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Bioassay of some plant oil against bacterial rot pathogens

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Abstract: This experiment was designed to assay for inhibitory *in vitro* and *in vivo* potential of some indigenous and medicinal plants' essential oil against some plant pathogens associated with sweet potato soft rot. Plant protection plays an important role in agriculture for food quality and quantity to ensure food security for the teeming populace. The diagnosis and identification of phytodiseases are essential prerequisites for their understanding and control. Among the plant pests, bacterial pathogens have devastating effects on plant yield and productivity. Essential oils were extracted from four local botanicals viz: *Azardirachta indica*, *Zingiber officinale*, *Cymbopogon citratus* and *Camellia sinensis* to inhibit three species of bacteria namely: *Ralstonia solanacearum Erwinia carotovora* and *Flavobacteria* sp isolated from diseased potato tuber. The disease causing abilities of these bacteria were successfully confirmed (pathogenicity test). All the plant oils assayed inhibited the bacterial growth at varying degrees compared with control (sterile water) experiment. *In vitro* bioassay of *Z. officinale* oil was found as the most inhibitive on *E. carotovora* (19.30mm), while in the *in vivo* studies, *E. carotovora* (17.75mm) was mostly inhibited by *C. sinensis* oil. The potential of the plant essential oils to serve as prospective bio control agent against phytobacteria rot of sweet potato can be explored further by isolating and characterizing the antimicrobial constituents as active principles without using crude oil.
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Keyword: sweet potato, plant oil, bacterial rot pathogens

Introduction:

Ipomea batatas (Sweet potato) is a dicotyledonous plant in the family convovulaceae. Potato is one of the most important food crops worldwide and represents a valuable source of nutrients in a balanced diet Viswanath**,** *et al.,* (2018). In terms of human consumption, the potato is the third most important food crop in the world, following only rice and wheat (Czajkowski, 2011). It is the third most important root/tuber crop after cassava and yam within the sub-Saharan African region Agrios (2007: Bhat *et al.,* 2010). Scoll *et al.,* (2000) reported that more 2billion people in Asia, Africa and America depend on potatoes for food and as animal feed. In Senegal, annual yield of 33.3ton per hectare was reported (FAOSTAT, 2011). There is presently a considerable vanguard in promoting the production of orange fleshed sweet potato varieties as a source of beta carotene, a precursor of vitamin A (VITAA, 2005). Sweet potato can be processed as juice, cakes, chips and chapattis (Kenyon, *et al*., 2006). Bacterial soft rot portend great challenge to potato productivity, availability and suitability for consumption, Bacterial disease can be found on crops in the field, in transit and in storage or during marketing. Soft rot causes greater total loss of produce than any other bacterial disease causing severe losses varying between 15-30% of the harvested crop (Viswanath**,** *et al.,* 2018). Tomlins *et al*., (2000) reported that transportation of sacks of potato over rough roads exposes the tubers to many minor impacts resulting to injury. Mechanical damage during post harvest storage or transportation has been implicated in predisposition of tubers to storage rot. The most critical factor in tuber decay is the natural opening or wounds which serve as passage way for pathogenic contaminations (Udoh *et al*., 2001). Extracellular enzymes are produced in advance of the bacterial cell or fungal hyphae of the attacking pathogens. Onifade *et al.,* (2004) substantially reported post harvest rot of potatoes tuber. The affected part becomes hydrotic and soft, turns brown, emits putrid odour and exhibits a sharp demarcation between uninfected intact tissue and a disease tissue (Snowdon, 2010). A wide variety of microorganism particularly moulds, have been implicated in tuber spoilage, relatively few are implicated as primary pathogens (Onifade *et al.,* 2004). Microbial infections on storage tubers usually affect the yield, aesthetics quality, storage life, and nutritional values of the tubers. Infection may be pre harvest through cracks and this

develops in the storage tuber. Disease severity is affected by the prevalent conditions of the field leading to harvest and dry weather which favours skinning of the tubers during harvesting, leading to an increased in the disease incidence (Loenbeistein and Thottapilly, 2009). The most adequate way to prevent disease before packing is to reduce wounding on sweet potato after harvest and to sufficiently cure them (Nelson, 2005). Fresh wounds and high moisture content are required for tuber infection, making washing and packing more favourable for disease development. Control may be achieved by including a mild bleach solution in the wash tank (Agrios, 2006). Control of disease of sweet potato has been extensively employed and several measures such as farming practices such as crop rotation, fallowing, planting of healthy materials and rouging as have been recommended (Duku 2005). Okigbo and Nmeka (2005) reported that extracts of *X. aethiopica* and *Z. officinale* controlled post harvest rot of sweet potato. Torto *et al*., (2010) and Golokumah (2007) have independently reported the effects of storage techniques on the shelf life of sweet potato. Sweet potato tubers under tropical conditions exhibit short shelf life of 2-3 weeks and that was attributed to storage condition and poor post harvest handlings has been reported by Rees *et al*., (2001). Amusa *et al*., (2003) reported the effects of chemical pesticides for the control of sweet potato pathogens. However, the possible harm posed by pesticide usage calls for more suitable option in phytodisease control strategy. Antimicrobial effects of essential oils such as purified eugenol from plants against human disease have been reported (Nakamurah*, et al.,* 2000). Biodegradability, availability, low cost and environmental friendliness of biopesticides make them preferable to synthetic chemicals.

