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RESEARCH ARTICLE

Effect of Selected Chicken Manure-Charged Biochar on Growth of *Ralstonia Solanacearum in Vitro*

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ABSTRACT

A study was conducted *in vitro* to assess the antibacterial effect of specific chicken manure-charged biochar extracts against *Ralstonia solanacearum* at the microbiology laboratory of Egerton University. The study tested charged biochar extracts from various feed stocks: maize cobs (MC), maize straw (MS), rice husks (RH), bean wastes (BW), and eucalyptus branches (EU). The extracts consisted of plain biochar and charged biochar with poultry manure. The physico-chemical properties of biochar were assessed according to established standard procedures. The antibacterial efficacy of charged biochar extract was evaluated by mixing nutrient agar (NA) in petri dishes with 0.5 ml of the extracts prior to solidification, followed by the spread of 0.1 ml of bacterial suspension on each plate. Sterile distilled water served as a negative control, while copper oxychloride was utilized as a positive control. The plates were incubated at 28°C for 48 hours in growth chamber. The experiment was arranged in a completely randomized design (CRD) with three replicates. Charged biochar exhibited higher nitrogen content than that of plain, with bean waste charged biochar having the highest at 1.5% and plain eucalyptus biochar lowest at 0.4%. For available phosphorus, maize cob charged biochar exhibited the highest with 1846 mg/kg while the lowest was plain rice husk biochar at 671.5 mg/kg. Organic carbon at 74.2% for maize cob charged biochar was the highest and EUPB exhibited the lowest 23.5%. Porosity was high in plain biochar as compared to charged, while charged biochar exhibited higher density than plain with the lowest 0.6 for MCPB and highest 1.0 for rice husk plain biochar. The antibacterial assay indicated that all biochar extracts significantly suppressed the growth of *R. solanacearum* compared to the negative control. Charged eucalyptus biochar reduced pathogen growth by 100% which was the highest inhibition while plain maize straw was lowest at 1.19%. These results proved that charging biochar, especially that derived from bean wastes and eucalyptus, with chicken manure not only increases the nutrient content but as well enhances its potential of controlling bacterial wilt.

Keywords: Charged biochar extracts, Chicken manure, *Ralstonia solanacearum*.

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1. Introduction

Ralstonia solanacearum, a soil-borne pathogen responsible for bacterial wilt in numerous species, is one of the soil-borne diseases that contributes to significant yield losses in vital plants globally [1]. It is an aerobic, gram-negative, motile bacterium characterized by a tuft of polar flagella, non-spore-forming, rod-shaped, and classified within the β-proteobacteria group, presenting a significant threat to the cultivation of many crop plants worldwide [2]. The pathogen is classified as a complex species comprising three species, five races (due to its ability to harm various crop species), and six biovars, as it may oxidize hexose, alcohol, sorbitol, and disaccharides. It comprises four phylotypes based on the geographical origin of the strains: phylotypes I from Asia, II America, III Africa, and IV Indonesia [3].

This pathogen is placed second among plant pathogenic bacteria in molecular plant pathology due to its global

scientific and commercial significance [4]. The Ralstonia population can attain 10^3 cfu/g–10^6 cfu/g in soil and plant tissue during peak infection [5]. The pathogen can cause yield losses in different crops; 91%, 90%, 30%, and 100% in tomato, potato, tobacco, and banana, respectively [6]. The pathogen's capacity to infect diverse plant families and its resilience in various environments including soil and water, results in significant diseases affecting agricultural crops globally, rendering various management and control methods less effective [7]. Numerous studies have established techniques for controlling the pathogen including biological, cultural, physical, chemical, and integrated biocontrol strategies [9]. Crop rotation and intercropping reduced the disease severity but cannot terminate it from the soil due the pathogen's ability to infect a wide range of hosts in the Solanaceae family and remain in soil for long time without a host [8]–[10]. Soil solarization has been documented as an effective method for controlling bacterial wilt, but its effectiveness is short lived, preventing its use throughout the year [11].

