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Effects of Culture Filtrates of Pathogenic Fungi on Seed Germination and Seedling Indices of Chrysophyllum albidum (G.Don)

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Abstract
This research was to evaluate the effects of culture filtrates of pathogenic fungi on seed germination and seedling indices of *Chrysophyllum albidum* (G.Don). The experiment was carried out at the Mycology Unit, Department of Forestry and Environment, Rivers State University, Nkpolu-Oroworkwo and was laid out in a Completely Randomized Design (CRD) and the treatment was replicated twenty times and the mean value separated using Duncan Multiple Range Test at probability of 5%. The biodeterioration of the fruits were caused by micro-organisms associated with the infected fruits obtained from three markets in Port Harcourt Metropolis. The micro-organisms isolated and identified include; *Apsergillus niger, Rhizopus stolonifer, Pseudomonas aeruginosa.* The results on frequency occurrence of micro-organisms in the three markets revealed that fruits obtained from Mile 1 market, *Rhizopus stolonifer* (8.0% ± 0.21) had the highest frequency occur, Mile 3 market, *Aspergillus niger* (12.0% ± 0.30) had the highest frequency, while from Fruit garden market, *Aspergillus niger* (15.0% ± 0.28) was the highest. Results on the mean percentage of frequency of micro-organisms isolated from infected fruits of *C. albidum* revealed that Mile 3 (51.6%) had the highest mean percentage followed by Fruit garden (48.3%) and Mile 1 (31.6%) was the lowest. Pathogenicity test results revealed that *Trichoderma harziarum* extract (29.5mm ± 0.28) caused significant ($p \leq 0.05$) rot damage on a relatively healthy fruits of *C. albidum* followed by *Aspergillus niger* (26.5mm ± 0.06) and the least were *Pseudomonas aeruginosa* (25.5mm ± 0.31) *and Rhizopus stolonifer* (25.0mm ± 0.25). Results on effect of culture filtrates on seed germination and seedling indices of *C. albidum* revealed that *T. harzianum* (70%) had the highest seed germination percentage and the lowest was *Pseudomonas aeruginosa* (20%). Results on vigour index showed that *T. harzianum* (13.8) had the highest index followed by the control (12.0), *R. stolonifer* (4.8), *A. niger* (4.2) and the lowest was *P. aeruginosa* (1.2). It was reported that culture filtrate of *A.niger* inhibited seed germination and subsequent seedling indices while culture filtrates of *T. harzianum* caused effective increase of seed germination and seedling indices. Results of this research therefore recommended *T. harzianum* extracts for improved seed germination and subsequent seedling performance of *C. albidum.*
Keywords: Culture filtrates, micro-organisms, germination, seedling indices
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INTRODUCTION

Fungal rot of fruits and vegetables is the predominant postharvest disease (Janiseiwicz and Korsten, 2002) and causes huge losses to the growers in terms of yield (Bhale, 2011). Certain fungal pathogens also synthesize certain mycotoxins or metabolites on their hosts which are harmful to the consumers and restrict their growth (Roy *et al*., 1972; Bhale, 2011). These fungal metabolites are substances that are discharged by the fungal species during their metabolic processes. The metabolites can be amino acids, phenols, terpenoids, plant growth regulators (Madhosing, 1995). Certain fungal genera like *Aspergillus, Penicillium, Alternaria, Rhizoctonia* produce mycotoxins and degrade the seed quality and their viability (Caster and Frederikson, 1980). These toxins are known to suppress the germination and sprouting of several seeds (Roy *et al.*, 1972; Kunwar *et al*., 1987). Strains of *Trichoderma spp* used as biological agents are capable of increasing plant growth even in absence of pathogen. The application of *Trichoderma* causes increase in germination, plant height, leaf area and dry weight (Lindsey and Baker, 1967; Inber *et al*., 1996).

Despite their importance, *C. albidum* have been greatly neglected, especially with respect to their regeneration and improvement. The yield of current crop of trees is decreasing due to old age and the fact that they have been harvested for decades. Thus, if the current practice of allowing *C. albidum* to grow in the wild (i.e. natural regeneration) is allowed to continue, the probability of obtaining its much-valued fruit on a sustained basis will be very low. Due to the lack of care and old age, a lot of the trees of the species have died or are in the process of doing so. In Nigeria, *C. albidum* is classified among the endangered tree species with a high possibility of going into extinction in the near future except something is done to conserve the species or increase their population, hence the use of culture filtrates to improve germination and seedling indices becomes very paramount.

