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Antifungal Activity of Neem (*Azadirachta indica*) Leaf Extract against Pathogens Associated with Tomato (*Solanum lycopersicum* L.) Fruit Spoilage

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ABSTRACT: The study was conducted to evaluate the antimicrobial activity of neem (*Azadirachta indica*) leaf extract against fungal phytopathogens isolated from diseased tomato (*Lycopersicum esculentum*) fruit. Diseased tomato fruits were obtained and to establish a mixed culture. Two distinctive fungi were identified on the mixed cultures and subculture into freshly prepared potato dextrose agar medium. The fungal isolates were identified using the cultural characterization. Neem (*Azadirachta indica*) leaves were obtained and used to prepare water extract. The antifungal activity of the neem leaf extract was evaluated using the poison plate method. Mycelial growth was measured and recorded. The results showed that two fungal pathogens were isolated from the diseased tomato fruit. The cultural characterization of the two isolates revealed the identity of the fungal isolates to be *Diaporthe* and *Xylaria* species. There was a significant reduction in the mycelia growth of *Diaporthe* species with values of 2.210±0.34, 1.42±0.37, and 0.61±0.16 cm for the 25, 50, and 100% neem leaf extract, respectively, compared to the control (3.67±0.34 cm), indicating antifungal activity of the neem leaf extract. Conversely, only the 25 and 50% neem extract showed antifungal activity against *Xylaria* species. The findings of the present study suggest that neem leaf extract could be used to preserve tomato fruits from fungal pathogens causing spoilage.

Keywords: Antifungal, *Azadirachta indica*, Pathogen, *Solanum lycopersicum*, spoilage

Introduction

Tomatoes are the major dietary source of the antioxidant lycopene, which has been linked to many health benefits, including reduced risk of heart disease and cancer. They are also a great source of vitamin C, potassium, folate, and vitamin K. The tomato fruit contain 85% water, 13% carbohydrates, 0.9% protein, 0.5% fat and oil, trace of minerals and vitamin (Kimura and Sinha, 2008).

Food spoilage is any change in the visual, smell and texture of the food that makes it unacceptable for human consumption (Samira, 2016). It is also the process in which food deteriorate to the point it is not edible to human (Samira, 2016). There are different forms of spoilage which is the physical spoilage (loss or gain of moisture), chemical spoilage and lastly microbial spoilage. 20% of all fruit and vegetables produced for human consumption are lost to food spoilage by the microbe (Samira, 2016; Katan, 2000). Food spoilage are caused by different factor which include light, chemical and phyto-pathogens (microbial contamination). Phytopathogen alone account for 20% of fruit and vegetables destruction. because of the nature of the tomato fruit in terms of pH, it doesn't support the growth of bacteria leaving fungi as the main phyto-pathogens causing fruit spoilage leading to the shortage of consumable tomato fruit, like vegetables fruit are nutrient rich substrate but the pH of fruit does not favour Bacteria

growth, as a result fungi like yeast and mold are more important in the spoilage of fruit (Savary, 2012; Dimphna, 2016). Fungi are one the leading factors of spoilage in food products. Fungi that reproduce by spores are important factor in food spoilage of many foods under different condition (Synder *et al*, 2019). There are different methods of preservation, which include: Salting, drying, refrigeration, canning and use of chemical that inhibit the growth of micro-organism. Neem is considered an effective source of environmentally-powerful natural pesticide and considered to be one of the most promising pesticides (Kumar and Parmar, 1996). It is believed to be one of the trees of the 21st century for its great potential in pest management, environmental protection and medicine (Wankar *et al.*, 2009).

