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Full Length Research Paper

Preliminary studies of inhibitions in *Aspergillus flavus* with extracts of two lichens and Bentex-T fungicide

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One ml aqueous concentrations at 0.1, 0.25, 0.5, 1.0, and 5.0 mg/ml of laboratory extracts of *Hypogymnia physodes* and *Ramalina farinacea* (Lichens), and Bentex-T, were incorporated separately in a basal broth culture medium inside conical flasks. Mycelial dry weight of *Aspergillus flavus,* grown on the medium with the extracts of the lichens, was inhibited between 70% - 80% compared to unincorporated control medium. Bentex-T inhibited the *A. flavus* between 60% - 65%. Sporulation of *A. flavus* was inhibited more by Bentex-T and the extract of *R. farinacea* by 60% - 75% than the 29% - 34% caused by *H. physodes*. Spores of *A. flavus* in control medium commenced germination after 2 h while it was mostly after 4 h or 6 h in the incorporated media. Inhibition of germination was significant at 5% level with 0.1 and 0.25 mg/ml and highly significant at 1% level with 0.5 to 5.0 mg/ml concentrations of the extracts and Bentex –T. The extract of *R. farinacea* appeared to be most inhibitory on the germination of the spore.

Key words: Lichen extracts, fungitoxic, *Aspergillus flavus.*

INTRODUCTION

Aspergillus flavus is one of the species of the genus known to produce aflatoxins, a group of acutely toxic and potentially carcinogenic and immunosuppressive mold metabolites *Saad* et al (1995) . The genus is widely distributed, and *A. flavus* spores are found in the air and in the soil (Alexopoulos, 1962). They thrive on any organic substance with little moisture. Products with moisture levels above 16% are capable of supporting the growth of *A. flavus*. In addition, their characteristic large number of enzymes enables them to utilize a variety of substances for food. Hence, they are often found on exposed foodstuffs, and cause decay. *A. flavus* is one of the storage fungi that develop on a wide variety of stored grains such as wheat, peanuts, soybeans, corn, and so on. Toxin production has been demonstrated on several food products. Under optimal conditions of growth, some toxins can be detected within 24 h, or generally within 4-10 days (Christensen, 1971).

Resistance by fungi has been reported for the systemic fungicides such as caboxine, fernfuran, pyrocarbolide and thiobendazole (Besri, 1992). Many lichens are known to produce one or more secondary metabolic products which have been characterized as weak phenolic acids (Mellanby, 1978; Ferry et al., 1973). The lichen metabolic products that have antibiotic activity may have the function of protecting the organism from attack by other fungi. Hale (1974) reported a retarding action of extracts of lichens on the growth of lower Phycomycetes and *Neurospora crassa*. In Finland, complex derivatives of usnic acid of lichens have been prepared with enhanced antibiotic activity (Richardson, 1975). It is effective against a wide range of bacteria and also some fungi which cause athlete's foot and ring worm. Sodium usnate has been successfully used by Ark et al. (1960), for the control of various fungal diseases of plants in the green house.

Cristan et al. (1978) and Dubey and Dwivendi (1991) have indicated the possible use of plant as well as lichen extracts in the control of plant diseases. The extraction, purification and concentration of the active ingredients of the lichen components could lead to large scale production of the compounds that could probably be used in the production of more effective fungicides. Furthermore, being easily biodegradable the adverse effects of the extracts on the environment are likely to be less.

MATERIALS AND METHODS

Extraction of lichens

Vegetable bunches of the lichens, *R. farinacea* and *H. physodes,* were cleaned free of any other plant materials and washed under running tap water. They were oven-dried at 70^0C for 72 h and ground into powder by the use of mechanical hand grinder. The powdered samples were stocked in sterilized specimen bottles until when needed.

Fifty gram (50 g) of each lichen powder was extracted in Soxhlet apparatus using 70% ethanol for 5 h. The ethanol was evaporated off in hot water bath regulated at 60^OC.

Preparation of fungicidal concentration from lichens and Bentex-T

The extracts were weighed out as 10, 25, 50, 100 and 500 mg respectively and, homogenized in 100 ml of sterile distilled water (giving 0.1, 0.25, 5.0, 1.0 and 5.0 mg/ml concentrations respectively). Bentex-T powder was also similarly weighed and made into 100 ml solution to give the same concentrations with the lichen extracts.

Inhibitory effects of lichen extracts and Bentex-T on *A. flavus*

Poisoned food techniques (Monger and Grover, 1991) were employed in the study. The experimental design was completely randomized.

