

Biocontrol Potential of Rhizospheric Fungi from *Moringa Oleifera*, their Phytochemicals and Secondary Metabolite Assessment Against Spoilage Fungi of Sweet Orange (*Citrus Sinensis*)

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ABSTRACT— *This investigative study aimed to assess the antifungal potential of Trichoderma viride and Penicillium chrysogenum isolated from Moringa oleifera rhizosphere against spoilage fungi of Citrus sinensis, as well as evaluate their phytochemical profile. The bio-agents (T. viride and P. chrysogenum) and the Citrus sinensis spoilage fungi were isolated following standard microbiological protocols. Initial in-vitro screening of the isolated bio-agents against the citrus pathogens in the confrontational assay was done. Phytochemical screening and antifungal activity of metabolites produced by the bioagents against the pathogens were also investigated. The inhibitory concentration (minimum/maximum: MIC and MFC) of the bioagent metabolites on the citrus pathogens was also assessed. The isolation screening investigation indicated that citrus pathogens isolated were P. digitatum, A. wentii, C. tropicalis and F. oxysporum and that P. digitatum had the highest frequency (43 %) of occurrence. The results also revealed that T. viride and P. chrysogenum significantly inhibited the pathogens on petri-plates using dual-confrontational assay. The phytochemical profile of the bioagents indicated there were flavonoids, cardiac glycosides, phenols, alkaloids, tannins, saponins and steroids present. Metabolites of the bioagents against the pathogens indicated that T. viride recorded the highest MIC against Fusarium oxysporum 77±1.0 and the highest MFC against Aspergillus wentii 97±1.0. Also, P. chrysogenum recorded the highest MIC against Fusarium oxysporum 59±1.0 and the highest MFC against Fusarium oxysporum 74.33±1.52. This study indicated the antagonistic potentials of using Trichoderma viride and Penicillium chrysogenum in controlling pathogens of citrus sinensis and this could be exploited further in formulating biopesticides to improve post-harvest qualities of Citrus.*

Keywords — Postharvest spoilt oranges, phyto-constituents, *Moringa oleifera*, antifungal agents, ethylacetate metabolites

1. INTRODUCTION

Citrus is a globally important perennial fruit crop, with a production quota of more than 124 million tons, cultivated in more than 140 countries thus making it the largest fruit crop [52].

Sweet orange (*Citrus sinensis*) is classified into rutaceae; the fruits are highly edible and they vary in morphology [41]. Although sweet orange is a perishable fruit, it has worldwide acceptability because of its value-added therapeutic properties and high nutrients composition [20]. In addition to its good sensorial characteristics, citrus contains high amount of therapeutic, nutritional and high dietary components that include antioxidants, flavanones, anthocyanins, vitamins, micronutrients, fibre and minerals which are of importance for human well-being [7, 43, 6].

There are various research reports on the benefits of fruit consumption that translates to a low incidence of diseases in man [21]. Consumption of fresh fruits has increased by between 26 – 36 % and exceed the observed increase for fruit that is processed. However, up to 20 % of fruits harvested are destroyed due to spoilage by microbial pathogens every year [6]. Post-harvest fruit spoilage pathogens are the major problem leading to fruit spoilage all over the world, and thus making fruits lose their aesthetic values especially in developing nations with less impressive storage facilities.

Sweet oranges are exposed to a diverse array of microbial diseases that are major as a result of fungi infestations, during the postharvest process of picking, packing, storing and transporting the fruit [26, 16, 54].

Postharvest deterioration and rot caused by various microorganisms is seen as the single most important factor militating against commercial orange production worldwide. Spoilage or rots of orange fruits may occur during the preharvest or postharvest stage and maybe be soft rot, dry or wet rot. Soil-borne pathogenic organisms are the main cause of Pre-harvest rots of oranges. Postharvest losses by fungal rot are sometimes up to 30% in orange fruits.

The ecology and climate of Africa are well suited for the commercial production of citrus.

Nigeria is estimated to produce 930,000 metric tons of citrus annually, mainly sweet oranges [35]. An agrarian developing country like Nigeria, possess the potential and comparative edge to be world-leading producer of citrus.

Nonetheless, these accruable potentials in terms of importance and economic benefits that could be exploited from citrus fruit production are being threatened due to many postharvest fungal diseases. Besides, this problem is further compounded due to improper handling and transportation, also, there are no good facilities for storage. This scenario is favorable to fungi pathogens causing devastating and significant economic losses [50, 4].

The approach that is currently used in combating postharvest diseases of citrus fruits is by applying synthetic fungicides like imazalil, thiabendazole, fludioxonil, pyrimethanil and others [13, 9, 39, 22].

However, there are serious concerns regarding using these synthetic fungicides, since it results to fungi acquiring resistance genes and becoming resistant to these antifungal chemicals, it also results to environmental pollution, it is costly and it also poses health hazards to mankind if some of these chemical residues remain on the orange fruit peels. Hence, the tendency worldwide is an increase in public demand for reduction in the use of fungicide, to produce healthy, and eco-friendly safe “fruits”, without chemical inputs for combating postharvest infestation of fruits. Therefore, a sustainable alternative is the use of microbial-based antagonist for the biocontrol of fruit spoilage by fungal pathogens, and this option appears to be an excellent choice [29, 23, 47].

The biocontrol approach is a natural and ecologically balanced alternative method that involves using microorganisms as antagonists to phytopathogens.

The market demand for biocontrol agents is experiencing continuous growth in recent times due to the drawback associated with synthetic pesticides on the environment [17, 11, 14, 51]. There are still a lot of prospects and opportunities in developing biological control agents that can effectively combat the menace of postharvest spoilage pathogens on economically important fruits like citrus.

Moringa oleifera (Moringaceae) also called the tree of life, is a well-known “plant”, and it is of vital importance globally due to its multifunctional properties; it is edible, medicinal and has industrial value [33]. *Moringa oleifera* plant is mostly found in India and Africa, more so it is an ecologically and therapeutically important “plant”, that is abundant in Nigeria.

The rhizosphere filamentous fungi of *Trichoderma* species and *Penicillium* species have been extensively studied for their plant disease suppression capabilities using different mechanisms among these, their capability in producing antagonistic metabolites (antibiotics, enzymes, proteins) as well as other volatile organic compound. Not only do these processes deployed by these rhizo-competent fungi improve plant growth, but they also stimulate plant respiration, thus enhancing photosynthetic efficiency, and increasing the ability of the plant to withstand environmental and biotic stresses [36, 13, 31].

However, there are few studies on the potential of secondary metabolites obtained from the fungi isolates as biocontrol agents in combating postharvest spoilage pathogens of citrus fruits in Nigeria.

Therefore, this study aimed at evaluating the biological control potential of secondary metabolites of indigenous fungi isolates from rhizosphere of *Moringa oleifera* plant against fungi pathogens of sweet orange (*Citrus sinensis*). This is undertaken to develop eco-friendly and sustainable control measures for combating the scourge of postharvest spoilage of sweet orange in Nigeria.

