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## Antimicrobial Potential of *Azadirachta indica* A. Juss Seed Oil Against Seed-Borne Pathogens of *Tectona grandis* L.f.

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### ABSTRACT

The antimicrobial potential of *Azadirachta indica* seed oil against seed-borne pathogens of *Tectona grandis* at different concentrations (2%, 4%, 6%, 8%, and 10%) was investigated. *T. grandis* seeds were obtained from the seed bank of FRIN Ibadan and taken to the Plant Pathology Laboratory of Forestry Research Institute of Nigeria Ibadan, for the isolation of fungal pathogens. The seeds were disinfected by dipping in sodium hypochlorite and placed on Potato dextrose agar (PDA) to isolate pathogenic fungi. Botanical oil extract was prepared from *Azadirachta indica* seed. The seed-borne fungi isolated were *Fusarium oxysporum*, *Curvularia lunata*, *Rhizopus nigricans*, *Aspergillus niger*, and *Trichoderma viride*. Results of the antifungal assay showed that neem seed oil extract at various concentrations inhibited the mycelial growth of the test organisms. However, the inhibitory effects of the oil extract increased with higher concentrations. The percentage inhibition for each organism at different concentrations varies, the highest percentage inhibition for *Trichoderma viride* was 100% at 6% concentration, *Rhizopus nigricans* was 100% at 8% concentration and *Cuvularia lunata* was 95.23% at 10% concentration respectively. This experiment, therefore, concludes that essential oil from neem seed contains secondary metabolites having the potential to serve as bio-fungicides in the management of *Trichoderma viride*, *Rhizopus nigricans*, and *cuvularia lunata*, seed-borne pathogens of *Tectona grandis* seeds.

### INTRODUCTION

*Tectona grandis* L.f. (teak) belongs to the family, Lamiaceae, It's one of the most important timbers in the world due to its outstanding properties (Robertson, 2002; Amadi and Saka, 2011). It is a fast-growing species and grows to about eighty-five meters (85m). The plant is distributed in parts of India, Myanmar, Thailand and West Africa. It has been widely established in plantations as an exotic species for producing high-quality poles and timber.

One of the challenges in establishing a teak plantation is the non-availability of healthy and viable seeds due to various pests and diseases (Robertson, 2002; Roychoudhury and Rajesh, 2021).

Seed-borne pathogens or Forest-tree seed diseases are primarily caused by fungi, some fungi are saprophytic in nature and have no effect on the quality of seed. However, most seeds contaminated with *Aspergillus* spp., *Bipolaris maydis*, *Fusarium* spp., *Penicillium* spp., and *Rhizopus* spp have led to low-field emergence, reduced crop vigour, increased seedling diseases, and low productivity (Gyasi *et al.*, 2020). Some seed-borne pathogens have the potential to cause diseases in plants such as damping-off, shoot dieback and cankers (Gupta *et al.*, 2017; Pedraza *et al.*, 2018).

The use of chemical fungicides as a seed treatment to tackle seed-borne pathogens is a common practice and this has been effective in reducing seed-borne pathogens and even improving seed germination (Nene *et al.*, 1969; Yorinori, 1994). However, extensive, and improper use of these fungicides can have adverse effects on the environment, humans, and animals and the development of fungicide-resistant pathogens (Hahn, 2014). There is a need for a natural antifungal agent with little or no safety concerns. The use of bio-fungicidal extracts against phytopathogens is recommended as an alternative to chemical fungicides (Amadi *et al.*, (2010); Badawy and Abdelgael (2014).

*Azadirachta indica* A. juss known as neem is an evergreen tree of large dimension which belongs to the family Meliaceae. It is a native of India and Burma but is now extensively grown throughout Nigeria, especially in Borno, Kano, and Sokoto states, where it is used as ornamental and shades.

