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In-vitro Studies on Aflatoxin Contamination and Management of Copra Quality

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ABSTRACT

Background: Post-harvest management of copra quality poses significant challenges due to improper handling by farmers and traders, a lack of awareness about mycotoxin contamination, and the associated health risks from metabolites produced by molds. Most traders noted that copra extraction happens at the farmers' level, which heightens the risk of mold infestation, as many farmers do not realize that improper drying can result in fungal contamination. Copra quality is found to be reduced by many fungi.

Methods: In the present study mycoflora isolated from storage copra. Potential biocontrol agents and chemical preservatives were screened against isolated mycoflora by using dual culture technique to enhance copra quality and to ensure better preservation methods in the industry.

Result: Isolation studies revealed that *Aspergillus flavus, A. niger, Rhizopus* spp*, Drechslera* spp*, Botryodiplodia* spp and *Penicillium* spp are the commonly associated mycoflora on copra during storage. *Aspergillus flavus* was predominant among the mycoflora with percent colonies ranging from 68 to 92 per each sample. Dual culture studies carried out with the three species of *Trichoderma* isolated from soils of coconut gardens revealed that *T.viride, T.harzianum* and *T.hamatum* were found very effective in inhibiting the mycelial growth of *A.flavus* under in vitro conditions. of them, *T.hamatum* was found very effective in controlling *A.flavus*. Among various chemical preservatives tested, Menadione showed the highest inhibition of *A. flavus* strains at 100 percent, followed by Potassium metabisulfite at 77.23 percent and Benzoic acid at 63.33 percent**.** Compatibility studies between *A.flavus* and the widely used chemical preservatives in food industry gives an idea of the nature of chemical preservatives to be used.

Key words: Aflatoxin, Biocontrol, Chemical preservatives, Coconut, Copra, *Trichoderma* spp.

INTRODUCTION

Coconut (*Cocos nucifera* L.) is a significant plantation crop in India. The crop is often called 'Kalpavriksha' due to its multifarious uses. Copra is a source for oil, cake and other edible products (Table 1). The copra is usually dried by air-drying, forced air-drying and by kiln drying methods. However, improper handling of any of these methods may lead to contamination with mycoflora which deteriorate the quality of copra and thus trade. The most widely used and cost-effective drying method in Asia is sun-drying, where grains are spread out in the open air for extended periods. However, this process also increases the risk of aflatoxin contamination (Lakshman *et al.,* 2022). A.*flavus* is the main species that is mainly responsible for aflatoxin production and crop contamination (Kakde, 2012). The present paper reviewed the research work on post harvest spoilage of copra and the possible aflatoxin contamination and a detailed account on the non pesticidal approaches for managing the post harvest spoilage of copra with emphasis on biocontrol of *A.flavus* the cause of aflatoxin contamination in copra and also the possible role of chemical preservatives in checking aflatoxin inducing *A.flavus* mold infestation in copra.

MATERIALS AND METHODS

Isolation of mycoflora from copra

Copra was cut into small bits of 0.1 cm size and surface sterilized with 0.1 per cent mercuric chloride for 1 min for isolation of mycoflora. Then copra bits were washed thoroughly in three changes of distilled water and plated again on potato dextrose agar and incubated at 28 ± 1°C temperature for a period of 7 days at department of Microbiology laboratory of SRR and CVR Govt Degree College (A), Vijayawada, Andhra Pradesh during the year 2022-23. The culture thus obtained was observed under compound microscope for presence of associated mycoflora with copra bits and further purified by single hyphal tip isolation.

Isolation of *Trichoderma* spp.

Isolation studies were carried out from soil samples collected from coconut gardens revealed the presence of

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three *Trichoderma* species and were subsequently identified as *T.viride, T.harzianu* and *T.hamatum* (Rifai, 1969).