2. MATERIALS AND METHODS

2.1. Soil sampling

Soil samples were collected into sterile polyethylene bags using soil auger at a depth of 4 – 15 cm from the rhizosphere of *Moringa oleifera* tree at Nigeria Stored Product Research Institute (NSPRI) Ilorin, Kwara State. They were conveyed to the laboratory for isolation of fungi “species” having potential as biological control agents.

2.1.1. Sweet Orange (*Citrus Sinensis*)

A meta-market survey of sweet orange (*Citrus sinensis*) fruits was done at different market in Ogbomoso, Oyo State. Spoiled oranges with rot symptoms and healthy oranges were bought and conveyed quickly to the laboratory for isolation of pathogenic fungi.

2.2. Isolation of biocontrol fungi species

For isolation of fungi species (potential biocontrol agents), a serial dilution technique was employed. This entails adding 1 gram soil sample into 9 ml of sterilized water to obtain a stock suspension of 10^{-1} dilution factor and continued up to 10^{-4} , followed by pour plate culturing method and final incubation at $28 \text{ }^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 1 week. Visual observation of plates was done daily, and a discrete colony counted as the colony formation unit (CFU). Individual colonies isolated were transferred into freshly prepared PDA petri-plates. Sterile McCartney bottles were used in preparing slants using PDA for the storage of axenic cultures at $4 \text{ }^{\circ}\text{C}$ [24, 3].

2.3 Isolation and identification of pathogens causing rot on sweet orange fruits.

The rotten oranges were cut into bits of between 0.5 - 1.0 cm. The bits were then surfaced sterilized by using a 3 % solution of sodium hypochlorite (NaOCl) solution for 2 min. The treated fruit section was rinsed 2 times in sterilized distilled water. The excess water on the surface of the tissues was removed by blotting on sterile absorbent paper, placed into petri-plates that contain PDA and then incubated for 7 d at $28 \pm 2 \text{ }^{\circ}\text{C}$. The identification of fungi was done based on their morpho-colonial and microscopic characteristics [5].

2.4. Pathogenicity test for the isolates of orange rot

The pathogenicity test for each rot fungi isolates was carried out on orange fruits. For this, the protocol as described by [45], involving fruits wound inoculation process was deployed. The control fruits received sterile water inoculation. The inoculated sweet oranges were incubated for 7 d. After which orange spots were subjected to examination for comparing the inoculated and un-inoculated oranges. Following the postulation, as described by Koch, the pathogenicity of the isolate was confirmed by re-isolating the fungus from the inoculated fruits and comparing the symptoms that it exhibits with the organism that was originally isolated [18].

2.5. In vitro biocontrol screening of fungal isolates

The potential biocontrol fungi isolated from *Moringa* trees rhizosphere were evaluated against fungal pathogens using the modified dual culture method [12]. Plugs from the margins of actively growing PDA (antagonists and pathogens) were taken and placed at 10-mm of plate edge in opposite ends into the plate. One plug of each pathogen fungus was maintained as control. The assays were replicated three times. Both the dual and alone cultures incubation was done at $28 \text{ }^{\circ}\text{C}$ for 4 d and measurement of colony diameters (in ml) were taken every 24 hours. Thereafter, percentage inhibition of pathogen fungus was determined by the formula;

$$\text{Inhibition of growth(\%)} = \frac{(M_1 - M_2)}{M_1} \times 100$$

M_1 = Mycelia growth rate of pathogen without biocontrol isolates (control).

M_2 = Mycelia growth rate of the pathogen in the presence of biocontrol isolates.

2.6. Extraction of secondary metabolite from bioagents

Bioagents' exhibiting antifungal potency, was deployed for the production of secondary bioactive metabolites by submerged fermentation. Fresh mycelia of *T. viride* and *P. chrysogenum* were multiplied on PDA petri-plates at $28 \pm 2 \text{ }^{\circ}\text{C}$ for upward of 3 – 6 d. The pure colonies of the fungi were then transferred to the potato dextrose broth and were agitated for 7 – 10 d. The broth was centrifuged to remove the fungal cells and mycelia (clarification). The supernatants were then collected in sterile flasks. A ratio 1:1 mixture of the broth with ethyl-acetate was ensued i.e. 300 ml of ethyl acetate and 300ml of broth was given time to settle for a day. The above protocol was repeated until almost all the metabolites was released into solvent phase and later obtained by the aid of separating funnel. After this step, ethyl acetate extracts were de-moisture using anhydrous sodium sulfate, before further drying by rotary evaporator to obtain the extracted crude metabolites [44].

2.7. Phytochemical screening of ethyl-acetate extracts

The concentrated extract obtained from the biocontrol agents was screened for phytochemical constituents using the method of [49]. The crude extracts were then reconstituted with 100mL of ethyl-acetate and screened for phytochemical constituent tests as follows:

Alkaloids: By using a single drop of ethyl acetate metabolite spotted on pre-coated TLC plate, and then followed with Dragendorff's reagent spray. The presence of alkaloids moiety is indicated with coloration that shows orange red/brown.

Tannins: 2 – 3 ml of ethyl acetate, 10 % alcoholic solution of ferric chloride was added. Coloration that shows dark blue or greenish-grey indicates that tannins are present.

Phenols: 1 drop of the extracted metabolite was initially spotted on filter paper, then phosphomolybdic acid spot follows. The presence of phenols is indicated by the appearance of a blue color.

Saponins: 2 ml of extracted metabolite, mixed with 5 ml of sterile water is shaken and subjected to heat up to boiling. Saponin is present if there is frothing (indicated by creamy color mix bubbles).

Flavonoids: 2 – 3 ml extracted ethyl acetate, a piece of magnesium strips and concentrated acid (HCl) addition. Flavonoids are present if the solution appeared as color red or pinkish-red.

Phlobatanins: 1 ml of extract is added with a solution of 2 % HCl solution. Phlobatanins are present if there is a red precipitate.

Steroids: 1 ml of extracted ethyl acetate metabolite is added to 1 ml of chloroform, then anhydrous acetic acid and finally drops of sulphuric acid, that is concentrated is added. Steroids are present by appearance of darkish green colour.

Terpenoids: 1 ml of ethyl acetate extract, is added to a few drops of sulphuric acid, acetic acid and chloroform. When the colour indicates darkish-pink, terpenoids are present.

Cardiac Glycosides: Hydrolyzation of the extract with HCl is followed by neutralization with a solution of NaOH. A mixture of Fehling's solution A and B was then added. Glycosides are present if reddish precipitates appear.

2.8. Antagonistic effects in vitro of the metabolites from *trichoderma viride* and *penicillium chrysogenum*

The antagonistic ability of the metabolites produced by *P. chrysogenum* and *T. viride* was evaluated in vitro against postharvest pathogens of orange. Crude extracts were assessed by plate and broth assays.

2.8.1. Plate assay

The biofungicidal metabolites were incorporated into PDA at the following increasing concentrations: 25 mg/ml, 50 mg/ml, 100 mg/ml and 200 mg/ml. The solidified media plates were inoculated with 9 mm mycelial discs of orange pathogenic fungi; incubation was done at 28 ± 2 °C for 7d. Petri plate containing the pathogen alone without the extracts, serves as a control. The bio-control activity was determined by the pathogen radial rate of growth in the presence of the metabolites compared with the mycelia rate of pathogen growth in the absence of extract.

