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Biocidal potential of Three Plant Extracts on *Rhizopus stolonifer*, Causal Organism of Irish Potato (*Solanum tuberosum* L.) Tuber Rot

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Abstract:- Research study was carried out to assess the biocidal effect of aqueous extracts of *Curcuma longa, Zingiber officinale, Citrus limon* peel and synthetic fungicide Mancozeb against *Rhizopus stolonifer* using the poisoned food technique on PDA. Various concentrations (50, 75, and 100%) of extracts from the rhizomes of *C. longa, Z. officinale,* the peel of *C. limon* and Mancozeb (0.002%) significantly inhibited the mycelia growth of *R. stolonifer* after 3 days. Effects of the synthetic fungicide (Mancozeb) comparative to the plant extracts were also determined. Although the extracts showed varying degrees of antifungal efficacy, 100% concentration of Z. *officinale* (58.96%) proved to be more potent against *R. stolonifer* than the other plant extracts but was lower and significantly different when compared with Mancozeb (73.31%) at (*P≤0.05*) after 3 days. Extracts of *C. longa* and *C. limon* peel showed a lower inhibition level ranging from 45.01% to 56.98% and 9.57% to 18.73% respectively and were significantly different when compared with Mancozeb at (P≤0.05). Inhibition of fungal growth increased with a corresponding increase in extract concentration and days. The plant leaf extracts effectively inhibited the mycelial growth of pathogen *in vitro* after 3 days. *In vivo* study was carried out using spore suspensions of *R. stolonifer*. Fresh, healthy and surface sterilized Irish potato tubers were inoculated with 6.4 x 10^4 spores/ml and treated with aqueous extracts of *C. longa, Z. officinale* and *C. limon* peel after 24 hours. The result showed that all plant extracts had significant effect on disease severity in tubers inoculated with *R. stolonifer.* However, 100% concentration of *Z. officinale* gave the best rot reduction caused by *R. stolonifer* with severity score of 0.33 but it was not significantly different at (p<0.05) from mancozeb which had a severity score of 0.67. However, they were significantly different at (p<0.05) from the inoculated control (3.33). There were variations in weight loss but no significant difference was observed among the various treatment methods adopted.

Keywords:- *Biocides, plant extracts, Rhizopus stolonifer, Irish potato Tuber Rot.*

I. INTRODUCTION

Irish Potato (*Solanum tuberosum* L.*)* is an annual swollen underground stem tuber crop belonging to the family Solanaceae. It is an important tuber crop ranked fourth most important crop in the world (Ugonna *et al.*, 2013; FAO, 2014). Since introduction, the production of potato has increased by over 120% in the past decade in Nigeria and it is still grossly below demand (FAO, 2014). Despite Nigeria being the highest producer of potato in Africa and second highest in the world, Nigeria is not an exporter of potato (ITC, 2016). This has been attributed to various constraints. According to Ugonna *et al.* (2013), some constraints which limit Irish potato production, processing and marketing in Nigeria include Poor diseases and pests' management, inadequate storage facilities and inadequate supply of good quality seeds

Chukwu *et al.*, (2008) stated that the availability of tuber crops has been limited because of the unavailability of good storage facilities and techniques. High percentage losses have been reported due to the in-effectiveness of traditional storage techniques such as pit storage and barn-heaping of the tubers. Losses between 40-60% have been attributed to poor storage methods and pathogens like *Mucor racemosus., Alterneria alternata., Rhizopus stolonifer, Phythophthora infestans, Pythium myriothylium, Botryodiplodia theobromae, Fusarium solani and Fusarium oxysporum* (Chuku *et al., 2005;* Ubalua and Chukwu, 2008). The effect of these losses can be translated to mean a decrease in revenue and threat to reliance on the crop as a major staple carbohydrate food crop in this present economic situation.

The use of chemicals have aided in the management of diseases but numerous problems such as, non-biodegradation, high cost, chemical residue retention in the plant produce, development of resistance in target organism, phytotoxicity, hazard to man and his environment and sometimes non-availability have rendered chemicals either difficult to adopt or farmers to have rejected them, in addition to various cultural and religious reasons (Abimbola *et al.*, 1993; Okigbo and Odurukwe, 2009). Hence, there is a pressing need to find alternatives to these existing methods with lesser or no side effects, readily available and less expensive (Khulbe and Sati, 2009).

