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Biological control of fruit rot of postharvest orange (*Citrus aurantium*) by aqueous plant extracts

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ABSTRACT

Post-harvest rot symptoms caused by complex airborne fungi are nowadays the most serious threatening disease affecting orange fruits in Nigeria and presumably in many African countries. Experimental trials were conducted at the Department of Biological Sciences (Akwa Ibom State, Nigeria) from January to December 2022 to ascertain the pathogens associated with postharvest fruit rot of oranges (*Citrus aurantium* L.). Results of the pathogenic test revealed that *Fusarium* sp. and *Penicillium* sp. are pathogenic and produced the highest lesion diameter (38.67 and 40.33 mm, respectively) and disease severity index (88.67 and 94.33%, respectively). The frequency occurrence of *Fusarium* sp. (69.14%) and *Penicillium* sp. (50.62%) was the highest. Two aqueous extracts of *Azadirachta indica* A. Juss. leaves and *Zingiber officinale* Roscoe rhizomes (at 80% concentration) were evaluated for their antifungal activities against the pathogenic isolates under *in vitro* and *in vivo* conditions. The results revealed that *A. indica* and *Z. officinale* aqueous extracts exhibited the highest mycelial growth inhibition (>90%) of *Fusarium* sp. and *Penicillium* sp. Similarly, under *in vivo* assay minimum lesion diameter (≤6.37 mm) and disease severity index (≤11.13%) were recorded in these aqueous extracts. In conclusion, the application of *A. indica* and *Z. officinale* can provide an alternative to fungicides against *Penicillium* sp. + *Fusarium* sp.

Keywords: *Azadirachta indica*, Bioassay methods, *Citrus sinensis*, Pathogenicity, Postharvest fruit rot, *Zingiber officinale*

Sweet orange (*Citrus aurantium* L.), from the family Rutaceae, is an important fruit tree crop grown in several regions of the world notably in Nigeria due to its nutritional value and socio-economic importance (Olife *et al.* 2015). Mechanical damage during harvest, microbial pathogens infection, poor storage, and post-harvest handling are major causes of post-harvest losses that threaten fruit production. Microbial pathogens responsible for post-harvest fruit spoilage are diverse and yield loss attributed to fungal infection reaches to 40-50% in developing and undeveloped countries (Singh and Sharma 2018). Specifically, the blue and green mold diseases are responsible for more than 30-70% losses on harvested orange fruits (Kahramanoglu *et al.* 2020). Fungal pathogens gain entry through injury on fruits and the spread of infection is facilitated by poor post-harvest handling and storage. *Fusarium* spp., *Mucor* spp., *Rhizopus* spp., *Penicillium* spp., and *Aspergillus* spp. represent the phytopathogens earlier reported to be associated with post-harvest rot of oranges in Nigeria (Kareem *et al.* 2020).

To date, the management of post-harvest rot has been effective through the use of conventional fungicides. However, the use of chemical fungicides is toxic to humans, animals, and environments (Singh *et al.* 2021, Rhouma *et al.* 2023). Aqueous extracts have emerged as a viable alternative to chemical fungicides and their potency as protectants has been reported (Chen *et al.* 2019). Neem (*Azadirachta indica* A. Juss) and ginger (*Zingiber officinale* Roscoe) represent the important medicinal plants available in many areas of the world and extracts from them have been reported to contain a long list of bioactive phytoconstituents, some of which have been evaluated for their antifungal, medicinal, insecticidal and antimicrobial potency (Chen *et al.* 2018, Ezeonu *et al.* 2018). Information on the fungi-toxic potency of neem and ginger extracts on postharvest spoilage fungi of orange fruits is necessary for incorporation into an integrated disease management strategy in the study area. The aim of the study was therefore to identify the fungal pathogens responsible for the postharvest decay of orange fruits and an *in vitro*

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evaluation of the fungistatic potential of the crude extract of neem leaves and ginger on these fungi.

MATERIALS AND METHODS

Orange fruit collection and fungal isolation: An experiment was conducted at the Department of Biological Sciences (Akwa Ibom State, Nigeria) from January to December 2022. Diseased orange fruits showing post-harvest rot symptoms were collected from three fields located in Akwa Ibom State of Nigeria (Abak, Etinan, and Uyo) (2022 and 2023). Small fruit pieces were sterilized in a 3% solution of NaOCl for 2 min and washed 3 times with sterilized distilled water. The samples were dried and placed on the surface of Petri dishes (7 fragments each with a total of 30 Petri dishes) containing potato dextrose agar medium amended with streptomycin (60 µg/ml). The plates were incubated in the dark at 25±2°C for 5–7 days and then examined to identify fungal colonies. The species were identified with phenotypic criteria and concerning different identification keys (Matrood *et al.* 2023).