The application of organic amendments as a sustainable alternative for mitigating soil-borne diseases is significant due to its environmental sustainability [12]. Organic additives are recognized to enhance microbial activity and increasing populations of particular microorganisms or groups of microorganisms to prevent disease invasion [13]. Soil supplements are frequently used in agriculture to raise or stabilize soil pH and are thought to positively influence soil health and plant growth [14]. Biochar is one of the soil additives applied to mitigate soil-borne diseases by improving soil and crop health. Different studies have been conducted to investigate the effect of biochar from different feed stock in management of bacterial wilt and other significant soil borne diseases in different crops [15]. A study conducted in China under greenhouse conditions revealed that the application of wheat straw and peanut shell biochar decreased the occurrence and severity of tomato bacterial wilt at 65.7% and 28.6% respectively and promoted the growth of tomatoes as well as the yield [16].

Rice husk biochar had performed well in management of root rot disease caused by *Fusarium solani* in ginseng (a perennial medicinal plant belonging to the Araliaceous family) by promoting the growth of beneficial organisms and suppressing the pathogenic fungi hence reduced the disease [25]. Bean waste biochar reduced the incidence and severity of bacterial wilt in tomatoes at 13% when applied as soil amendment in sandy soil [17]. Field trials conducted in North Central Nigeria has shown that Eucalyptus biochar mixed with saw dust performed well in management of parasitic nematodes associated with Beniseed (*sesamum indicum*) where it has reduced the population of nematodes at 29.7% and improved yield of Beniseed [18].

The decomposition of organic waste in biochar by microbes, releases inhibitory chemicals into the soil limiting the resources accessible to pathogens and thereby affecting their resilience [19], [20]. Biochar enhances soil structure and improves nutrient retention. The increment of nutrient availability to plants results in healthier crops that exhibit improved disease resistance [21]. The incorporation of these renewable resources is consistent with the ideas of the bio-circular economy, fostering sustainable agriculture practices and environmental conservation [22].The act of charging biochar with chicken manure is to enhance its properties for agricultural application. Chicken manure is known for its capacity to reduce soil borne diseases by increasing the population of beneficial micro-organisms like: Bacillus, Pseudomonas, Streptomyces, Chaetomium, and Mycothermus that help in controlling soil-borne diseases. Chicken manure can prevent soil borne pathogens when used as soil fumigant and this is ecofriendly compared to chemical fumigants [23]. Chicken manure can increase the organic matter content of the soil and also increase microbial activities by improving their respiration and soil conditions [24].

2. Materials and Methods

2.1. Preparation of Biochar and its Extracts

Biochar was produced in the Engineering Department of Egerton University using a modified kiln. The raw materials used were sourced from various places; Bean waste from KALRO Njoro, Eucalyptus (*E. saligna*), maize cobs and maize straw were gathered from Field 7, Egerton University. Materials were heated in a kiln at a temperature of 400°C in a pyrolysis process for two hours for bean waste and maize straw and four hours for maize cobs and eucalyptus branches. Rice husk biochar was sourced from Mwea county, Kenya supplied by Safi Organics Company Ltd while chicken manure was obtained from a commercial battery cage poultry farm in Njoro.

Biochar was activated by mixing with chicken droppings in a 1:1 ratio and watering it consistently for four weeks before use to promote nutrient transfer from the manure to the biochar [25]. A charged biochar extract was made by grinding the biochar to fine powder then mixed with sterile distilled water in a ratio of 1:4. The mixture was shaken for 30 minutes with a magnetic stirrer, left to settle for 72 hours, and subsequently filtered using Whatman filter paper of 125 mm to remove solid residues and obtain extracts [26]. The extracts were sterilized using autoclave to avoid any contamination that can be caused by microorganisms from the charged biochar. Afterwards, biochar extracts were stored in sealed glass bottles in refrigerator.

2.2. Physical Properties

Density and porosity were determined by applying the formulae described by Downie *et al*. [27] and [28].

2.3. Chemical Properties

The following properties were analyzed and calculated using the respective methods and protocols: Total Nitrogen (TN) by Kjeldahl Digestion Method, Available phosphorus (AP) using Mehlich III method, percentage organic carbon (% OC) by Wet digestion method, pH determined by electrometric method and measured by pH meter with buffers of pH 7 and pH 4 and cation exchange capacity (CEC) was determined by Ammonium Acetate Exchange method [29], [30].

