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Pathogenicity and Prospective Control of *Xanthomonas vesicatoria* WI02 Isolated from Infected Tomato Fruit

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Abstract: Species of *Xanthomonas* cause the black rot disease of tomato, a disease reported to be responsible for over 15% annual loss of tomatoes in Nigeria. Strains of *Xanthomonas* species were isolated with sucrose peptone agar from infected tomato fruits; only *X. vesicatoria* WI02 exhibited a positive pathogenicity test result on tomato plant with a severity measure of 5. Subsequently, the pathogenicity and prospective control of this pathogen were further investigated. *X. vesicatoria* WI02 had a narrow phytopathogenicity spectrum; tomato and pepper were the most susceptible plants to this pathogen with minimum infective doses of 0.4×10^8 and 0.8×10^8 cfu/mL respectively. Aqueous extracts of Kocide (microbial pesticide), *Azadirachta indica* and *Alcalypha indica* had similar inhibitory scores against *X. vesicatoria* WI02 on agar well diffusion plates; subsequently, the minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of these extracts and chemical pesticide were determined. Kocide (MIC: 0.025g/mL; MBC: 0.05g/mL), extracts of *Azadirachta indica* (MIC: 0.0125g/mL; MBC: 0.0125g/mL) and *Alcalypha indica* (MIC: 0.025g/mL; MBC: 0.05g/mL) all exhibited significant antimicrobial activities against *X. vesicatoria* WI02. With an MBC/MIC range of 1-2, all the investigated extracts appeared to possess a bactericidal activity. Aqueous extract of *Azadirachta indica* leaves could therefore be further investigated as a better and natural alternative to chemical pesticides like Kocide in the control of *Xanthomonas* infections on tomato.

Keywords: *Xanthomonas vesicatoria, Azadirachta indica, Alcalypha indica*

1. Introduction

Bacterial spot or rot disease of tomato (*Lycopersicon esculentum* Mill.) is usually caused by *Xanthomonas campestris* pv. *vesicatoria*, it is a very serious disease of this economically important crop, especially in Nigeria; its virulence is generally observed on the seeds, leaves and fruits of this plant. X. *vesicatoria* is destructive to tomato and pepper seedlings [1], resulting in economic losses in warm and humid areas due to the persistence of *Xanthomonas vesicatoria* in these weather conditions [2]. Signs of this disease on tomato begin as small circular to irregular greasy spots usually conspicuous on the surface of the fruits. As the infection progresses, the water-soaked regions enlarge, grow

into a mass, and finally change colour from dark green to purple or gray, characterized by a distinctive black center. This bacterium can survive on tomato seeds for a period of 10 years and its manifestation can reduce seed germination [3],

cause the defoliation of tomato leaves and has been documented to reduce the weight of harvested fruits by up to 52% [4]. While this pathogen attacks both fruits and other plant parts, characteristic lesions are more distinguishing on tomato fruits.

The use of copper compounds (especially the copper hydroxide based Kocide pesticide), antibiotics and other chemicals as microbial pesticides in the control of agricultural pests and diseases is gradually being restricted due to public health concern over toxic residues (with some already proven to be mutagenic, teratogenic and carcinogenic), increment in diseases resistance, affordability and accessibility to farmers [5; 6]. Consequently, there has been an increased interest among researchers to develop suitable alternative control measures to combat microbial plant pests. Farmers in Nigeria are increasingly adopting the use of different medicinal plant extracts (like Neem plant and *Acalypha indica*) to treat several livestock diseases. The utilization of such plants materials, due to their safety, affordability and abundant distribution, offer a potential practical and convenient approach to developing alternative antimicrobial control measures against infections of agricultural significance.

Neem (*Azadirachta indica*) leaf extract is used as a source of many therapeutic agents in different countries, including Nigeria. It is used for the treatment of diabetes because of its potential anti-diabetic properties and has also been indicated to possess anti-hyperglycemic, anti-inflammatory, antimicrobial and antiviral properties [7]. The root, stem and leaf of *Acalypha indica* have also been documented to possess herbal activities; the plant is traditionally used as an expectorant against asthma and pneumonia and also has antimicrobial, emetic, emmenagogue and anthelmintic properties [8]. This current research screened the extracts of *Azadirachta indica* and *Acalypha indica* as potential alternatives to chemical-based antimicrobial pesticide in the control of *X. vesicatoria* associated with tomato.