Fungal pathogenicity and aggressiveness on orange fruits: The *in-vivo* assay was based on the methods described by El-Shahir *et al.* (2022). Healthy orange fruits were superficially disinfected and wounded with a sterilized cork borer (6 mm diameter and 5 mm depth with three wounds/fruit), inoculated separately by spraying with 50 µl of fungal strains spore suspension (10^6 spores.m/l). One control has been performed; by treating the fruits with distilled water (negative control). Each treatment consisted of 36 fruits per replicate (3 replicates), and the experiment was performed twice. Six inoculated fruits were placed in plastic containers on sterile wet paper and incubated at 25±2°C for 7 days (El-Shahir *et al.* 2022). After 7 days of incubation, the lesion diameter (LD) (mm) and disease severity index (DSI) (%) were assessed according to Hajji-Hedfi *et al.* (2023).

Preparation of aqueous plant extracts: A total of two aqueous plant extracts were evaluated for laboratory assays. *A. indica* and *Z. officinale* were performed by the method of Matrood and Rhouma (2021). The extracts of the leaf (neem) and rhizome (ginger) were prepared by crushing each plant material (dried at room temperature) with distilled water at a ratio of 1:1 (w/v). The powdered mass of leaves and rhizomes was squeezed through 75 µm sieves and the extracts were centrifuged at 3000 rpm for 20 min. The supernatants were filtered on the Whatman filter paper. The retained material was collected in Erlenmeyer flasks (250 ml) (Matrood and Rhouma 2021).

In-vitro evaluation of aqueous extracts: The relative efficacy of each aqueous extract on mycelial growth inhibition of phytopathogens was assessed *in vitro* using the poisoned food method according to the method described by Sharma *et al.* (2017). The percentage of mycelial growth inhibition (I) was evaluated according to Matrood and Rhouma (2021):

$$I\ (\%) = (1 - Cn/C0) \times 100$$

where, Cn, radial growth diameter of the pathogen in the presence of the treatment; C0, radial growth diameter of the pathogen in the control treatment.

In-vivo evaluation of aqueous extracts: Healthy orange fruits were soaked in NaOCl (2%) for 15 min, washed (two times) with distilled water, dried at room temperature, and wounded with a sterilized cork borer (three wounds/fruit). The orange fruits were dipped separately in prepared aqueous extracts (at a concentration of 80%) of neem leaves and ginger rhizomes for 30 min. After 2 h, the treated fruits were inoculated separately by spraying with 50 µL of spore suspensions of selected phytopathogens. Two controls were conducted; positive control (by inoculating the fruits separately with selected phytopathogens only), and negative control. Each treatment consisted of 36 fruits per replicate (3 replicates), and the experiment was performed twice. An average of six treated fruits were placed in plastic containers on sterile wet paper. The containers were enclosed in a plastic bag to maintain high humidity and subsequently incubated for 7 days in a growth chamber at 25°C. After the incubation time, LD and DSI were evaluated (Hajji-Hedfi *et al.* 2023).

Statistical analysis: Statistical analysis was performed using the mean values of the replicates. The data were analyzed by ANOVA using SPSS version 20.0 statistical software (SPSS, SAS Institute, USA). Differences between treatments, homogeneity of variances, and normality were checked by applying Duncan's Multiple Range Test. All statistical tests were performed with a significance level of 1% ($P \leq 0.01$).

RESULTS AND DISCUSSION

Surveys and fungal species isolation: Surveyed orange fruits located in Abak, Etinan, and Uyo (Akwa Ibom State, Nigeria) showed typical symptoms of post-harvest rot. These consisted of small, light-to-dark irregular, and sunken lesions (Fig 1). The most frequent pathogens identified from lesions on symptomatic fruit were *Fusarium* sp. (69.14%), *Penicillium* sp. (50.62%), and *Aspergillus* sp. II (48.15%). *Rhizopus* spp. (2 species) and *Aspergillus* sp. I were also detected but low in frequency (Fig 2).