The study was aimed at assessing the effects of fungi culture filtrates on seed germination and seedling indices of *Chrys*ophyllum albidum.

Specific objectives of this study are to;

1. isolate and identify fungi from diseased fruits of *Chrysophyllum albidum.*
2. determine the effect of fungi culture filtrates on seed germination and seedling indices of *C. albidum.*
3. evaluate the pathogenicity of fungi culture filtrates on the relatively healthy fruits of *C. albidum.*

MATERIALS AND METHODS

The Study Area

This research was carried out in the laboratory of the Department of Forestry and Environment (Forest Pathology and Mycology unit), Faculty of Agriculture, Rivers State University, Nkpolu-Oroworukwo, Port Harcourt, Rivers State, Nigeria, located at Latitude 4.5°N and Longitude 7.0°E, on an elevation of 18m above sea level, a mean annual temperature of 27°C and rainfall of 2000 - 2467mm (Chukunda, 2014).

Source and Collection of African Apple Fruits

A total of two hundred (200) standardized ripe African star apple fruits of average weight 20g were purchased from three different local markets; Mile 3, Mile 1 and Fruit garden all in Port Harcourt Metropolis, Rivers State, Nigeria. The fruit samples selected were at the peak of their freshness. The fruits were packaged with black polythene bags and brought to the Forestry Laboratory (Mycology Unit) for further analysis and studies. The fruits were washed with potable water, sorted to distinguish infected and un-infected fruits.

Isolation and Identification of Microorganisms

Infected fruits of *Chrysophyllum albidum* were surface sterilized for 2 minutes with 70% ethanol and rinsed twice in sterile distilled water. The infected parts were cut into small pieces (3 x 4mm) in size with the help of sterilized knife. The pieces were placed in Petri dishes, replicated 20 times and incubated at room temperature 27°C for 5 – 7 days. Fungi isolates were sub-cultured on PDA and identified based on their morphological characteristics (Garuba *et al*., 2014; Chukunda *et al*., 2019). The fungi were examined and the disease incidence (frequency) was calculated. The percentage of diseased fruit parts per total number of fruits incubated. Percentage disease incidence was identified and calculated using the formula of (Mamatha *et al*., 2000; Chukunda *et al*., 2019).

$$D_I = \frac{D_o}{D} \times \frac{100}{1} \quad \text{…… …… Equation I}$$

Where;

D_I = Disease incidence

D_o = Number of diseased fruits

D = Total number of fruits plated

For bacterial isolation, infected portions were transferred into sterile distilled water from which serial aliquot of 0.1ml of dilution was plated on nutrient agar (NA) and incubated at 37°C for 24 hours, discrete colonies were observed and further sub-cultured. The pure isolates were characterized and identified using the method of (Holt *et al*., 1994). Infected parts of *Chrysophyllum albidum* (Plate 2).