2. Materials and Methods

2.1 Isolation of *Xanthomonas vesicatoria* from Infected Tomato

Tomato fruits showing typical and similar *Xanthomonas* spot lesions were collected in farms within Ibadan, Oyo State, Nigeria and at Apata market, Ibadan between May and June, 2016. Isolation of *Xanthomonas* was accomplished using the Tavga isolation procedure. Five mm^2 segments were taken from the edge of infected samples, surface sterilized with 1% NaOCl for two minutes, rinsed twice with sterile distilled water and then dried on sterilized filter papers. This procedure was modified by macerating the prepared samples in 2 mL of sterile distilled water using a sterile spatula, and the resulting suspension was stirred in a vortex mixer to obtain a turbid bacterial suspension. A loopful of the resulting aqueous suspension in each case was streaked onto sucrose peptone agar and incubated at 30˚C for 48 hours. Distinct colonies were thereafter subcultured and identified [9]. Xanthomonads identification's done using biochemical, carbon utilization and chemical sensitivity assays with a Biolog GENIII 96 microplate (Biolog, Omnilog, US).

2.2 Pathogenicity Test

Pathogenicity test (to establish Koch's postulate) was carried out using the isolated strains of *Xanthomonas* spp. to infect young, healthy tomato leaves. Suspension of pure *Xanthomonas* cells was used for the standardization of each *Xanthomonas* spp. for tomato infection; microbial doses of each bacterium (in colony forming units per milliliter) were extrapolated from a light absorption standard curve of each strain using a spectrophotometer at wavelength of 600 nm (OD_{600}). Tomato leaves were inoculated with 1ml spray of 1.7 x 10^8 CFU/mL cell suspension dose of each *Xanthomonas* spp. Control plants were inoculated with sterile distilled water. Inoculated plants were kept humid for 48 hrs and left in the greenhouse for observation. The manifestation of signs associated with bacterial rot disease was observed 1-3 weeks post-inoculation. *Xanthomonas* cell were isolated from diseased areas of inoculated leaves (as previously described) to reconfirm the etiology of this disease [10]. Disease severity evaluations were made according to the rating scale of Asare-Bediako *et al.* [11].

2.3 Pathogenicity Spectrum

To investigate the spectrum or range of selected plants susceptible to this bacterium, pathogenicity spectrum of *Xanthomonas vesicatoria* WI02 on selected economically important plants was determined as described for pathogenicity test. While the minimum infective dose (MID) was determined using predetermined inoculating densities (CFU/mL) of *Xanthomonas vesicatoria* WI02 on young leaves of growing plants (mostly seedlings) and defined as the lowest concentration or density of test bacterium capable of

infecting and subsequently generation signs of infection peculiar to black rot on tomato.

2.4 Extraction of Plant Materials

Fresh leaves of *Azadirachta indica* and *Acalypha indica* were collected in June, 2016 at the Institute of Agricultural Research and Training, Ibadan, Oyo State, while the collected leaf samples were further identified at the Herbarium Department, Forestry Research Institute of Nigeria, Ibadan. The collected leaves were oven dried at 45^0C till constant weight of the leaves were obtained. The dried leaves were coarsely powdered and 50g each was used for successive extraction in 250 ml sterile distilled water. The aqueous extraction was carried out using a Soxhlet extractor. The liquid extracts, concentrated with a rotary evaporator, were subsequently evaporated to dryness and stored at 4°C till they were required in preparations of varying concentrations [7].

2.5 Agar well Antimicrobial Screening of Kocide and Plant Extracts

Agar well inhibition assay, as described by Adedire *et al.* [12], was used to screen for the inhibitory potentials of plant extracts and the chemical microbial pesticide (Kocide); 0.5mL of *X. vesicatoria* WI02 cell suspension (1.7×10^8 CFU/mL) was inoculated into 15mL of cooled, molten sucrose peptone soft agar. This preparation was poured into a petri dish and allowed to set, then a sterile cork borer of 6mm diameter was used to make wells on the medium, 0.1mL each of different concentrations of extracts and kocide was introduced into the appropriately labeled wells. The inoculated plate preparations were incubated at 30^0C for 24 - 48 hours with daily observation of inhibition zone. Inhibition was measured and scored positive (significant) if the zone is ≥ 2mm diameter against the indicator (*X. vesicatoria* WI02) lawn.

