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Efficacy of *Azadirachta Indica* Leaves Extract in, Management of Fungal Diseases of Sweet Pepper (*Capsicum Annuum* L.) Fruit in Gusau Local Government, Zamfara State, Nigeria

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ABSTRACT

Isolation of fungal species and efficacy of *Azadirachta indica* Ethanol leaves extract in the management of fungal pathogens of sweet pepper (*Capsicum annuum* L.) fruit was conducted. Samples were collected from three locations (Geba, Gidan Malammai, and Chakal) of Gusau local government in sterile polythene bags and transported to the laboratory of Biological Sciences, Sokoto State University for fungal isolation. The frequency of occurrence was investigated and pathogenicity test was carried out. Four fungal species of *Rhizopous stolonifer, Rhizopous oryzae, Aspergillus niger*, and *Mucor hiemalis* were isolated and found to be pathogenic to sweet pepper. Aspergillus niger had the highest frequency of occurrence and *Rhizopous oryzae* exhibited the highest level of virulence. The Ethanol extract of *Azadirachta indica* showed statistically ($p \leq 0.05$) significant inhibitory activity against the isolated fungus. The extract showed more inhibitory activity at the highest concentration. In conclusion, all fungal species are pathogenic to sweet pepper. It is recommended that more research on the fungal species affecting sweet pepper should be conducted in other local government areas of Zamfara State.

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Introduction

The genus *Capsicum* is part of the large *Solanaceae* family, which is among the more than 90 genera and 2500 species of flowering plants, and includes commercially important vegetables such as tomato, potato, and eggplant. This genus is native to tropical and subtropical America, in a wide region comprising Mexico and northern Central America, the Caribbean, the lowland Bolivia, the northern lowland Amazonia, and the mid-elevation southern Andes, where archaeglogical evidence suggests the use of this spice crop since BC. At the beginning, fruits were exchanged for black pepper (piper nigrum), a species similar in taste (though not in appearance) although not phylogenetically related to *Capsicum*. For this reason, it was incorrectly named ''pepper''. It was Fuchs, who proposed for the first time in 1543, the botanical term *Capsicum*, which was adopted later in 1753 by Linneo. The name would be the Neolithic derivation of Greek ''*Capsa*'', which refers to the peculiar shape of the fruit [1,2].

The crop was first introduced in Europe by Christoper Columbus during his travels after the discovery of America in the fifteenth century and later spread to Africa and Asia. Early imported varieties belong to C. Chinese which most probably was the most consumed during that time. *Capsicum* species are reported, five of which namely, *C. annuum, C. baccatum, C. chinense, C. frutescens*, and *C. Pubescens* have been domesticated and widespread with different terms depending on the region of cultivation [3].

The National Programme for Agriculture and Food Security (NPAFS) (2009) reported that the yield of pepper between 1999 and 2004 declined from 2939 tonnes to 2912 tonnes and 2744 tonnes to 2469 tonnes. Between 2007 and 2009 despite the increased hectrage from 22.5 hectares to 26.92 hectares of land, the yield was not commensurate (5532 tonnes to 6063 tonnes). The decline according to has been attributed to biotic and abiotic factors like weeds, pests, diseases and environmental stress. The present worth of dry pepper is 3.8 billion dollars, while fresh pepper contributes 30,208 billion dollars. For both the increase observed over the past 25 years is four times higher in dry pepper and six times higher in fresh pepper. Peppers are also an extremely good source of compounds exerting antioxidants and responsible for fruit pigmentation [4-6].

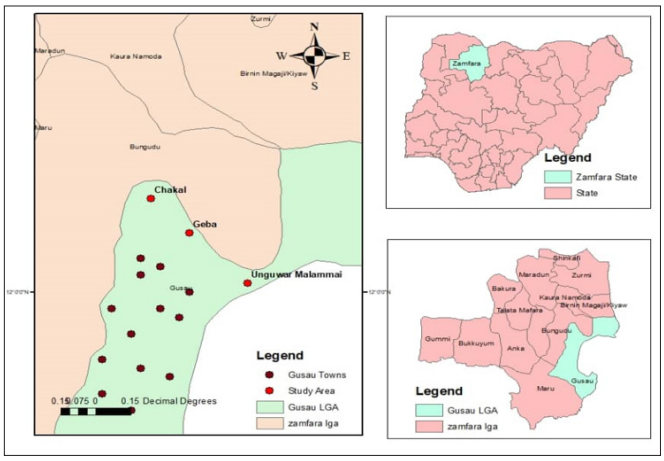
Fungal disease is one of the most important diseases of bell pepper (*Capsicum annum* L.) in tropical and subtropical regions. In mild to warm climates and rainy seasons, fruit production losses can reach

