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In vitro Bioprospecting of Botanicals Towards Inhibition of Microbial Pathogens of Rice (*Oryza sativa* L.)

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Abstract: The global call on the use of botanicals sequel to control of phytopathogenic microorganisms causing diseases of food crops most especially on rice has not been given attention in the tropics. A study was conducted to evaluate the antagonistic effect (in-vitro) of aqueous and ethanol extracts of *Eucalyptus lameldulensis* and *Moringa oleifera* leaves at different concentration (5, 10, 20, 40 and 160mg/ml) on fungal and bacterial pathogens affecting the aerial part of rice. The isolated fungal pathogens were *Curvularia* sp., *Helminthosporium* sp., *Monascus rubber* and *Pyricularia grisea* while that of bacterial pathogens were *Xanthomonas oryzae* and *Corynobacterium xerosis*. The aqueous extract from the botanicals showed less inhibition compared to the ethanol extract. All the tested concentration levels with respect to aqueous and ethanol extracts were significantly ($p<0.05$) effective on the fungal and bacterial pathogens. The highest inhibitory activity was observed on *Curvularia* sp. for fungi and *Xanthomonas oryzae* for bacteria. *Eucalyptus lameldulensis* and *Moringa oleifera* leaves extracts are therefore recommended for management of microbial pathogens of rice.

Key words: Botanicals · Fungal pathogens · Bacterial pathogens · Rice · Inhibition

INTRODUCTION

Rice (*Oryza sativa* L.) is an important cereal crop consumed all over the world in various forms [1]. Under normal conditions; rice is a crop which rarely suffers from main yield losses through pests and diseases due to the fact that rice in many areas is cultivated only once in a year [2]. However, rice is affected by pest and diseases which included weeds, pathogens or inocular, insects, rodents and bird [3]. A large number of other microbes (fungi, bacteria, nematodes and virus) also occur in the rice field which could from time to time cause problems locally, coupled with the fact that, disease management largely depends on variety selection and good fertilizer management [4]. Some varieties are more tolerant than others as the control measures includes planting resistant varieties and draining fields with history of the disorder just prior to internodes elongation [5].

To control pests, diseases and weeds, several biological methods are being used; aqueous management are very important because of their direct effect on crop health and because of their effect on the growing conditions of the pest, disease or weed. Apart from these preventive measures, pests and diseases can also be directly controlled by applying botanical pesticides [6]. Botanical extracts including Rice husk, Bamboo and Wood extract have been established to play significant roles in combating phytopathogenic fungi *In vitro* and *In vivo* [7, 8], though, at different concentrations. Aqueous extracts of some plants are known to have toxic properties; roots, leaves and other parts of some plants contain chemicals which when present in sufficient concentration exerts toxic effect on the plant pathogens [9]. Therefore, this study evaluates (*In vitro*) the antagonistic properties of *Moringa oleifera* leaf and *Eucalyptus lameldulensis* leaf on fungal and bacterial pathogens affecting the aerial part of rice.

MATERIALS AND METHODS

Sample Collection, Preparation, Identification, Occurrence and Pathogenicity: The samples were collected from Kware Lake in Kware Local Government Area of Sokoto State, Nigeria. Samples were collected

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from two farms, debris were removed from the leaves and dipped in 70% ethanol for 2 minutes, rinsed in several changes of sterile distilled water prior utilization. Thereafter, already sterilized leaves of the diseased rice were cut into small pieces with sterile surgical blades and transferred with the aid of forceps to the solidified media for fungal isolation. For bacteria, the infected folia part of rice was grounded with 10ml of sterile distilled water in a sterile mortar and pestle. Serial dilution was then carried out on 1ml of the suspension and inoculated on nutrient agar plates, followed by incubation at 28±2°C. The cultures were sub cultured to obtain a pure culture for identification. The biochemical tests, viz, gram staining, catalase test, starch hydrolysis, casein hydrolysis, growth in 4% NaCl, gelatin hydrolysis and sugar fermentation following the standard protocol [10] were performed on pure cultured bacteria. Microscope slides were prepared from the pure culture of each fungal isolates and identified according to the published description of fungi [11, 12]. The frequency of occurrence was calculated for the fungal and the bacterial isolates using the following formula:

$$\text{Frequency of occurrence (\%)} = \frac{\text{Number of times a fungus / bacterium occurred}}{\text{Total number of fungal / bacterial isolates}} \text{ x } 100$$

Pathogenicity test was carried out in accordance with the procedure of [13] and re-isolation procedure was again carried out to verify the authenticity of the pathogenic activity [14, 15].