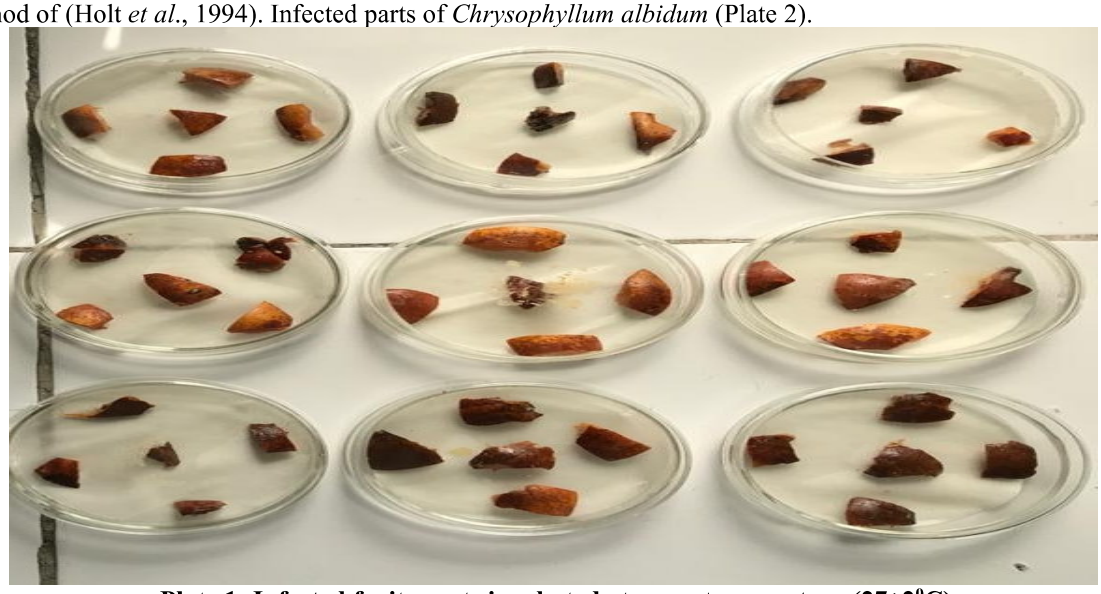


Plate 1: Infected fruits parts incubated at room temperature (27±2^{0}C)

Preparation of Culture Filtrate

The fungal organisms used in this study were grown on Potato dextrose broth (PSB) of 100ml and were transferred to 250ml conical flasks and inoculated with fungi inoculum taken from the edge of growing hyphae of 7 days old culture grown on PDA medium. The flasks were incubated at 27±2°C for 10 days. The mycelial mat was then removed by filtering through Whatmans's filter paper. The filtrates were centrifuged to obtain a cell free culture filtrates. The filtrate was collected in a pre-sterilized conical flasks and used to determine its effects on seed germination and seedling growth indices (Chukunda *et al*., 2006b).

Effect of Culture Filtrates on Seed Germination and Seedling Indices of *Chrysophyllum albidum*

Seeds of *Chrysophyllum albidum* soaked in culture filtrates. The effect of fungi culture filtrates of fungal pathogens was determined on seed germination. Relatively healthy seeds were selected, and surface sterilized using 70% ethanol and then rinsed with distilled water. Sterilized seeds were soaked in the culture filtrates for 2 hours. These seeds were removed from the culture filtrates and placed on moist filter paper in pre-sterilized Petri dishes. The Petri dishes were then sealed with masking tape and kept at room temperature (27± 2°C). Seed soaked in distilled water served as control. Five (5) replicates were taken for each treatment. Percentage of germination of seeds were observed, recorded a and calculated using the formula;

$$\text{Percentage seed germination} = \frac{\textit{Total number of germinated seeds}}{\textit{Total Number of seeds planted}} \times 100 \quad \ldots\ldots\ldots\ldots\ldots \text{Eqn II}$$

After 14 - 21 days of incubation period germination and subsequent seedling indices were measured using a graduated meter rule to determine the root length and shoot length and then vigour index calculated using the formula of (Suthar *et al*., 2014; Chukunda, 2014).

Vigour Index = Shoot length (cm) + Root length (cm) x Germination percentage ….Equ. III

Pathogenicity Test

Fresh ripe African star apple fruits were purchased from Mile 3 market, Mile 2 and Fruit garden markets in the Port Harcourt metropolis. The fruits were surface sterilized by swabbing with 70% ethanol. A sterile cork borer (5mm in diameter) was used to remove a tissue core from each of the surface sterilized fruit. 2ml of a 3 days old cultures of fungal extracts of the isolates were measured using a syringe and was inoculated in the hole created by scooping out the fruit tissue. The inoculated portion of the fruits were covered with moist cotton wool to maintain relative humidity and incubated at 27°C for 7 days. The inoculated fruits were then enclosed in a container. Five fruits were inoculated per isolate. The extent of rot was determined by measuring the size of infection (Chukunda *et al.*, 2020).

Experimental Design and Statistical Analysis

This experiment was designed in a One – way Analysis of Variance using SPS- 5 version in a Completely Randomized Design (CRD). The treatments were replicated 20 times and mean separated using Duncan Multiple Range Test at 5% probability.

RESULTS

Isolation and Identification of Fungi from Diseased *Chrysophyllum aldidum* Fruits