In many parts of the world, scientists have discovered the many possibilities in using plants and their extracts for treating plant diseases (Sofowora, 1984; Okigbo and Igwe, 2007). These higher plants have shown amazing potency as sources for new drugs although it is still largely under utilized. (Ahmedulla and Nayar, 1994). These plants derivatives are being used in several forms such as; liquid, powder, mixtures, spice, ointments, etc., (Apata, 1979; Wee yeow Chin, 1992). Plant-based products are generally affordable, readily available, non-phytotoxic and easily biodegradable. Moreover, they are ecofriendly and stand as alternative to chemical fungicides as reported by different scholars (Akhilesh *et al.,* 2012; Okigbo and Omodamiro, 2006 and Okigbo and Igwe 2007). This study was aimed at evaluating the biocidal potentials of aqueous extracts of *Curcuma longa*, *Zingiber officinale*, *Citrus limon* peel against *Rhizopus stolonifer*, the causal organism of Irish potato tuber rot.

- *Sample Collection*

Irish potato *(Solanum tuberosum)* tubers with rot symptoms were procured from Rumuokoro market in Obio -Akpor Local Government Area, Rivers State, South-South Nigeria. Fresh and apparently healthy tubers were procured from the same market. Rhizomes of Ginger (*Zingiber officinale),* Turmeric *(Curcuma longa)* and fruits of Lemon (*Citrus limon)* were procured from the Fruit Garden Market in D/line area of Port Harcourt, Rivers State. Synthetic fungicide (Mancozeb) was obtained from Agricultural Development Programme unit (Rivers ADP) of the Rivers State Government.

- *Isolation of Fungal Pathogens from Irish Potato Tubers*

Infected potato tubers were surface sterilized in 5% Sodium hypochlorite and then rinsed thrice in sterile distilled water (SDW) according to the method of (Ritchie, 1991). Approximately 2mm cubes were cut from the tissue at the junction between healthy and infected portion of potato tubers using a sterilized scalpel. These sections were inoculated to Petri dishes containing sterilized Potato Dextrose Agar (PDA) and incubated at 28±2ºC for 7 days and then examined for the development of fungi growth.

- *Subculturing, Purification and Identification of Test Fungi Pathogens.*

When growth was established, subcultures were prepared using inocula from the different fungi in the mixed cultures in order to obtain pure culture. This was done by transferring the hyphal tips from the colony edge of the mixed cultures to fresh plates of Potato Dextrose Agar using flame sterilized inoculating needle. After sub-culturing, the plates were incubated at 28 ± 2^{0}C for 7 days. The process was repeatedly done until pure cultures were obtained. The resulting pure cultures were used for characterization and subsequent identification of the fungal isolates based on their characteristics, using identification guides by (Barnett and Hunter, 2008). Stock cultures were maintained on agar slants in McCartney bottles and stored at 4^{0}C in the refrigerator.

- *Pathogenicity Test*

Pathogenicity test was carried out according to the methods described by Chuku *et al,* (2005) and Nwachukwu and Osuji (2008). A mycelia disk (5mm) of the test fungus (*Rhizopus stolonifer*) from pure culture was cultured for 14 days in a sterilized Potato Dextrose Agar (PDA) broth containing 1g of PDA mixed in 140ml Sterile Distilled Water. The culture was filtered using No 1 Whatman filter paper and transferred into a 50ml distilled water containing 10% glucose.

The mixture was properly agitated and sprayed on fresh surfaced sterile uninfected potato tubers. The tubers were kept at room temperature (28±2°C) for 14 days to examine for symptoms of rot and thus prove that the test fungi were able to cause infection.

- *Preparation of Stock Solution of the Plant Extracts*

15 grams of each plant powder was mixed in 100ml of Sterile Distilled Water, stirred vigorously and kept for 24 hours in other to obtain a stock solution. The extracts was decanted, filtered through a No. 1 Whatman filter paper and stored in a refrigerator at 4^{0}C and used within 48 hours. The stock solution of the plant extracts were diluted to 50%, 75% and 100% concentration respectively. 50% was got by diluting 50ml of the stock solution to 50ml of distilled water; 75% was obtained by diluting 75ml of stock solution in 25ml of distilled water; whilst 100% was 100ml of stock solution only and was used in the *in vitro* experiment.

- *In vitro Bioassay: Effect of Plant Extracts and Mancozeb on Diametric growth of the Pathogen-Rhizopus stolonifer*

Thirty nine (39g) of PDA powder was dispensed into 1000ml of sterile distilled water in a conical flask and the mouth was covered with non-absorbent cotton wool and aluminum foil. This was autoclaved at a temperature of 121°C and pressure of 15atm for 15 minutes. About 2ml of the different concentration of the plant extracts was mixed with 10 ml PDA and dispensed into the Petri dish, the medium was uniformly mixed and allowed to cool and solidify. For positive control, 2ml of 0.002% of fungicide (Mancozeb), obtained by diluting 2gms of fungicide in 1000ml of distilled water was mixed with 10ml PDA poured into Petri dish and allowed to solidify.