Screening of antagonistic effect of *Trichoderma* spp. (Gams *et al.*, 1980)

Antagonistic effect of *Trichoderma* spp. on *A. flavus* under *in vitro* conditions

The isolated native *Trichoderma* spp. were screened for antagonism under *in vitro* conditions against *A.flavus* on PDA by using dual culture technique. Eight mm diameter discs of 3 day old growing culture of the pathogen and the antagonistic fungi were inoculated at opposite ends in a Petri-dish containing PDA. A control plate with only test fungus was simultaneously maintained. The Petri-plates were incubated at 28±1°C for 7 days and the ability of the antagonist to inhibit the pathogen was recorded by periodic observations. The per cent growth reduction was calculated by using the formulae as given by Vincent (1947).

$$\% \text{ inhibition} = \frac{\text{Mean growth in control - Mean growth in treatment}}{\text{Mean growth in control}} \times 100$$

Effect of volatile metabolites of *Trichoderma* spp. on *A. flavus*

The production and inhibitory effect of volatile antibiotics by the antagonists were tested against the test pathogen by using the procedure as given by Dennis and Webster (1971). The antagonists were grown on PDA for a period from 0 to 25 days and its effect on growth of *A.flavus* was tested by exposing inverted plates of freshly inoculated test pathogens to plates containing antagonistic cultures and sealing together by cello tape. The pathogen growth was measured after 4 days after incubation at 28±1°C and per cent inhibition was calculated.

Effect of non-volatile metabolites of *Trichoderma* spp. on *A.flavus*

The antagonists that have shown inhibition in dual culture studies were grown on potato dextrose broth to test the effect of the culture filtrates (non-volatile antibiotics) on the test pathogen by poisoned food technique (Khara and Hadwan, 1990). The culture filtrates were purified either by autoclaving at 15 PSI for 15 min. The sterilized filtrate was incorporated in the medium for observing fungal growth and inhibition at different concentration (10%, 20%, 50% and 100%). The PDA mixed filtrate were poured (20 ml each) into sterilized Petri-dishes and the plates were inoculated with fresh disc of the test pathogen *i.e*., *A.flavus* and per cent inhibition was calculated after 7 days of incubation.

Effect of chemical preservatives on *A. flavus* strains and *Trichoderma* spp.

The effect of commonly used chemical preservatives at 100 ppm and 500 ppm concentrations were tested against *A.flavus* strains and *Trichoderma* spp. on PDA by poisoned food technique under *in vitro* conditions. Then inoculated plates with 8 mm discs of three days old cultures of *A.flavus* strains and *Trichoderma* spp. separately and incubated at 28±1°C temperature. The observations on mycelial growth of *A.flavus* strains and *Trichoderma* spp. were recorded at 24 hours intervals for a period of seven days. Three replicates were maintained for each treatment.

RESULTS AND DISCUSSION

Association of mycoflora with copra

Isolation studies indicated that the mycoflora commonly found on copra during storage includes *Aspergillus flavus, A. niger, Rhizopus* spp. *Drechslera* spp. *Botryodiplodia* spp. and *Penicillium* spp. (Table 2 and Plate 1). *Aspergillus flavus* was predominant among the mycoflora with per cent colonies was ranging from 68 to 92 per each sample. This was followed by *Penicillium* spp. with a range of 61 to 69 per cent colonies. *Aspergillus niger* was recorded to a tune of 46 to 64 per cent colonies whereas other species of *Aspergillus* were recorded to an extent of 47 to 57 per cent.

***In vitro* antagonistic studies**

Dual culture studies carried out with three species of *Trichoderma* isolated from soils of coconut gardens revealed that *T. viride, T. harzianum* and *T. hamatum* were found very effective in hindering the *A. flavus* growth under *in vitro* conditions (Plate 2). Of them, *T. hamatum* was found very effective in controlling both the isolates. This was followed by *T. harzianum* and *T. viride* with insignificant differences in per cent of inhibition (Table 3). A clear inhibition zone was noticed with all the three *Trichoderma*

Table 1: Threat of aflatoxin contamination among various food and feed stuffs of copra.