Azadirachta indica is one of the most widespread introduced tree species in Nigeria and is extensively naturalized in drier parts of Nigeria and the most successful shade and fuel plantation tree. More than 135 compounds have been isolated from different parts of the tree. They have been divided into isoprenoid and non-isoprenoid compounds (Kumar and Parmar, 1996; Dastagir and Haq, 1997; Biswas *et al*., 2002,). A good number of plants possess therapeutic properties against bacterial infections (Kayode and Kayode, 2011). The antimicrobial activity of neem leaves extracts against *Staphylococcus* spp., *Streptococcus* spp., *Pseudomonas* spp., *E. coli*, and some fungal strains have been reported (Koona and Budida, 2011; Valarmathy *et al*., 2010). Extract of neem leaf is known to have antibacterial activity (Akhter and Sarker, 2019).

The aim of the study was to investigate the antifungal activity of neem (*Azadirachta indica*) against phytopathogens isolated from diseased tomato fruit which will achieved by isolating fungal pathogens from diseased tomato fruit, characterizing the isolated fungal pathogens, preparing neem leaf extract and determining the antifungal activity of the isolated isolates.

Materials and methods

Source of tomato fruits: Tomato fruits were bought from the popular Uselu Market, Uselu Quarters, Benin City.

Preparation of potato dextrose agar: Potato dextrose agar (PDA) was prepared according to the manufacturer's instruction. The PDA was prepared by dissolving 39 g of powdered PDA in 1 liter. The media were then sterilized using a pressure cooker for 20 min under pressure. After sterilization, the media were then cooled to 45-50 °C and then aseptically dispensed into sterile Petri dishes. Before pouring, an antibiotic (chloramphenicol) was added to the PDA to inhibit bacteria growth.

Isolation of fungal pathogens from diseased tomato plants: Tomato fruits were prepared by cutting them into smaller pieces, followed by surface sterilization with alcohol to remove surface contaminants. The sterilized tomato fruits were inoculated into the PDA. The cultures were incubated 37 °C for 72 h.

Sub-culturing of fungal isolates: Single isolated mycelia of the fungi were picked up with the help of sterilized wire loop and were streaked on fresh potato dextrose agar medium. The potato dextrose agar plates were incubated at 37 °C for 72 h.

Characterization and identification of fungal isolates: The fungal isolates were identified using macroscopy and microscopy. The morphological characteristics were observed and described. The isolates were also observed on the microscope after staining with lactophenol. A drop of lactophenol blue stain was dropped on a clean grease free sterilized glass slide and after this, a sterile inoculating wire loop was used to pick the mycelium unto the glass slide from the culture. The mycelium was spread evenly on the slide. Teasing was carried out to separate the mycelium in order to get a homogenous mixture and the mixture was then covered with cover slips gently and then allowed to stay for some seconds before observing under the microscope at x40 magnification. This was then compared with a laboratory manual for fungal identification.

Preparation of aqueous neem leaf extract: Aqueous neem leaf extract was prepared according to the method described by Agbenin and Marley (2006). Fresh neem leaves were weighed, sterilized (1% sodium hypochlorite), and were washed three times with sterile distilled water. The leaves were ground in a mortar and mixed with 10 ml of sterile distilled water.

In vitro antimicrobial activity of neem leaf extract: The antimicrobial activity of neem leaf extract on the fungal isolates was tested using the agar well diffusion method. Three different concentrations corresponding to 100, 50, and 0% of neem leaf extract were prepared. The 100% neem leaf extract was taken to be the stock solution while the 50% was prepared by dispensing 50 ml of sterile distilled water into 50 ml of neem leaf extract. The 0% was the control without neem leaf extract. 1ml of the neem leaf extract for the different concentrations was added to 9 ml of PDA after pouring under sterile conditions. This was then stirred carefully and allowed to gel. After solidifying, the

medium was inoculated by picking a culture plug of the fungal culture and placing it at the center. This was then incubated and the fungal mycelia growth was measured for a period of five days. The same process was repeated for the different concentrations (0, 50 and 100%) were prepared accordingly. The rate of inhibition of fungal growth by the neem leaf extract treatment was calculated using the following formula:

$$\text{Inhibition rate} = \frac{\text{Average mycelia growth of control} - \text{average mycelia growth of the treatment}}{\textit{Average mycelia growth of control}} \times 100$$