Growth of *A. flavus*

A. flavus was cultured in a basal liquid medium of Punja and Jenkins (1984), which was separately incorporated with 1 ml of the different concentrations of the extracts of the lichens and Bentex-T, in replicates of three flasks. Control set up was with 1 ml of sterile distilled water. The inoculated media were incubated at room temperature (27 ± 2° C) on a culture shaker. Dry weight of the fungal mycelium (that was recovered) was measured on laboratory Mettler Balance (P163) after 7 days of incubation. The average of their constant dry weight at 70°C was the measure of growth attained in response to the effect of the poisoning, or none in the control. Percentage inhibition was calculated as difference between control and treatment, divided by the control value and, multiplied by 100 (Abdulsalam et al., 1990).

Sporulation of *A. flavus*

Effect of the concentrations of the lichen extracts and Bentex-T on the ability of the fungus to sporulate was evaluated on potato dextrose agar (PDA) medium. One ml of each concentration of the lichen extracts and Bentex-T was mixed into each of three replicate plates of PDA, just before it solidified. The control was just plain PDA. The plates were then centrally inoculated with single 2 ml diameter mycelia plug of *A. flavus* obtained from maintenance plain PDA. They were incubated at room temperature. After seven days of incubation, five 2 ml diameter mycelia plugs from a plate of each of the concentrations of the lichens, Bentex-T, and of the control, were randomly harvested. The harvests per plate of every concentration were immersed in 5 ml distilled water in test tube, which was shaken to dislodge the spores. The number of spores from the replicates of every concentration was counted with hemocytometer (Oke, 1990), and calculated. The ability of the fungus to sporulate with reference to the fungicidal concentrations of the lichen extracts and Bentex-T compared to control are presented as percentage inhibition.

Germination of the spores of *A. flavus*

Petri dishes containing very thin layer of PDA mixed with l ml of the concentrations of the lichen extracts, Bentex-T, and sterile distilled water were prepared for the studies on spore germination. The spores of *A . flavus* were recovered from its culture grown on maintenance medium into 50 ml of sterile distilled water in a beaker. Using a camel hair brush, the spores were streaked on the thin PDA plates containing the concentrations of the lichen extracts, Bentex-T, and the control. A 2 ml diameter cork borer was used to cut a disc of the streaked media at 2 hourly intervals and observed under light microscope (*400 objective) for the emergence of germ tube. The spores that produced germ tube were counted for germination.

RESULTS

The extracts of the lichens and Bentex-T suppressed mycelial growth, sporulation and germination of the spores of *A. flavus,* compared to the control. Data on mycelial dry weight and sporulation are presented in percentage inhibition, while germination in number is directly compared to control.

Dry weight of the mycelia mat of *A. flavus*

Growth inhibition of the fungus was effective with as low as 0.l mg/ml concentrations of the extracts of the lichens and Bentex-T. Inhibition was most severe with the extracts of *H . physodes* (75%) followed by the extract of *R. farinacea* (70%) and mildest with Bentex-T (60%). Higher concentrations of 1.0 mg/ml to 5.0 mg/ml of the fungicidal substances imposed higher inhibitions on the mycelia growth (Figure 1).

Sporulation of *A. flavus*

In Figure 2, the extract concentrations of *R. farinacea,* and of Bentex-T impsed much higher percentage inhibit-tion on the sporulation of *A. flavus* than the extract of *H. physodes* . Percentage inhibition on the sporulation of *A. flavus* by *R. farinacea* and Bentex- T ranged between 60 and 75%, while it ranged from 30 to 34% for the extract of *H. physodes.*

Germination of the spores of *A. flavus*

Comparative number of germinated spores of *A. flavus*, at the different concentrations of the extracts of the lichens, Bentex-T, and control are presented in Figures 3, 4, 5, 6, and 7. Generally, lower numbers of spores were found to germinate in the media incorporated with the extracts of the lichens, and Bentex-T than on the plain agar media. The extracts of the lichens, and solutions of