The plant has been reported to have antimicrobial and pesticidal efficacy that has been demonstrated on a wide spectrum of insects and some pathogenic fungi (Roychoudhury, 2016). Over 300 phytochemicals and diverse chemicals have been extracted and isolated from different parts of this tree (Gupta *et al.*, 2017). The essential oil from neem is used as an

antipyretic, natural insecticide, antimicrobial, and antimalarial agent and for the treatment of leptospirosis (Akeel, 2017; Del Serrone, 2015). Thus, this work aimed to identify the seed-borne mycoflora of *T. grandis* and to also determine the antifungal potential of neem oil on the seed-borne pathogens isolated from *T. grandis*.

## MATERIALS AND METHODS

### Study Area:

The research was carried out at the Plant Pathology Laboratory of Forestry Research Institute of Nigeria (FRIN), Ibadan, Oyo state located on longitude 03°51'20" E to 03°53'43" E and latitude 07°23'18" N to 07°23'43" N. Neem seeds were collected from Ejigbo in Ejigbo Local Government Area of Osun state, Nigeria. The matured seeds were harvested during July and August 2021. *T. grandis* seeds were obtained from the seed bank of FRIN Ibadan.

### Seed Samples:

*T. grandis* seeds were examined for impurities such as plant debris, sclerotia, and gall sand and symptoms such as discolouration and malformation. The seeds were disinfected by dipping in 1% sodium hypochlorite solution for 3-5 minutes and rinsed in 3 changes of sterile distilled water. After this, the seeds were kept in the laminar flow for 5-10mins to air dry (Chrapacien *et al.*, 2023)

### Isolation and Identification:

The method of seed health testing recommended by the International Seed Testing Association (ISTA, 2014) uses the blotter method. 100 seeds were randomly selected from the prepared disinfected seeds and plated in three layers of moistened filtered paper in 9cm diameter sterilized Petri plates at 5 seeds per plate. The Petri plates were incubated for 7 days at  $25 \pm 2^\circ\text{C}$  in the laboratory, filter papers were aseptically moistened with sterile distilled water. After 7 days, the plates were examined, and the percentage of seeds infested, and different colonies observed were documented. Mycelia growths were

aseptically transferred to Petri-dishes containing potato dextrose agar (PDA) for purification and identification. The isolated fungal pathogens were identified morphologically and microscopically using standard methods (Jurgen *et al.*, 1978; Barnett and Hunter, 1987; Labbe and Garcia 2001). The percentage incidence was calculated using the formula.

$$\text{Incidence \%} = \frac{\text{number of organisms}}{\text{total number of organisms}} \times 100 \text{ ----- (1)}$$

#### Preparation of Neem Oil:

The extraction of oil was carried out at the chemistry laboratory, Federal University of Agriculture, Abeokuta, Nigeria. Neem seeds were manually sorted, dehulled, and winnowed before drying. One thousand and five hundred grams (1500g) of dried neem seeds were pulverized into fine particles using a blending machine. The grounded sample was placed in a conical flask and soaked with n-hexane for 24 hrs. The n-hexane-oil mixture was sieved through muslin cloth and heated with a regulated water bath at 70°C. The extract was separated from the n-hexane through the distillation method (Oladipo and Betiku, 2019). After extraction, 20 ml of neem oil was recovered. The chemical properties in terms of peroxide, saponification, iodine, and acid values were already identified (Hussein *et al.*, 2021).

#### Bioassay:

The neem oil was diluted using the formula  $C_1V_1 = C_2V_2$  to obtain 2%, 4%, 6%, 8%, and 10% concentrations. The different neem oil concentrations were added to the PDA medium after cooling to 45°C, gently agitated, and allowed to solidify. Mycelia discs of *Curvularia lunata*, *Rhizopus nigricans*, and *Trichoderma viride* were prepared using a sterilized 5 mm diameter cork borer from 7 days cultures and placed at the centre of the 9cm PDA dishes supplemented with neem seed oil. Each

treatment was replicated 4 times, plates containing PDA media without neem oil served as control. The radial growths of the mycelia were measured and recorded at 7 days after inoculation (DAI) when the mycelia growth from control had covered the plates. The percentage of mycelia growth inhibition was calculated using the formula described by Choudhary *et al.*, (2017) thus:

$$I = \frac{C-T}{C} \times 100 \text{ ----- (2)}$$

Where, I= Percent Inhibition

C= Colony diameter in the Control plate

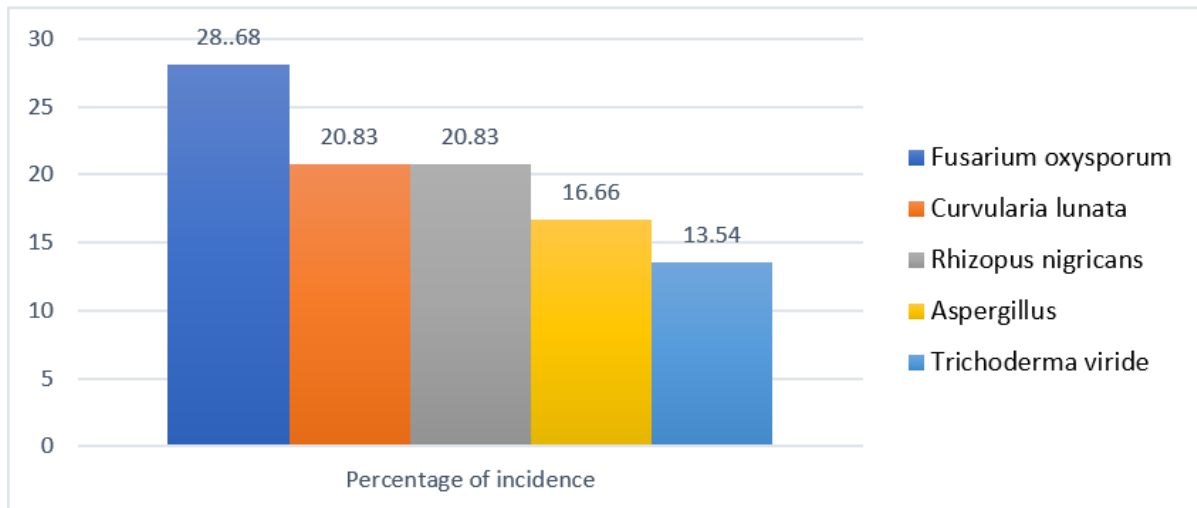
T= Colony diameter in Treated plate