Fungal pathogenicity and aggressiveness on orange fruits: Pathogenic ability of *Rhizopus* spp., *Aspergillus* spp., *Penicillium* sp., and *Fusarium* sp. was conducted to confirm their virulence and to define the most aggressive species causing serious damage on orange fruits. Results (Table 1) showed that the tested species were pathogenic with varying degrees on the orange fruits as they significantly ($P<0.01$) increased disease severity. It showed that *Penicillium* sp. and *Fusarium* sp. were the most aggressive species. The effect of the tested species on lesion diameter (38.67 and 40.33 mm, respectively) and disease severity index (88.67 and 94.33%, respectively) was highly observed in *Penicillium* sp. and *Fusarium* sp. (Table 1).

In-vitro evaluation of aqueous extracts: Data (Table 2) indicated clearly that the aqueous extracts of neem leaves and ginger rhizomes exerted a higher significant reduction ($P<0.01$) in the radial mycelial growth of *Rhizopus* sp. I, *Rhizopus* sp. II, *Aspergillus* sp. I, *Aspergillus* sp. II,

Fig 1 Symptoms on orange fruits caused by post-harvest rots: Brown and dark lesions and stem-end rot.

Penicillium sp. and *Fusarium* sp. after 7 days of incubation using poison food technique. The different treatments with aqueous extracts exhibited a significant reduction in mycelium growth. The highest inhibition percent was registered for *Aspergillus* sp. I, *Aspergillus* sp. II, *Penicillium* sp. and *Fusarium* sp. and obtained data ranging from 90.11% (*Aspergillus* sp. I) to 94.90% (*Fusarium* sp.) for aqueous *A. indica* extract, and from 92.67% (*Fusarium* sp.) to 94.56% (*Aspergillus* sp. II) for aqueous *Z. officinale* extract. However, the lowest inhibition was recorded for *Rhizopus* spp. (<25.90%). It can be concluded that the treatment with aqueous extracts against *Aspergillus* spp., *Penicillium* spp., and *Fusarium* sp. seemed to be the most effective with an inhibition rate above 90% under laboratory conditions (Table 2).

In-vivo evaluation of aqueous extracts: ANOVA revealed significant differences (P<0.01) in DSI and LD observed between the treated fruits and controls. All aqueous extract applications significantly decreased the aggressiveness of *Penicillium* sp. and *Fusarium* sp. (Table 3). Aqueous extracts of neem leaves and ginger rhizomes revealed their efficacy in decreasing the lesion diameter of *Penicillium* sp. (5.60 and 4.57 mm, respectively) and *Fusarium* sp. (6.37 and 2.83 mm, respectively) than positive controls (37.17 and 40.40 mm, respectively) (Table 3). Similarly, the lowest DSI was obtained from the orange fruits treated with neem leaves and ginger rhizomes and the values ranged between 2.68% (*Z. officinale* rhizomes + *Fusarium* sp.) and 11.13% (*A. indica* leaves + *Fusarium* sp.). However, the orange fruits inoculated only with *Penicillium* sp. (93.99%) and *Fusarium* sp. (98%) showed the highest DSI (Table 3).

The obtained results revealed that the diversity of fungal pathogens associated with post-harvest rot of orange fruits shows the identification of 6 fungal species: *Rhizopus* spp. (2 species), *Aspergillus* spp. (2 species), *Penicillium* sp. and

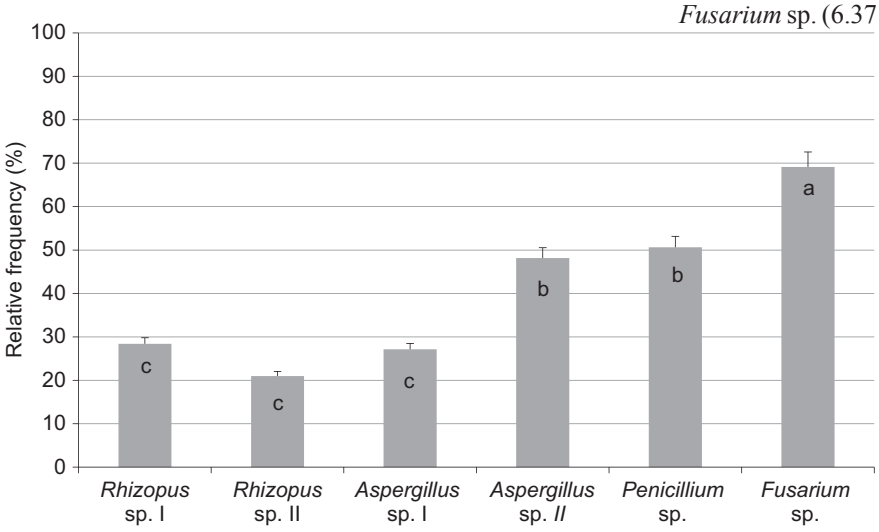

Fig 2 Relative frequency of the fungal species isolated from the orange fruits. Different letters above bars indicate statistically significant differences between treatments within the experiments (P≤0.5) according to the Duncan's multiple range tests.