Two intersecting lines were drawn at the bottom of the petri dish to determine the centre of the plate. A disc (5mm diameter) of a 3 - 4 day old culture of the fungal pathogen (*Rhizopus stolonifer*) was placed at the centre of each plate amended with various concentrations of plant extracts and fungicide respectively. The Completely Randomized Designed experiment had 3 replications per treatment. The control experiment had no plant extract treatment. Inoculated plates were incubated at temperature of 28±2ºC. The diameter of the growth of the test fungi was measured daily for three days and then used to detect the fungal toxicity levels of the extracts using the formula according to Chuku *et al.* (2005).

% growth inhibition = (DC-DT) / (DC) x 100/1
Where DC = is the farthest diametric distance of pathogen in control plate
DT = is the farthest diametric distance of pathogen colony in extract incorporated plates

➢ *In vivo Test Using Plant Extracts.*

This was carried out according to the methods of Nwauzoma *et al.*, (2017). About three 5mm mycelia disk of the pathogen (*R. stolonifer*) from a 4 days old pure culture was grown for 14days in a PDA broth containing 1g of PDA mixed in 200 ml sterile distilled water. The culture was filtered using No. 1 Whatman filter paper and transferred into 300 ml distilled water containing 10 % glucose. The mixtures were properly agitated and 6.4 $x10^4$ (spores/ml) suspension was obtained using a haemocytometer. This was sprayed separately on fresh surface sterile uninfected Irish potato at 10 ml per tuber. Plant extracts at various concentrations of 50%, 75% and 100% was sprayed on the tubers 24 hours later, using a sterile manual hand spray pump. 10ml of 0.002% Mancozeb, (fungicide) was applied as positive control. Also, inoculated and un-inoculated controls were kept. The Completely Randomized Design experiment was replicated thrice. The infected tubers were kept for 5 weeks at a temperature of 28 ± 2^0 C in order to access the disease severity.

The severity of the infection after 5 weeks was assessed by visual observation and scoring was done according to Nwachukwu and Osuji (2008) based on a 0 – 4 scale.
0-no infection
1-slight infection
2-moderate infection (50% of tuber infected.)
3-severe infection (75% of tuber infected)
4-complete rot (100% infection).

Also, Weight loss was observed weekly for five (5) weeks and percentage weight loss was calculated using the formula,
% Weight loss = (IW-FW/IW) x 100/1
IW= Weight before inoculation
FW = Final weight after 7, 14, 21, 28 and 35days

II. RESULT

Treatments	Percentage Inhibition (%)		
	Day 1	Day 2	Day 3
Control	0.00^a	0.0000^a	0.00^a
Mancozeb	66.23^h	69.99^j	73.31^i
***Z. officinale* 50%**	22.46^c	39.41^e	45.01^e
***Z. officinale* 75%**	29.96^{de}	42.35^f	47.81^f
***Z. officinale* 100%**	38.67^f	47.07^h	58.96^h
***C. limon* Peel 50%**	13.77^b	7.05^b	9.57^b
***C. limon* Peel 75%**	19.99^c	11.76^c	14.35^c
***C. limon* Peel 100%**	27.49^d	16.47^d	18.73^d
***C. longa* 50%**	27.49^d	44.71^g	45.013^e
***C. longa* 75%**	33.71^e	48.24^h	48.21^f
***C. longa* 100%**	44.97^g	51.17^i	56.98^g

N.B. Means within the same column with different super script (^{a,b,c,d}) are significantly different (P<0.05) according to DMRT
Table 1:- Effect of Plant Extracts and Mancozeb on Diametric Growth of *R. stolonifer* after 3 Days