#### Statistical Analysis:

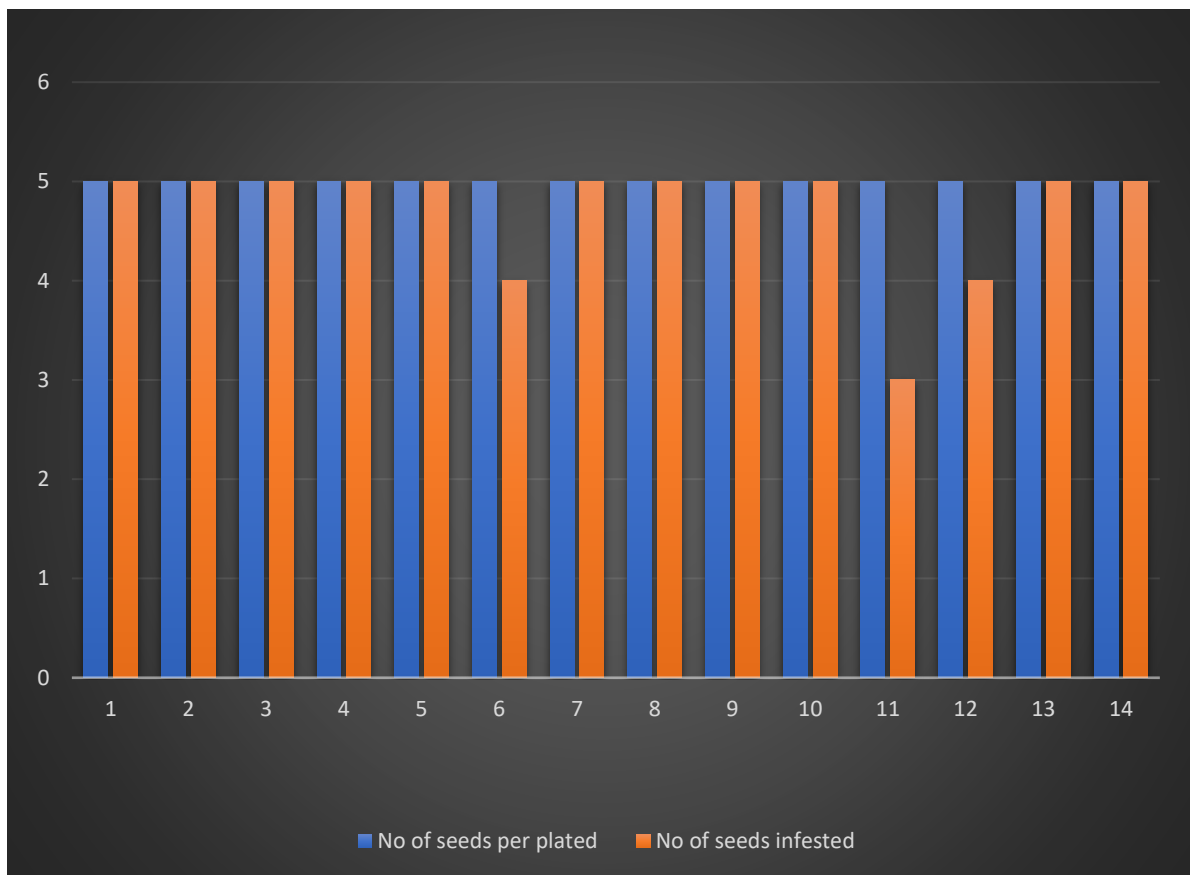
The data collected were subjected to analysis of variance (ANOVA) followed by Duncan's Multiple Range Test to separate the treatment means at  $P < 0.05$  using GenStat Discovery Edition 4.