2.6 Minimum Inhibitory Concentration (MIC) of Kocide and Plant Extracts

The MIC of plant extracts and chemical pesticide were determined through the microtiter broth dilution technique. Standardized suspension (1.7×10^8 CFU/mL) of the indicator organism (*X. vesicatoria* WI02) was inoculated into the microtiter plate wells, including one positive growth and one negative sterility control. Sucrose peptone broth containing equal volume of plant extracts or chemical pesticide in varying concentrations of 0.1, 0.05, 0.025, 0.0125, 0.00625 and 0.003125 g/ml were prepared, introduced into the wells (at 50% v/v) and incubated at 37 ^{O}C for 24 hours. After the overnight incubation, these tubes were observed for turbidity with microplate reader. The lowest plant extract or kocide concentration corresponding to microtiter plate showing no turbidity was recorded as the MIC for these antimicrobial components [13].

2.7 Minimum Bactericidal Concentration (MBC) of Kocide and Plant Extracts

The MBCs were determined using the MIC broth mixture. A 0.5mL mixture from each stirred tube was diluted and subcultured (pour-plate) onto fresh, extract free, sucrose peptone agar plates; the inoculated plates were subsequently incubated at 30 ^{O}C for 24 hours. The MBC was defined as the least extract or kocide concentration at which no colony growth was observed on subcultured plate.

3. Results and Discussion

3.1 Pathogenicity Test

Three *Xanthomonas* species (*Xanthomonas* spp. WI01, *Xanthomonas* spp. WI02 and *Xanthomonas* spp. F01), with similar macroscopic morphological appearance, were isolated from infected tomato and subsequently subjected to pathogenicity test on tomato plants. Only *Xanthomonas* spp. WI02 exhibited a positive pathogenicity test result on this plant with a severity measure of 5 (Table 1); this strain was further identified as *X. vesicatoria* WI02 and subsequently investigated. Cao *et al.*[14] had reported bacteria as plant pathogens causing severe economically damaging diseases, ranging from spots, mosaic patterns, pustules on leaves and fruits, smelly tuber rot to plant death (as eventually observed for tomato plant infected with 1.7×10^8 CFU/mL *X. vesicatoria* WI02).

3.2 Pathogenicity Spectrum

Table 2 below shows the virulence spectrum of *X. vesicatoria* WI02 on selected economically important plants within Ibadan, south-west Nigeria. Of all the screened plants, only four plants were susceptible to this pathogen with infection signs varying from mild necrosis to total leaf wilt and the minimum infective dose of *X. vesicatoria* WI02 was subsequently determined on each of these plants. Tomato (*Lycopersicon esculentum* Mill.) and pepper (*Capsicum annuum*) were most susceptible to this pathogen with minimum infective doses of 0.4×10^8 and 0.8×10^8 cfu/mL respectively.

3.3 Agar well Antimicrobial Screening of Kocide and Plant Extracts

Agar well inhibitory screening of antimicrobial properties of pesticide and plant extracts revealed that Kocide had a significantly higher inhibition zone at lower concentration (0.5mg/ml) than each plant extract; but as concentrations increased, Kocide and plant extracts had similar inhibitory strength (Table 3). *Azadirachta indica* extract exhibited the widest average inhibition zone (23mm) against *X. vesicatoria* WI02 at 2mg/ml; however, this inhibition was not statistically different from those generated by *Acalypha indica and* Kocide at the same concentration against the pathogen. Akbary [15] also recorded a tremendous increment in antimicrobial activity of *Camellia sinensis* tea extract with an increment in concentration.

3.4 Minimum Inhibitory Concentration (MIC) of Kocide and Plant Extracts

Minimum inhibitory concentration is often used to determine the dosage of an antimicrobial component; it reveals the lowest concentration of such component capable of generating the desired selective toxicity against target pathogen. The minimum inhibitory concentrations of Kocide, *Azadirachta indica* and *Acalypha indica* extracts were recorded at 0.025, 0.0125 and 0.025g/ml respectively (Table 4). Extract of *Azadirachta indica* best inhibited the growth of *X. vesicatoria* WI02 in broth mixture; this extract had the lowest MIC against the indicator strain (*Xanthomonas vesicatoria* WI02) and as such exhibited the highest inhibitory properties against this pathogen.