Food products	
a. Wet meat or kernel	**b. Dry kernel or copra**
Coconut milk (CM)	Coconut Oil
Coconut jams (CJ) *and* Coconut syrup (CS)	Coconut Cake
Coconut skim milk (CSM)	
Coconut flour (CF)	**c. Coconut water**
Desiccated coconut (DC)	**d. Coconut toddy**
	Coconut Jaggery
	Coconut vinegar

* a and b are prone to aflatoxin contamination.

species and the inhibition zone was prevailed up to one week duration. Biological control of aflatoxins is a cost-effective and eco-friendly approach for the reducing aflatoxin contamination in food and feed and as well as minimizing the contamination throughout the food value chain (Bandyopadhyay *et al.,* 2019). Aflatoxins can be absorbed directly by microorganisms, either by binding to their cell wall components (Motawe *et al.,* 2014) or by being absorbed into the cells of dead microorganisms (Mwakinyali *et al.,* 2019).

Further *in vitro* studies carried out to determine the potentiality of *Trichoderma* spp. against *A. flavus* isolates revealed that volatile metabolites of 30 day old cultures of *T. viride, T. harzianum* and *T. hamatum* were inhibitory to *A. flavus*. While, 0 and 15 day old cultures of all the three *Trichoderma* spp. were found infective in inhibiting the mycelial growth of *A. flavus* through volatile metabolites (Plate 3). Among the *Trichoderma* spp. of 30 days old, maximum inhibition of *A.flavus* isolates was obtained with *T. viride* (66.67%), followed by *T. harzianum* and *T. hamatum* with an inhibition of 61.11% (Table 4).

For non-volatile metabolites, a rising trend in the inhibition of *A. flavus* was observed as the concentration of *Trichoderma* spp. culture filtrate was increased. Among the *Trichoderma* spp. maximum inhibition in mycelial growth of *A.flavus* was obtained by *T.hamatum* (57.6 per cent) (Plate 4), followed by *T. harzianum* (42.3 per cent) and *T. viride* (30.7 per cent) at 100 per cent concentration of the culture filtrate (Table 5). However, inhibition of the test fungus to a notable extent was also achieved by all the three *Trichoderma* spp. at culture filtrate concentrations of 75, 50 and 20 per cent respectively. Recent studies indicated that various fungal species, including *Trichoderma* sp., *Alternaria* sp., *Peniophora* sp., *Phoma* sp., *Armillariella tabescens*, *Mucor* sp., *Rhizopus* sp., *Pleurotus ostreatus* and *Phanerochaete chrysosporium*, can degrade aflatoxins produced by aflatoxigenic species into less or non-toxic metabolites (Wu *et al.,* 2009; Verheecke *et al.,* 2016; Adebo *et al.,* 2017).

Studies on the inhibition effect of chemical preservatives *viz.,* Menadione, Potassium meta bisulphite, Benzoic acid, Sodium benzoate, L-Ascorbic acid, Propionic acid and Acetic acid (glacial) on *A. flavus* strains (AF2 = Highly virulent and Aggressive strain; AF3 = Moderately virulent strain) showed that all the tested chemicals decreased the linear growth of aflatoxin-producing molds from moderate to significant levels at a concentration of 500 ppm and to some extent at 100 ppm as well (Table 6). A significant positive correlation was noticed among majority of the chemicals with respect to increase in dosage from 100

Table 2: Nature and extent of fungal infection on copra collected from traders, East Godavari district Andhra Pradesh.

Fungus	Number of colonies /sample (%)
Aspergillus flavus	68-92
Penicillium spp.	61-69
Aspergillus niger	46-64
Aspergillus spp.	47-67
Rhizopus spp.	33-44
Drechslera spp.	20-31
Botryodiplodina spp.	11-18

Table 3: Dual culture studies between *Trichoderma* spp. and *A.flavus.*

Antagonist	Per cent inhibition of *A.flavus* copra isolate	Remarks
Trichoderma viride	84.4[b]	Inhibition zone was observed after 4 days followed by mycoparasitism.
T. harzianum	83.3[b]	
T. hamatum	88.8[a]	

* Numbers in each column followed by the different letter are significantly different.