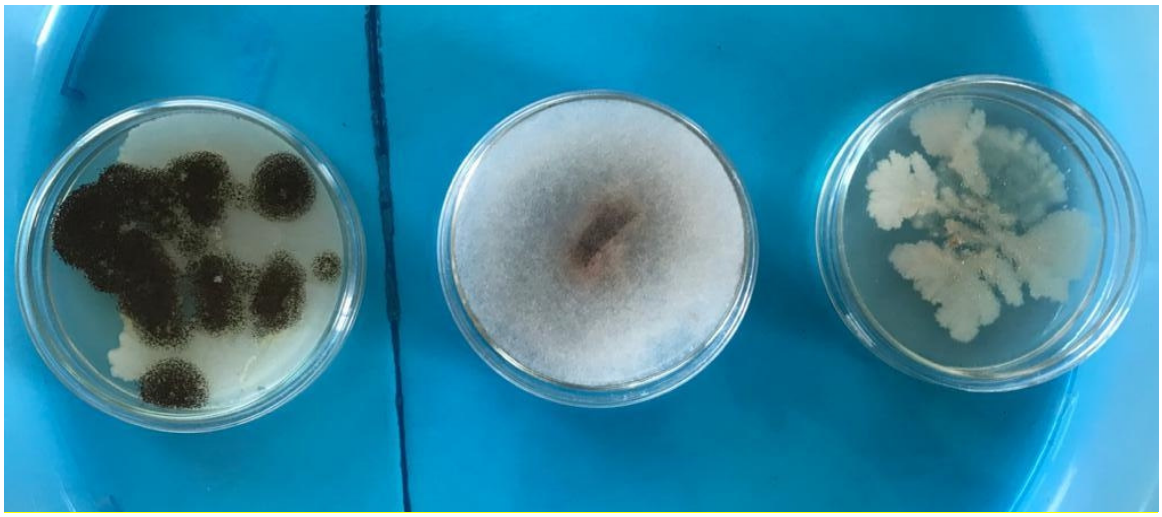
Isolation and identification of fungi from diseased *C. aldidum* are presented in Table 1 and Plate 2. The result showed that three micro-organisms were identified from the diseased fruits of *Chrysophyllum albidum* obtained from the three different markets in Port Harcourt Metropolis, namely; *Rhizopus stolonifer*, *Aspergillus niger*, and *Pseudomonas aeruginosa.* Both *R. stolonifer* and *A. niger* belong to the Fungi family, while *P.* aeruginosa belong to the bacteria family.

In Mile 1 market, *Rhizopus stolonifer* (8.0% ± 0.21) had the highest frequency of micro-organisms isolated from the diseased fruits of *C. albidum* followed by *Aspergillus niger* (6.0 ± 0.35) and *Pseudomonas aeruginosa* had the lowest with (5.0% ± 0.08). In Mile 3 market, *A. niger* (12.0% ± 0.30) had the highest frequency followed by *R. stolonifera* (10.0% ± 0.24) and *P. aeruginosa* (9.0% ± 0.25) was the lowest, while in Fruit garden market, *A. niger* (15.0% ± 0.28) was the highest followed by *R. stolonifera* (8.0 ± 0.21) and *P. aeurignosa* (6.0% ± 0.21) was the least. Results on the mean percentage of the frequency of micro-organisms isolated from infected diseased fruits of *C. albidum* revealed that Mile 3 market had the highest mean percentage of 51.6% followed by Fruit garden market with 48.3% and Mile 1 market was the least (31.6%).

Table 1: **Frequency of Micro-organisms Isolated from Diseased Fruits of *C. albidum***

Micro-organisms	Markets in Port Harcourt Metropolis (Mean ± SD)		
	Mile 1	**Mile 3**	**Fruit Garden**
Rhizopus stolonifer	$8.0^{a} \pm 0.21$	$10.0^{b} \pm 0.24$	$8.0^{b} \pm 0.21$
Aspergillus niger	$6.0^{b} \pm 0.35$	$12.0^{a} \pm 0.30$	$15.0^{a} \pm 0.28$
Pseudomonas aeruginosa	$5.0^{c} \pm 0.08$	$9.0^{c} \pm 0.25$	$6.0^{c} \pm 0.21$
Grand Total Mean (%)	31.6	51.6	48.3

Mean values with the same subscripts (a, b, c..) in the same column are not significantly ($P \leq 0.05$) different using Duncan Multiple Test ($P \leq 0.05$). (n = 20)



Aspergillus niger ***Rhizopus stolonifer*** ***Pseudomonas aeruginoa***

Plate 2: Pure Culture of the Microorganisms Isolated

Effects of Fungi Culture Filtrates on Relatively Healthy Fruits of *Chrysophyllum albidum* Obtained from Port Harcourt Metropolis