2.8.1.1. Broth assay

Each biofungicidal metabolite was incorporated into potato dextrose broth (PDB) flasks at: 25 mg/ml, 50 mg/ml, 100 mg/ml and 200 mg/ml concentrations. This was then inoculated with 5 mm mycelial discs (each flask had 4 discs) of orange pathogenic fungi and incubated at 28 ± 2 °C for 7 d. Harvesting of the pathogen and estimation of its growth was done using the method of dry mycelial weight quantification.

2.8.2. Determination of mic and mfc of the biocontrol metabolites

The microdilution method according to [52] was performed to evaluate the MIC and MFC of the biocontrol metabolites.

2.8.2.1. Determination of minimum inhibitory concentration (mic)

To determine the MIC, the broth dilution protocol was used. Concentrations of the biocontrol metabolites ranging from 10.0 mg/ml to 100 mg/ml were used. 0.5 ml of each concentration of the extracts was dispensed to 9 ml of PDB containing 0.5 ml of each extract concentration was distributed to 9 ml of PDB containing 0.5 ml of the fungal pathogenic test organism.

Controls were equally set up by using solvents and test organisms without the treatments. The test tubes are aerobically incubated at 28 ± 2 °C for 72 h. Controls were also formed without the treatments by using solvents and test species. The tube that has the least concentration, and showing no intensity or any form of growth is the minimum inhibitory concentration (MIC).

2.8.2.2. Determination of minimum fungicidal concentration (mfc)

The tubes used in evaluating Minimum Inhibitory Concentration (MIC), and that displayed no turbidity or any growth sign was used to determine the MFC. 0.5 ml aliquot sample was dispensed into PDA petri-plate surface respectively as inoculum, and incubation was done at $28 \pm 2^\circ\text{C}$ for growth. The least concentration treatment, which inhibited fungal pathogen was taken as the (MFC).

2.9. Data analysis

The experiment was done with complete randomization design. Data from the study were evaluated using analysis of variance (ANOVA) and the Fishers Least Significant Difference (FLSD) was deployed in separating means at 5 % significant level.

3. RESULTS

3.1. Isolation and identification of biocontrol agents

Four (4) fungi species were isolated in this study, out of this, two isolates were used for further analysis after the preliminary antagonistic screening. The two isolates gave a very high biocontrol activity compared to the other isolates found. Isolated colonies on PDA plates were characterized and sub-cultured to obtain pure cultures. The two (2) biocontrol fungi isolates were identified morpho-culturally and microscopically and the result indicated that the biocontrol agents were *Trichoderma viride* and *Penicillium chrysogenum*. This is shown in (Table 1 and Figure 1)

Table 1: COLONIAL, micro-morphological characteristics of biocontrol isolates.

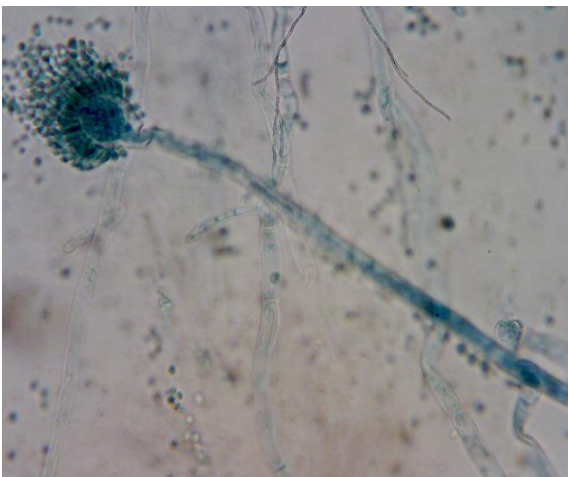
S/No	Microscopic characteristics	Macroscopic characteristics	Fungus species
1	Conidiophores were erect, compact, wooly and penicillate branched. Conidia ($3.0 \times 2.6 \mu\text{m}$, Length-L and Width-W) hyaline, globose, to sub shape like an oval and smooth-walled.	They grow very well on all media compare to other species of <i>Trichoderma</i> . Its initial colour on PDA plate was whitish (1 - 2 days), then turned light green and watery in the centre.	<i>Trichoderma viride</i>
2	Conidia length and wide is ($2.8 - 2.5 \mu\text{m}$, L x W) smooth finely roughened, and subglobose conidia shape. The Phialides has a flask shape.	They possessed yellow-orange colour on PDA which later change to pale brown colour on the plate.	<i>Penicillium chrysogenum</i>



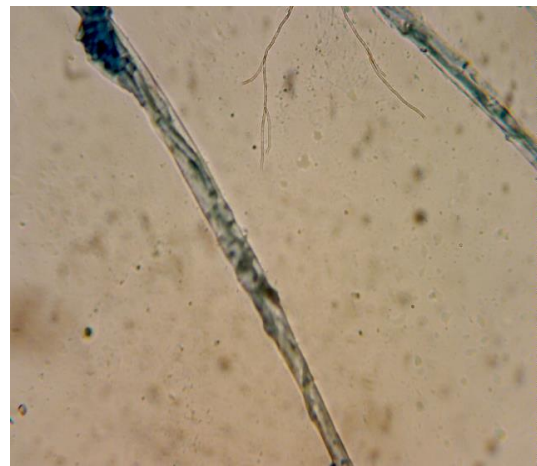
Penicillium chrysogenum



Trichoderma viride



Microscopic observation of *Penicillium chrysogenum*



Microscopic observation of *Trichoderma viride*

Figure 1: MACROSCOPIC and microscopic observation of the biocontrol agents

3.2. Isolation of pathogens fungi causing storage rots on sweet oranges (*Citrus sinensis*)

Isolation was made from orange fruit that had shown spoilage/rot disease symptoms. Morphological and cultural identification of the pathogens were carried out. The following fungi isolate: *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Mucor species*, *Aspergillus wentii*, *Penicillium digitatum*, *Fusarium oxysporum*, *Candida tropicalis*, *Aspergillus tropicalis*, *Saccharomyces cerevisiae* were identified. The occurrence rate of each spoilage organisms from the orange fruits showed that *Penicillium digitatum* (43 %) had the highest incidence of spoilage organism, while *Aspergillus wentii* (25 %), *Fusarium oxysporum* (20 %), *Candida tropicalis* (4 %) showed moderately high level of occurrence while others showed low-level of occurrence. This result is shown in (Table 2; Figures 2 and 3).

Table 2: COLONIAL, micro-morphological characteristics of Fungi causing spoilage of orange fruits.