Results

When the surface sterilized tomato fruits were inoculated into the PDA, two distinct type of fungi were observed in the culture. Plate 1 shows the mixed culture obtained. When the mixed cultures were sub-cultured into a freshly prepared PDA and incubated for three days, pure cultures of the fungal isolates were obtained. Plate 2 shows the outcome of the sub-culturing. Isolate 1 is opaque, with white pigmentation, and rough texture, while isolate 2 is also opaque, with white pigmentation and smooth texture. The results indicate that the two suspected fungal isolates were *Diaporthe* sp. and *Xylaria* sp.

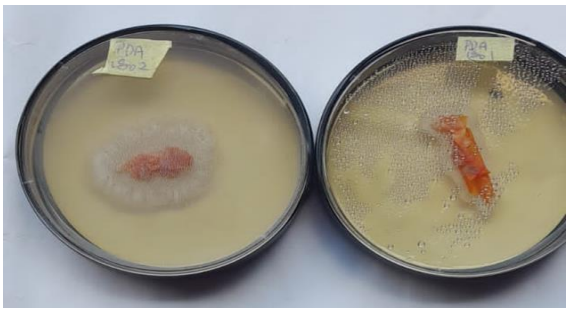

Plate 1: Mixed fungal cultures from diseased tomato fruits

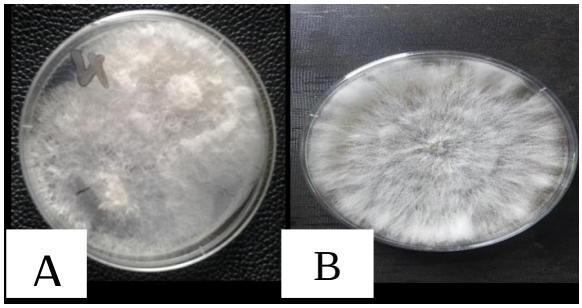

Plate 2: Pure cultures of isolated fungal pathogens grown on PDA for 3 days. A: Isolate 1 (*Diaporthe* sp.); B: Isolate 2 (*Xylaria* sp.)

The morphological descriptions of fungal pathogens isolated from the diseased tomato fruit after three days of culture at room temperature is shown in Table 1.

Table 1: Morphological description of fungal isolates associated with diseased tomato plants

Morphology	**Isolate 1**	**Isolate 2**
Margin	Rough	Rough
Elevation	Raised	Raised
Size	Large	Large
Texture	Rough	Smooth
Pigmentation	White	White
Optical property	Opaque	Opaque
Suspected organism	*Diaporthe* sp.	*Xylaria* sp.

The results of the antifungal effect of neem leaf extract on mycelia growth of *Diaporthe* sp. are presented in Table 2. Each value is a mean ± standard error of 3 replicates. There was significant difference between the control (0 % concentration of neem leaf extract) and the other treatments (25, 50, and 100 % concentration of neem leaf extract) in the six days of observation. The highest mycelia growth of 3.67±0.34 cm was observed in the control (0% concentration of the neem leaf extract) after 6 days of incubation, while the lowest value of 0.61±0.16 cm was recorded for the 100% neem leaf extract treatment. There was a progressive increase in mycelial growth in all the treatments and control.

Table 2: Effect of neem leaf extract on the mycelia growth of *Diaporthe* sp.

Concentration (%)	Mycelia growth (cm)				
	Day 2	Day 3	Day 4	Day 5	Day 6
0	1.61±0.64	1.89±0.54	2.56±0.44	2.79±0.53	3.67±0.34
25	0.89±0.54	1.27±0.37	1.98±0.28	2.33±0.26	2.210±0.34
50	0.38±0.29	0.49±0.02	0.65a±0.08	1.19±0.18	1.42±0.37
100	0.262±0.13	0.37±0.13	1.50±0.03	0.49a±0.16	0.61±0.16

Plate 3 shows the mycelia growth of *Diaporthe* sp. after 6 days of culture incubation.