The effect of plant extracts on diametric growth of the pathogen after 24 hours is presented in table 1. Mancozeb as well as the plant extracts inhibited the growth of *R. stolonifer* by 66.23%. However it was significantly different at (p<0.05) from the plant extracts. Among the extracts, 100% concentration of *C. longa* recorded a percentage inhibition of 44.97% and it was significantly different at (P<0.05) from 100% concentrations of *C. limon* peel and *Z. officinale* with percentage inhibition of 27.49% and 38.67% respectively after 24 hours. Also, Mancozeb had the highest percentage inhibition of the growth of *R. stolonifer* with a percentage inhibition of 69.99 after 48 hours. However it was significantly different (p<0.05) from the plant extracts. Among the extracts, 100% concentration of *C. longa* recorded a percentage inhibition of 51.17% and it was significantly different (P<0.05) from 100% concentrations of *C. limon* peel and *Z. officinale* with inhibition percentage of 16.47% and 47.07% respectively. The 100% concentration of *C. limon* peel (18.73%) had the lowest percentage inhibition among the plant extracts and was statistically different (p<0.05) from 100% concentration *Z. officinale* with a percentage inhibition of 58.96% after 3 days. The percentage inhibition of *10*0% concentration of *Z. officinale* (58.96%) was higher than and statistically different (p<0.05) from other plant extracts but was lower and statistically different (p<0.05) from Mancozeb (73.31%) after 3 days.

Table 2 shows disease development and severity under each treatment method adopted after 5 weeks. The rate of rot caused by *Rhizopus stolonifer* was reduced by the botanicals and mancozeb at each treatment method used. The 100% concentrations of *Z. officinale* gave the best rot reduction caused by *R. stolonifer* with severity

score of 0.33 but it was not significantly different ($p<0.05$) from 100% concentration of *C. longa*, *C. limon* peel and mancozeb with severity scores of 0.67, 1.33 and 0.67 respectively. However, they were better and significantly different ($p<0.05$) from the inoculate control with a score of 3.33.

Treatments	Rot Development
Z. *officinale* 50%	3 ± 0^{c}
Z. *officinale* 75%	0.33 ± 0.58^{a}
Z. *officinale* 100%	0.33 ± 0.58^{a}
***C. limon* Peel 50%**	1.67 ± 0.58^{b}
***C. limon* Peel 75%**	1.33 ± 0.58^{ab}
***C. limon* Peel 100%**	1.33 ± 0.58^{ab}
***C. longa* 50%**	1.67 ± 1.15^{b}
***C. longa* 75%**	1.33 ± 0.58^{ab}
***C. longa* 100%**	0.67 ± 0.58^{ab}
Mancozeb	0.67 ± 0.58^{ab}
Inoculated control	3.33 ± 0.58^{c}
Uninoculated control	0.67 ± 0.58^{ab}
P value	<.0001*
Significant Difference	Yes

N.B. Means within the same column with different super script (^{a,b,c,d}) are significantly different ($P<0.05$) according to DMRT.

Table 2:- Effects of Plant Extracts and Mancozeb on Rot Development after 5 Weeks Incubation

Table 3 shows the percentage weight loss in Irish potato tubers inoculated with *R. stolonifer* recorded weekly for 5 weeks. The 75% concentration of *C. limon* peel recorded the highest percentage weight loss 9.65%, 12.73%, 16.39% for weeks 1, 2 and 3 respectively while inoculated control 19.12% and 25.44% had the highest percentage weight loss for week 4 and 5 respectively. *C. longa* at 100% concentration had a percentage weight loss of 4.64%, 6.25%, 8.64%, 9.47% and 10.51% in week 1, 2, 3, 4 and 5 respectively. Mancozeb had a percentage weight loss of 6.4%, 8.5%, 12%, 13.03% and 13.93% in week 1, 2, 3, 4 and 5 respectively. There was no statistical difference ($P\leq0.05$) in percentage weight loss in all treatments and all weeks.

Treatment					
	Week 1	**Week 2**	**Week 3**	**Week 4**	**Week 5**
Z. *officinale* _50	4.72±3.58	7.07±5.66	11.55±10.57	13.35±12.87	15.57±15.77
Z. *officinale* _75	2.08±0.43	3.08±0.43	4.84±0.51	5.33±0.59	5.87±0.62
Z. *officinale* _100	5.54±5.22	7.44±6.91	10.85±9.82	11.87±10.93	13.11±12.03
***C. limon* Peel _50**	4.53±3.21	5.68±3.63	7.63±4.01	9.57±4.76	8.54±4.22
***C. limon* Peel _75**	9.65±7.9	12.73±10.57	16.39±12.67	18.88±15.84	21.54±19.19
***C. limon* Peel _100**	3.73±2.01	5.19±2.59	7.57±3.29	8.16±3.54	8.76±3.8
***C. longa* _50**	6.02±4.06	7.91±5.13	11.71±8.13	17.39±16.39	24.58±27.48
***C. longa* _75**	4.97±2.95	6.72±3.68	10.13±5.32	13.55±5.84	16.39±8.48
***C. longa* _100**	4.64±3	6.25±3.75	8.64±4.9	9.47±5.38	10.51±5.94
Mancozeb	6.4±4.07	8.5±5.65	12±8.21	13.03±9.21	13.93±10.27
Inoculated control	4.04±0.59	6.02±0.5	10.05±3.26	19.12±17.66	25.44±28.07
Uninoculated control	5.41±5.17	7.44±6.64	11.22±8.83	12.6±9.68	13.63±10.64
P Value	0.8019	0.8428	0.9169	0.9063	0.8592
Significant Difference	NO	NO	NO	NO	NO

