EVALUATION OF ANTIBACTERIAL ACTIVITY OF SOME PLANT EXTRACTS AGAINST *PECTOBACTERIUM CAROTOVORA* SUBSP. *CAROTOVORA*

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SUMMARY

Bacterial soft rot of sweet potatoes tubers, induced by Pectobacterium spp. (previously called Erwinia spp.), results in substantial reduction in quality and thereby reduces its market value and consequently low income for potato growers. Out of 45 bacterial isolates from infected sweet potato tubers collected from Gombe, Billiri and Kashere town, Gombe State Nigeria, thirty-eight (38) were pathogenic on potato tubers. The six isolates selected as test organisms revealed the same results in morphological and biochemical tests hence, identified as Pectobacterium carotovora subsp. carotovora (previously called Erwinia carotovora subsp. carotovora). Antibacterial potential of aqueous extracts of Allium sativum L., Zingiber officinale, Capsicum annum var. chinense, Capsicum annum var. Cayenne and Allium cepa were examined at 25, 50 and 75% concentrations against the growth and survival of Pectobacterium isolates. The treatments consisted of five plant extracts with 3 concentrations each. Streptomycin (0.2%) served as positive control while sterile distilled water served as negative control. The experiment was laid out in a completely randomized design (CRD) with three replicates. All the plant extracts were most effective at 75% concentration. Isolates Pcc-1 and Pcc-2 were most susceptible at 75% concentration. The extracts exhibited their highest potency at 72 hours after incubation (HAI). All the extracts significantly reduced the growth of potato soft rot organisms. It was also found out that the higher the concentration and incubation period, the greater the effect of the extracts on the test isolates.

Keywords: Biopesticides, *in-vitro*, isolates, pathogen, pathogenicity

The principal factor responsible for losses during storage of sweet potatoes, *Ipomoea batatas*, has been reported to be due to infection by microorganisms resulting in tuber decay mainly by bacteria, causing rot in potato, due to the activity of a wide range of hydrolytic enzymes such as cellulase, xylanase, and protease. These enzymes are also responsible for tissue maceration and cell death (Olivieri *et al*., 2004).

Most of these rots cause substantial reduction in yield or quality (Zitter and Loria, 2002) and losses in transit and storage particularly in the warm regions where temperature is high (Bdliya and Dahiru, 2006). Tuber rot in potato is caused by a variety of bacteria and fungi each causing its own type of rot. In potato, the evident rots are the dry rot induced by *Fusarium* spp., soft rot by *Pectobacterium carotovora*, pink rot by *Phytophthora erythroseptica* and ring rot by *Corynebacterium sepedonicum* (Bdliya and Dahiru, 2006). The activities of these microorganisms usually lead to the changes in appearance, deterioration in texture and possibly flavour or taste of the potato (Amienyo and Ataga, 2007).

Bacterial soft rot is one of the most common potato diseases in the tropics that induces quick and heavy spoilage losses. It is caused by *Pectobacterium* spp., principally *Pectobacterium carotovora* subsp. *carotovora*, *Pectobacterium carotovora* subsp *atrosiptica or Pectobacterium chrysanthemi*

(Czajkowski *et al*., 2011). It is the one of the most important and widespread bacterial diseases of a variety of plants either in the field or in storage (Harrison and Nielson, 1990; Hajhamed *et al*., 2007). Bacterial soft rot accounts for 30–50% loss (Czajkowski *et al*., 2011). Indiscriminate use of chemical pesticides to control diseases of crop plants is causing health hazard to both man and the environment. Recent studies on the use of plant extracts have opened a new opportunity for the control of tuber diseases. Several other methods of the disease control such as hot water treatment (Shirsat *et al*., 1991) and air-drying of tubers (Bartz and Kelman, 1985) have been tried with varying scale of success. Green plants are a huge reservoir of various effective chemotherapeutics and could serve as an environment-friendly natural alternative to toxic chemical pesticides. The lack of appropriate storage facilities in Nigeria has led to high losses of potato tubers in transit and storage. Therefore, the search for a more effective and cheaper method of controlling the disease becomes very essential.

Natural plant extracts are environment-friendly, easy to apply and relatively inexpensive. The use of plants extracts which are rich in biologically active ingredients have proven better than synthetic chemical in effectively controlling diseases. Therefore, this work was out to isolate and identify *Pectobacterium carotovora* subsp. *carotovora*, causal agent of potato tuber soft rot and to determine the effects of some plant extracts on the pathogen *in-vitro*.