as high as 100% when adequate control measures are not applied. Disease symptoms may occur either during the crop development in the field or after harvest, but only the fruit exhibits symptoms. Infection begins with the appearance of small, circular, depressed lesions that rapidly expand and have no defined diameter. Bacterial wilt is ranked as one of the most important plant diseases in the entire world as it causes cent percent yield losses in solanaceous vegetables [7-9].

Materials and Methods

Study Area

Gusau local government is the capital of Zamfara state, Northwestern Nigeria, with a population of 226,857 located at Latitude: 12 10' 12.86''N Longitude: 6 39' 50.83'' E. Gusau covers a total land area of approximately 3469 square kilometers in 1927 and is now a major collecting point for cotton and peanuts (groundnuts) grown in the surrounding area. Gusau town attracted attention as an important agricultural and commercial centre. The vegetable crops cultivated in the local Government include Groundnuts, Sweet pepper, Onions, and Sweet potatoes which serve as sources of income and employment to rural communities. The town's Hausa and Fulani peoples also raise cattle, sheep, donkeys, horses, and camels and trade in millet, sorghum, rice, cowpeas, beans, and floodplain-grown vegetables [10-12].



Map of Gusau Local Government, Zamfara State, showing sample collection area

Sample Collection

The samples of affected sweet pepper fruits were collected from the three locations in Gusau local government. The locations Geba, Gidan Malamai and Chakal and Ten samples were collected in sterile polythene leather, each location making a total of thirty samples (30 samples). The samples were transported to the biology laboratory of Sokoto State University for isolation and identification of fungal organisms responsible for sweet pepper fungal diseases in Gusau local government [13].

Preparation of Media (PDA)

Thirty nine grams (39g) of PDA were suspended in 1000ml of distilled water in a conical flask. The medium was dissolved by closing the conical flask with a cotton plug and heated completely. It was then sterilize by autoclaving at 121ᴐC for 15 minutes and allowed to cool to 45ᴐC. To prevent bacterial growth, 0.2g of streptomycin was added and was allowed to solidify and stored until needed. The preparation of the medium was carried out according to manufacturer's instructions.

Isolation of Fungal Pathogens

Portion of sweet pepper fruits were cut aseptically with the aid of sterile scissors in to small pieces (5mm). It was then placed centrally on petri dishes containing solidified PDA. The plates were then incubated at room temperature in the dark for 72hours in August 2023. The fungal colony grown from the incubated plates was sub-cultured in to fresh medium until pure culture was obtained [14,15].

Identification of Fungal Pathogen (phenotype)

A sterile needle was used to take a little portion of the hyphae containing spores on the sterile glass slide, stained with lacto phenol cotton blue, and then examined under the microscope for fungal structures. The microscopic/culture features and the microscopic characteristics observed were then compared with the fungal identification atlas for the identification of the fungi. An isolated fungus was identified based on colony and morphological characteristics, such as colour and shape observed with the microscope [16,17].

Infection/Pathogenicity Test

Fresh and apparently healthy sweet pepper fruits were surfaced-sterilized with methanol for 30 seconds and then rinsed three times in distilled water. With a 7mm diameter flame-sterilized cork borer, cylindrical cores were removed from each fruit. An equal diameter of fungal mycelium was punctured and used to inoculate the apparently healthy fruits earlier punctured. Vaseline jelly was smeared to completely seal the surface of each of the inoculated pepper fruit to prevent external infection and then incubated for 10 days in triplicate. The control was inoculated with disc of solidified potato dextrose agar medium. The symptoms developed with different fungal isolates was compared to the control. The pathogen was re-isolated and identified using the same procedures described earlier [18-20].