Extraction of Plant Materials: *Moringa oleifera* and *Eucalyptus lameldulensis* leaves were grounded and sieved to obtain the powdery form. For aqueous extraction, about 200g of the powder was weighed then soaked in 4 litres of distilled water in separate containers for each of the botanicals, allow standing for 24 hours and then sieved with Muslin cloth and labelled. The filtrates were heated separately with respect to the botanicals, evaporation occurred and the dry extract was finally scrapped off using a spatula and kept until required. While for the ethanol extraction, 100g of the powdered botanical leaves were weighed and transferred into 2 litres of ethanol separately. This was then shaken thoroughly, allowed to stay overnight. The solution was sieved and then heated using water bath at 100°C until the aqueous content evaporated totally. The dry extract were collected separately and weighed in varying concentrations viz., 5, 10, 20, 40 and 160 mg/ml.

***In vitro* Phytofungicidal Activity of Botanicals:** Five milliliters each of varying concentrations of the two botanical extracts were separately incorporated into molten PDA plates. The mixture was allowed to solidify for about 15 - 20 minutes, before inoculating the fungi. Control was set up for each fungus without the addition of extracts. Two lines bisecting one another in a perpendicular form were ruled underneath the petri plates with a marker. A cork borer 5 mm in diameter was used to cut the fungal pathogens from the periphery of 7 days old culture. This was inoculated in the center of each petri plate [16] with the help of the ejector attached to the cork borer. The cut disc was placed upside down to enhance direct contact between the mycelia and the agar. The control without plant extract was also inoculated and all treatments were replicated thrice in a complete randomized design (CRD). The petri plates were incubated at 28±2°C and observations were recorded. The percentage mycelia growth inhibition was calculated using the following formula adopted from [17].

$$\% \text{ MI} = \frac{\text{Mc} - \text{Mt x } 100}{\text{Mc}}$$

where;
Mc = Average mycelial growth in the control.
Mt = Average mycelial growth in the treatment.
MI = Mycelial inhibition.

In vitro Phytobactericidal Activity of Botanicals: Paper disc of 5mm were also made from the filter paper using paper puncher and were put into a 100 ml conical flask. The discs were sterilized, counted and placed into different concentrations of the test plants (*Moringa oleifera* and *Eucalyptus lameldulensis* leaves extracts) already prepared and allowed to stand for 24 hours. Bacteria pure cultures for each isolates were streak on fresh nutrient agar plates, impregnated with paper disc from the various botanical concentrations and incubated at 37±2°C. After 24 hours, the plates were observed for any zone of inhibition and measured with the help of meter rule in millimeter.

RESULTS

Identification, Occurrence and Pathogenicity: The isolated and identified fungi from foliar part of rice were *Curvularia* sp., *Monascus rubber*, *Pyricularia grisea* and

Table 1: Frequency of occurrence of fungi and bacteria isolated from folia part of rice

	Frequency of isolation	Percentage frequency of occurrence (%)
Isolated Fungi		
Curvularia sp.	64	39.02
Helminthosporium sp.	4	2.44
Monascus rubber	44	26.83
Pyricularia grisea	52	31.71
Isolated bacteria		
S. aureus	54	32.93
X. oryzae	45	27.44
S. intermedius	24	14.63
C. noryi	2	1.22
P. putida	1	0.6
C. xerosis	34	20.73
B. macerans	4	2.44

Table 2: Percentage inhibition of botanical extracts on *Curvularia* sp.

	Aqueous extract		Ethanol extract	
Concentration (mg/ml)	*Moringa oleifera*	*Eucalyptus lameldulensis*	*Moringa oleifera*	*Eucalyptus lameldulensis*
5	0.0c±0.0	0.0c±0.0	32.4c±1.3	62.6d±0.9
10	0.0c±0.0	0.0c±0.0	39.8bc±4.4	63.2d±1.2
20	0.0c±0.0	0.0c±0.0	41.3bc±3.9	68.2c±0.1
40	62.8b±0.9	65.1b±0.2	45.9b±3.3	72.2b±0.8
160	66.0a±0.6	71.4a±1.2	57.6a±2.0	78.4a±0.7
Control	0.0c±0.0	0.0c±0.0	0.0d±0.0	0.0e±0.0

[a, b, c] means in a column with different superscripts are significantly different ($p<0.05$), values are means ± standard error of 3 replications

Table 3: Percentage inhibition of botanical extracts on *Pyricularia grisea*

	Aqueous extract		Ethanol extract	
Concentration (mg/ml)	*Moringa oleifera*	*Eucalyptus lameldulensis*	*Moringa oleifera*	*Eucalyptus lameldulensis*
5	0.0c±0.0	0.0c±0.0	32.8c±1.3	31.3d±0.9
10	0.0c±0.0	0.0c±0.0	34.1c±1.1	39.6c±4.4
20	0.0c±0.0	0.0c±0.0	49.5b±3.1	54.6b±2.3
40	49.5b±3.1	68.2b±0.1	62.9a±1.1	59.5b±1.5
160	62.9a±0.9	72.2a±0.8	66.0a±0.6	70.5a±0.8
Control	0.0c±0.0	0.0c±0.0	0.0d±0.0	0.0e±0.0