Table 3:- Effects of Plant Extracts and Mancozeb on Weight of *R. stolonifer* Inoculated Tubers after 5 Weeks Incubation

III. DISCUSSION

Rhizopus stolonifer is an important fungal pathogen of Irish potato in Nigeria. It has been reported to cause extensive rot of Irish potato tubers in storage (Clark and Hoy 1994; Onuegbu, 2002; Muhammed *et al*., 2004; Oyewale, 2006; Ameinyo and Ataga, 2006; Salami and Popoola, 2007; Mohammed *et. al*., 2017). Tuber losses of up to 25% has been attributed to this organism (Owunbiko and Mbanaso, 2005).

The inhibition of *R. stolonifer* by extracts of Z. *officinale*, *C. longa* and *C. limon* peel may be attributed to the presence of antimicrobial substances, tannins,

flavonoids and saponin found in the extract. This result is in agreement with the findings of Giriraju and Yumus (2013), who reported that Z. *officinale* extracts showed antimicrobial activities against some plant pathogens. Also, various studies of Z. *officinale* showed that gingerol an important root extract of the plant, and shagaols, the dehydrated form of gingerols significantly inhibited the growth of some fungal and bacteria pathogens (Mahady *et al.,* 2003; Azu and Onyeagba, 2007; Chen *et al.,* 2008, Ali *et al* 2008; Jiang *et al* 2006). In same way, extracts of *C. longa* have shown antimicrobial activity against molds and bacteria such as *Aspergillus niger*, *Penicillium digitatum, Aspergillus flavus, Penicillium javanium, Curvularia oryzae, Trichophyton mentagrophytes, Escherichia coli, Staphylococcus aureus, Salmonella typhi* and *Candida albicans* and have been reported by earlier workers (Kapoor 1997; Arora and Kaur 1999, Gal and Bakht, 2015). Citrus plants have been reported to display a wide spectrum of antibacterial and antifungal activity against microorganisms such as *Fusarium oxysporium, Penicillium chrysogenum, Aspergillus niger, Trichophyton rubrum* and *Candida albicans* (Akhilesh *et al.,* 2012; Junab *et al.,* 2017).

The in vivo study revealed the effects of the plant extracts on rot development and weight loss in Irish potato tubers inoculated with *R. stolonifer*. The extract of the test plants reduced tuber rot of Irish potato when applied 24 hours after inoculation with *R. stolonifer*. There was no statistical difference ($p<0.05$) when compared with synthetic fungicide (mancozeb) and uninoculated control respectively. This result indicates that the extracts of the test plants could effectively reduce rot development in Irish potato tubers at test concentrations. The ability of plant extracts to reduce rot in tuber crops have been reported by earlier researchers (Nwachukwu and Osuji, 2008; Strivastava and Kshma, 2014: Boungab *et al.* 2015; Nwauzoma *et. al.,* 2017). Also, Plant extracts have been reported to reduce weight loss in tubers. Eze *et. al.* (2015) reported that *Cassia alata* reduced weight loss and rot in cocoyam cormels in Enugu State, Nigeria. Similar results on the reduction of weight loss using ashes from the bark of kola nut tree, neem tree, and inflorescence of oil palm have been reported (Eze, 1991). In a related study, Nwauzoma *et al.* (2017) reported that leaf extracts of *Carica papaya, Chromolaena odorata* and *Azadirachta indica* significantly reduced rot in *Sclerotium rolfsii* inoculated tubers after 14 days in storage.

IV. CONCLUSION

The inhibitory effects of extracts on growth of pathogen *in vitro* and *in vivo* varied with concentration of the plant extract. Increase in concentration and days had a corresponding increase in percentage inhibition of growth of the pathogen *in vitro*. This is not unconnected with the increase in the amount of phytochemical constituents.

RECOMMENDATION

It is recommended that the use of extracts of Z. *officinale, C. longa* and *C. limon* peel should be encouraged as part of an integrated approach for the control of Irish potato tuber rot caused by *R. stolonifer*. It is also recommended that further investigations should be done on the chemical nature of the active principles of the plants; further investigations can combine the plant extracts for possible synergistic effect.

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