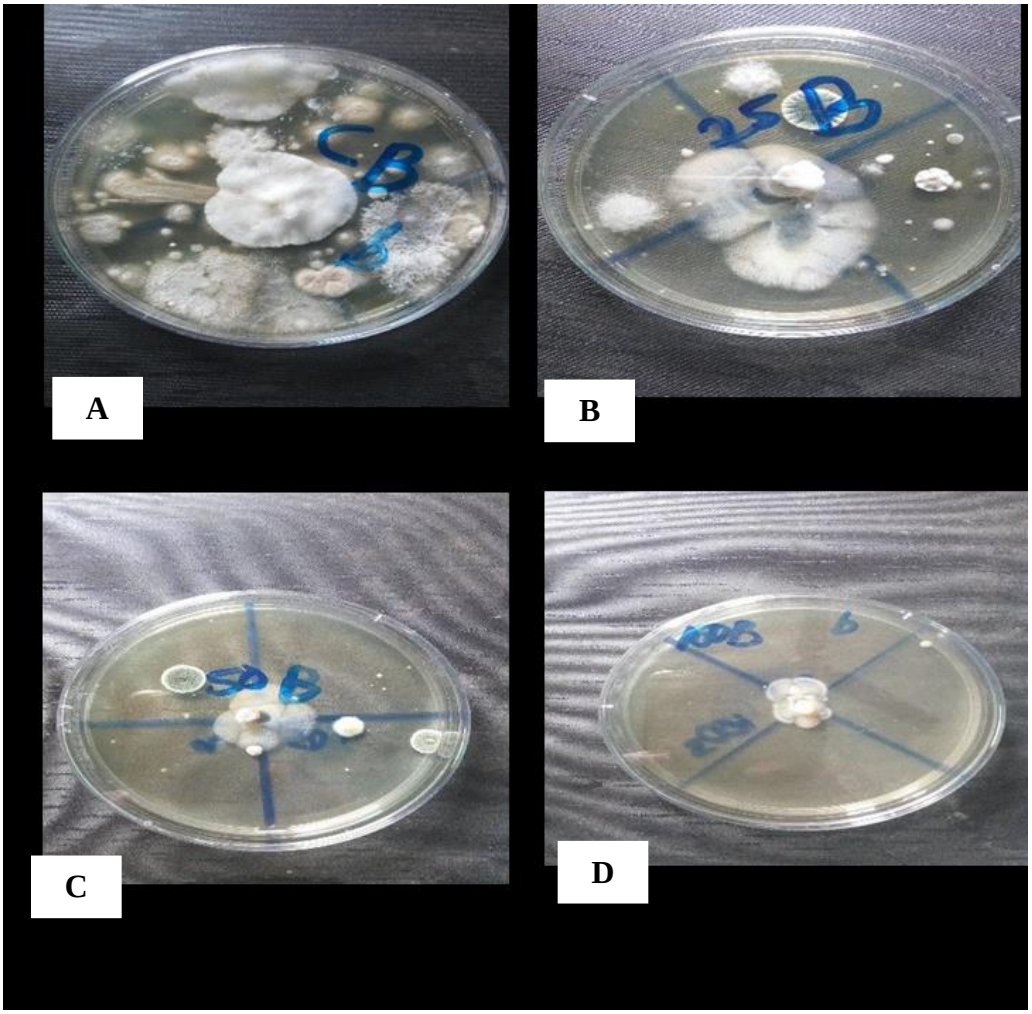

Plate 3: Effects of neem leaf extract on mycelia growth of *Diaporthe* sp. after 6 days of incubation. A: Control (0%; Water); B: 25 % neem leaf extract; C: 50 % neem leaf extract; D: 100 % neem leaf extract.