[a, b, c] means in a column with different superscripts are significantly different ($p<0.05$), values are means ± standard error of 3 replications

Table 4: Percentage inhibition of Botanical extracts on *Monascus rubber*

	Aqueous extract		Ethanol extract	
Concentration (mg/ml)	*Moringa oleifera*	*Eucalyptus lameldulensis*	*Moringa oleifera*	*Eucalyptus lameldulensis*
5	0.0±0.0	0.0±0.0	0.0±0.0	27.8c±0.2
10	0.0±0.0	0.0±0.0	0.0±0.0	38.7b±4.4
20	0.0±0.0	0.0±0.0	0.0±0.0	43.3b±4.1
40	0.0±0.0	0.0±0.0	0.0±0.0	65.1a±1.0
160	0.0±0.0	0.0±0.0	0.0±0.0	71.4a±1.2
Control	0.0±0.0	0.0±0.0	0.0±0.0	0.0d±0.0

[a, b, c] means in a column with different superscripts are significantly different ($p<0.05$), values are means ± standard error of 3 replications

Table 5: *In vitro* effect of Botanical extracts on *Xanthomonas oryzae.*

	Aqueous extract		Ethanol extract	
Concentration (mg/ml)	*Moringa oleifera*	*Eucalyptus lameldulensis*	*Moringa oleifera*	*Eucalyptus lameldulensis*
5	0.0c±0.0	0.0c±0.0	20.0c±0.5	12.0c±0.46
10	0.0c±0.0	0.0c±0.0	22.0b±0.4	13.0b±0.1
20	0.0c±0.0	0.0c±0.0	23.0b±0.3	13.0b±0.0
40	7.00b±0.5	10.0b±0.2	25.0a±0.2	14.0ab±0.2
80	9.00a±0.5	11.0a±0.3	26.0a±0.5	15.0a±0.1
Control	0.0c±0.0	0.0c±0.0	0.0d±0.0	0.0d±0.0

[a, b, c] means in a column with different superscripts are significantly different ($p<0.05$), values are means ± standard error of 3 replications

Table 6: *In vitro* effect of Botanical extracts on *Corynebacterium xerosis.*

	Aqueous extract		Ethanol extract	
Concentration (mg/ml)	*Moringa oleifera*	*Eucalyptus lameldulensis*	*Moringa oleifera*	*Eucalyptus lameldulensis*
5	0.0±0.0	0.0c±0.0	14.0c±0.0	18.0d±0.2
10	0.0±0.0	0.0c±0.0	14.0c±0.4	20.0c±0.4
20	0.0±0.0	0.0c±0.0	16.0b±0.0	20.0c±0.1
40	0.0±0.0	9.0b±0.1	17.0b±0.2	23.0b±0.2
160	0.0±0.0	10.0a±0.0	15.9a±0.3	25.0a±0.2
Control	0.0±0.0	0.0d±0.0	0.0d±0.0	0.0e±0.0

[a, b, c] means in a column with different superscripts are significantly different ($p<0.05$), values are means ± standard error of 3 replications

Helminthosporium sp. *Curvularia* sp. occurred more frequently with a percentage frequency of 39.02%, followed by *Pyricularia grisea* with 31.71%, *Monascus rubber* with 26.83%, while the least was *Helminthosporium* sp. with 2.44% (Table 1). The bacteria isolated from the foliar part of rice were *Staphylococcus aureus*, *Xanthomonas oryzae*, *Staphylococcus intermedius, Clostridium noryi, Pseudomonas putida, Corynoebacterium xerosis* and *Bacillus macerans*. However, *Staphylococcus aureus* occurred more frequently with 32.93%, followed by *Xanthomonas oryzae* (27.44%), *Corynoebacterium xerosis* (20.73%), then *Staphylococcus intermedius* (14.63%), *Bacillus macerans* (2.44%) and *Clostridium noryi* (1.22%), while *Pseudomonas putida* was recorded as the least with 0.61% (Table 1). For pathogenicity, *Pyricularia grisea*, *Monascus rubber* and *Curvularia* sp. manifested symptoms on the folia part of rice within 3 days of pairing while *Helminthosporium* sp. did not show any symptoms. For bacteria, *Xanthomonas oryzae* and *Corynoebacterium xerosis* showed considerable tissue maceration within 2-3 days compared to *Staphylococcus aureus* and *Staphylococcus intermedius* that did not show any effect after pairing for 2-5 days.