2.4. *Ralstonia Solanacearum Isolation and Identification*

Potato (Shangi variety) showing bacterial wilt symptoms was collected from field seven Egerton University research farm, washed thoroughly in tap water for 10–15 minutes to remove adhering soil debris then the branches removed leaving only the stem and main roots. It was surface sterilized using 4% sodium hypochlorite followed by dipping it into 70% ethanol for 30 seconds [31]. Using sterile scalpel, it was cut into small pieces (15 cm) then submerged into sterile distilled water for 30 minutes under laminar flow (hood), to allow the bacterial ooze from the cut end. The inoculation loop was streaked on nutrient agar media after being dipped in the ooze. The streaked plates were incubated in growth chamber for 48 hours at 30°C to obtain the bacterial culture [32]. single spore technique was done from the 48 hours old bacteria to obtain pure culture. Pathogen was identified by looking at size, colour, shape and gram staining reaction was performed and further Polymarase chain reaction (PCR) molecular identification was done.

2.5. *DNA Extraction*

DNA was extracted by organic extraction method (phenol-chloroform extraction) [33].Bacterial cells were harvested by centrifugation of 24 hours old bacteria at 10000 rpm for five minutes at room temperature. 1 ml of bacterial suspension was mixed with 30 µl of Sodium dodecyl sulfate(SDS) and 3 µl of proteinase k incubate for one hour at 37°C. 100 µl of 5M Sodium chloride (Nacl) and mixed. 80 µl of cetyl trim ethyl ammonium bromide (CTAB) was added, mixed and incubated for 10 minutes at 65°C. 80 µl of chloroform lisomyl was added, spun for 10 minutes at 14000 rpm at room temperature. Viscous aqueous supernatant was carefully collected into fresh test tube. 80 µl of Isopropanol was added to precipitate the DNA, centrifuged at 14000 rpm at room temperature for 10 minutes then mixed from bottom to top and spun for 10 minutes at 14000 rpm at room temperature. DNA precipitate was washed with 500 ml of 70% ethanol and centrifuged for 10 minutes at 14000rpm at room temperature. The supernatant was carefully removed with tip to remain with DNA pellet. The DNA pellet was re-dissolved in Tris-EDTA(TE) buffer and stored at 4°C for the next day. Agarose Gel Electrophoresis was done to see the DNA fragment from the bacterial sample [34].

1000 bp DNA ladder was used to confirm the isolate by Polymarase chain reaction (PCR) technique. The pair of species-specific primers used are as shown below:

760F 5'GTC GCC GTC AGC AAT GCG GAA TCG -3'

759R 5'-GTC GCC GTC AAC TCA CTT TCC-3'

2.6. *Inhibitory Effect of Selected Chicken Manure Charged Biochar on Ralstonia Solanacearum in Vitro*

Experiment was conducted at Egerton University microbiology laboratory. Bacterial suspension was prepared by pouring sterile distilled water over the stored 48 hour old bacterial growths on nutrient agar [35]. The effects of charged biochar extracts was determined by mixing 0.5 mls of sterile biochar extract with the media (nutrient agar) on plates before the media solidified. Bacterial suspension of 0.1 mls with a concentration of 6×10^5 Cfu/ml adjusted by the hemocytometer under light microscope was spread on each plate and incubated for 72 hours at 28°C to allow bacterial growth. The experiment was laid out in completely randomized design (CRD) with three replications.

2.7. *Collection and Analyses*

Data on bacterial growth was collected after 72 hours of incubation; colonies were counted on each plate (solid media) using a colony counter. The colony counts from each plate were analyzed using analysis of variance (ANOVA) with R software version 4.4.3 to assess the inhibitory effect of charged biochar extracts on *Ralstonia solanacearum*. The means of treatments were separated with Least Significant Difference (LSD) test at a significance level of 0.05.

3. Results

3.1. *Physical and Chemical Properties of Biochar*

3.1.1. *Physical Properties*

Plain biochar exhibited greater porosity than charged biochar with plain maize cob highest at 76.50% and charged rice husks lowest at 70.50%. The density of plain maize cobs was lowest 0.6 g/ml while most charged biochar had densities of 1 g/ml (Table I).