#### RESULTS

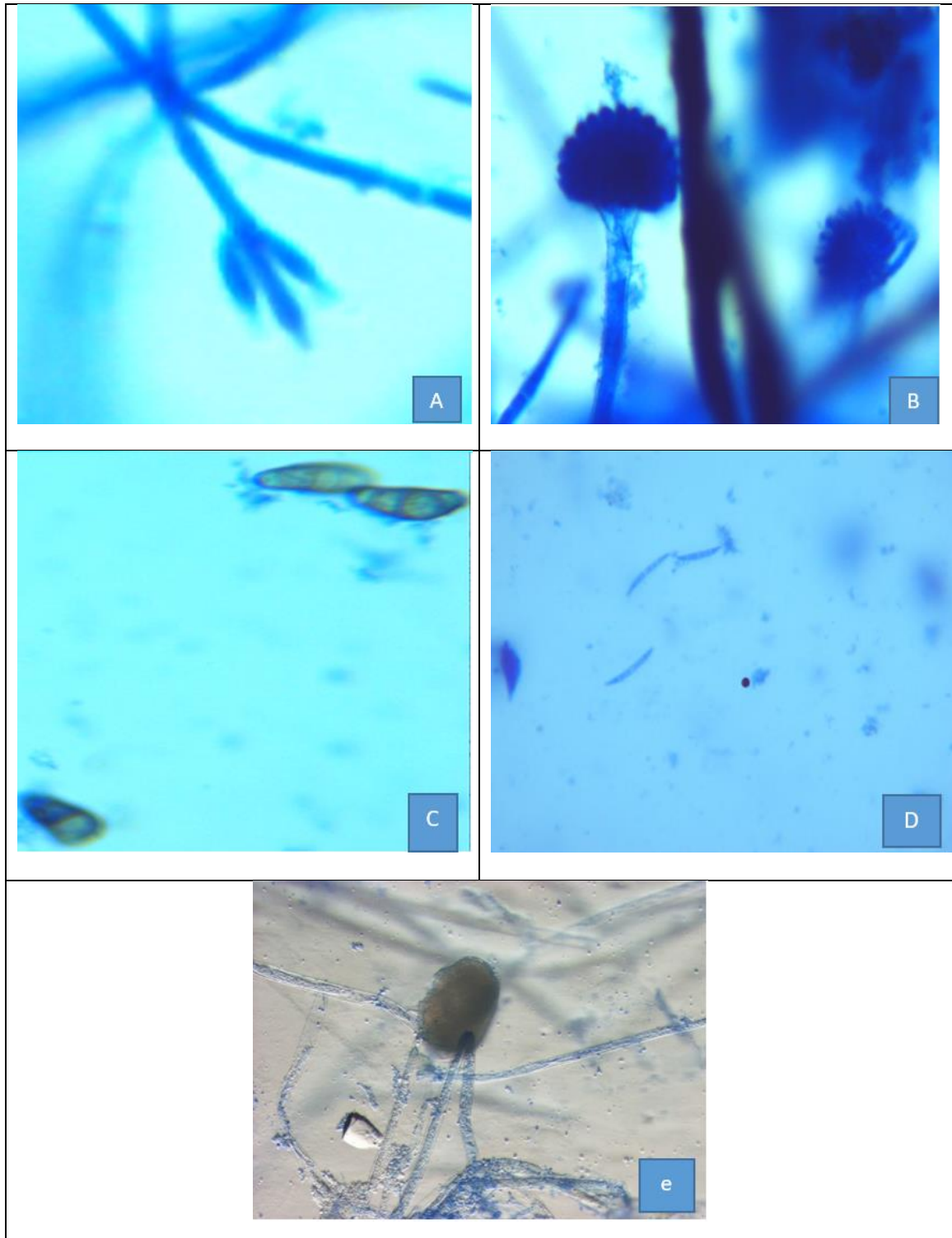
After 7 days of incubation, 5 fungal species were isolated and identified from *T. grandis* seeds as shown in Figure 3. The results of percentage incidence show that *Fusarium oxysporum* has the highest percentage incidence of 28.68 %, followed by *Curvularia lunata* at 20.83% and *Rhizopus nigricans* at 20.83%, *Aspergillus niger* at 16.66% and *Trichoderma viride* with 13% as shown in Figure 1. However, Figure 2 shows that 96% of *T. grandis* seeds tested were infested with seed-borne pathogens. The mean effect of different conc. of neem oil, on the inhibition of the three test pathogens as shown in Table 1, shows that among the 6 concentrations of neem oil screened, 10% and 8% inhibited more than 98% and 88% of the growth of the organisms, which indicate a high antifungal activity. The least concentration is 2% with 15.11%.



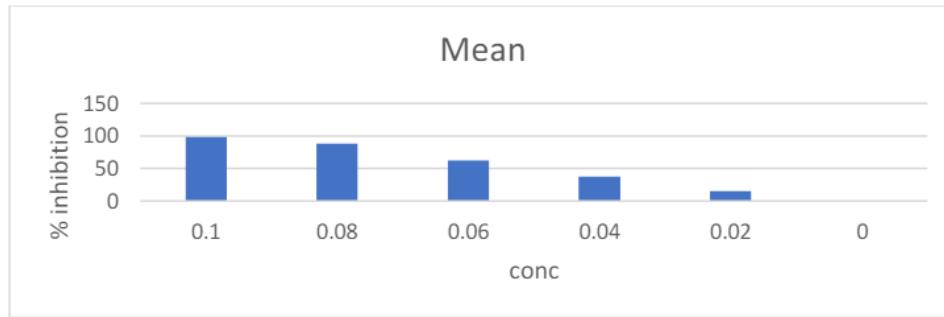
**Fig .1:** Percentage incidence of seed-borne pathogens isolated from *T. grandis*.



