



Pathological and biochemical descriptors of *Ralstonia solanacearum* of tomato (*Solanum lycopersicum*) in Tanzania

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ABSTRACT

An experiment was conducted to identify bacterial wilt disease-causing isolates of *Ralstonia solanacearum* (Smith) in Tanzania. The pathological and biochemical descriptors were used to determine the virulence and biovars of 40 bacterial isolates originating from 200 wilted tomato (*Solanum lycopersicum* L.) stems collected from the main agro ecological zones of Tanzania. Results revealed that 53% of the investigated isolates were pathogenic, whereas 90% of isolates belonged to biovar 3 and the rest (10%) were biovar 2. Biovar 3 was present in all of the surveyed agro-ecological zones, whereas biovar 2 prevailed in Southern zone only and is reported for the first time in Tanzania. This is an alert for plant health officers to implement standard phytosanitary measures in preventing new introduction and spread of *Ralstonia* to uninfected locations. Descriptors adopted successfully identified biovars of *Ralstonia*, thus can be integrated with other diagnosis tools to manage bacterial wilt disease.

Key words: Carbohydrate oxidation, Pathogenicity, *Ralstonia solanacearum*, TTC medium, Virulence

Tomato (*Solanum lycopersicum* L.) is the most important vegetable crop for nutrition and income in Tanzania. Tomato contains a substantial amount of protein and essential vitamins, minerals and trace elements to enhance human diet (Wang *et al.* 2015, URT 2016, Schreinemachers *et al.* 2018). Tomato is a crop of great economic significance as it is commonly cultivated for income generation in different Tanzanian agro ecological zones. It contributes 64% of the total annual vegetable production in Tanzania but production is low (19 t/ha) and not according to the crop potential (40–100 t/ha) (URT 2016). Several factors are responsible for its low yield and diseases are one of them. The bacterial wilt disease (BWD) caused by *Ralstonia solanacearum* (*R. solanacearum*) is one of the major diseases of tomato.

Various management strategies are used to manage BWD (Yuliar and Koki 2015) but the diversity of the causative pathogen challenges effectiveness of the management strategies (Aloyce *et al.* 2017). Use of resistant cultivars for instance is considered the main management strategy but the stability of resistance is highly affected by the biology of the causative pathogen (Wang *et al.* 2013). *R. solanacearum* is a species complex plant pathogen with broad genetic and physiological diversity. Based on host range, it was classified into five races (Pegg and Moffet 1971, He *et al.* 1983). Pathologically, *R. solanacearum*

isolates can be classified as virulent and pathogenic or avirulent and non-pathogenic (Janse and Ruissen 1988). On the other hand, biochemically *R. solanacearum* isolates are classified into groups known as biovars depending on the type of carbohydrate oxidation (Hayward 1964, Hayward 1991, Hayward and Hartman 1994).

There are different approaches developed to identify *R. solanacearum* which vary from molecular, biochemical, pathological and cultural approaches (Yuliar and Koki 2015). The effectiveness and efficiency of each approach is reduced by associated application challenges (Aloyce *et al.* 2017). The pathological and biochemical approaches used in this study were selected to improve effectiveness and efficiency of determining biovars of *R. solanacearum*. The information generated could contribute to the improvement of diagnosis tools and management strategies of *R. solanacearum*.

MATERIALS AND METHODS

A field survey was conducted from September 2017 to December 2018 to determine biovars of *R. solanacearum* causing BWD in Tanzania. Purposive sampling was adopted by selecting 10 districts namely Arumeru, Babati, Manyoni, Nyamagana, Kilolo, Temeke, Chake Chake, Mbeya Urban, Kibondo and Kongwa to cover the major agro-ecological zones of Tanzania. A multistage random sampling procedure was used in selecting the wards, villages and the farms. Four wards were selected at random in a district and five farmers' fields were sampled from each village. Within the farm, five plots of 50 m² were sampled by critically observing symptoms of BWD. For a quick field diagnosis,

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the streaming of milky white masses of bacterial cells distinguished BWD caused by *R. solanacearum* from vascular wilts caused by fungal pathogens and nematodes.

Data on wilt incidence were recorded in five plots within a field by counting number of plants with BWD symptoms in a plot. Then the per cent wilt incidence was calculated on a 1 – 5 scale according to Hyakumachi *et al.* (2013) and Sang *et al.* (2016) whereby 1= No symptom, 2 = Top young leaves wilted, 3 = Two leaves wilted, 4 = Four or more leaves wilted and 5 = Plant dies.