3.5 Minimum Bactericidal Concentration (MBC) of Kocide and Plant Extracts

Samples from broth mixtures of *X. vesicatoria* WI02 and each antimicrobial component were inoculated on fresh sucrose peptone agar plates and the minimum bactericidal concentrations of Kocide, *Azadirachta indica* and *Acalypha indica* extracts were recorded as 0.05, 0.0125 and 0.05 g/ml respectively (Table 5).

The determination of MIC and MBC values allows a better evaluation of antibacterial effect of bioactive compounds. Pankey [16] postulated that an antimicrobial substance is bacteriostatic only when the ratio MBC/MIC is greater than 4, otherwise, such substance is bactericidal. With the determined MBC/MIC values of antimicrobials against *X. vesicatoria* WI02 in this study, it appeared aqueous extracts of *Azadirachta indica* (MBC/MIC of 1), Kocide (MBC/MIC of 2) and *Acalypha indica* (MBC/MIC of 2) all exhibited bactericidal activities.

4. Conclusion

Taking cognizance of the pathogenicity spectrum of *X. vesicatoria* WI02 identified in this study, prevention and potential control measures are very important in farm plots containing any of the identified susceptible plants, especially Tomato and Pepper. Extract of *Azadirachta indica* leaves (with MIC, MBC and MBC/MIC values of 0.0125g/mL, 0.0125g/mL and 1 respectively) appeared to be a better and natural alternative to chemical pesticides like Kocide in the control of *X. vesicatoria* WI02 isolated from tomato fruit.

Table 1: Pathogenicity Test of Isolated *Xanthomonas* Strains

Strains of *Xanthomonas vesicatoria*	**Severity**
Xanthomonas spp. WI01	0
Xanthomonas spp. WI02	5
Xanthomonas spp. F01	0

Table 2: Pathogenicity Spectrum of *Xanthomonas vesicatoria* W102

	Dose of *Xanthomonas vesicatoria* WI02 (CFU/mL) / Severity				
Plants	**3.0 x 10^8**	**1.5 x 10^8**	**0.8 x 10^8**	**0.4 x 10^8**	**0.2 x 10^8**
Citrus sinensis	4	3	0	0	0
Magnifera indica	0	0	0	0	0
Solanum melongena	0	0	0	0	0
Corchorus olitorius	4	2	1	0	0
Talinum triangulare	0	0	0	0	0
Lycopersicon esculentum	5	5	3	2	0
Manihot esculenta	0	0	0	0	0
Musa acuminate	0	0	0	0	0
Carica papaya	0	0	0	0	0
Capsicum annuum	5	4	2	0	0

Table 3: Agar well Antimicrobial Screening of Kocide and Plant Extracts

Concentration (mg/ml)	**Extracts/Inhibition(mm)**		
	Azadirachta indica	***Acalypha indica***	**Kocide**
0.5	11.67c	8.33c	13.00b
1.0	17.67b	13.33b	18.00ab
2.0	23.00a	18.33a	21.67a

Table 4: Minimum Inhibitory Concentration (MIC) of Kocide and Plant Extracts

Concentration (g/ml)	**Extracts/Turbidity**		
	Azadirachta indica	***Acalypha indica***	**Kocide**
0.1	-	-	-
0.05	-	-	-
0.025	-	-	-
0.0125	-	+	+
0.00625	+	+ +	+ +
0.003125	+ +	+ + +	+ + +

(+ + +) High turbidity; (++) Average turbidity; (+) Low turbidity; (-) No turbidity

Table 5: Minimum Bactericidal Concentration (MBC) of Kocide and Plant Extracts

Concentration (g/ml)	Extracts/*X. vesicatoria* (CFU/mL)		
	Azadirachta indica	***Acalypha indica***	**Kocide**
0.1	0	0	0
0.05	0	0	0
0.025	0	1.95×10^2	4.10×10^3
0.0125	0	2.34×10^5	3.21×10^6
0.00625	1.24×10^3	1.11×10^7	1.82×10^9
0.003125	1.36×10^9	1.09×10^{10}	5.44×10^9

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