Materials and Methods

The test plants for this study: C. *citratus Z. officinale C. sinensis* and *Azardirachta indica* were sourced locally within the vegetation premises of Ekiti State University and the neighboring town. The sweet potato was sourced from a local market Akure, Ondo State. Bacho nutrient agar used for this research was prepared in tandem with the manufactures recommendation as 28g of the powdered agar was dispensed into 1 litre of distilled water in a flask, mixed and heated in water bath until the agar melted to form a homogenous mixture. The PH was measured and adjusted to standard for 7.2+ 0.2. The prepared media was sterilized in the autoclave at 121^0C for 15min. The sterile media was allowed to cool down to 45^0C before it was aseptically dispensed in 15ml aliquot into sterile Petri dish where it was finally allowed to cool and solidify room temperature before used for culturing bacterial isolates.

Preparation and extraction oil from plant samples

Each of the plant materials was washed very well in running water, drained and air dried at 30^0C room temperature until they were bristle enough for pulverization. The samples were pulverized to increase surface area in order to enhance oil extraction. Oil extraction method of Amadioah, (2002) was adopted for this finding. This method involved weighing 200g and soaked same in excess acetone. This was vigorously shaken, this was allowed to stand for 24hr, this was filtered using cheese cloth, and the filtrate was moved into oil extraction flask, connected to a Soxhlet extractor. The solvent (acetone) was systematically removed each time the Soxhlet reflexed filled up. This was continued until no more solvent was left in the flask. The oil extracted then transferred to a screw capped bottle and stored at 32^0C and his was used within 72 hr of extraction.

Bacterial isolation from diseased potato tubers

Collected samples of diseased potato tubers were surface sterilized with 0.1% sodium hypochlorite solution and the infected tissue was macerated in sterile water to make a bacterial suspension. A drop of resultant suspension was spread on Crystal violet pectate, a semi selective medium (CVP). The type of colonies which upon flooding with 1% hexadecyltrimethyl ammonium bromide(precipitant solution) formed halo zones around them on Crystal violet pectate medium (CVP) were selected for sub culturing on nutrient agar and were tested for pathogenicity.

Pathogenicity test using potato slice method

Potato tubers were first surface sterilized with sodium hypochlorite solution (0.5%) and cut into slices (1.0 cm in thickness) with sterile blade. These slices were inoculated by smearing a loop full of bacteria at the centre, on the surface of healthy tuber slice. The inoculated tuber slices were incubated for 24-48 h at 28 ± 2oC in Petri plates having sterile filter paper at the bottom of Petri plate soaked in 5ml of sterile water, kept in such a way that the tuber slices should not come in direct contact with the water by placing a glass slide at the bottom of the slice (Okigbo and Ikediugwu (2000)). Tuber slice inoculated with sterile water in one Petri plate was kept as control. Softening of the inoculated tuber slices was taken as a positive reaction. From the softened/macerated slice tissue, bacteria was re-isolated and compared with the original isolate of inoculated pathogen swabbing the bacterial suspension on them (Shashirekha *et al.,* 1987). In the second set of tubers, after giving the pinpricks, application of plant extracts was done simultaneously. In third case, the pinpricked tubers were first inoculated by the pathogen by swabbing bacterial suspension on them and 12

hours afterwards they were treated with plant extracts for 10 minutes. One set of potato tubers which were inoculated with only pathogen (no treatment) served as inoculated control. Other set of tubers inoculated and treated with antibiotic (streptomycin @150ppm) were kept as positive control. Five potato tubers constituted 1 replication and total of 5 replications were maintained in each treatment. The tubers were kept in sterile air tight plastic bags and were stored at 30±1°C. Observations on soft rot incidence and severity were recorded on 2nd, 4th and 6th day of incubation.