Collection of Plant Sample

The leaves of *Azadirachta indica* were collected from Geba and Gidan malammai of Gusau Local Government, Zamfara State by hand picking. The plant was identified at biology laboratory department of Biological Science Sokoto State University Sokoto [21].

Extraction of Plant for the Assay

In preparation of Ethanol and Aqueous Extracts, the leave samples of Azadirachta indica was rinsed in clean water and dried at room temperature 25°C to 3 °C for two weeks. The dried leaves sample was ground into powder using a mortar and pestle. An equal measurement of 100grams of *Azadirachta indica* dried powdered leaves was soaked separately in 1000ml of ethanol and distilled water. The mixtures of each extract were allowed to stand for 24 hours. The extract was then squeezed and then filtered with a muslin cloth. The extracts were then placed in to hot air oven to evaporate.After evaporation, each extract was grinded to powder using mortar and pestle and then 100mg, 300mg and 500mg of each was dissolved in 5ml of their respective solvent (ethanol and distilled water) in a test tube, to produce 40, 60 and 80mg/ml solution respectively [22].

Determination of the Antifungal Activity of the Leaves

Agar well diffusion method as described by was adopted for the evaluation of the plant extract. Spore suspension method was carried out according to,in which 10ml of sterile distilled water was inoculated into Petri plates of each of the isolated fungi using sterile syringes. Sterile syringes were also used to inoculate 10ml

of suspended fungal spores on sterile PDA, homogenized and poured into a sterile Petri plate where a uniform depth of 4mm was maintained. The Petriplates were then allowed to solidify. A sterile cork borer of 7mm in diameter was used to make a well at the centre of each Petri plate. A 100µl of each concentration (30mg/ml, 60mg/ml, and 100mg/ml) of leaves extract was loaded into wells for each of the isolated fungi. Sterile distilled water was loaded in the well of a separate Petri plate as negative control while 100µl of standard antifungal Fluconazole (100mg/ml) was loaded into the well of separate Petri plate as positive control. The plates were then incubated at 32°C for 24 hours. After the incubation period, the plates were observed for the zones of inhibition. Antifungal potential was evaluated by measuring inhibition zone diameters in millimetres (mm) with the help of the transparent meter rule. Each treatment was repeated three times. Average zones of inhibition was calculated and presented in standard deviation [23].

Frequency of Occurrence of the Fungal Isolates

The number of times each fungus occurs on the samples was recorded. The percentage frequency of occurrence was calculated [24].

Number of times the fungus was encountered

$$\text{Frequency} = \frac{\text{Number of times the fungus was encountered}}{\text{Total fungal isolations}} \times 100$$

Data Analysis

The data obtained in this study was analysed using SPSS software version 13. Data entry was done using Microsoft Excel version 23.0. Data entry was used to verify the distribution of the obtained data. One-way analysis of variance (ANOVA) was conducted. Statistical significance was considered for $p \leq 0.05$ was statically significant.

Results

Isolation and Identification of Fungal Pathogens

The result of isolation and identification of fungal species associated with sweet pepper fruit fungal diseases in Gusau Local Government investigated in this research shows that, four species of two genera of fungal pathogens that include Aspergillus and Mucor were identified. The genera of *Aspergillus* include: *Aspergillus niger* and *Rhizopous stolonifer*. The genera of *Mucor* include: *Rhizopous oryzae* and *Mucor hiemalis* Table 1.

Percentage Occurrence of Fungal Isolates

Among four fungal pathogens that were identified in this research (*Rhizopous stolonifer, Rhizopous oryzae, Aspergillus niger, andMucor hiemalis*), *Rhizopous stolonifer* and *Aspergillusniger* has the highest frequency of occurrence in all the sweet pepper investigated with 44%, and Rhizopous oryzae and Mucor hiemalis with 22% respectively Table 2.

Pathogenicity Test

All four fungal Isolates, (*Rhizopous stolonifer, Rhizopous oryzae, Aspergillus niger, andMucor hiemalis*) were found to be pathogenic to the sweet pepper fruit in Gusau Local Government. *Rhizopous oryzae* exhibited the highest level of virulence (i. e., mycelia and or rot covering more 54.13% of the fruit), followed by *Aspergillus niger* rated as medium levels having a pathogenic effect covered 50% and the least was *Mucor hiemalis* with virulence (i.e. mycelia/ or rot covering 33.83% of the fruit) Table3.