S/No	Cultural features	Micro-morphological characteristics	Fungal species
1	Colonies with loose white to yellow mycelium, rapidly turning dark brown and eventually black on the development of conidia.	Vesicles were light yellow-brown. Phialides growing radially along the periphery of vesicles. Primary phialides and secondary phialides are both brown	<i>Aspergillus niger</i>
2	The colonies are usually yellowish brown to black.	Conidia are one celled, smooth or rough walled, hyaline pigmented and produced in long chain.	
3	The texture of the colonies is wooly to cottony to granular.	Conidiophores smooth tinted greenish, up to 300 µm long, 20-30 µm in diameter. Hyphae are septate and hyaline.	<i>Aspergillus flavus</i>
4	The colony appeared wooly resembling cotton.	<i>Mucor</i> has broad and aseptate hyphae. Sporangiophores are long, branched and terminate in sporangia (20 - 300 µm). <i>Penicillium mycelia</i> were arranged very irregular and asymmetrical with branches of various lengths. Its conidia are smooth and relatively short. Sparse and irregular metulae with Phialides on them.	<i>Aspergillus fumigatus</i>
5	Colonies were velvety, greenish yellow.	They produced microconidia on richly branched conidiophores and oval in shape.	<i>Mucor species</i>
6	The mycelium grew white with sparse floccose.	They are cylindrical facilitating phialides growing along whole periphery. Primary phialides and secondary vesicles are both brown	<i>Penicillium digitatum</i>
7	Colonies with loose white to yellow mycelium rapidly to brown and dark brown on the development of conidia.	They possessed hyphae (pseudo-hyphae) and no germ tube production.	<i>Fusarium oxysporum</i>
8	Yeast size is 4 – 6 x 10 µm. The colony is dark blue.	They form bud (yeast).	<i>Aspergillus wentii</i>
9	Colonies are white to cream smooth, globose and yeast like in nature.	They form bud (yeast). Budding yeast (blastoconidia), 3.0 -10.0 x 4.5 - 21.0 µm. The diameter is 2 - 8 µm and length is 3 – 5 µm.	<i>Candida tropicalis</i>
			<i>Saccharomyces cerevisiae</i>

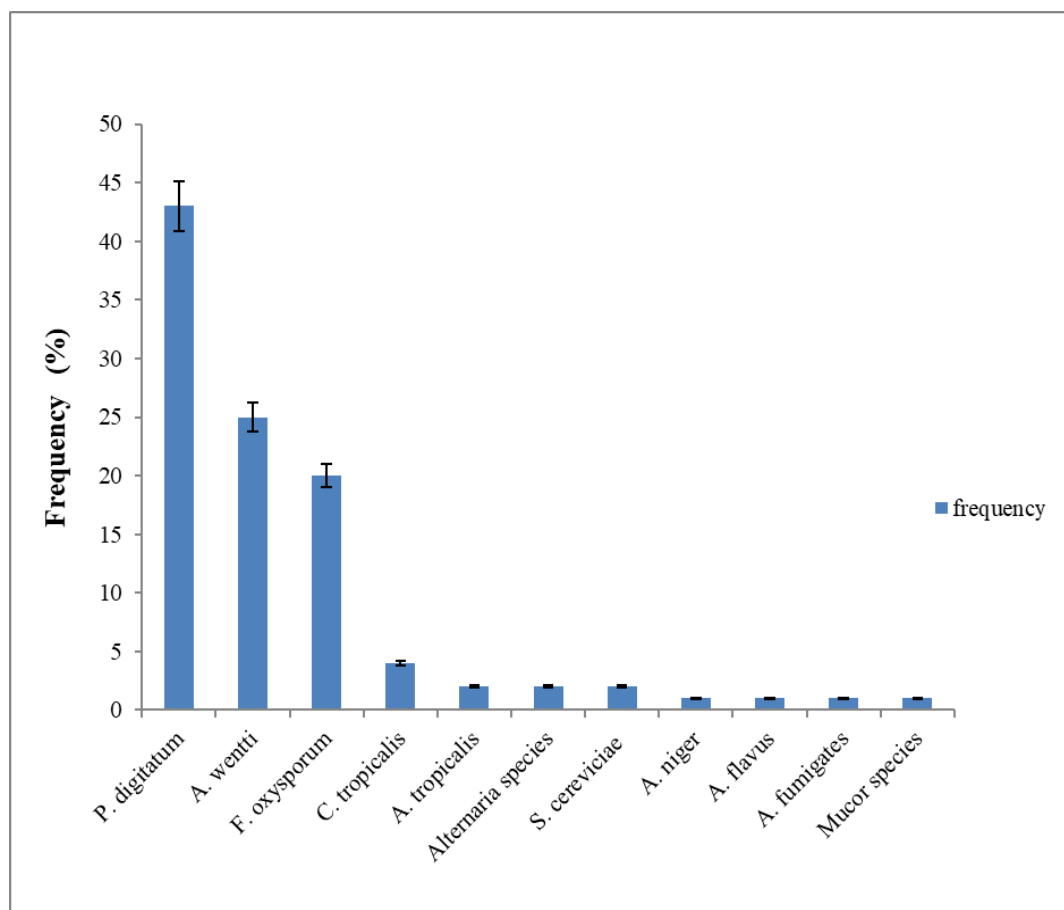


Figure 2: FREQUENCY of occurrence of spoilage fungi causing rot of orange fruits.

3.1.1 Pathogenicity tests of fungal isolates of fruits

The pathogenicity tests of the most frequent spoilage fungi of orange fruits organisms on orange fruits, showed that the highest percentage of pathogenicity corresponded to *P. digitatum* ($83 \pm 2.0\%$), followed by *Aspergillus wentii* ($76 \pm 1.0\%$) and *Fusarium oxysporum* ($62 \pm 1.1\%$) and the least percentage pathogenicity obtained was in *Candida tropicalis* ($54 \pm 1.5\%$) as shown in (Figure 3).