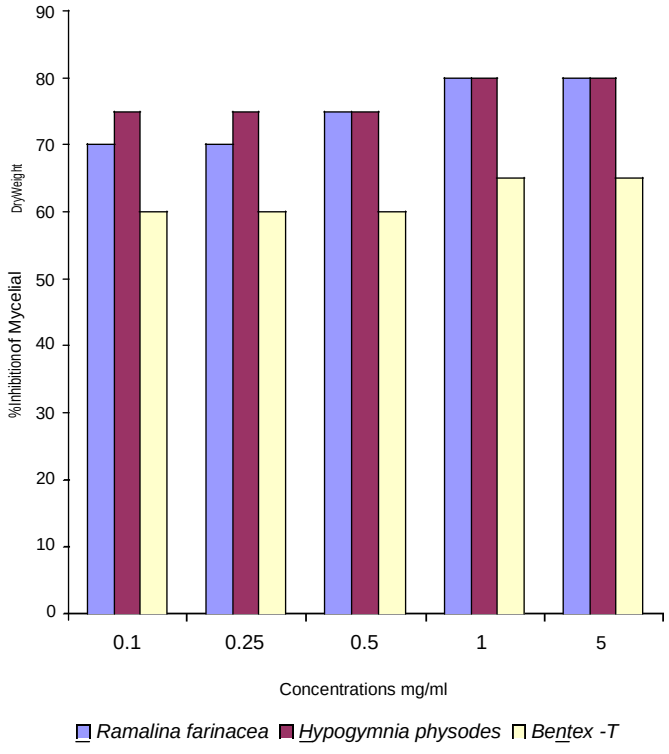

Figure 1. Percentage inhibition of mycelial dry weight of *A. flavus* in concentrations of the extracts of the Lichens.

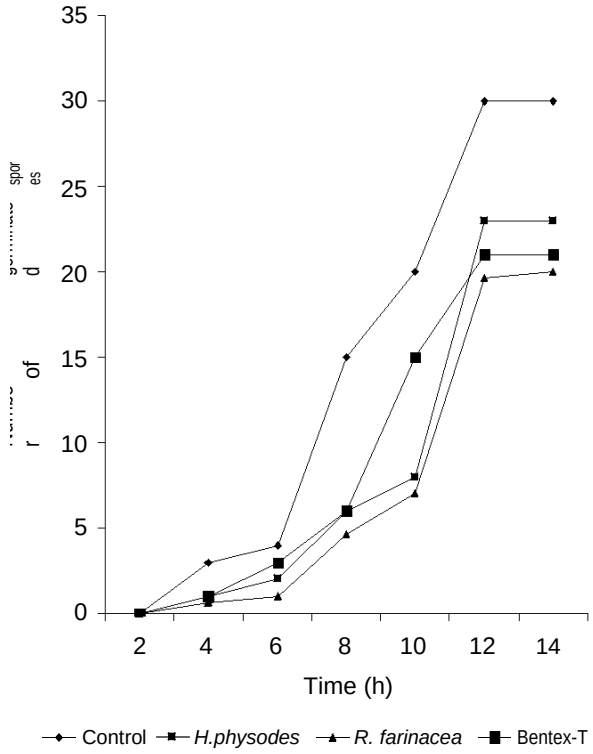

Figure 3. Number of germinated spores of *A. flavus* at 2 hourly microscopy in 0.1 mg/ml extracts.

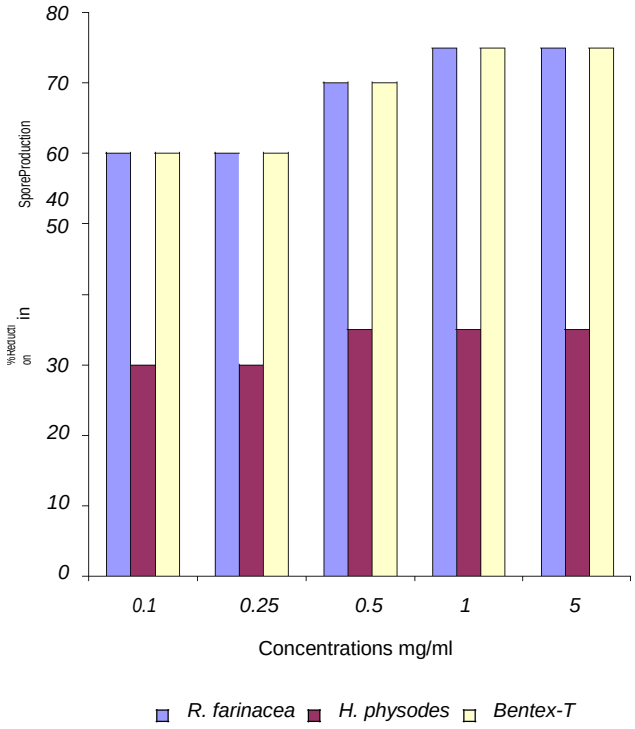

Figure 2. Percentage inhibition of spore production in *A. flavus* by the extracts of the Lichens at different concentrations.