MATERIALS AND METHODS

Sample collection

Diseased/rotten sweet potato tuber samples were sourced from Billiri, Gombe and Kashere farmers' farms and markets; six locations. Garlic clove *(Allium sativum* L.), ginger rhizomes (*Zingiber officinale*), bonnet pepper fruits (*C. annum* var. *chinense*), cow horn pepper fruits (*C. annum* var. *cayenne*) and onion bulbs (*Allium cepa*) were sourced from Kashere market. Clean and disease-free botanicals were washed under clean running tap water, surface sterilized with 70% ethanol for 5 minutes, and rinsed in three changes of sterile distilled water (SDW). The plant materials were wiped with paper towel and packed into polythene bags before using them for extracts preparation.

Treatments and experimental design

The experiment was carried out in Biological Science laboratory, Federal University of Kashere, Gombe State. The treatments consisted of five plant extracts with 3 concentrations each (25, 50 and 75% (w/v)). Streptomycin (0.2%) served as positive control while SDW served as negative control. Completely randomized design (CRD) was used with three replicates. Six *Pectobacterium* isolates from rotten potato tubers were used as test organisms.

Isolation and selection of soft rot bacteria

The diseased sweet potato tubers were surface sterilized with 1% sodium hypochlorite solution for two minutes. The tubers were cut open with sterilized knife to reveal area between diseased and healthy portion. Bits were cut out from the margin of rotten tissues of the infected tubers, put in 10 ml of SDW in a beaker and crushed with sterile scalpel. The beaker was kept undisturbed for 1 hour to release bacteria associated with rotted tissues. A loopful from the suspension was aseptically streaked on Crystal violet pectate (CVP) and incubated at 28±2^{o} C for 48 hrs. Colonies that formed cavities or pits on CVP medium were sub-cultured**.** The pure cultures obtained were

kept in agar slant at 4° C. Serial dilution was carried out on 24 hr-old cultures and six representative isolates, one from each location, with highest $CFUml^{-1}$ were selected as test organisms and were further subjected to biochemical tests.

Pathogenicity test

The test was carried out according to the method of Ganiyu *et al*. (2017). Healthy potato tubers were washed with SDW and disinfected by mopping the entire surface with cotton wool moistened with 70% ethanol. Cylindrical discs were removed from the disinfected tubers with a sterile cork borer (5 mm diameter). One milliliter (1 ml) suspension ($1x\ 10^6\ CFUml^{-1}$) each of the respective test organisms were inoculated into the holes (1 cm deep) created and allowed to sink into the tuber before replacing the cylindrical discs. Inoculation points were sealed with petroleum jelly to disallow extraneous pathogens from entering through the wounds. Control tubers were inoculated with sterile distilled water. After incubation at 28±2° C for the period of two weeks, tubers were cut open in transverse direction. The organisms were re-isolated and identified as previously isolated organisms in which colonies appeared round, convex, and creamy-translucent on Nutrient agar (NA) and deep red on MacConkey agar (MAC) and formed pits on CVP.

Characterization of the pathogenic bacterial isolates

Pathogenic isolates were submitted to morphological tests such as colony characteristics on Nutrient agar (NA) and MacConkey agar (MAC) and biochemical tests such as Gram staining, growth at 37° C, oxidase, catalase, sensitivity to erythromycin, indole production and arginine dihydrolase tests were performed.

Preparation of the plant extracts

Garlic clove *(Allium sativum L.*), ginger rhizomes (*Zingiber officinale*), bonnet pepper fruits (*C. annum* var. *chinense*), cow horn pepper fruits (*C. annum* var. *cayenne*) and onion bulbs (*Allium cepa*) were washed under running water, surface sterilised with 70% ethanol for 5 minutes, rinsed with three changes of SDW, aseptically chopped into pieces and air dried at room temperature for 14 days. Dried plant materials were ground into powder with aid of electric blender (MasterChef and Crownstar, MC-BL3302, China). One hundred gram (100 g) of dried powder was mixed with 100 ml SDW (1:1 w/v). The suspension was filtered through double layer of sterile cheese cloth. This concentration (100%) was reconstituted to 75, 50 and 25% and were used for the experiment.