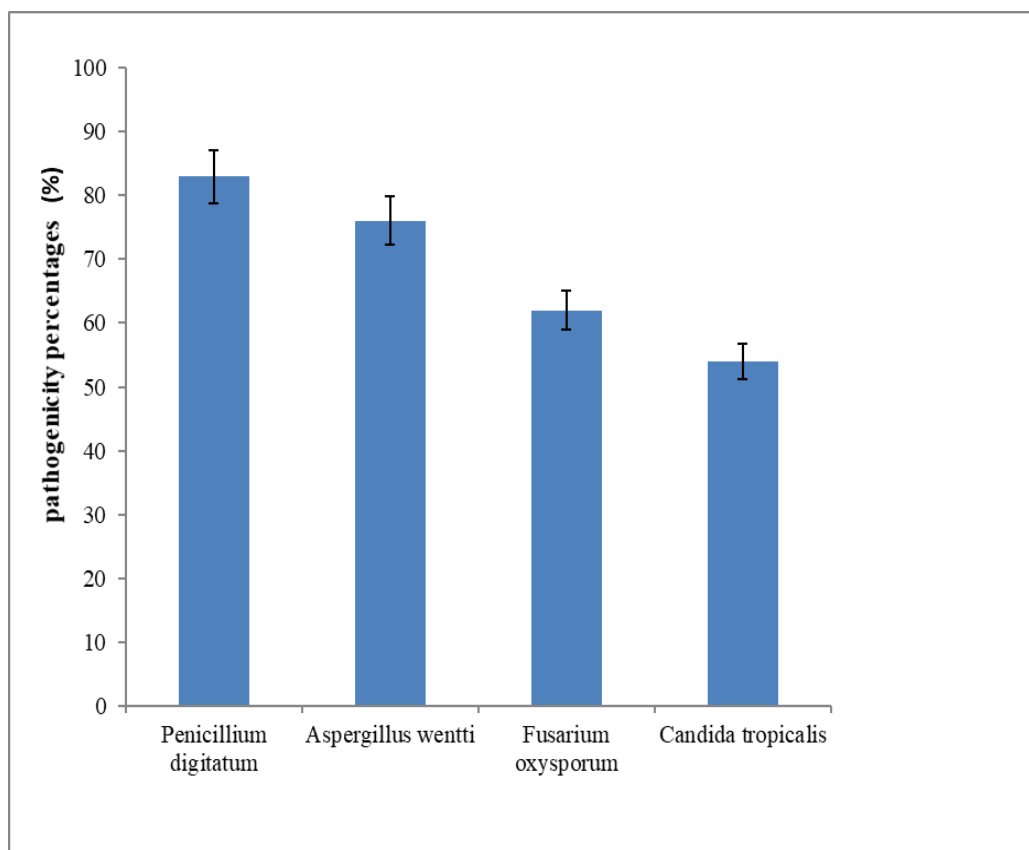


Figure 3: PATHOGENICITY percentage of the spoilage fungal in inoculated orange fruits after 7 d.

3.3. *In vitro* biocontrol screening of rhizospheric fungi

The dual culture confrontational assay method was used in screening the biocontrol ability of rhizospheric fungus *T. viride* against pathogens fungi of orange. The results showed that the highest radial growth was recorded against *A. wentii* (4.2 ± 0.15 cm), followed by *F. oxysporum* (4.0 ± 0.17), *P. digitatum* (3.8 ± 0.06 cm) and the lowest was *C. tropicalis* (2.6 ± 0.2 cm). This consequently means that *T. viride* had the best inhibition effect on *C. tropicalis*. More so, the result of *P. chrysogenum* against the spoilage organism followed the prior order with the highest radial growth being recorded for *A. wentii* (3.9 ± 0.18 cm), followed by *P. digitatum* (3.1 ± 0.34 cm) and the lowest was *C. tropicalis* (2.4 ± 0.17 cm). This also means that *P. chrysogenum* had the best inhibition effect on *C. tropicalis*. This was statistically significant as shown in (Table 3).

Table 3: ANTAGONISTIC effects of the biocontrol agents against citrus spoilage fungi.

Biocontrol	Radial mycelia growth (cm)			
	<i>A. wentii</i>	<i>P. digitatum</i>	<i>F. oxysporum</i>	<i>C. tropicalis</i>
<i>T. viride</i>	4.2 ± 0.15	3.8 ± 0.06	4.0 ± 0.17	2.6 ± 0.2
<i>P. chrysogenum</i>	3.7 ± 0.26	2.4 ± 0.26	1.7 ± 0.15	2.1 ± 0.3
Control	6.0 ± 0.0	4.8 ± 0.11	6.3 ± 0.01	3.4 ± 0.02
Mean	3.9 ± 0.18	3.1 ± 0.34	2.85 ± 0.5	2.4 ± 0.17
P level	***	***	***	***

Values are mean of three replications. *** = Mean squares significant at $P < 0.05$, Ns = Mean squares not significant ($P < 0.05$)

3.4. Phytochemical screening of metabolites isolated from biocontrol isolates

The screening was carried on the crude extract of the two biocontrol agents *Penicillium chrysogenum* and *Trichoderma viride*. The results of the detection tests of the metabolites isolated from *P. chrysogenum* and *T. viride* are as shown in (Table 4).

Table 4: PHYTOCHEMICAL screening of metabolites isolated from biocontrol isolates

Secondary metabolites	<i>Trichoderma viride</i>	<i>Penicillium chrysogenum</i>
Alkaloids	+++	++
Flavonoids	+++	+++
Phenols	+++	+++
Tannins	+	+
Cardiac glycosides	--	--
Steroids	++	+
Saponins	++	+
Terpenoids	--	--
Phlobatannins	+	+

KEY: +++: Largely present ++: Moderately present +: Present: -Absent.

3.5 Antifungal inhibitory activity of metabolites from crude extract

Antimicrobial activity of the crude extract obtained from *P. chrysogenum* and *T. viride* was carried out against rot pathogens from orange fruits using agar well diffusion method. The antifungal inhibitory activities of *T. viride* secondary metabolite was investigated using varying concentration. The inhibitory activities of *T. viride* extracts at 200 mg/ml concentration showed the highest inhibition against all the pathogens having 52.1 ± 0.1 mm mycelial growth of pathogen *F. oxysporum*, *A. wentii* (43 ± 1.0 mm), *P. digitatum* (23.3 ± 0.2 mm) and *Candida tropicalis* (14.6 ± 0.2 mm). The lowest inhibition of 4.33 ± 0.15 mm was observed against *Candida tropicalis* at 25 mg/ml. The result is illustrated in Table 5.

Also, antifungal inhibitory activity of the extracted crude metabolite of *P. chrysogenum* against spoilage organisms are shown in Table 6. At 200 mg/ml concentration, *P. chrysogenum* had the highest inhibitory activities against *F. oxysporum* with 46.5 ± 0.1 mm, *P. digitatum* (38.6 ± 0.2 mm), *A. wentii* (29.2 ± 0.1 mm) and *Candida tropicalis* (22.7 ± 0.1 mm). The lowest inhibition of 1.17 ± 0.15 mm was also observed against *Candida tropicalis* at 25 mg/ml. Generally, these results revealed that there was variation in the antimicrobial activity of the crude extract of the bioagents at different concentrations.

Table 5: ANTIFUNGAL effect of secondary metabolite of *Trichoderma viride* on citrus rot pathogens.

Pathogens	Zones of Inhibition (mm)				
	200 mg/ml	100 mg/ml	50 mg/ml	25 mg/ml	Control
<i>Aspergillus wentii</i>	43 ± 1.0^b	26.0 ± 1.0^b	11.13 ± 0.15^c	8.0 ± 0.2^b	--
<i>Penicillium digitatum</i>	25.3 ± 0.2^c	12.47 ± 0.31^c	9.6 ± 0.2^d	6.3 ± 0.2^c	--
<i>F. oxysporum</i>	52.1 ± 0.1^a	32.17 ± 0.29^a	16.0 ± 0.2^b	10.6 ± 0.1^a	--
<i>Candida tropicalis</i>	14.6 ± 0.2^d	10.2 ± 0.1^d	72.0 ± 0.2^a	4.33 ± 0.15^d	--

-- = No inhibition; Values are means \pm standard deviations. Means with the same superscripts within the same column are not significantly different ($P < 0.05$).

Table 6: ANTIFUNGAL influence of metabolite of *Penicillium chrysogenum* on citrus rot pathogens.