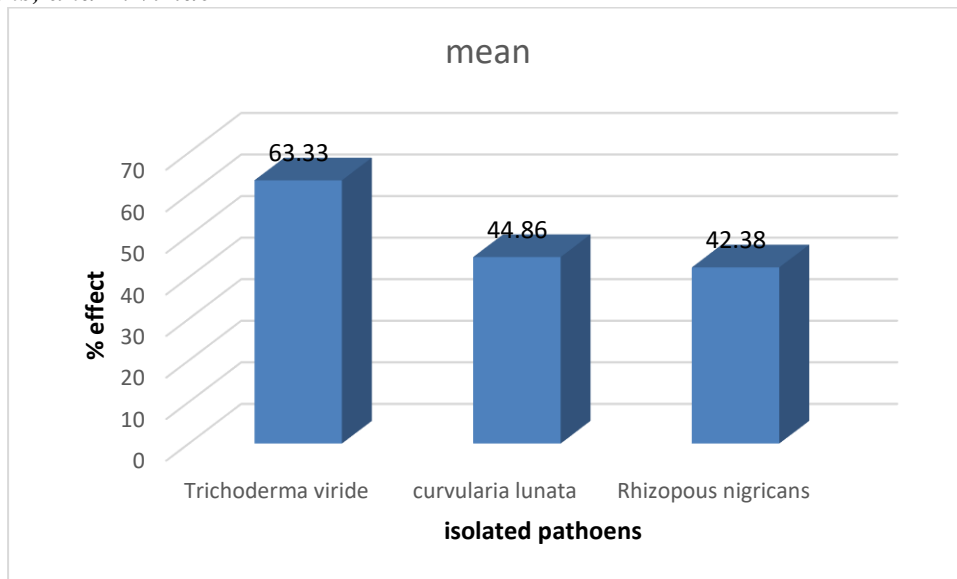
**Fig. 2:** Showing the number of infested *T. grandis* seeds per plate.



**Plate 1.** Photomicrograph of *T. grandis*. A: *T. viride*. B: *Aspergillus spp.* C: conidia of *C. lunata*. D: conidia of *F. oxysporium*. E: *Rhizopus spp*



**Fig. 3:** Mean effect of different concentrations of neem oil on the inhibition of *C. lunata*, *R. nigricans*, and *T. viride*



**Fig. 4:** Mean effect of neem seed oil on *C. lunata*, *R. nigricans*, and *T. viride*

**Table 1:** Effect of different concentrations of neem oil on *C. lunata*, *R. nigricans*, and *T. viride*

Oil Conc %	Pathogens	% inhibition
0	Cuvularia	0 <sup>h</sup>
0	Rhizopous	0 <sup>h</sup>
0	Trichoderma	0 <sup>h</sup>
2	Cuvularia	17.6 <sup>f</sup>
2	Rhizopous	7.72 <sup>g</sup>
2	Trichoderma	20 <sup>f</sup>
4	Cuvularia	36.42 <sup>d</sup>
4	Cuvularia	36.42 <sup>d</sup>
4	Rhizopus	16.6 <sup>f</sup>
4	Trichoderma	60 <sup>bc</sup>
6	Cuvularia	55.25 <sup>c</sup>
6	Rhizopus	29.98 <sup>e</sup>
6	Trichoderma	100 <sup>a</sup>
8	Cuvularia	64.65 <sup>b</sup>
8	Rhizopus	100 <sup>a</sup>
8	Trichoderma	100 <sup>a</sup>
10	Cuvularia	95.23 <sup>a</sup>
10	Rhizopus	100 <sup>a</sup>
10	Trichoderma	100 <sup>a</sup>

Means in columns marked with the same letter are not significantly different at the level  $\alpha = 0.05$ , according to the Newman-Keuls test.

The mean effect of the three test organisms reveals that *T. viride* had the highest percentage inhibition followed by *C. lunata*, and *R. nigricans* as seen in Fig 2. Results on the effect of different concentrations of neem oil on *C. lunata*, *R. nigricans*, and *T. viride* are shown in Table 3. Results show that neem oil produced significant ( $P \leq 0.05$ ) levels of inhibition of mycelia growth of all the test organisms at various concentrations. However, the inhibitory effect on mycelia growth increased with higher concentrations.