Values represent the means of 3 replicates.

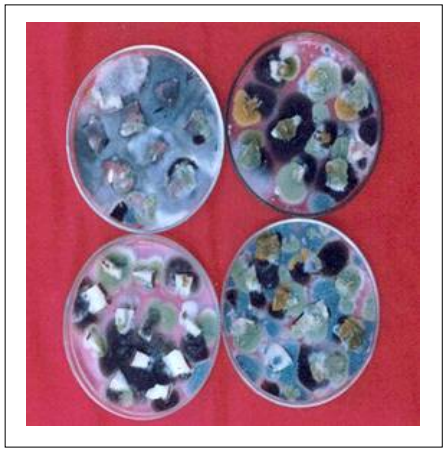

Plate 1: Mycoflora associated with copra during improper storage condition.

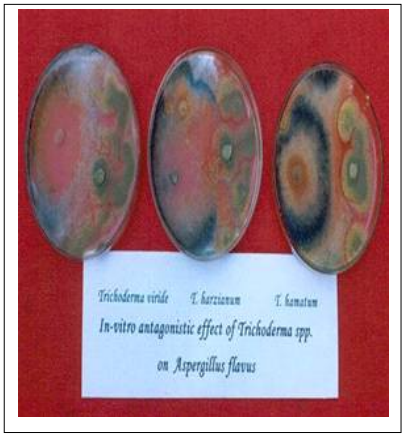

Plate 2: *In vitro* inhibition of linear spread during improper storage of *A.flavus* by *Trichoderma* spp.

ppm to 500 ppm with respect to *A.flavus* strain inhibition in terms of linear growth (Plate 5). The inhibition of linear growth for the highly virulent strain of *A. flavus* (AF2) was varied from 6.67 to 100 per cent, while the moderately virulent strain AF3 was inhibited between 7.00 and 100 per cent. Since, chemicals are targeted against all the strains *i.e.,* right from aggressive to moderately to less aggressive strains; the current discussion on the average per cent inhibition of *A.flavus* is apt. Among the various chemicals tested, Menadione exhibited the highest level of inhibition against *A.flavus* strains at 100 per cent, followed by Potassium metabisulfite and Benzoic acid which showed inhibition rates of 77.23 and 63.33 per cent respectively. The preservatives, Sodium Benzoate and Ascorbic acid also performed well in inhibiting the *A.flavus* strains by more than 50 per cent *i.e.,* 57.78 and 53.89 per cent respectively. However, the efficacy of Propionic acid is also notable with an inhibition of 43.33 per cent on *A.flavus* strains. On the other hand, Glacial acetic acid had a mild inhibitory effect with an inhibition of 6.84 per cent on *A.flavus* strains. The same chemical preservative even did prove ineffective against both the aflatoxin producing molds at 100 ppm with no inhibitory effect (Table 6). Food preservatives were also found effective in preventing rot, potassium metabisulphite 0.5 per cent followed by sodium benzoate 0.5 per cent proved most effective against the rot in both pre- and post-inoculation treatments. Manjunatha *et al.,* 2022; Kumar *et al.,* 2019 found that there was a drastic decrease in both the morphological growth and the aflatoxin biosynthesis of *A. parasiticus* in anoxic state. Seyedjafarri (2021) reported that the yoghurt bacteria (*S. thermophilus* and *L. delbrueckii* subsp. *Bulgaricus)* are able to reduce the levels of AFM1 in milk during the fermentation process.