Table 3 shows the effect of neem leaf extract on the mycelia growth of *Xylaria* sp. Each value is a mean ± standard error of 3 replicates. The fungal mycelia completely covered the entire plate in both the control and 100% neem leaf extract treatment. There was a sharp increase in mycelial growth throughout the six days of the incubation period. On the sixth day, there were 2.65±1.26 and 3.67±0.98 cm mycelial growth values recorded in the 25 and 50% neem leaf extract treatment groups, respectively.

Table 3: Effect of neem leaf extract on the mycelia growth of *Xylaria* sp.

Concentration (%)	Mycelia growth (cm)				
	Day 2	**Day 3**	**Day 4**	**Day 5**	**Day 6**
0	2.98±1.54	5.76±1.76	6.57±1.11	7.56±1.51	8.16±0.39
25	0.08±0.00	0.18±0.00	0.87±0.10	1.78±0.17	2.65±1.26
50	0.09±0.00	0.45±0.89	0.65±0.12	2.45±0.58	3.67±0.98
100	0.03±0.00	0.54±0.23	2.76±0.25	4.77±0.65	7.71±0.54

Plate 4 shows the mycelia growth of *Diaporthe* sp. after 6 days of culture incubation.

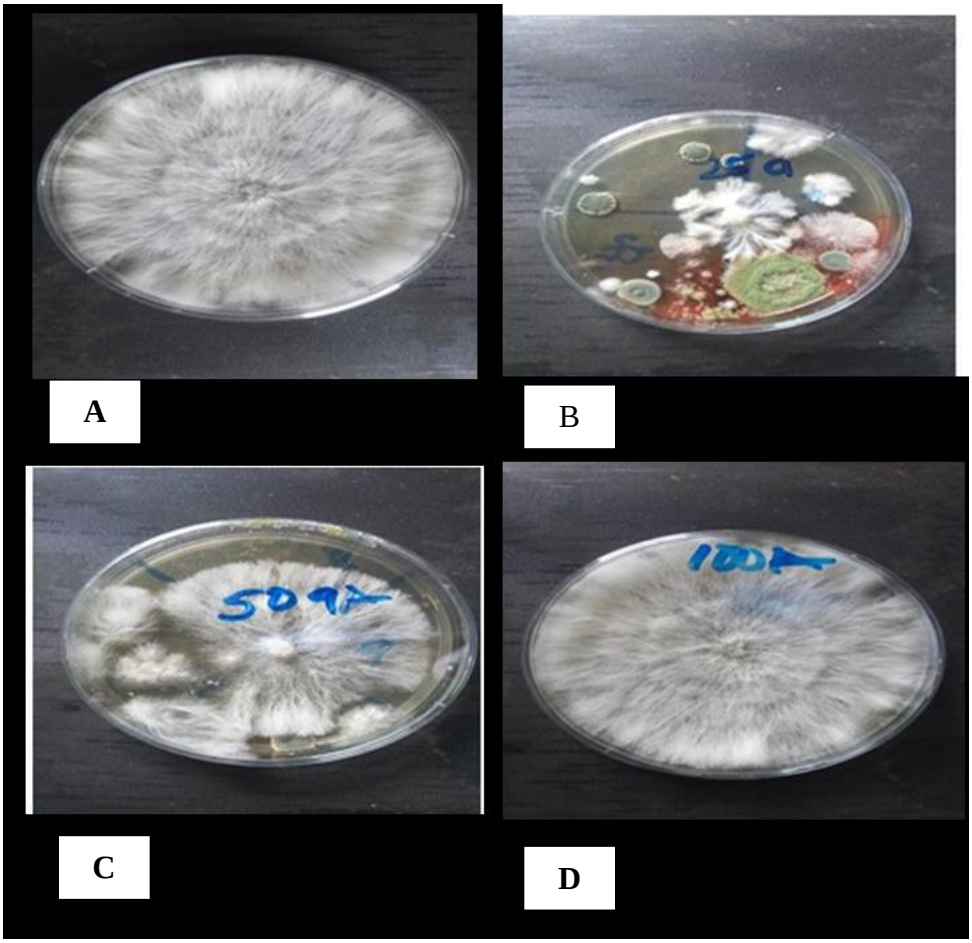

Plate 4: Effect of neem leaf extract on the mycelia growth of *Xylaria* sp. after 6 days of incubation. A: Control (Water); B: 25 % neem leaf extract; C: 50 % neem leaf extract; D: 100 % neem leaf extract.