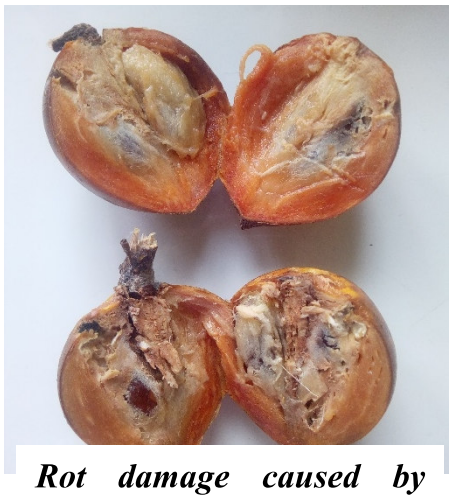
The results on the pathogenicity test of culture filtrates on relatively healthy fruits of *C. albidum* are presented in Table 2 and Plate 2. The results revealed that *Trichoderma harzianum* (29.5mm ± 0.28) extract caused significant ($P \leq 0.05$) rot damage on the healthy fruits followed by *Aspergillus niger* (26.5mm ± 0.06) and the least are *Pseudomonas aeruginosa* and *Rhizopus stolonifer* which had 25.5mm ± 0.31 and 25.0mm ± 0.25 respectively.

Table 2: **Pathogenicity Test of Fungi Culture Filtrates on Relatively Healthy Fruits of *Chrysophyllum albidum***

Micro-organisms	Rot Fungi (mm) Mean ± SD
Rhizopus stolonifer	$25.0^{c} \pm 0.25$
Aspergillus niger	$26.5^{b} \pm 0.06$
Trichoderma harzianum	$29.5^{a} \pm 0.28$
Pseudomonas aeruginosa	$25.5^{c} \pm 0.31$

Mean values with the same subscripts (a, b, c..) in the same column are not significantly ($P \leq 0.05$) different using Duncan Multiple Test ($P \leq 0.05$).

Fruit injected with *Pseudomonas aeruginosa* culture Filtrate



Rot damage caused by Pseudomonas aeruginosa

Fruit injected with *Rhizopus stolonifer* culture Filtrate

Rot damage caused by Rhizopus stolonifer

Fruit injected with *Aspergillus niger* culture Filtrate

Rot damage caused by Aspergillus niger

Plate 3: Pathogenicity Test of Microorganisms

Effect of Fungi Culture Filtrates on Seed Germination and Seedling Indices of *Chysophyllum albidum*

The effect of fungi culture filtrates on seed germination and seedling indices of *C.albidum* are presented in Table 3 and Plate 4. From the results it was observed that seed germination of *Trichoderma harzianum* (70%) significantly ($P \leq 0.05$) higher than Control (60%) followed by *Aspergillus niger* (40%), *Rhizopus stolonifer* (30%) and the last was *Pseudomonas aeruginosa* (20%). Results on root length of the seedlings revealed that *T. harzianum* (9.7cm ± 0.35) was the highest followed by Control (9.2cm ± 0.21), *R. stolonifer* (6.5cm ± 0.08), *A. niger* (4.6cm ± 0.18) and *P. aeruginosa* (2.6cm± 0.012) was the lowest. While the shoot length showed that Control (10.8cm ± 0.28) had the highest shoot length followed by *T. harzianum* (10.0cm ± 0.20), *R. stolonifer* (9.5cm ± 0.25), *A. niger* (5.9cm ± 0.10) and *P. aeruginosa* (3.5cm ± 0.07) was the least. However, the result for vigour index revealed that *T. harzianum* (13.8) had the highest vigour index followed by Control (12.0), *R. stolonifer* (4.8), *A. niger* (4.2) and *P. aeruginosa* has the lowest vigour index of 1.2.

Table 3: Effect of Culture Filtrates on Seed Germination and Seedling Indices of *C. albidum*

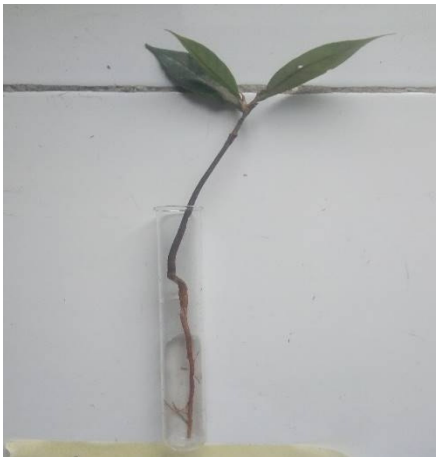
Microorganisms	Seedling Indices (Mean ± SD) cm			
	Seed Germination (%)	Root Length (cm)	Shoot Length (cm)	Vigour Index
Control	60	$9.2^{b} \pm 0.21$	$10.8^{a} \pm 0.28$	12.0^{b}
Trichoderma harzianum	70	$9.7^{a} \pm 0.35$	$10.0^{b} \pm 0.20$	13.8^{a}
Aspergillus niger	40	$4.6^{d} \pm 0.18$	$5.9^{d} \pm 0.10$	4.2^{d}
Rhizopus stolonifer	30	$6.5^{c} \pm 0.08$	$9.5^{c} \pm 0.25$	4.8^{c}
Pseudomonas aeruginosa	20	$2.6^{e} \pm 0.012$	$3.5^{e} \pm 0.07$	1.2^{e}