3.1.2. *Chemical Properties of Selected Charged Biochar*

Chemical analyses revealed that percent organic carbon (% OC) content in all charged biochar was nearly double that of plain biochar with charged maize cob biochar highest at 74.2% and plain eucalyptus biochar lowest at 23.5%. Charged biochar exhibited elevated levels of available phosphorus (AP) with the highest for charged maize cobs at 1846 mg/kg and plain rice husk with lowest at 671.5 mg/kg. For total nitrogen (TN) the highest content was seen in charged bean waste biochar with 1.51% while the lowest was 0.4% for plain eucalyptus biochar, for cation exchange capacity (CEC), charged maize straw biochar exhibited the highest value of 68.47 meq/100 g while maize cobs with 24.47 meq/100 g is the lowest. All selected biochar exhibited alkaline properties with the highest pH

TABLE I: Physical Properties of Biochar

Treatments	Porosity (%)	Density (g/cm^3)
BW PB	72.7b	0.8b
BW CB	70.5c	1.0a
EU PB	70.5c	1.0a
EU CB	70.5c	1.0a
MC PB	76.9a	0.6c
MC CB	72.7b	0.8b
MS PB	72.7b	0.8b
MS CB	70.5c	1.0a
RH PB	72.7b	0.8b
RH CB	70.5c	1.0a

Note: BW: Bean waste, EU: Eucalyptus, MC: Maize cobs, MS: Maize straw, RH: Rice husk, PB: plain Biochar, and CB: Charged biochar. Means followed by the same letter in the same column are not significant at $p \leq 0.05$.

TABLE II: Chemical Properties of Selected Charged Biochar

Treatments	pH	%OC	AP (mg/kg)	TN (%)	CEC in meq/100 gg
BW PB	10.2b	51.8d	825.0g	0.9d	41.3e
BW CB	10.3a	70.4b	1815.8b	1.5a	44.3d
EU PB	9.6e	23.5h	1053.0	0.4g	48.3c
EU CB	9.7d	71.1b	1587.3d	1.3b	54.3b
MC PB	10.2b	44.3e	714.5h	0.9d	24.5i
MC CB	10.2b	74.2a	1846.0a	1.2c	40.6f
MS PB	9.9c	41.4f	1352.3e	0.5	54.2b
MS CB	10.4a	70.0b	1765.2c	1.2c	68.4a
RH PB	5.4g	31.3g	671.5i	0.5f	29.8h
RH CB	7.2f	60.3c	864.5f	0.6e	36.1g

Note. BW: Bean waste, EU: Eucalyptus, MC: Maize cobs, MS: Maize straw, RH: Rice husk, PB: plain Biochar, and CB: Charged biochar. Means followed by the same letter in the same column are not significant at $p \leq 0.05$.

TABLE III: Anti-Bacterial Assay of Selected Charged Biochar on R. Solanacearum In Vitro

Treatment	Dist. water	MS PB	MS CB	MC PB	MC CB	RH PB	BW PB	RH CB	DAP	BW CB	COPER OXY	EU BP	EUCB
Colony count	168^{a}	164^{b}	133^{c}	90^{d}	71^{e}	63^{f}	49^{g}	21^{h}	18^{i}	3^{j}	0^{k}	0^{k}	0^{k}
% inhibit^{-o}	0^{k}	1.2^{j}	81^{e}	45^{h}	57^{g}	12^{i}	70^{f}	87^{d}	92^{c}	98^{b}	100^{a}	100^{a}	100^{a}

Note: Means followed by the same letter in the same row are not significant at $p \leq 0.05$.

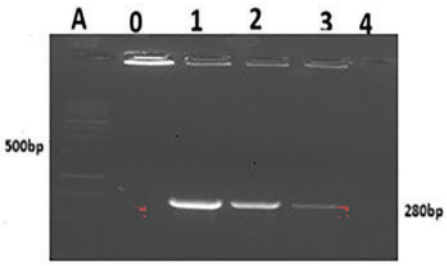

Fig. 1. Amplified 280 bp DNA fragments from *R. solanacearum* with specie -specific primers. A = 1000 bp DNA ladder, 0 = Positive control, 1,2,3 = the replicates of the isolates, 4 = Negative control.

values of 10.35 for charged maize straw biochar and the lowest of 5.3 for plain rice husk biochar (Table II).

3.1.3. Identification and Confirmation of R. solanacearum

The pathogen appeared white creamy on plate with NA media, small size 0.6 µm in width and 1.8 µm length, rod-shaped and appeared pink under microscope after gram staining which confirmed the bacteria to be gram negative [6], [36], [37]. Further confirmation was done and PCR showed the positive result for *R. solanacearum* as presented and shown in Fig. 2.