Table 1: Identified Fungal Isolates and their Colonial and Microscopic Characteristics

Specimen	Appearance on petri plate		Description	Organism Identified
A	White yellow and then black	White to yellow	Simple conidiophores that terminate in the globose swelling. Hyphae is septate	*Aspergillus niger*
B	Cottony, rapid grower	Brown to gray	Clear, nonseptate hyphae. Spores borne inside large spherical structures called sporangia. Similar to Rhizopus spp. but lacking rhizoids.	*Mucor hiemalis*
C	Gray to brown to black colony	White to yellow	Similar to Mucor spp. except foot-like structures (rhizoids) at base of spore bearing hyphae (see arrows). Spores in sporangium clear, coenocytic hyphae	*Rhizopus oryzae*
A	White cottony	White to yellow	Mycelium with black sporangia containing sporangiospores	*Rhizophus stolonifer*

Table 2: Frequency of Fungal Species of Sweet Pepper, Cultivated in Gusau Local Government Area, Zamfara State, Nigeria. August 2023-09-22

	Percentage frequency occurrence			
	Geba	Chakal	U/malammai	Total%
Rhizopus stolonifer	0.33	0.66	0.33	0.44%
Rhizopus oryzae	0	0.66	0	0.22%
Aspergillus niger	0.66	0.33	0.33	0.44%
Mucor hiemalis	0.33	0	0.33	0. 22%
Total	1.32	1.65	0.99	132

Table 3: Length and Percentage of the Mycelia Growth of Fungi in Treatment Replica

Fungal species	Original length of the fruit (mm) (A)	length of mycelia growth in sweet pepper (mm)			Average (B)	% Damage (B/A) × 100
		Fruit 1	Fruit 2	Fruit 3		
Rhizopus stolonifer	9	4.5	2.9	3.5	3.63±0.57	40.33%
Rhizopus oryzae	8	3.5	4.5	5.0	4.33±0.62	54.13%
Aspergillus niger	7	4.5	2.5	3.5	3.50±0.82	50%
Mucor hiemalis	6	2.5	1.8	2.7	2.33±0.38	33.83%

To perform the ANOVA and determine the statistical differences between the treatment groups (30 mg/ml, 60 mg/ml, and 100 mg/ml) and the 100 mg/ml fluconazole positive control group, on the effects of methanol extract of *A. indica*,the data was organized and then analyzed. Fungal Species are *Rhizopus oryzae*, *Mucor hemalis*, *Rhizopus stolonifer*, *Aspergillus niger* and Treatment Groups are 100 mg/ml, 60 mg/ml,30 mg/mland Fluconazole positive control (100 mg/ml). Data was structured into a format suitable for ANOVA and one-way ANOVA was performed to compare the means of the inhibition zones across the different treatment groups. When the ANOVA shows significant differences, a post-hoc test was conducted to identify which groups are significantly different from each other.

Considering a p-value ≤ 0.05 as statistically significant, the ANOVA test yields a p-value of approximately 0.00021, which is much less than the threshold of 0.05. This indicates that there are statistically significant differences between the means of the inhibition zones across the different treatment groups. This indicates that the inhibition zones differ significantly between some of the treatment groups, particularly when comparing the lower concentration (30 mg/ml) and the fluconazole control Table 4.

Table 4: Summary of the Mean Differences, P-Values, and Confidence Intervals for Pairwise Comparisons between the Treatment Groups

Group 1	Group 2	Mean Difference (Meandiff)	Adjusted p-value (p-adj)	Lower Bound	Upper Bound
100 mg/ml	30 mg/ml	-0.865	0.001	-1.3124	-0.4176
100 mg/ml	60 mg/ml	-0.4183	0.0895	-0.8657	0.0291
100 mg/ml	Fluconazole 100 mg/ml	0.2217	0.3938	-0.2257	0.6691
30 mg/ml	60 mg/ml	0.4467	0.0682	-0.0007	0.8941
30 mg/ml	Fluconazole 100 mg/ml	1.0867	0.001	0.6393	1.5341
60 mg/ml	Fluconazole 100 mg/ml	0.64	0.001	0.1926	1.0874