Pathogens	Zones of inhibition (mm)				
	200 mg/ml	100 mg/ml	50 mg/ml	25 mg/ml	Control
<i>Aspergillus wentii</i>	29.2 ± 0.1^c	13.43 ± 0.21^c	9.23 ± 0.15^c	4.3 ± 0.1^b	--
<i>Penicillium digitatum</i>	38.6 ± 0.2^b	21.13 ± 0.15^b	11.23 ± 0.15^b	8.6 ± 0.1^a	--
<i>F. oxysporum</i>	46.5 ± 0.1^a	38.3 ± 0.1^a	14.2 ± 0.1^a	3.33 ± 0.15^c	--
<i>Candida tropicalis</i>	22.7 ± 0.1^d	12.43 ± 0.1^d	9.10 ± 0.1^c	1.17 ± 0.15^d	--

-- = No inhibition; Values are means \pm standard deviations. Means with the same superscripts within the same column are not significantly different ($P < 0.05$).

3.6. Fungicidal inhibitory minimum and maximum concentration of biocontrol secondary metabolites.

The (MIC and MFC) of *T. viride* and *P. chrysogenum* crude extract against the spoilage organisms were determined. *T. viride* crude extract metabolite strongly inhibited *Candida tropicalis* at the lowest MIC and MFC value of 23.67 ± 1.5 mg/ml and 54 ± 0.11 mg/ml respectively. The highest MIC value was obtained against *Fusarium oxysporum* (77 ± 1.0 mg/ml), while the highest MFC was obtained against *Aspergillus wentii* (97 ± 1.0 mg/ml).

Also, the MIC and MFC results obtained from antifungal inhibitory activities of secondary metabolites of *Penicillium chrysogenum* against citrus rot pathogens indicated that *Candida tropicalis* had the lowest MIC and MFC value of 17.67 ± 1.8 mg/ml and 43.33 ± 1.6 mg/ml respectively. Meanwhile, the highest MIC and MFC value of 59 ± 1.0 mg/ml and 74.33 ± 1.52 mg/ml respectively were obtained against *Fusarium oxysporum*. This result is shown in (Table 7).

Table 7: FUNGICIDAL inhibitory minimum and maximum concentration from secondary metabolite of *T. viride* and *P. chrysogenum* on citrus pathogens

rot pathogen	<i>T. viride</i> metabolite		<i>P. chrysogenum</i> metabolite	
	MIC (mg/ml)	MFC (mg/ml)	MIC (mg/ml)	MFC (mg/ml)
<i>Aspergillus wentii</i>	69 ± 1.0^b	97 ± 1.0^a	39 ± 1.0^b	53 ± 1.0^c
<i>Penicillium digitatum</i>	44 ± 2.0^c	64 ± 1.0^c	41.67 ± 1.53^b	61 ± 1.0^b
<i>Fusarium oxysporum</i>	77 ± 1.0^a	82 ± 1.0^b	59 ± 1.0^a	74.33 ± 1.52^a
<i>Candida tropicalis</i>	23.67 ± 1.5^d	54 ± 0.11^d	17.67 ± 1.8^c	43.33 ± 1.6^d
P level	***	***	***	***

Values expressed as mean \pm standard error of mean (SEM) *** = Means square significant at $P < 0.05$

4. DISCUSSION

This investigative experiment involves two indigenous fungal isolates viz *Trichoderma viride* and *Penicillium chrysogenum* from the rhizosphere of *Moringa oleifera* plants that were investigated for their potentials as biocontrol agents against rot pathogens of sweet orange fruit. Different fungi were isolated from the rotten fruit of *Citrus sinensis*. Frequency of occurrence and percentage pathogenicity values of fungi are considered to be the most prevalent fruit spoilage agents world-wide as reported by past researchers [10,1,32,48,38].

The fungal specie *Aspergillus niger* is implicated in experiments done by [2,37], had the highest prevalence in terms of citrus spoilage. Also, in a study on pathogenic fungi associated with orange fruits spoilage of some selected fruit garden, [25] reported *Aspergillus niger* as having the highest incidence of occurrence among fungal implicated in orange fruits spoilage. However, the obtained results in our investigation differs a bit from findings indicating that *Penicillium digitatum* was the most pathogenic, closely followed by *Aspergillus niger*.

In this study, only a few isolated fungi are pathogenic when inoculated in healthy oranges. The explanation for this is not clear, but this could be subjected to further studies.

The results of the direct antagonistic confrontational activity of *T. viride* and *P. chrysogenum* against citrus spoilage pathogens confirmed their antifungal inhibitory ability. It has been suggested that rhizo-competent rhizospheric organisms like *T. viride* and *P. chrysogenum* use different mechanisms to parasitize other phytopathogenic fungi. The various mechanisms that they deployed over phytopathogens include; directly antagonizing pathogens, producing antibiosis, inducing resistance and inactivation of the different pathogen's enzyme. Also, well established is that *Trichoderma species* can secrete potent bioactive metabolites like trichodermol, trichodermin, and harzianolide, which helps in reducing the harmful effect of pathogens.

Our results obtained in this study using rhizospheric *T. viride* and *P. chrysogenum* as biocontrol agents on orange pathogens fungi are in agreement with the observed result in the study of [15], that reported in a study that several *Trichoderma spp* and *Penicillium spp* are considered to be highly antagonistic to other phytopathogenic fungi such as *A. niger*, *A. flavus*, and *S. cerevisiae*.

The rapid growth of *T. viride* and *P. chrysogenum* against the pathogenic fungi in this study was probably a result of high competition for space and nutrients with the pathogenic fungi. Both fungi species are agro-biotechnologically important microorganisms, that are applied as an antagonist against phytopathogenic fungi. Although the two bioagents were isolated from the rhizosphere of the highly beneficial moringa plant, they are free-living fungi and are mostly found in the diverse ecological domain like forest, agriculture, desert soil, marsh, and planted crop soil. Therefore, they are readily available for utilization as biocontrol agents [40]. Other studies by [46,42], reported that

diverse *Trichoderma* species were effective in suppressing phytopathogens. Moreover, *T. viride* was reported as biological control agent against *P. digitatum* causing green rots of citrus

The phytochemical analysis of extracts obtained from *T. viride* and *P. chrysogenum* obtained in our study is similar to the reported phytochemical profile in studies by [8,28]. This phyto-constituents' presence may be the reason for the antifungal inhibitory potency of the bioagents against the citrus pathogens [34].

The ethylacetate secondary metabolites derived from the rhizospheric fungi isolates assessed for their antifungal ability showed effectiveness in controlling the post-harvest deterioration caused by the citrus rot pathogenic fungi.

The lowest MIC and MFC values for both two extracts were obtained against *Candida tropicalis*, which means that *T. viride* and *P. chrysogenum* are potent agent that can be used to inhibit *Candida tropicalis*. While the highest MIC value was obtained against *Fusarium oxysporum*, and the highest MFC was obtained against *Aspergillus wentii*, which means *T. viride* has poor inhibition effect against them. Meanwhile, the highest MIC and MFC value was obtained against *Fusarium oxysporum* by *P. chrysogenum*, which means that *Penicillium chrysogenum* has poor inhibitory action against *Fusarium oxysporum*.