Incidence of soft rot disease = $\frac{\text{Number of tubers infected}}{\text{Total number of tubers assessed}} \times 100$

Severity of tuber rot

Severity of the disease was calculated using 0-5 scale Bdliya and Langerfeld (2005)

0= No symptoms of rot, **1=** 1-15% tuber rot, **2=** 16-30% tuber rot, **3=** 31-45% tuber rot, **4=** 46-60% tuber rot, **5=** ≥61% tuber rot

The severity was calculated using formula:

Tuber rot severity = $\Sigma nv \times 100 / N \times G$

Where,

Σ = Summation, v = Disease score, n = Number of tubers showing a particular score.

N = Number of tubers examined, G = Highest score.

Characterization of and identification of isolated pathogens

Identification of the bacterial isolates was based on examination of different characteristic with reference to existing taxa in a standard manual and he Bergeys manual of determinative bacteriology (Bdliya, 1995, Safrinet, 2000, Buchaman and Gibbrons, 2004, Bdliya and Haruna, 2007). Four levels of characterization were outlined as microscopic examination, colony features, biochemical test and biochemical reaction.

Effects of oil on bacterial pathogens

Inhibitory effect of each plant of oil against each pathogen was assayed on healthy *I. batatas*. Cores were made with the aid of flamed cork borer and bacterial inoculums was dipped inside oil before placing inside the created hole, the removed core was replaced at the point of inoculation, this was smeared with sterile petroleum jelly. Control was designed in which pathogen was inoculated into another potato tuber without plant oil. In order to observe for rot development, all the tubers were excised open through the locus of inoculation. the extent of decay was determine by measurement using transparent meter rule. The degree of growth inhibition of the pathogen was measured in respect to rot signs, the control (without oil). Antibiotic streptomycin (@ 150ppm) was used as the standard (Las llagas *et al.,* 2014). Diffusion technique of Cheesbrough, (2000) was engaged to evaluate the effects of botanical oil against bacterial pathogens in plates. Forty eight hours culture of each pathogenic isolate was introduced by spread plate method. Five (5 mm) circular paper discs were punched out from a filter paper; the discs were made sterile using 70% ethanol and these were oven dried. More discs were placed in the plant oil and these were left to absorb the oil. The oil bearing discs were placed in triplicates on the inoculated plate using flamed pair of forceps, maintaining 4mm spacing between discs, these were compressed carefully, and allowed to stay for 5min prior to incubation. For 48hr after which examination for growth development and inhibition were made. This was carried out for each isolate to notice the clear zone of inhibition around the oil disc. A check experiment was done by following protocols explained above using standard industrial antibiotic multidisc. Control was set up using different plate inoculated with the bacterial isolate placed with the disc immersed in sterile distilled water in lieu of oil. Data were obtained by determining the diameter of zone of inhibition (zone clearing). Percentage (%) growth inhibition was determined using the formula of Amadioha (2004) as:

Inhibition %= DC-DT x100

Where:

DC=colony diameter of control, DT= colony diameter of treated tubers

Results

The result of pathogenicity test carried out showed that the bacterial isolates caused tuber rot at room temperature. Soft rot signs commenced after small water soaked necrosis. The inoculated area became soft and watery as the surface became depressed and discoloured, tissue within the area got slimy (Walker, 2004). Ephinesstone and John, (2010) reported characteristic black border that separated the disease area from healthy tissue. The occurrence/incidence and pathogenicity of the isolates from both healthy and infected sweet potato tuber: each of bacterial isolate was inoculated into disease free potato tubers, these were observed for soft rot sign within two weeks, and all the bacterial isolates induced rot with varied degree of rot initiation. *R. solanacearum* and *E. carotovora*, were mostly distributed with 80% each while Flavobacterial was 60%. The result shows huge relative occurrence of *E. carotovora* on sweet potato tuber which can perhaps be ascribed to availability of this bacterium in the soil as part of soil micro flora.

Identification and characterization of bacterial pathogen

Characterization was carried out on oxidase and nitrate (biochemical reaction), microscopic test (gram stain, spore nature, flagella type and motility). Identification of the phytobacterial isolates was authenticated according to Buchaman and Gibbons (2004). *E. carotovora* was found to be rod shaped creamy white as described by Gupta and Thind, (2006), glistering colony, a gram negative and after staining smear culture with a drop of hydrogen peroxide (H202).