Discussion

Phytopathogens affect different parts of tomato plants growing on the field and even the harvested ones and cause diseases. The protection of crop plant against diseases has a major role to play in meeting the food demand of the growing human population. With an increase in antibiotic resistance, comes a growing interest in developing new antimicrobial agents (Gold *et al.*, 1996). Fungal pathogens can infect crop plants causing diseases to the stem, leaves and flowers. Agricultural products such as fruits, seeds, tubers etc. can be contaminated while on the plants or after harvest. The contamination after harvest could be due to poor handling of these agricultural products through transportation and in the hands of market women. The contaminating phytopathogens are responsible for the

spoilage of these agricultural products. In the present study, phytopathogens were isolated from tomato fruits and used to prepare pure cultures. The pure cultures were subjected to cultural characterization.

Based on the cultural characteristics, the suspected fungal phytopathogens isolated from the diseased tomato fruit were *Diaporthe* sp. *and Xylaria* sp. (Table 1). *Xylaria* sp is made up of diverse species ranging from saprophytic, endophytic to parasitic organisms (Krittapong *et al.,* 2016). *Diaporthe* sp. have often been reported as plant pathogens, non-pathogenic endophytes or saprobes, commonly isolated from a wide range of hosts (Gomes *et al.,* 2013; Zong *et al*., 2014), causing root and fruit rots, dieback, cankers, leafspots, blights, decay and wilts. Both pathogens have been reported to cause significant damage to agricultural products.

The antifungal activity of neem was investigated by preparing neem leaf extract. The extract was used to perform antimicrobial sensitivity testing on test pathogens. The results show that the antimicrobial activity of neem leaf extract on *Diaporthe* sp. Is concentration dependent as the antifungal activity increased with an increasing concentration of extract as evident in the corresponding decrease in the mycelial growth. Phytochemicals such as phenol, tannins, saponins, and others present in the leaf extract have been implicated for the antimicrobial activity observed (Vignesh, 2015; Sravanthi *et al*., 2015). The 100% showed the highest antimicrobial activity probably due to the fact that it contained more phytochemicals compared to the other concentrations. The least amount of antifungal activity was observed in the control due to the fact that it does not contain any phytochemicals. The results of the antimicrobial testing agree with the studies carried out by Mahmoud *et al.,* (2011). Zong *et al.* (2014) discovered that neem leaf plant extract has antimicrobial activity against certain pathogens which include *Aspergillus niger, Aspergillus flavus, Aspergillus terrues, Aspergillus fumigatus* and *Candida albicans*.

Conversely, neem leaf extract has little antimicrobial effect on mycelia growth of *Xylaria* sp. There was a significant mycelial growth in the control and 100% neem leaf extract. Some level of antimicrobial activity of the 25 and 50% neem leaf extract was observed on the *Xylaria* sp. This observation shows that the antifungal activity of neem extract on *Xylaria* sp. Is not concentration-dependent. The 50% neem leaf extract had the highest antimicrobial activity, followed by the 25%. By comparing the results of the two pathogens, Xylaria seems to be more aggressive than *Diaporthe* sp.

From all the results shown in this study, the finding is that the neem plant leaf extract possesses some antifungal activity against the two pathogens that were studied. It can be used to preserve tomato fruits and by extension all other fruits against fungal pathogens that can cause food spoilage.

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