Compatibility studies between *Trichoderma* spp. that are isolated *viz., T. viride*, *T. harzianum* and *T. hamatum* and chemical preservatives that inhibit growth of *A. flavus* revealed that sodium benzoate, Ascorbic acid and Potassium meta bisulphate were safe with regard to the growth and multiplication of *Trichodrerma* spp. and can be used in conjunction with biocontrol management of copra spoilage. In contrast, Menadione, Propionic acid, Benzoic Acid and acetic acid reduced the mycelial growth of *Trichoderma* spp. at 500 ppm concentration (Plate 6). All

Plate 3: Antagonistic activity of *Trichoderma* spp. volatile metabolites on *A.flavus*

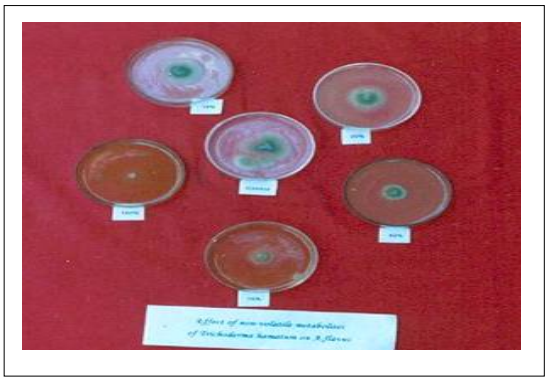

Plate 4: Antagonistic activity of non-volatile metabolites *Trichoderma* spp. on *A.flavus*

Table 4: *In vitro* inhibition effect of volatile metabolites of *Trichoderma* spp. on *A.flavus.*

Antagonist	Per cent inhibition of *A.flavus* Age of *Trichoderma* spp.		
	0 days	15 days	30 days
Trichoderma viride	0	0	66.67[a]
T. harzianum	0	0	61.11[b]
T. hamatum	0	0	61.11[b]

* Numbers in each column followed by the different letters are significantly different.

**Values represent the mean of 3 replicates.

Table 5: *In vitro* inhibition effect of non-volatile metabolites of *Trichoderma* spp. on *A.flavus.*

Antagonist	Per cent inhibition of *A.flavus* Concentration of culture filtrate of antagonist				
	10%	20%	50%	75%	100 %
Trichoderma viride	3.81[b]	7.6[c]	11.5[c]	30.7[c]	30.7[c]
T. harzianum	3.84[b]	23.0[b]	23.0[b]	38.4[b]	42.3[b]
T. hamatum	19.2[a]	34.6[a]	46.1[a]	48.0[a]	57.6[a]

* Numbers in each column followed by the different letters are significantly different.

**Values represent the mean of 3 replicates.

these chemicals were relatively safe at 100 ppm with respect to *Trichoderma* spp. growth inhibition (Table 7).

Comparison was drawn with respect to chemicals that inhibited *A.flavus* growth and *Trichoderma* spp. growth at 500 ppm concentration under *in vitro* conditions. The results indicated that Menadione though effective in controlling *A.flavus* population, is also adverse in terms of *Trichoderma* spp. growth. The results with respect to Potassium meta bisulphate, Benzoic acid, Sodium benzoate and Ascorbic acid were encouraging in the sense that only *A.flavus* growth was reduced whereas *Trichoderma* spp. growth is almost unaffected under *in-vitro* conditions (Fig 1). However, the efficacy of Propionic acid in terms of reduction of both *A.flavus* strains and *Trichoderma* spp. is almost on par with each other. With regard to Acetic acid, a poor mold inhibitor was also doubly disadvantageous with its inhibitory effect on *Trichoderma* spp. The compatibility studies of chemical presvatives on *Trichoderma* spp. were only a study taken up keeping in view the precautions to be adopted while applying preservatives to the copra. Potassium meta bisulphate, Benzoic acid, Sodium benzoate and Ascorbic acid can be recommended to be applied on copra along with in godowns where copra is stored, the soils of which may inhabit *Trichoderma* spp. Whereas, in order to have a dual check of mold growth by biocontrol agents as well, the chemical Menadione has to be applied only to the copra