Table 1: shows the distinct biochemical features of the bacterial isolates

Test/features	*R. solanacearum*	*E. carotovora*	Flavobacteria
Results of microscopic investigation: spore gram stain motility flagella nitrate oxidase Colony nature	-ve -ve -ve -ve +ve +ve regular and slimy ring green colour colonies	-ve -ve -ve -ve +ve +ve creamy white, faintly glistering and raised, rod shape	-ve -ve -ve -ve +ve +ve yellow tints, ring colonies plus smooth surface

key: +ve =positive response, -ve= negative response

The result of *in vivo* sensitivity (inhibition) and percentage growth reduction of the bacterial isolates of 20% concentration of *C. sinensis* oil was most inhibitive against *E. carotovora* (17.75) *R. solanacearum* (12.75) and *Flavobacteria* (14.00) (Table 2).

Table 2: shows the *in vivo* test and the inhibition of the plant oil on different bacterial pathogens in potato tuber

	E. carotovora	*R. solanacearum*	Flavobacteria
Antibiotic (@ 150ppm)	7.55	5.00	5.50
Sterile distilled H20 (control)	30.00	22.75	24.00
Plant oils:			
C. sinensis	17.75	12.75	14.00
A. indica,	14.00	10.75	13.00
Z. officinale	14.75	10.75	12.25
C. citratus	11.75	9.75	10.25
LSD($p \leq 0.005$)	3.215	1.540	2.386

Various *in vitro* inhibitory effects of plant oil exhibiting varying levels of growth reduction against the bacterial pathogens. *Z. officinale* inhibited *E. carotovora* (19.30mm) most followed by the inhibitory effect of oil of *A. indica* on *E. carotovora* (16.30mm) while the antibiotic effect against *A. carotovora* was 28.68mm. *R. solanacearum* (25.68mm) and *Flavobacteria* (20.68mm) was found to be more active against *A. indica* while the effect of antibiotic was 20.68 and 25.68 against *Flavobacteria* and *R. solanacearum* respectively. There were differences in the growth reduction values of plant oils and antibiotic (streptomycin) at in vitro investigation (Table 3).

Table 3: shows the *in vitro* inhibitory effects of various plant oils against bacterial pathogens isolated from rotten potato tuber.

	E. carotovora	*R. solanacearum*	Flavobacteria
Antibiotic(@ 150ppm)	28.68	25.68	20.68
Sterile distilled H_20 (control)	0.00	0.00	0.00
Plant oils:			
C. sinensis	11.68	13.75	9.38
A indica,	16.30	23.68	12.90
Z. officinale	19.30	14.30	9.25
C. citratus	12.58	12.25	8.80
LSD($p \leq 0.005$)	1. 076	1.435	8.677

Discussion

Essential oils were extracted from four locally sourced botanicals viz: *C. sinensis*, *A. indica*, *Z. officinale* and *C. citratus* against three species of isolated bacteria from rotten potato tuber viz: *R. solanacearum E. carotovora* and *Flavobacteria* sp. The pathogenicity of the isolated bacteria from diseased potato tuber was authenticated. Different techniques (microscopy, serology, biochemical, physiological, molecular tools and culture propagation) are currently used to detect and identify bacterial pathogens. Detection and identification are critical steps for the appropriate application of phytosanitary measures. The "harmonization of phytosanitary regulations and all other areas of official plant protection action" mean the good practices for plant protection and plant material certification. The prevention of diseases progression and spread by early detection are a valuable strategy for proper pest management and disease control. For this purpose, innovative methods aim achieving results within a shorter time and higher performance, to provide rapidly, accurately and reliably diagnosis. In this review, we focus on the techniques for plant bacterial diagnosis and on the regulations for harmonizing plant protection issue. Phytopathogenic bacteria might survive in diverse environments: in plants as pathogens and outside their hosts as saprophytes and epiphytes. Adverse environmental conditions might reduce bacterial survival and compromise disease initiation and dissemination. On the contrary, the infection cycle might occur when the condition become favorable. Rainfall, contaminated farming equipment and/or plant material and insects promoted the dissemination of phytobacteria. Disease symptoms caused by bacteria include leaf spots, blights, wilts, scabs, cankers, tumors and soft rots of roots, storage organs and fruit and overgrowth. The protection of natural and managed plant systems from alien and emerging indigenous pests is a strategic socio-economic issue Smith, (2013). Globalization, climate change and landscape modification facilitated the dissemination and introduction of alien pests, the evolution of new races, biotypes and strains of indigenous pests; this dramatic change of scenario causes emerging diseases everywhere. Plant productivity, sustainability and biodiversity are compromised by pathogens Agrios, (2005). All the bacterial isolated from spoilt potato tuber caused bio-deterioration on the tuber with varied degree of rot. It was also noticed that *R. solanacearum* and *E. carotovora* in the test sample have been reported as potato bacteria soft rot pathogens by Agrios (2007). Olivier *et al*, (2004) and Mahmoud *et al*., (2008) reported *E. carotovra* bacterial rot pathogen of potato tuber.

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