***In-vitro* assay**

Each of the test bacterial isolates (24 hr-old culture) was inoculated by the spread plate technique. A cork-borer (5 mm diameter) was used to cut out paper discs from Whatman No.1 filter paper and sterilized before use. Paper discs were impregnated with extracts of different concentrations. Paper discs were placed accordingly into Petri plates containing inoculated bacterial isolates. Plates were incubated at 28±2° C for 24, 48 and 72 hrs and the presence or absence of zones of inhibition was observed at interval. Streptomycin (0.2%) served as positive control while SDW served as negative control. Each treatment was replicated three times. Record of inhibition was obtained by measuring the diameter of inhibition zone in each case with diameter of paper discs inclusive.

Data analysis
Data collected were subjected to analysis of variance (ANOVA) and means separated by LSD at $p \leq 5\%$ using GenStat Discovery Edition 4.

RESULTS

Locations, isolation, and characteristics of pathogenic bacterial isolates

Locations, isolation media and colony characteristics of *Pectobacterium* isolates from rotten potato tubers are presented in Table 1. Pathogenicity test carried out on potato tubers showed positive reaction for 35 isolates and were therefore pathogenic. None of the tubers in the control treatments showed symptoms of soft rot. Colonies appeared round convex creamy-translucent on NA and deep red on MacConkey agar and formed pits on CVP. Table 2 presents the biochemical characteristics of the selected six bacterial isolates. Catalase test produced gas bubbles after mixing a loopful of bacterial culture on a glass slide. All the isolates were able to grow at 37^{o}C and tested negative for Gram staining, oxidase, and arginine dihydrolase, sensitivity to erythromycin and indole production. All these bacterial isolates showed the characteristics of *Pectobacterium carotovora* subsp. *carotovora* (*Pcc*).

Effect of plant extracts, concentrations, and reactions of Pectobacterium carotovora subsp. *carotovora*

The antimicrobial effects of plant extracts, concentrations, and reactions of *Pectobacterium carotovora* subsp. *carotovora.* isolates on the diameter of inhibition zones at 24, 48 and 72 hours after incubation, are outlined in Table 3. Plant extracts had inhibitory effect on diameter of inhibition zones ranging from 10.85 to 16.32 mm for the period of data collection. The least inhibition of 10.85 mm diameter was recorded at 24 hours after incubation when *C. annum* var. *chinense* was applied and the value was significantly lower ($p \leq 0.05$) than 11.37, 11.40- and 12.82-mm diameter of inhibition zones observed in plates treated with *Allium sativum*, *Zingiber officinale* and *C. annum* var. *cayenne* respectively, but not significantly different ($p \geq 0.05$) from 10.90 mm diameter of inhibition zones recorded in *Allium cepa.* At 72 hours after incubation, zones of inhibitions ranged from 14.54 to 16.32 mm. *C. annum* var. *cayenne* exhibited the highest significant effect ($p \leq 0.05$) on zone of inhibition (16.32 mm) followed by *Zingiber officinale* with 14.86 mm zone of inhibition. However, there was no significant difference among plant extracts at 24 hours after incubation. Aside streptomycin (0.2%) and SDW, there were varying degrees of inhibitory activities among the concentrations (Table 2). At 24, 48 and 72 hours after incubation, 75% concentration performed best. Concentration at 75% had highly significant ($p \leq 0.05$) inhibitory effect of 14.38, 18.03- and 19.67-mm diameters at 24, 48 and 72 hours after incubation followed by 50% concentration.

The pathogens reacted differently to the application of plant extracts. At 24 hours after incubation, *Pcc*-1 was the most sensitive to the application of plant extracts with inhibition zone of 12.84 mm which was significantly different ($p \leq 0.05$) from 10.38 mm observed in *Pcc*-6. There was also no significant difference ($p \geq 0.05$) among the isolates at 48 hours after incubation. Zones of inhibitions ranged from 12.39 to 14.31 mm at 48 HAI. At 72 HAI, *Pcc*-2 was most sensitive with 15.73 mm and was not significantly different ($p \geq 0.05$) from 15.52 mm diameter of inhibition zone observed from *Pcc*-1 but significantly different ($p \leq 0.05$) from 15.35 mm recorded in *Pcc*-5.