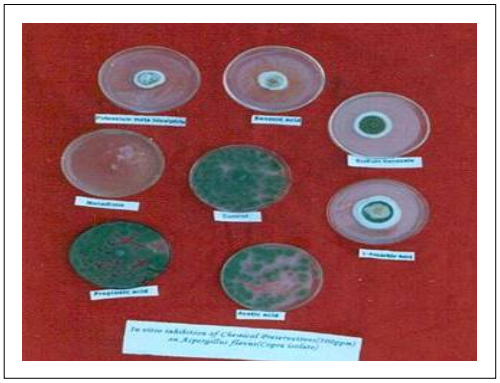

Plate 5: *In vitro* inhibition of chemical preservation on the linear growth of *A.flavus* at 500 ppm concentration.

Table 6: Effect of chemical preservatives on the linear growth of *Aspergillus flavus* strains on PDA incubated at 28°C for 96 hrs.

Name of the chemical	AF2 (*A. flavus*) % Inhibition at		AF3 (*A. flavus*) % Inhibition at		Average inhibition (%) *A. flavus* strains at
	100 ppm	500 ppm	100 ppm	500 ppm	500 ppm
Menadione	77.78 (20[a])	60.00 (36[b])	54.44 (41[c])	54.44 (41[c])	55.55 (40[c])
Potassium meta bisulphite	0.00 (90[d])	0.00 (90[d])	100 (0[a])	77.78 (20[b])	64.44 (32[c])
Benzoic acid	53.33 (42[d])	53.33 (42[d])	22.22 (70[e])	6.67 (84[f])	85.56 (13[a])
Sodium benzoate	64.44 (32[b])	58.89 (37[c])	54.44 (41[d])	53.33 (42[d])	0.00 (90[e])
L-ascorbic acid	0.00 (90[e])	100 (0[a])	76.67 (21[b])	62.22 (34[c])	62.22 (34[c])
Propionic acid	54.44 (41[d])	64.44 (32[c])	7.00 (83.7[e])	100	77.23
Acetic acid glacial	63.33	57.78	53.89	43.33	6.84
Control	90 mm				

* Means followed by similar letters are not different statistically (P=0.05) by Duncan's multiple range test.

** Values in parentheses are the linear growths of fungi in millimeters.

Table 7: Effect of chemical preservatives on the linear growth of *Trichoderma* strains on PDA incubated at 28°C for 96 hrs.

Name of the chemical	*T. viride* % Inhibition at		*T. harzianum* % Inhibition at		*T. hamatum* % Inhibition at		Average inhibition (%) *A. flavus* strains at
	100 ppm	500 ppm	100 ppm	500 ppm	100 ppm	500 ppm	500 ppm
Menadione	68.89 (28[a])	100 (0[a])	68.89 (28[a])	100 (0[a])	65.56 (31[a])	100 (0[a])	100
Potassium meta bisulphite	0.00 (90[d])	0.00 (90[e])	0.00 (90[c])	0.00 (90[e])	0.00 (90[c])	0.00 (90[e])	0
Benzoic acid	0.00 (90[d])	11.11 (80[c])	0.00 (90[c])	30.0 (63[c])	0.0 0 (90[c])	27.77 (65[c])	22.96
Sodium benzoate	0.00 (90[d])	0.00 (90[e])	0.00 (90[c])	0.00 (90[e])	0.00 (90[c])	0.00 (90[e])	0
L-ascorbic acid	0.00 (90[d])	0.00(90[e])	0.00 (90[c])	0.00 (90[e])	0.00 (90[c])	0.00 (90[e])	0
Propionic acid	18.89 (73[b])	53.33 (42[b])	5.55 (85[b])	44.44 (50[b])	11.11 (80[b])	53.33 (42[b])	50.36
Acetic acid glacial	0.67 (89.4[c])	7.11 (83.6[d])	0.00 (90[c])	6.66 (84[d])	0.28 (89.74[c])	7.66 (83.10[d])	7.14
Control	90 mm						

* Means followed by similar letters are not different statistically (P = 0.05) by Duncan's multiple range test.