The highest (95.23%) percentage inhibition of mycelia growth on *C. lunata* was produced at 10 % concentration. while the least (17.6%) percentage inhibition of mycelia growth was recorded at 2% conc. The highest (100%) percentage inhibition of mycelia growth on *R. nigricans* was produced at a concentration of 8 % conc. while the least (7.2%) percentage inhibition of mycelia growth was recorded at a concentration of 2%. The highest (100%) percentage inhibition of mycelia growth on *T. viride* was produced at concentrations 6, 8, and 10 % respectively while the least (20%) percentage inhibition of mycelia growth was recorded at a concentration of 2%

### DISCUSSION

The study was designed to identify seed-borne fungal genera present in *T. grandis* seeds and evaluate the potential of neem seed oil on the isolates. The isolation and identification procedures using the agar plating method and microscopy revealed an array of fungal seed-borne pathogens in the seed samples collected. Five (5) fungi species were isolated from *T. grandis* seeds namely *F. oxysporium*, *C. lunata*, *R. nigricans*, *A. niger* and *T. viride*. The results for the isolation and identification of seed-borne fungal pathogens associated with *T. grandis* seeds were in agreement with the findings of other scholars (Amadi and Saka 2011; Kamatou *et al.*, 2013). Some of these organisms have been established to cause

diseases in several forest species (Olasupo *et al.*, 2020; Avasthi *et al.*, 2018).

Neem seed oil extracted with n-hexane had been observed to have completely inhibited the growth of *C. lunata*, *R. nigricans*, and *T. viride* at an increased concentration. This can be attributed to the antimicrobial properties of the oil. However, the inhibition observed on *R. nigricans* contradicts the findings of Adepoju *et al.*, (2014), that petroleum ether extract of neem seed oil affected other test organisms but no positive effect on *Rhizopus spp.* This may be due to the difference in the solvent of extraction used. Nonetheless, several studies have reported the potential of plant extracts and essential oils to inhibit the growth of several pathogens in vitro (Adekunle *et al.*, 2021; Olugbenga *et al.*, 2021; Geosel *et al.*, (2014).

Furthermore, it was observed that at an increased concentration all the test pathogens were inhibited, this was according to the findings of Adekunle *et al.*, (2021) where the antifungal activity of neem leaf extract on *L. theobromae* and *M. phaseolina* biomass shows 73 % and 71% growth inhibition at 50% concentration. These observations are also in agreement with the findings of (Gowda *et al* 2004: and Mariana *et al*, 2019) where it was reported that neem oil inhibited more than 82% of the test pathogens at 0.5% concentration while at 0.1% concentration, low antifungal activity of 52% and 36% was recorded.

Many chemicals and biologically active compounds have been identified from *A. indica* (e.g phytol, octadecatrienoic acid, methyl ester, hexadecanoic acid, methyl ester, etc.) (Hossain *et al.*, 2013) which may be the reason for its fungicidal effect.

### CONCLUSION

It can be concluded that almost all the seeds used were infested with pathogens, which are known to have adverse effects on seed germination and growth. The results of this experiment show that the antifungal



effects of neem oil at an appropriate concentration are very effective in managing *C. lunata*, *R. nigricans*, and *T. viride* isolated from *T. grandis* seeds. This reveals that essential oil from neem seeds possesses antifungal properties which are very effective on fungal pathogens and could be recommended as an alternative to fungicides in seed treatment since they are environmentally safe. Further studies should be encouraged to determine the active ingredient most effective against each specific fungus because knowledge of the fungi will help in controlling this seed-borne mycoflora which will assist in providing a remedy to the loss of genetic resources and management.

**Declarations:**

**Ethical Approval:** It is not applicable.

**Conflicts of Interest:** The author declares no conflicts of interest.

**Authors Contributions:** All authors were responsible for the study design, experiment execution, data analysis, and manuscript drafting.

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**Availability of Data and Materials:** All datasets analyzed and described during the present study are available from the corresponding author upon reasonable request.

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