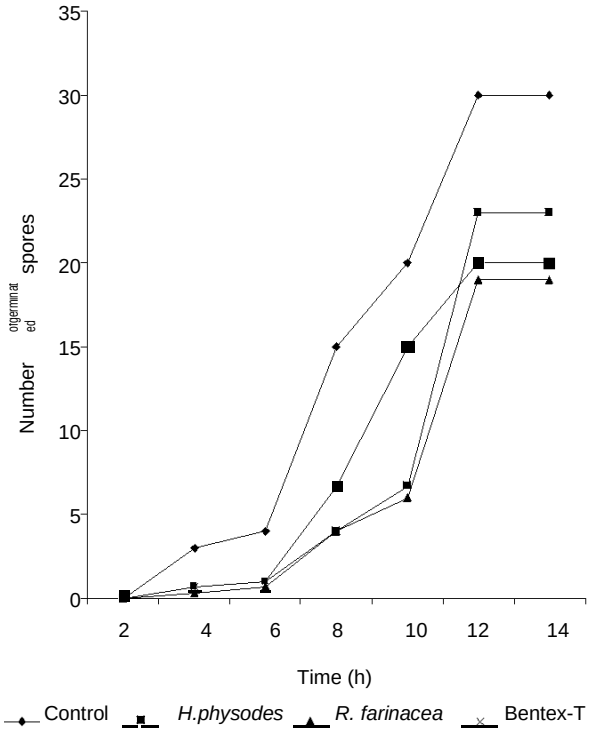

Figure 4. Number of germinated spores of *A. flavus* at 2 hourly microscopy in 0.25 mg/ml.

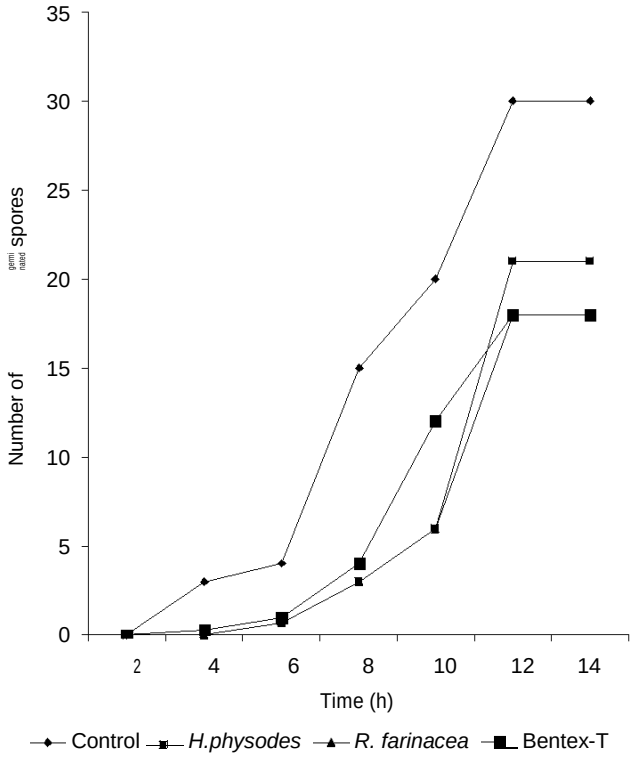

Figure 5. Number of germinated spores of *A. flavus* at 2 hourly microscopy in 0.5 mg/ml.

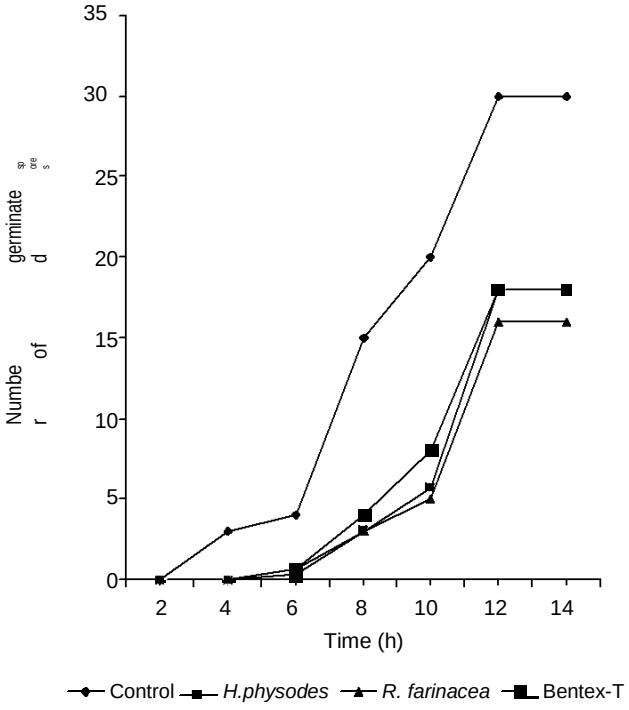

Figure 6. Number of germinated spores of *A. flavus* at 2 hourly microscopy in 1.0 mg/ml.