Table 1 Effect of preventive treatments of two aqueous extracts on aggressiveness of isolated phytopathogens on orange fruits

Treatment	Lesion diameter (mm)	Disease severity index (%)
Rhizopus sp. I	4.13±0.58b[a]	20±0.73c
Rhizopus sp. II	5.13±0.32b	24.67±0.55bc
Aspergillus sp. I	4.43±0.33b	29.33±0.21b
Aspergillus sp. II	3.80±0.26b	8±0.79d
Penicillium sp.	38.67±0.52a	88.67±0.44a
Fusarium sp.	40.33±0.47a	94.33±0.61a
Untreated control	0c	0e
P-value[b]	<0.01	<0.01

[a]Duncan's Multiple Range Test, values followed by various superscripts differ significantly at P≤0.05; [b]Probabilities associated with individual F tests.

Table 2 Effect of two aqueous extracts on mycelial growth inhibition of isolated phytopathogens after 7 days of incubation at 25±2°C under *in vitro* conditions

Treatment	*Azadirachta indica* leaves	*Zingiber officinale* rhizomes
Rhizopus sp. I	2.29±0.05d[a]	19.48±0.75b
Rhizopus sp. II	0.33±0.18e	25.90±0.11b
Aspergillus sp. I	90.11±0.27c	94.48±2.07a
Aspergillus sp. II	92.86±0.11b	94.56±1.68a
Penicillium sp.	93.62±0.09ab	93.76±1.27a
Fusarium sp.	94.90±0.12a	92.67±1.09a
P-value[b]	<0.01	<0.01

[a]Duncan's Multiple Range Test, values followed by different superscripts are significantly different at P≤0.05; [b]Probabilities associated with individual F tests.

Table 3 Effect of preventive treatments of two aqueous extracts on aggressiveness of *Penicillium* sp. and *Fusarium* sp. on orange fruits

Treatment	Lesion diameter (mm)	Disease severity index (%)
Negative control	0±0e	0±0g
Positive control *(Penicillium* sp.)	37.17±0.45b	93.99±0.53b
Positive control (*Fusarium*sp.)	40.40±0.95a	98±0.63a
A. indica leaves + *Penicillium* sp.	5.60±0.44c	6.87±0.37d
A. indica leaves + *Fusarium* sp.	6.37±0.38c	11.13±0.78c
Z. officinale rhizomes + *Penicillium* sp.	4.57±0.31cd	4.57±0.32e
Z. officinale rhizomes + *Fusarium* sp.	2.83±0.25d	2.68±0.49f
P-value[b]	<0.01	<0.01

[a]Duncan's Multiple Range Test, values followed by various superscripts differ significantly at P≤0.05; [b]Probabilities associated with individual F tests.

Fusarium sp. Pathogenicity test revealed that *Penicillium* sp. and *Fusarium* sp. were the most aggressive species. Kareem *et al.* (2020) pointed out that the main pathogens found in the post-harvest rots were *Penicillium* sp., *Fusarium* sp., *Lasiodiplodia theobromae* and *Penicillium digitatum*, which were surpassed by *Colletotrichum gloeosporioides* in *Citrus reticulata* × *sinensis*. In related studies by Mendonça *et al.* (2017), *Alternaria alternata* and *Colletotrichum musae* were reported as the major pathogens responsible for post-harvest rots and compromised citrus fruit quality, while members of the *Aspergillus* spp. were classified as secondary colonizers (Ezeonu *et al.* 2018). Kwon-Ndung *et al.* (2022) highlighted that *Curvularia* sp. (37.50%), *Aspergillus* sp. (25%), *Colletotrichum* sp. (25%) and *Alternaria* sp. (12.50%) were among the major fungi responsible for post-harvest losses on orange fruits and verified by the results of the pathogenicity assay. Some of the isolated phytopathogens have been reported to be associated with several diseases in animals and humans. Toxic metabolites and mycotoxins produced by *Aspergillus* spp. and *Penicillium* spp. are detrimental to human health (Hajji-Hedfi *et al.* 2023).