Table 1: Locations, isolation media and colony characteristics of the bacterial isolates from rotted potato tubers

S/N	Isolate	Location	Isolation medium*	Colony characteristic	Pathogenicity test
1.	*Pcc*-GF	Gombe Farm	NA	Round, convex, creamy-translucent	+
2.	*Pcc*-GF	Gombe farm	MAC	Deep red	+
3.	*Pcc*-GF	Gombe Farm	NA	Round, convex, creamy-translucent	+
4.	*Pcc*-GF	Gombe Farm	MAC	Deep red	+
5.	*Pcc*-GF	Gombe Farm	NA	Round, convex, creamy-translucent	+
6.	*Pcc*-GF	Gombe farm	NA	Round, convex, creamy-translucent	+
7.	*Pcc*-GF	Gombe Farm	NA	Round, convex, creamy-translucent	+
8.	*Pcc*-GM	Gombe market	MAC	Deep red	+
9.	*Pcc*-GM	Gombe market	MAC	Deep red	+
10.	*Pcc*-GM	Gombe market	NA	Round, convex, creamy-translucent	+
11.	*Pcc*-GM	Gombe market	NA	Round, convex, creamy-translucent	+
12	*Pcc*-GM	Gombe market	MAC	Deep red	+
13	*Pcc*-KF	Kashere farm	NA	Round, convex, creamy-translucent	+
14	*Pcc*-KF	Kashere farm	MAC	Deep red	+
15	*Pcc*-KF	Kashere farm	NA	Round, convex, creamy-translucent	+
16	*Pcc*-KF	Kashere farm	MAC	Deep red	+
18	*Pcc*-KM	Kashere market	NA	Round, convex, creamy-translucent	+
19	*Pcc*-KM	Kashere market	MAC	Deep red	+
20	*Pcc*-KM	Kashere market	NA	Round, convex, creamy-translucent	+
21	*Pcc*-KM	Kashere market	NA	Round, convex, creamy-translucent	+
22	*Pcc*-KM	Kashere market	MAC	Deep red	+
23	*Pcc*-KM	Kashere market	MAC	Deep red	+
24	*Pcc*-KM	Kashere market	NA	Round, convex, creamy-translucent	+
25	*Pcc*-BF	Billiri farm	MAC	Deep red	+
26	*Pcc*-BF	Billiri farm	NA	Round, convex, creamy-translucent	+
27	*Pcc*-BF	Billiri farm	NA	Round, convex, creamy-translucent	+
28	*Pcc*-BF	Billiri farm	NA	Round, convex, creamy-translucent	+
29	*Pcc*-BF	Billiri farm	MAC	Deep red	+
30	*Pcc*-BF	Billiri farm	NA	Round, convex, creamy-translucent	+
31	*Pcc*-BF	Billiri farm	MAC	Deep red	+

32	*Pcc*-BM	Billiri market	NA	Round, convex, creamy-translucent	+
33	*Pcc*-BM	Billiri market	MAC	Deep red	+
34	*Pcc*-BM	Billiri market	MAC	Deep red	+
35	*Pcc*-BM	Billiri market	NA	Round, convex, creamy-translucent	+
36	*Pcc*-BM	Billiri market	NA	Round, convex, creamy-translucent	+
37	*Pcc*-BM	Billiri market	MAC	Deep red	+
38	*Pcc*-BM	Billiri market	MAC	Deep red	+

*NA: Nutrient agar, MAC: MacConkey agar

Table 2: Biochemical characteristics of the selected bacterial isolates

Test	Isolates of *Pectobacterium carotovora* subsp. *carotovora* (*Pcc*)					
	Pcc-1	*Pcc*-2	*Pcc*-3	*Pcc*-4	*Pcc*-5	*Pcc*-6
Gram reaction	-	-	-	-	-	-
Oxidase	-	-	-	-	-	-
Catalase	+	+	+	+	+	+
Arginine dihydrolase	-	-	-	-	-	-
Growth at 37° C	+	+	+	+	+	+
Sensitivity to erythromycin	-	-	-	-	-	-
Indole production	-	-	-	-	-	-

Table 3: Effect of plant extracts, concentrations, and reactions of *Pectobacterium carotovora* subsp. *carotovora* isolates on the diameter of inhibition zones at 24, 48 and 72 hours after incubation