Our results obtained in this study concerning MIC and MFC of the metabolites of *Trichoderma viride* and *Penicillium chrysogenum* against citrus spoilage organisms confirmed the antifungal antagonistic efficacy of these indigenous bioagents against sweet orange pathogens fungi: thus, these results are similar to obtained results by [27] *Trichoderma harzianum* was investigated as an antagonist against different spoilage organisms isolated from different sources of spoilt fruits with varying degrees of inhibition and MIC ranges from 100 - 150 µl / ml. The results showed that *T. harzianum* was an effective biocontrol agent. In addition to this, in a report of work done by [30] on the potentials of using extracellular secondary metabolites from *Penicillium* species to inhibit fungi pathogens, it was established that the metabolites were effective as an antifungal against fungi phytopathogens.

5. CONCLUSION

The highlighted results from our study, proved that *T. viride* and *P. chrysogenum* can successfully reduce the level of post-harvest spoilage of citrus fruits. Both biocontrol agents showed high inhibition against the isolated pathogens causing fruit rot of sweet orange. This study is an addition to existing knowledge that indigenous rhizospheric fungi of *Moringa oleifera* plants can be a source of antifungal secondary metabolites that can be used to formulate biopesticide to control citrus spoilage pathogens.

However, there is a need for further research to determine the bioactive metabolites responsible for the antifungal activity and characterize their structure.

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7. REFERENCES

1. Akinmusire, O.O. (2011). Fungal species associated with the spoilage of some edible fruits in Maiduguri northern eastern Nigeria. *Adv Environ Biol.* 5(1):157–61
2. Akintobi, A.O, Okonkwo, I.O, Agunbiade, S.O, Akano, O.R, Onianwa, O (2011). Isolation and identification of fungi associated with the spoilage of some selected fruits in Ibadan, South-western Nigeria. *Academ. Arena* 3(11):1–10
3. Babu, K.N., Pallavi, P.N., 2013. Isolation, identification and mass multiplication of *Trichoderma* an important bio-control agent. *Int. J. Pharm. Life Sci.* 4(1): 2320-2323.
4. Ballester, A.R., Izquierdo, A., Lafuente, M. T., and Gonzalez-Candalas, L. (2010). Biochemical and molecular characterization of induced resistance against *Penicillium digitatum* in Citrus fruit. *Postharvest Biol. Technol.* 56(1), 31-38.
5. Barnett, H. L. and Hunter, B. B. (2003). *Illustrated Genera of Imperfect fungi* 3rd edition Burges Publishing Company, Minnesota. 56:45-50
6. Barth, M., Hankinson, T. R., Zhuang, H., and Breidt, F. (2009). Microbiological Spoilage of Fruits and Vegetables. In *compendium of the microbiological spoilage of foods and beverages*. pp 135-183. Springer, New York, NY.
7. Bello, O. B, Habib, U., Olawuyi, O.J, Opeyemi A.S, Alase, A.H., Owoade, T.A. (2016). Microorganisms causing post-harvest tomato (*Solanum lycopersium* L.) fruit decay in Nigeria. *J. Entomol. Zool. Stud.* 4:374-377.
8. Bhardwaj, A., Sharma, D., Jadon, N., and Agarwal, P.K., 2015. Antimicrobial and phytochemical screening of endophytic fungi isolated from spikes of *Pinus roxburghii*. *Arch. Clin. Microbiol.* 6(3), 1-9.
9. BI, Y (2016). *The principle and control of postharvest diseases of fruits and vegetables*, science press, Beijing, china, in Chinese.
10. Bukar, A, Mukhtar, M.O, Adamu, S. (2009). Isolation and identification of postharvest spoilage fungi associated with sweet orange (*Citrus sinensis*) traded in Kano metropolis. *Bayero J Pure Appl. Sci.* 2(1):122–124.
11. Butt, T. M., Jackson, C. W. and Magan, N. (eds). (2001). *Fungi as Biological Control Agents: Progress, Problems and Potential*. CAB International. Wallingford, U. K. 54-62.