** Values in parentheses are the linear growths of fungi in millimeters.

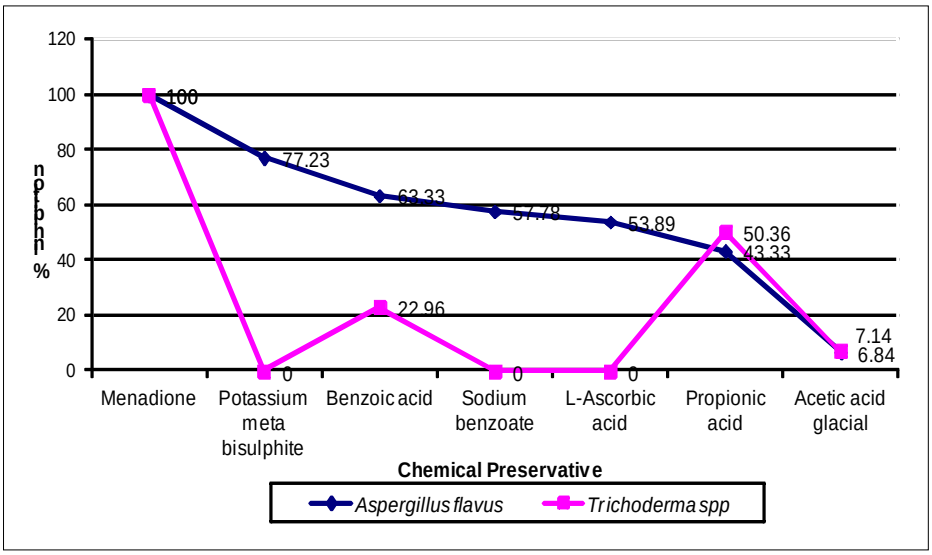

Fig 1: *In vitro* efficacy of chemical preservatives in inhinting the linear growth of *A.flavus* by *Trichoderma* spp. at 500 ppm concentration.

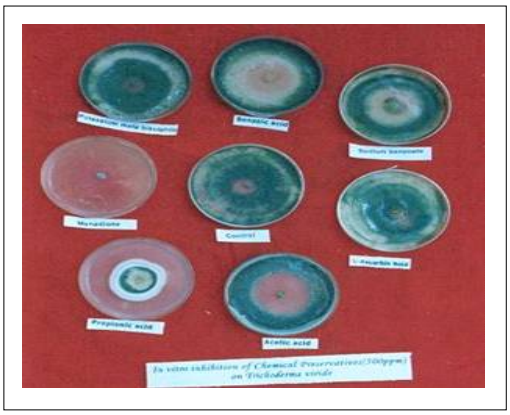

Plate 6: *In vitro* inhibition of chemical preservation on the linear growth of *T.viride* at 500 ppm concentration.

and not to the godowns as a general. From the present studies, it can be inferred that chemical preservatives offer a feasible and an ecofriendly approach in managing the post harvest spoilage of copra especially the aflatoxin problem.

CONCLUSION

This study highlights the significant impact of fungal contamination on copra quality, with *Aspergillus flavus* identified as the most prevalent species. The isolation of various fungi, including *A. niger, Rhizopus* spp*. Drechslera* spp*. Botryodiplodia* spp. *and Penicillium* spp. under scores the need for effective management strategies during storage. The efficacy of *Trichoderma* species, particularly *T. hamatum* in inhibiting the growth of *A. flavus* demonstrates the potential for biological control measures. Additionally, chemical preservatives such as Menadione, Potassium metabisulfite and Benzoic acid showed promise in reducing fungal growth. However, further research is essential to explore these findings in practical, *in-vivo* studies. An integrated approach to post-harvest technology could significantly enhance copra quality and ensure better preservation methods in the industry.

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Disclaimers

The authors are responsible for the accuracy and completeness of the information provided.

Conflict of interest

No conflicts of interest regarding the publication of this article.

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