The aqueous extracts used in the study had varying inhibitory effects on the mycelial growth of each of the fungal species (except *Rhizopus* spp.) which were significantly different from that of the non-treated plates. The variation in the degree of inhibition could be due to the nature and quantity of chemical composition in the extracts. Plant extracts are known to contain several bioactive compounds with antifungal properties, but their activities are dependent on the mode of extraction, concentrations, quantity used, and susceptibility of the fungi to the extracts (Matrood and Rhouma 2021). Appressorium is an important fungal structure during the penetration process; therefore, the aqueous extracts show inhibition of spore germination and blocking of appressorium formation (Matrood and Rhouma 2021). *A. indica* and *Z. officinale* have several phytochemicals that have shown their effectiveness in plant disease management (Shahid *et al.* 2021, Xi *et al.* 2022).

Research conducted by Ezeonu *et al.* (2018) reported the ability of aqueous leaf extract of neem (at 5% concentration) to inhibit the mycelial growth and colonization of *Aspergillus* spp. and *Fusarium* spp. Khan *et al.* (2021) documented that aqueous extracts of neem leaves showed the strongest antifungal activity against *Rhizopus* spp. and *Aspergillus* spp. Shrivastava and Swarnkar (2014) gave a similar report on the effectiveness of neem leaf extract against *A. flavus*, *Alternaria* spp., *Bipolaris sorokiniana, Fusarium oxysporum, Thielaviopsis* sp., and *Helminthosporium* sp. This suggests that these phytochemical components might have been responsible for the observed antifungal properties of the test plant used (Baby *et al.* 2022). It has been previously reported that the active ingredients of neem constitute mostly triterpenoides (Nimbin, Nimbidine, and Azadirachtin) (Baby *et al.* 2022).

Bordoh *et al.* (2020) indicated that ginger rhizome extract (at 10 mg/ml) revealed good antifungal activity against *Colletotrichum gloeosporioides* under laboratory conditions; it suppressed conidial germination (88.48%) and mycelial growth (87.50%). Shahid *et al.* (2021) found the effectiveness of the aqueous extracts of ginger rhizome in controlling *Alternaria alternata* under *in vitro* conditions. A previous study demonstrated that ginger rhizome extract (at 20 mg/ml) completely inhibited the mycelium growth of *Fusarium solani* (Xi *et al.* 2022). The aqueous ginger rhizome extract is rich in gingerone, dehydroshogaol, dihydrogingerone terpenes, and terpenoid, which could be responsible for its antifungal activity against many phytopathogens (Mao *et al.* 2019). As stated by Beristain-Bauza *et al.* (2019) the antifungal activity could be attributed to the presence of phenolic compounds (gingerol and shogaol); which are the active ingredients in the ginger rhizome. The effectiveness of ginger rhizome extract depends on the chemical composition, extraction solvent, and method (Beristain-Bauza *et al.* 2019).

This study proved that both aqueous extracts of neem leaves and ginger rhizomes controlled the disease severity index and lesion diameter compared to the positive controls. Our results are close to those obtained by Kwon-Ndung *et al.* (2022), who depicted that neem leaf extract greatly lowered the lesion diameter of *Penicillium* sp. and *Fusarium* sp. when applied to orange fruits under storage conditions. As stated by Khan *et al.* (2021), spraying fruits with aqueous extracts of neem leaves significantly decreased the lesion diameter and disease incidence of different postharvest diseases. El-Samawaty *et al.* (2021) and Xi *et al.* (2022) depicted that aqueous extracts of *Z. officinale* rhizomes revealed the strongest antifungal activity against *Penicillium* spp. *Fusarium* spp. under storage conditions. The penetration process involves appressorium (Salas-Gomez *et al.* 2023) and the aqueous extracts exhibit inhibition of mycelial growth (Al-Otibi *et al.* 2023), sporulation (Cruz-Cerino *et al.* 2023) and conidial germination (Al-Zaben *et al.* 2023) and blocking of appressorium formation (Salas-Gomez *et al.* 2023). Otherwise, Baby *et al.* (2022) and Xi *et al.*

(2022) reported that phytochemicals present in rhizomes (*Z. officinale*) and leaves (*A. indica*) extracts could be toxic to the *Penicillium* sp. and *Fusarium* sp., thereby causing its growth inhibition.

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