Discussion

The result of isolation and identification of fungal species associated with sweet pepper fruit diseases in Gusau Local Government investigated in this research shows that four species of two genera of fungal pathogens that include *Aspergillus* and *Mucor* genus were identified. The genera of *Aspergillus* include: *Aspergillus niger* and *Rhizopous stolonifer*. The genera of *Mucor* genusinclude: *Rhizopous oryzae* and *Mucor hiemalis*. These findings are in line findings of who isolated, it is also in accordance of findings the result of isolation and identification of fungal species associated with sweet pepper fruit diseases in Bungudu Local Government investigated in this research shows that, four species of fungal pathogens that include Mucor and Aspergillus genus were identified. The genera of *Mucor genus* include: *Rhizopous oryzae and Mucor hiemalis*. The genera of *Aspergillus* include *Aspergilus niger* and *Rhizopous stolonifera* [25].

Four fungal pathogens (*Rhizopous stolonifer*, *Rhizopous oryzae*, *Aspergillus niger*, and *Mucor hiemalis*) were found to be pathogenic to sweet pepper fruit in Gusau local Government, Zamfara State, Nigeria. *Rhizopous stolonifer* and *Aspergillusniger* has the highest frequency of occurrence in all the sweet pepper investigated with 44%, followed by *Rhizopous oryzae* and *Mucor hiemalis* with 22%. These findings are in line findings of. Four fungal pathogens (*Aspergillus niger*, *Rhizopous oryzae*, *Mucor hiemalis*, and*Rhizopous stolonifer*) were found to be pathogenic to sweet pepper fruit in Gada Local Government, Sokoto State, Nigeria. *Rhizopous stolonifer* has the highest frequency of occurrence in all the sweet pepper investigated with 44%, followed by *Rhizopous oryzae* with 41.5%, followed by *Aspergillus niger* with 39.3% and Mucor hiemalis with 22% [20].

Among the four Isolates, *Rhizopous oryzae* exhibited the highest level of virulence (i. e., mycelia and or rot covering more 54.13% of the fruit). *Aspergillus niger* rated as medium levels having a pathogenic effect covered 50% followed by *Rhizopous stolonifer* having 40.33% of the fruit. *Mucor hiemalis* was rated the least virulence (mycelia/or rot covering 33.83% of the fruit). These findings are in line findings of. Among the four isolates, *Aspergillus niger* exhibited the highest level of virulence (i.e., mycelia and or rot covering more with 60.23% of the fruit). *Rhizopous stolonifer* rated as medium levels having a pathogenic effect covered 55% followed by *Mucor hiemalis* having 32.30% of the fruit. *Rhizopous oryzae* was rated the least virulence (mycelia/or rot covering 19.22% of the fruit) [26].

Determination and comparison of the antifungal efficacies of the methanol and aqueous extracts of *A. indica* revealed that the ethanol extract of *A. indica* showed the highest statistically significant inhibitory activity against sweet pepper pathogenic fungi at the highest concentration i. e., 80mg/ml. In line with these

findings,in their study used methanolic extract of Acacia nilotica bark and leaf and tested using disc diffusion assay. Demonstrated that crude extracts from stem bark and root bark of Azadirachta indica, V. amygdalina and Cochlospermum planchonii due to the presence 23 of compounds like alkaloids, flavonoids, glycosides, saponins and tannins, has exhibited strong fungitoxicity against *Colletotrichum capcisi* [27-31].

Conclusion

1. There are four fungal species that are found to be pathogeneic to sweet pepper in Gusau Local Government.
2. The fungal species are *Rhizopous stolonifer*, *Rhizopous oryzae*, *Aspergillus niger* and *Mucor hiemalis*.
3. *Rhizopous stolonifer* and *Aspergillus niger* has the highest number of occurrence among the four isolates.
4. *Rhizopous oryzae* was found to be more pathogenic to sweet pepper (*Capsicum annum* L.) with highest number of virulence (54.13%).

Recommendation

- It is therefore recommended that the Farmers of Chakal, Geba and Gidan mallamai should take measures on management of fungal diseases sweet pepper.
- More research on the fungal species affecting sweet pepper should be conducted in the other local government areas of Zamfara State.
- Conducting field trials with plant extracts in sweet pepper cultivating areas in local government for management of fungal diseases of sweet pepper.

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