Average disease severity per district was calculated as:

$$\text{Severity (\%)} = \frac{5A + 4B + 3C + 2D + E}{5N} \times 100$$

where A = number of plants on scale 5; B = number of plants on scale 4; C = number of plants on scale 3; D = number of plants on scale 2; E = number of plants on scale 1; N = total number of plants evaluated.

Twenty samples of tomato stems with typical bacterial wilt symptoms were collected from each district (200 samples in total) and sent to the laboratory for the isolation and characterization of *R. solanacearum*.

Data of disease incidence and severity were pooled together by calculating the average of incidence and severity of each ward in a district; this resulted into 40 samples which were thereafter subjected to the analysis of variance (ANOVA). The mean separation were carried out by using the least significant difference (LSD) procedure ($P = 0.05$). The cost at data analysis software program facilitated analyses. From the 200 samples of wilted tomato stems originating from the agro ecological zones, eight from each zone were randomly picked to represent a ward in the study area and coded accordingly. A total of 40 samples were thus selected for the biovar (s) determination. Selected samples were washed with running water from the tap to remove soils and then immersed in 70% ethanol for 2 to 3 minutes to remove any saprophytic or epiphytic bacteria from stem surfaces. Surface-sterilized stems of each isolate were macerated in sterile water to obtain a bacterial suspension.

The virulence, pathogenesis and biovars of *R. solanacearum* isolates were tested by using the pathological and biochemical approaches (Kinyua *et al.* 2014). Solution of TTC and mineral media, disaccharides and sugar alcohols were prepared (Table 2). D-Trehalose, L- Tryptophan and D-Ribose were included in the test to differentiate between the sub-phenotypes 2A and 2T of biovar 2. Negative controls were set up without any carbohydrate, where salicin and sterile water were used. Dextrose, the most commonly utilized carbohydrate by all biovars was included as a positive control. Bacterial suspension was streaked into the TTC medium agar plates and incubated at 28°C for 48 h. Single growing colonies were picked and sub-cultured onto a fresh medium to obtain pure cultures. Identification of presumptive colonies were made when typical colonies of virulent showed a characteristics light red colored center and whitish margin while those of avirulent isolates were smaller, off-white and non-fluidal. The virulent isolates were

Table 1 Bacterial wilt disease incidence and severity in main agro-ecological zones of Tanzania

Zone	Isolate code	Disease incidence (%)	Disease severity (%)
Northern	NAA1	23.89b	70.97fg
Northern	NAA2	21.02b	48.19c
Northern	NAA3	18.89a	44.27bc
Northern	NAA4	24.11b	63.19ef
Northern	NMB1	25.80b	68.99f
Northern	NMB2	20.67a	51.33de
Northern	NMB3	21.55b	40.57bc
Northern	NMB4	23.52b	66.67f
Southern	SIK1	29.73c	82.40a
Southern	SIK2	32.47c	77.38g
Southern	SIK3	30.11c	82.04g
Southern	SIK4	37.00d	89.43i
Southern	SMM1	26.03bc	74.00fg
Southern	SMM2	23.67b	64.43ef
Southern	SMM3	24.02b	60.57ef
Southern	SMM4	23.79b	59.33e
Central	CSM1	24.11b	54.00de
Central	CSM2	21.50b	52.31de
Central	CSM3	23.44b	69.00f
Central	CSM4	20.80b	53.09de
Central	CDK1	23.06b	59.19e
Central	CDK2	17.02ab	39.31b
Central	CDK3	22.78b	60.33ef
Central	CDK4	22.50a	69.65f
Lake	LMN1	22.96b	57.49e
Lake	LMN2	21.47b	60.05ef
Lake	LMN3	20.67b	54.10de
Lake	LMN4	12.96a	22.47a
Lake	LKK1	20.42ab	50.33de
Lake	LKK2	18.99ab	30.63b
Lake	LKK3	12.78a	22.65a
Lake	LKK4	23.49b	57.91e
Coastal	CZC1	12.00a	42.16b
Coastal	CZC2	13.63a	26.89ab
Coastal	CZC3	24.09b	73.07fg
Coastal	CZC4	21.20b	45.80cd
Coastal	CDT1	23.07b	23.16a
Coastal	CDT2	22.00b	61.63ef
Coastal	CDT3	12.63a	37.97b
Coastal	CDT4	21.99b	57.02e
Mean		21.45	51.47
F test		*	**
SD		4.07	3.19

**, * = Means are significant at $P \leq 0.01$ and 0.05 respectively. Mean of incidence and severity with the same letter(s) within the column are not significantly different based on LSD test ($P=0.05$).

