in Tanzania. Findings revealed that the isolate of tomato BWD is diverse and composed of 2 species and 3 phylotypes namely phylotypes I and III of *Ralstonia pseudosolanacearum* and phylotype II of *Ralstonia solanacearum*. The pathogen diversity reported in this research contributes towards developing sustainable management measures based on prevailing isolates in particular locations and alerting plant health regulators globally to implement strong standard phytosanitary measures to avert disease introduction or spread to uninfected areas. Follow-up research is thus suggested to develop sustainable management measures including the use of microbial-based bactericides.

ACKNOWLEDGMENTS

This study was partially funded by the German Academic Exchange Service Program (DAAD) through the In-Country/In-Region Scholarship Programme Tanzania 2016 (grant number 91637162) and the Centre for Research, Agriculture Advancement, Teaching Excellence and Sustainability (CREATES) in Food and Nutrition Security (grant number 02090107-048-301-4001-P044-J01S01-C42) of the Nelson Mandela African Institution of Science and Technology (NM-AIST).

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