12. Chan, Z and Tian, S. (2005). Interaction of antagonistic yeasts against postharvest pathogens of apple fruit and possible mode of action. *Postharvest Biol. Technol.* 36, 215-223
13. Chen, J., Shen, Y., Chen, C., and Wan, C. (2019). Inhibition of key Citrus postharvest fungal strains by plant extracts *in vitro* and *in vivo*: A review. *Plants*, 8(2), 26. Doi: 10.3390 / plants 8020026.
14. Dinham, B. (2005). Agrochemical markets soar – pest pressures or corporate design? *Pesticides News*, **68**: 9–11.
15. Doley, K., and Jite. P.K (2012). *In vitro* Efficacy of *Trichoderma viride* against *Sclerotium rolfsii* and *Macrophomina phaseolina*. *Not. Sci. Biol.* **4**(4):39-44.
16. Droby, S. (2005). Improving quality and safety of fresh fruit and vegetables after harvest by the use of biocontrol agents and natural materials. In international symposium on natural preservatives in food systems, **709**: 45-51.
17. Droby, S., Wisniewski, M., Macarasin, D. and Wilson, C. (2009). Twenty years of postharvest biocontrol research: is it time for new paradigm? *Postharvest Biol. Technol.* **52**: 137-145.
18. Faarrag, S. H. E. (2011). First record of *Cercospora* leaf spot disease on okra plants and its control in Egypt. *Plant Pathol. J.* **10**(4): 175 – 180.
19. Farrar K, Bryant D, Cope-Selby N. (2014). Understanding and engineering beneficial plant-microbe interactions: plant growth promotion in energy crops. *Plant Biotechnol. J.* 12(9) 1:193–206. Doi: 10.1111/pbi.12279.
20. Gorinstein, S., Martin, B. O., Park, Y., Haruenkit, R., Lojek, A., Milan, I., Caspi, A., Libma, I. and Traktenberg, S. (2001). Comparison of some biochemical characteristics of different citrus fruits. *Food Chem.* **74**(3): 309-315.
21. Hung, H. C., Joshipura, K. J., Jiang, R. H., Hunter, D. and Smith, S.A. (2004). Fruits and Vegetable intake and the risk of major chronic disease. *J. Natl Cancer Inst.* **95**:157- 164
22. Ismail, M. and Zhang, J. (2004). Postharvest citrus diseases and their control. *Outlooks of pest management*, **15**: 29 – 35.
23. Jamalizadeh. M, Etebarian. H.R., Amirian. H, and Alizadeh. A. (2011). A review of mechanisms of action of biological control organisms against Post-harvest fruit spoilage”, *EPPO Bulletin*, 41(1), 65-71.
24. Kator, L., Kalu, O.J., and Oche, O.D. (2015). Assessing the Biocontrol Potential of *Trichoderma* species on Sclerotia rot disease of Tomato Plants in Chile Island (Makurdi). *IOSR J. Environ. Sci. Toxicol. Food Technol.* **9**:51-58.
25. Khan, A., Iram, S., Rasool, A. (2015). Pathogens identification and characterisation that compromised citrus fruit quality in selected orchards of Sargodha. *Int. J. Environ. Sci. Toxicol. Res.* 3(4):54–59.
26. Ladanyia, M., and Ladanyia, M. (2010). *Citrus fruit: Biology, Technology and Evaluation*; Academic Press: San Diego, CA, U.S.A.
27. Leelavathi, M.S., Vani, L., and Reena, P. (2014). Antimicrobial activity of *Trichoderma harzianum* against bacteria and fungi. *Int. J. Curr. Microbiol. App. Sci.* 3(1): 96-103.
28. Leylaie, S., and Zafari, D. (2018). Antiproliferative and antimicrobial activities of secondary metabolites and phylogenetic study of endophytic *Trichoderma* species from vinca plants. *Front. Microbiol.* 9
29. Liu, P., Chen, K., Li, G., Yang, X., and long, C.A., (2016). Comparative transcriptional profiling of orange fruit in response to the biocontrol yeast *Kloeckera apiculata* and its active compounds. *BMC Genomics*, 17(1), 17.
30. Mejdoub-Trabelsi B, Rania Aydi Ben A, Nawaim A, Mejda Daami R (2017) Antifungal Potential of Extracellular Metabolites from *Penicillium* spp. and *Aspergillus* spp. Naturally Associated to Potato against *Fusarium* species. Causing Tuber Dry Rot. *J. Microb. Biochem. Technol.* 9:181-190. Doi: [10.4172/1948-5948.1000364](https://doi.org/10.4172/1948-5948.1000364)
31. Mendes R, Garbeva P, Raaijmakers J.M. (2013). The rhizosphere microbiome: significance of plant beneficial, plant pathogenic and human pathogenic microorganisms. *FEMS Microbiol. Rev.* 37(5) 6:34–63. Doi: 10.1111/1574-6976.12028
32. Mohammed SSD, Kumar N, Damisa D, Bala E, Muhammad IL, Muhammad RG. (2013). Fungi associated with decayed sweet oranges (*Citrus sinensis*) collected from Lapal, Niger State, Nigeria. *Indian J. L. Sci.* 2(2):123–31.
33. Moyo, B., Masiaka, P. J., Hugo, A. and Muchenje, V. (2011). Nutritional characterization of Moringa (*Moringa oleifera* Lam.) leaves. *Afr. J. Biotechnol.* **10**: 12925 – 12933.
34. Muthu, C., M. Ayyanar, N. Raja and S. Ignacimuthu, (2006). Medicinal plants used by traditional healers in Kanchipuram district of Tamil Nadu, India. *J. Ethonobiol. Ethnomed.* **2**: 43-43. Doi: [10.1186/1746-4269-2-43](https://doi.org/10.1186/1746-4269-2-43)
35. Olife IC, Ibeagha OA, Onwualu A.P (2015). Citrus fruits value chain development in Nigeria. *J Biol. Agric. Health.* 5(4): 36-47.
36. Omomowo I.O, Akinmusire, O.O, Omomowo, I.O. Olaifa, K.W and Fadiji, A.E. (2018). Biocontrol potential of endophytic fungi from Moringa oleifera against tomato postharvest rot fungi (*Solanum lycopersicum* L.). *Research Journal of Science* Volume 18(1): 96-105.
37. Onuorah S, Obika I, Okafor U. (2015). Filamentous fungi associated with the spoilage of post-harvest sweet orange fruits (*Citrus sinensis*) sold in Awka major markets, Nigeria. *Bioeng. Biosci.* 3(3): 44–9.
38. Oviasogie, F. E., Ogofure, A. G., Beshiru, A., Ode, J. N. and Omeje F. I. (2015). Assessment of fungal pathogens associated with orange spoilage. *Afr. J. Microbiol. Res.* **9** (29): 1758-1763.
39. Palou, L., Smilanick, J.L. and Droby, S. (2008). Alternatives to conventional fungicides for the control of citrus postharvest green and blue moulds. *Stewart Postharvest. Rev* 4, 1–16.
40. Pandya, J. R., Sabalpara A. N. and Chawda, S. K. (2011). *Trichoderma*: A particular weapon for biological control of phytopathogens. *J. Agr. Technol.* 7: 1187–1191.

41. Parle, M., Chaturvedi, D. (2012). Orange: Range of benefits. *Int. Res J. Pharm* 3(7): 59-63
42. Pathak, A.K., Muralia, S. and Pathak, S. (2007). *Biocontrol of plant diseases*. Jaipur, Aavishkar Publisher, India, 89-111.
43. Platiana, C, Restuvccia, C, Muccilli, S and Cirvilleri, G. (2012) “Efficacy of killer yeasts in the biological control of *Penicillium digitatum* on Tarocco orange fruit (*Citrus sinensis*). *Food Microbiol.* 30(1) 219-225.
44. Ramesha, M. and Srinivas, A. (2014). Pathogenicity and Pectinase Activity of Some Facultative Mycoparasites Isolated from *Vicia faba* diseased leaves in Relation to Photosynthetic Pigments of Plant. *J. Plant Pathol. Microbiol.* 3(6): 1-7.
45. Rivera-Vargas, L.I., Y. Lugo-Noel., R.J. McGovern., T., Seijo and Davis, M.J., 2006. Occurrence and distribution of *Collectotrichum* spp. On mango (*Mangifera indica* L.) in Puerto rico and Florida, USA. *Plant Pathol. J.*, 5: 191-198.
46. Sharfuddin, C. and Mohanka, R. (2012). In vitro antagonism of indigenous *Trichoderma* isolates against phytopathogen causing wilt of lentil. *Int. J. L. Sci. Pharma Res.* 2: 195–202.
47. Sharma, R.R., Singh, D., and Singh. R., (2009). “Biological control of Postharvest diseases of fruits and vegetables by microbial antagonists: a review”, *Biological control*, vol 50, no. 3, pp. 205-221.
48. Tafinta, I.Y., Shehu, K., Abdulganiyu, H., Rabe, A.M., Usman, A. (2013). Isolation and identification of fungi associated with the spoilage of sweet orange (*Citrus sinensis*) fruit in Sokoto State. *Nig. J. Basic Appl. Sci.* 21(3):193–6.
49. Tapwal, A., Purnima, P. and Suresh, C. R. (2015). Antimicrobial activity and phytochemical screening of endophytic fungi associated with *Cassia fistula*. *Int. J. Chem. Biol. Sci.* 2(7):2349–2724.
50. Terao, D., de Lima Nechet, K., Ponle, M.S., de Almeida Halfeld-Vieira, B., de Holanda Nunes Naia, A., de Almeida Angos V.D. (2017). “Physical post-harvest treatments combined with antagonistic yeast on the control of orange green mold. *Sci. Hortic.* 224, 317-323.
51. Verma, M., Brar, S. K., Tyagi, R. D. (2007). Antagonistic fungi, *Trichoderma* spp. Panoply of biological control. *Biochem. Eng. J.* 37: 1–20.
52. Wani, A.H. (2011). An overview of the fungal rot of tomato. *Mycopath.* 9(1): 33-38.
53. Xu Q, Chen, L.L., Ruan, X., Chen, D., Zhu, A., Chen, C., Bertrand, D., Jiao, W., Hao, B., Lyon, M.P. (2013). The draft genome of sweet orange (*Citrus sinensis*), *Nat. Genet.* 45 (1):59–68.
54. Zhu, S. J., Noureddine, B. and Norio, S. (Eds.) (2006). Non-chemical approaches to decay control in postharvest fruit. In: *Advances in Postharvest Technologies for Horticultural Crops*. Research Signpost, Trivandrum, India, pp. 297–313.