Table 2 Biovars and isolates of *R. solanacearum* of tomato with bacterial wilt in Tanzania

Isolate	Maltose	Lactose	Cellobiose	Mannitol	Dulcitol	Sorbitol	Ribose	Trehalose	Tryptophan	Dextrose	Salicin	DW	Biovar
NAA1	+	+	+	+	+	+	+	+	+	+	-	-	3
NAA4	+	+	+	+	+	+	+	+	+	+	-	-	3
SIK1	+	+	+	+	+	+	+	+	+	+	-	-	3
SIK4	+	+	+	-	-	-	+	+	+	+	-	-	2 (2T)
CSM4	+	+	+	+	+	+	+	+	+	+	-	-	3
LMN1	+	+	+	+	+	+	+	+	+	+	-	-	3
LKK1	+	+	+	+	+	+	+	+	+	+	-	-	3
CZC1	+	+	+	+	+	+	+	+	+	+	-	-	3
CZC3	+	+	+	+	+	+	+	+	+	+	-	-	3
CDT4	+	+	+	+	+	+	+	+	+	+	-	-	3
XYZ	+	+	+	-	-	-	+	+	+	+	-	-	2 (2T)

+ Positive reaction; - Negative reaction; 2T, Sub-group of biovar 2.

selected for the subsequent experiment.

Seeds of tomato variety called Tanya, a commonly cultivated but susceptible variety to BWD in Tanzania were used in this experiment. The seeds were sown in a 1L pot filled with forest soil and sand at 3:1. The pots were placed in screen-house at the average temperature of 25°C and watering was conducted after every other day. After two weeks, seedlings were inoculated with the inoculum of the isolates which had presumptive *R. solanacearum* colony appearance on TTC medium (Janse and Ruissen 1988). Three seedlings in replicate were inoculated with suspension of isolates at the rate of 10^9 cfu/ml with punctures made with a sterile needle in a stem between the two cotyledons. Three replicates per bacterial suspension were used so that a total of nine seedlings were inoculated with each bacterial isolate. Seedlings inoculated with sterile water were included as negative control. Prior to inoculation, seedlings were not irrigated for 24 h.

Experiment was designed in a completely randomized design with three replications and held at 25°C in screen-house. Development of wilting symptoms was observed and severity was recorded weekly (He *et al.* 1983, Horita and Tsuchiya 2001) on a 0 to 5 scale where 0=no symptoms; 1= leaf above inoculation wilted; 2=two leaves wilted; 3= three leaves wilted; 4= four or more leaves wilted and 5=plant died. Score of BWD severity were related with the BWD severity recorded in the field by computing a correlation coefficient by using CoStat data analysis software.

When typical symptoms were observed, re-isolation of the bacteria was made on TTC medium. After 48 h incubation at 28°C, presence of *R. solanacearum* looking colony was examined and recorded. Isolates with *R. solanacearum* colony characteristics were subjected to biovar(s) determination. Ten isolates identified as the most virulent according to Koch's rule (culturing and pathogenicity test) were used. Single colony of each isolate was streaked on TTC medium and incubated for 48 h at 28°C. A loop-full of each isolate was taken and mixed into 1ml sterile water in a 2.0 ml centrifuge tube (Eppendorf)

to make suspension containing about 10^9 cfu/ml. After autoclaving, the media was cooled to 65°C. 10 ml of carbon source was each mixed with 90 ml of the mineral medium to make sugar/alcohol amended medium. 300 µl of sugar/alcohol-amended medium were dispensed in each 2.0ml centrifuge tube and inoculated with 10 µl of suspension of each isolate then incubated at 28°C. Observations were recorded on changing pH as indicated by colour change for 7 days incubation (Schaad 1988).

RESULTS AND DISCUSSION

Results indicated that on average bacterial wilt disease was present in 55% of the visited farmers' tomato fields. The mean of disease incidence and severity in the study area ranged from 12-37% and 19.65-89.43% respectively. Results showed that out of 40 isolates of *R. solanacearum* evaluated for virulence from different zones 29 produced typical colonies of virulent isolates on TTC medium (Table 1). In pathogenicity test, 19 isolates showed typical wilting, whereas the remaining 11 were non-pathogenic and could be rated as saprophytic although they had similar colony appearance with *R. solanacearum*. Subsequently, when the 19 isolates were re-isolated on TTC medium 10 produced virulent colonies of *R. solanacearum*.