	Diameter of inhibition zone (mm)		
Treatment	24HAI	48HAI	72HAI†
Extract			
Allium sativum	11.37	14.01	14.83
Zingiber officinale	11.40	13.11	14.86
C. annum var. *chinense*	10.85	12.85	14.62
C. annum var. *cayenne*	12.82	14.43	16.32
Allium cepa	10.90	12.80	14.54
LSD $_{(0.05)}$	0.37	Ns	0.31
Concentration (%)			
25	9.00	10.75	12.70
50	11.30	13.45	15.63
75	14.38	18.03	19.67
SDW	5.00	5.00	5.00
Streptomycin (0.2%)	17.65	19.97	22.18
LSD $_{(0.05)}$	0.37	1.44	0.31
Isolate			
Pcc-1	12.84	14.31	15.52
Pcc-2	12.37	14.06	15.73
Pcc-3	11.12	12.60	14.21
Pcc-4	11.01	14.01	14.82
Pcc-5	11.07	13.25	15.35
Pcc-6	10.38	12.39	14.59
LSD $_{(0.05)}$	0.41	Ns	0.34

†HAI = Hour after incubation

DISCUSSION

The experiment evaluated the antibacterial activity of aqueous extracts of *Allium sativum, Zingiber officinale, C. annum* var. *chinense, C. annum* var. *cayenne* and *Allium cepa* at 25, 50 and 75% concentrations against *Pectobacterium carotovora* subsp. *carotovora,* a causal agent of potato soft rot disease.

The isolated bacterial isolates were identified as *Pectobacterium carotovora* subsp. *carotovora* based on their morphological and biochemical tests used in this study. The findings of this experiment were in agreement of Oliveira *et al.* (2003) and Yap *et al*. (2004) who reported that all *Pectobacterium carotovora* subsp. *carotovora* (previously, *Erwinia carotovora*) isolates formed pits on CVP medium. The catalase test performed on the isolates used was positive, which was similar to the findings of Himel *et al.* (2016), Rahman *et al.* (2012) and Schaad *et al*. (2001). Gram staining result was negative for all the isolates and was in consonance with the report of Oliveira *et al.* (2003). All the isolates used in this study grew at 37^{o} C. Oliveira *et al.* (2003), Rahman *et al.* (2012) and Khedr (2019) reported similar results. The results of sensitivity to erythromycin and indole production tests agreed with the work of Hyman (1995) and Schaad *et al*. (2001). Likewise, oxidase and Arginine dihydrolase tests revealed similar results to the findings of Schaad *et al*. (2001) and M'hamed *et al.* (2018), respectively.

According to the findings of Gwa and Nwankiti (2018), *Z. officinale* inhibits the growth of pathogenic organisms *in-vitro*. Also, in this study, plant extracts used hindered the growth of the test isolates. *Allium sativum* extract had antibacterial effect on the isolates and this confirmed the statement of Ahmed and Agnihotri (1990) that *Allium sativum* had broad-spectrum activity on bacterial organisms (Ahmed and Agnihotri, 1990). Antibacterial properties of *Capsicum* spp. used in this study was in consonance with the findings of Soetarno *et al.* (1997). They confirmed that the main antimicrobial compound in many *Capsicum* fruits was capsaicin. Antibacterial effect of plant extracts, used in this study, on *Pectobacterium carotovora* subsp. *carotovora* might be accountable to the presence of phytochemicals in the plant materials. This substantiated the report of Suman *et al.* (2014) that the presence of phytochemicals like alkaloids, safonins, tannins, flavonoids, cumonins, phenols in Coleus leaf extract was responsible for its antimicrobial activity (Suman *et al.*, 2014).

Six bacterial isolates (*Pcc*-1, *Pcc*-2, *Pcc*-3, *Pcc*-4, *Pcc*-5 and *Pcc*-6), were identified as *Pectobacterium carotovora* subsp. *carotovora* based on conventional morphological and biochemical analysis. Molecular analysis would be needed to draw a solid conclusion. Extracts of botanicals used in this study demonstrated promising potentials for the management of soft rot disease induced by *Erwinia* spp. on sweet potato tubers which could serve as substitutes for chemical pesticides. Application of these botanicals at 75% concentration would be able to reduce the destructive effect of infection caused by *Pectobacterium* spp. on sweet potato tubers. Evaluation of these extracts for the management of soft rot disease of potatoes *in-vivo* is recommended.

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