Mean values with the same subscripts (a, b, c….) in the same column are not significantly ($P \leq 0.05$) different using Duncan Multiple Test ($P \leq 0.05$). (n = 10)

Control

Trichoderma harzianum

Rhizopus stolonifer



Aspergillus niger

Pseudomonas aeruginosa

Plate 4: Effect of Culture Filtrates on Seed Germination and Seedling Indices of *C. albidum*

DISCUSSION

Effect of Culture Filtrates on Seed Germination and Subsequent Seedling Indices of *C. albidum*

In this study, it was reported that the culture filtrates of the pathogenic fungi caused inhibition in germination of seeds as well as seedling growth of *Chrysophyllum albidum*. Similar results were obtained by (Garuba *et al*., 2014; Alwakeel, 2013; Jalander and Gachande, 2012; Haikal, 2008; Kunwar and Mehrotra, 1988) studied the effect of culture filtrate of storage fungi on germination and sprouting of wheat grains and reported that culture filtrate of all fungi species screened inhibited germination and sprouting of grains, with complete inhibition by culture filtrate of *Aspergillus clavatus* and *Penicillium urticae*. Chukunda *et al*. (2006a) investigated on the effects of the culture filtrates of *Macrophomina phaseolina* on Okra seedlings and reported that culture filtrates of *M. phaseolina* reduced germination and vigour performance of the seedlings. Similarly, Umecharuba and Nwachukwa (1997) reported that *Aspergillus niger*, *Aspergillus flavus*, *Fusarium moniliforme* and *Penicillium* sp. produce various metabolites that are that are known to reduce the seed germination percentage and seedling development of bean seeds. Jalander and Gachande (2012) reported that the culture filtrate of *Aspergillus niger* inhibit seed germination of several pulses and cereals. Haikal (2008) reported that the culture filtrate of *Aspergillus niger*, *Fusarium culmorium*, *Penicillum* sp. and *Rhizoctonia solani* inhibited seed germination and seedling development of soya beans, representing that these fungi produced some toxic substances in the culture media in which they were grown, inhibiting or reducing the germination percentage of seeds. Inhibition or stimulation of seed germination may be related to the certain substances that these fungi produce extracellularly, that can regulate the activity of hydrolytic enzymes (Negi *et al*., 1983).

This study also depicted that the culture filtrate of *Trichoderma harzianum*, have a pronounced effect on the seed gemination of *Chrysophyllum albidum*. *Trichoderma harzianum* effectively increased the seed germination percentage of the seeds. This result is in agreement with Kunwar and Mehrotra (1988) who reported that culture filtrates of antagonistic fungi like *Trichoderma harzianum, Trichoderma viride*, and *Trichoderma asperellum* effectively increased the seed germination of *solanum lycopersicum*, *Brassica rapa*, *Raphanus sativus* and *Trigonella melongena*. Cole and Zvenyika (1988) reported that seeds of few plants like Tobacco grew better when *T. harzianum* was added to the soil. Gupta and Sharma (1995) reported that the culture filtrate of *Trichoderma* spp. increased the seed germination of black gram.

CONCLUSION AND RECOMMENDATIONS

The inhibitory or stimulatory effect by the culture filtrate on the seed germination can be attributed to the presence of certain mycotoxins, enzymes, released by the respective fungal species. This study reveals that *Aspergillus niger*, *Rhizopus stolonifer*, and *Pseudomonas aeruginosa* inhibit seed germination of *Chrysophyllum albidum*. whereas *Trichoderma harzianum* has shown to effectively increase seed germination percentage and subsequent seedling performance of *Chrysophyllum albidum*.

Recommendations

1. *Trichoderma harzianum* is recommended for seed germination and subsequent seedling indices of *Chrysophyllum albidum*
2. Consumption of infected fruits of *C. albidum* is risky and should be avoided.

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