Results showed that all the tested bacterial isolates were able to oxidize the four basic carbon sources (Dextrose, sucrose, mannitol and lactose) in 3 days and nine isolates oxidized disaccharides (sucrose, lactose, and maltose) and sugar alcohols (mannitol, sorbitol and dulcitol) while an isolate SIK4 was not able to utilize the sugar alcohols (Table 2). The isolate coded as XYZ was isolated from the wilted round potato stems and was include as an out group, it behaved similarly as isolate SIK4. On the other hand, all the control plates of carbon sources (dextrose and salicin) and DW remain unchanged. BWD is widely spread in Tanzania affecting more than 55% of farmers' field at significantly ($P \leq 0.05$) different levels of disease incidence and severity. The level of virulence and pathogenesis among isolates significantly varied within and across the agro ecological

zones. Variations in the incidence and severity of BWD in the agro ecological zones may be attributed to factors such as diversity of *R. solanacearum* isolates soil types and the production environment (Rahman *et al.* 2010). In terms of production environment, hot weather and high (80-90%) relative humidity favour survival of *R. solanacearum* hence accelerating disease incidence and severity. Such environmental conditions are common in most screen-houses especially in developing countries and therefore prevention measures are critically important to avoid introduction of *R. solanacearum* to such environments. *R. solanacearum* survives well in moist soils with a pH values from 6-7. Since soils in the agro ecological zones of Tanzania are not homogenous, variations of bacterial wilt disease incidence and severity may be caused by differences in soil moisture and pH levels. Studies conducted by Wang *et al.* (2013) revealed that higher relative humidity and changes in soil pH affected the severity and incidences of bacterial wilt disease respectively.

The pathological descriptors adopted were able to distinguish virulent from avirulent isolates and further pathogenic from non-pathogenic isolates. Moreover, the use of biochemical descriptors successfully differentiated *R. solanacearum* isolates into biovars as previously reported by Hayward (Hayward 1964, He *et al.* 1983, Kumar *et al.* 1993) who observed and reported that biovar 3 oxidizes both disaccharides and sugar alcohols, biovar 2 oxidizes only disaccharides, whereas biovar 1 utilizes hexose alcohols only and biovar 4 oxidizes only alcohols. The isolates of *R. solanacearum* utilize various carbon sources for maintenance and growth (Dhital *et al.* 2001). Out of 10 isolates of *R. solanacearum* used in this study, 90% were identified as biovar 3, while the remaining (10%) were recognized as biovar 2 (2T). Biovar 3 was recorded from all the surveyed zones while biovar 2 was present in the Southern zone of Tanzania. To the best of our knowledge, this is the first report of the prevalence of biovar 2 of *R. solanacearum* in Tanzania, and hence is an alarm to the plant health regulators globally to design and implement management strategies that prevent introduction and/or spread to other uninfected geographical locations. Biovar 2 of *R. solanacearum* infects both tomato and potatoes and thus considered to be of more economic importance. The prevalence and survival of *R. solanacearum* biovar 2 in the Southern agro ecological zones of Tanzania could be associated with the continuous cultivation of host plants such as round potato, tomato, pepper and eggplants (Alvarez *et al.* 2010). This indicates that the farmers are not aware of the demerits of continuous cultivation of the same host plant in the same piece of land every season. It therefore calls upon the extension service providers to help farmers to adopt plant protection practices such as crop rotation for improved disease management.

Descriptors used in the study were selected based on their effectiveness in terms of availability of the ingredients, affordability, simplicity in application and time serving to obtain the diagnosis results. All ingredients used were

locally and reasonably available and can be prepared by the plant protection officers and technician. The descriptors have demonstrated their effectiveness in identification of *R. solanacearum*. Average time required to obtain results for pathological descriptors is from 24 h for virulence test and 3-7 days for pathogenicity tests. On the other hand, the results of biochemical test was obtained in 0-3 days of incubation with some carbon sources such as Dextrose (utilized by all biovars) providing results in 1 h (< a day). Therefore they have a potential to be used to develop a quick diagnosis kit for *R. solanacearum*.

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