3.1.4. Anti-Bacterial Assay of Selected Charged Biochar on R. Solanacearum in Vitro

All the treatments (both charged and plain biochar) significantly inhibited *R. solanacearum in vitro* compared to distilled water used as negative control whereas eucalyptus registered 100% inhibition of bacterial growth same as copper oxychloride used as a positive control as shown in Fig. 2 and Table III).

4. Discussion

The physical and chemical features of the selected chicken manure-charged biochar indicate its high quality, consistent with findings by Razia *et al*. [38], who revealed that biochar from various feed stocks is alkaline and nutrient-rich. The inhibitory effects exhibited by the tested biochar extracts are supported by numerous studies on biochar's efficacy in managing crop diseases, despite the absence of study specifically on biochar extracts. Oni *et al*. [19] stated that biochar and vinegar produced by pyrolysis contain phenolic compounds and organic acids, which are also found in biochar extracts and are recognized for their antibacterial properties, damaging the cell membranes of pathogens. The low porosity and high density in charged biochar compared to plain biochar is due to the filling of biochar pores with organic and inorganic compounds introduced by chicken manure used to charge biochar. The higher pH in biochar extracts is the primary characteristic that generates adverse conditions for pathogens, hence resulting in their suppression [39]. The findings related to the selected biochar extracts in the study are consistent with those of prior research. Eucalyptus which exhibited 100% inhibition of pathogen growth is due to its antibacterial properties, attributed to its abundance of essential oils and terpenes that effectively suppress bacterial proliferation. Additionally, tannins and polyphenols present in the biochar extracts possess potent antibacterial characteristics, disrupting microbial protein synthesis as confirmed by Oni *et al*. [19].

Volatile Organic Compounds (VOCs) included in Eucalyptus-derived biochar exhibit inhibitory effects on pathogens due to their volatility and capacity to disrupt cellular processes. The reported findings of bean waste inhibiting the growth of *R. solanacearum* are attributed to the alkaloids present in bean waste biochar, recognized for their antibacterial and antifungal effects. Previous research demonstrated that phenolic compounds, organic acids, and flavonoids found in maize cobs, maize straw, and rice husks contributed to their antibacterial efficacy [20], [40].

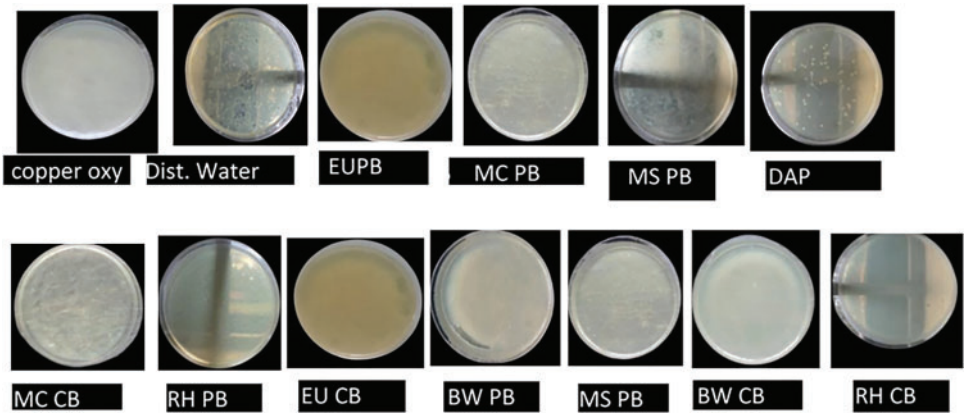

Fig. 2. Effects of selected charged biochar on growth of *R. Solanacearum in vitro*.

5. Conclusion

The study's findings indicated that all examined biochar extracts inhibited the development and proliferation of *Ralstonia solanacearum* in vitro, attributable to their alkaline properties and chemical makeup. The complete inhibition of bacterial growth by eucalyptus was due to its high concentration of essential oils, tannins, polyphenols, and organic volatile chemicals. The alkaloids in bean waste biochar allowed it to limit pathogen development by 98%. It is advised that farmers recycle their crop residue after harvest in order to make biochar, which can be utilized not only for crop production but for crop protection as well. Research on foliar spray of biochar extracts in management of bacterial wilt can be done in the future.

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Conflict of Interest

There are no conflicts of interest either personal, professional or financial.

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