Bentex – T caused significant inhibition to germination of the spores; lower concentrations of 0.1 and 0.25 mg/ml

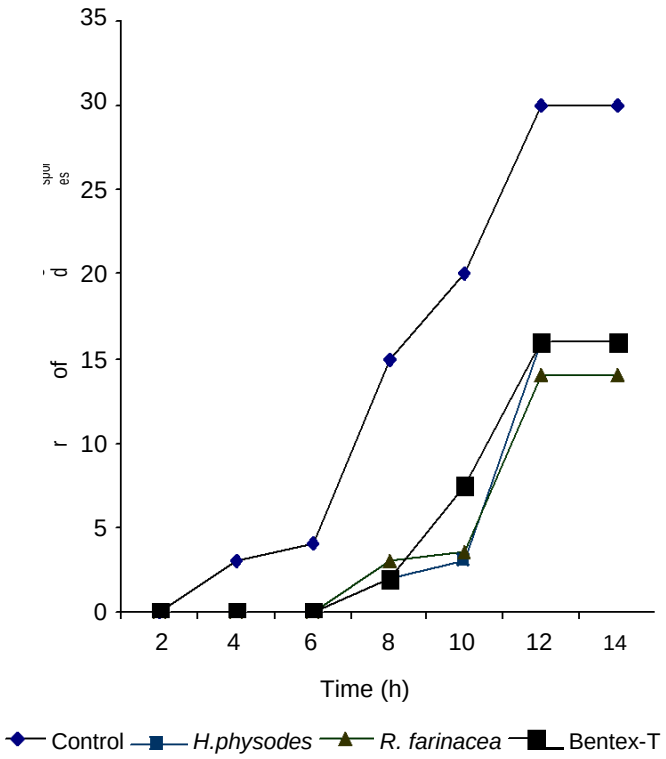

Figure 7. Number of germinated spores of *A. flavus* at 2 hourly microscopy in 5.0 mg/ml.

were significantly lower than control at 5% level, and the higher concentrations at 1%.

DISCUSSION

The extracts of *R. farinacea* and *H. physodes* seem to contain active fungicidal chemical substances because of their inhibitory influence on the growth, sporulation and germination of spores of *A. flavus*. The higher the concentration of the extracts of the lichens and Bentex-T, the higher the percentage of inhibition of the mycelial growth, implying lower dry weight of the fungal mycelium. Fungicidal and bactericidal properties of plant extracts have been reported by Aggarwal and Mehrotra (1988). Aside from the genetic attributes, fungal metabolisms are influenced by substrate compositions (Deacon, 1980). This should be true of the components in the lichen extracts, or the synthetic fungicide that affected the fungal growth in this study. Aggarwal and Mehrotra (1988), studying the effect of synthetic fungicides on *Phytophthora colocasiae,* observed a correlation between mycelial growth inhibition and inhibition in the rate of respiration.

As obtained in this study, lichen extracts and the synthetic fungicide inhibited spore production. The extract of *R. farinacea* and Bentex-T seem to contain more

effective inhibitory substances on the sporulation of *A. flavus* than *H. physodes*. Perhaps the inhibitory substance may be the volatile property known to inhibit spore production (Dubey and Dwivendi, 1991; Mangamma and Sheeramulu, 1991). It follows that the inhibitory volatile property is higher or more effective in *R. farinacea* and Bentex-T than in *H. physodes*.

The extracts of the lichens and Bentex-T generally caused a lag in germination of the spores. Lower concentrations were less inhibitory on the spores while the higher ones caused corresponding higher inhibition on germination. Moore and Atkins (1977), and Dubey and Dwivendi (1991) have reported inhibition of germination of spores of *Macrophomina phaseolina* by plant extracts.

Since lichens are composite organisms of definite algal and fungal components, their by-products are different. This difference is observed in the lichen extracts which exhibited different degrees of growth, sporulation and spore germination inhibitions.

The inhibitory potentials of the extracts of the lichens are established by the results on the mycelial growth, sporulation, and germination of spores of *A. flavus*. In this study certain characteristics of the fungus were even more inhibited by the extracts of the lichen than the synthetic fungicide. In the search for alternative control agents of fungal pathogens, as resistance to existing artificial fungicide develop, this study has shown that extracts of lichen are fungi toxic. This opens the possibility of extracting fungi toxic chemicals from lichens.

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