Effect of Botanical Extracts on Mycelial growth of Fungal Pathogens: The phytofungicidal activity of ethanol extract of *Eucalyptus lameldulensis* showed significant ($p<0.05$) inhibition at all concentrations. The percentage of mycelia inhibition at 160 mg/ml concentration on *Curvularia* sp. was significantly ($p<0.05$) high (78.39%) compared to other fungal pathogens (Table 2). Similarly, *Pyricularia grisea* and *Monascus rubber* were also significantly ($p<0.05$) inhibited with percentage mycelia inhibition of 70.53% and 71.44% respectively at 160 mg/ml (Table 2). Other concentrations i.e., 5, 10 and 20 mg/ml did not show any significant ($p<0.05$) effect on the fungal pathogens.

Effect of Botanical Extracts on Growth of Bacterial Pathogens: *Moringa oleifera* ethanol extract exhibited phytobactericidal effect (in-vitro) on the two pathogenic bacteria (*Xanthomonas oryzae* and *Corynoebacterium xerosis*). The significant ($p<0.05$) effect of *Moringa oleifera* ethanol extract on *Xanthomonas oryzae* were similar with respect to all the concentration levels tested (Table 3), while that of aqueous extract of *Moringa oleifera* was only effective at higher concentrations (> 40 mg/ml). The aqueous extract of *Moringa oleifera* was not effective on *Corynoebacterium xerosis* at all the concentration levels (Table 4) compared to their ethanol extracts that showed significant ($p<0.05$) effect on *Corynoebacterium xerosis* at all the concentration levels. Similar observation was recorded for aqueous extract of *Eucalyptus lameldulensis*. High significant ($p<0.05$) zone of inhibition was recorded for *Xanthomonas oryzae* most especially at higher concentration levels (>40 mg/ml) in comparison to low

concentration levels (5mg/ml, 10mg/ml and 20mg/ml). However, the low concentration levels were observed to be insignificant on *Xanthomonas oryzae* and as well on *Corynoebacterium xerosis* (Table 4). Ethanol extract of *Eucalyptus lameldulensis* showed considerable phytobactericidal effect (in-vitro) as high zone of inhibition was obtained for *Xanthomonas oryzae* and as well for *Corynoebacterium xerosis* (Table 4) at > 40 mg/ml concentration level.

DISCUSSION

Based on our study, it was observed that a number of pathogenic fungi (*Curvalaria sp, Helminthosporium sp Monascus rubber* and *P. grisea*) affect the foliar part of rice. Our understanding of fungal pathogenicity in this study agreed with the report of [1] that, fungal pathogens may move into the field through the seeds which could be responsible for a number of rice diseases, right from the nursery to the field. Our observation on *Curvalaria* sp commemorated with the report of [18] that, *Curvalaria* sp is one of the fungal pathogens causing diseases of rice. *Xanthomonas oryzae* has been reported [19] as one of the bacterial pathogens of rice, while our study justified the occurrence of *Corynoebacterium xerosis* as one of the bacteria causing foliar disease of rice, though, probably reported for the first time in Northern part of Nigeria.

The established report by [20] that, higher plants contained antifungal compounds for the control of plant disease is well justifiable in our study. Various plant extracts have been evaluated for their antifungal property against different pathogens [21] which included microbial pathogens of rice reported in this study. A close study into the antifungal and antibacterial activity of the aqueous extract of *Moringa oleifera* and *Eucalyptus lameldulensis* did not exhibit significant effect at low concentrations, but with increase in concentrations of the extracts, showed inhibition on some of the identified microbial pathogens. This conformed to the findings of [22] that high concentration of *Nicotinia tabacum* significantly controls *Colletotrichium destructivum*. The highest inhibitory effect was recorded at 40 mg/ml to 160 mg/ml compared to the less effect recorded for *Monascus rubber* at all concentrations. This corroborated with the reported work of [23] that, aqueous extract has the ability to control pathogenic fungi of rice at high concentrations.

Consistent observation in our study showed that at low concentrations, plant extracts showed less inhibitory effect on both fungal and bacterial foliar pathogens of rice compared to the higher concentrations. Further observation showed that, ethanol extract of the test plant extracts particularly *Eucalyptus lameldulensis* exhibited satisfactory inhibitory effect on both fungi and bacterial pathogens of rice. This reconfirmed the report of [23] that, *Eucalyptus* possesses strong antifungal activity on several plant pathogens. With respect to our observation, it could be deduced that, *Eucalyptus lameldulensis* and *Moringa oleifera* have antimicrobial properties against fungi and bacteria reported to be associated with foliar diseases of rice in this study. Thus, proper utilization and application of *Eucalyptus lameldulensis* and *Moringa oleifera* will not only discouraged the use of chemical pesticides but it will also contributes to sustainable production of rice.

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