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Effect of Different Antifungals on the Control of Paper Biodeterioration Caused by Fungi

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The inhibition of some fungal strains responsible for paper biodeterioration is reported. Antimicrobials (butylated hydroxytoluene, BHT and butylated hydroxyanisole, BHA), azole antifungals (econazole, miconazole and ketoconazole) and chitin synthase inhibitors (uridine, 5-fluorouridine, 2-deoxyuridine) have been assessed for efficacy against *Penicillium chrysogenum* Thom, *Aspergillus terreus* Thom, *Stachybotrys atra* Corda and *Chaetomium elatum* Kunze, fungal strains isolated from deteriorated papers. Our results have shown that the most evident inhibiting effect on fungal growth has been obtained with miconazole and econazole at 10^{-3} M. © 1997 Published by Elsevier Science Limited

INTRODUCTION

Microorganisms, particularly the fungi, because of their cellulolytic activity are potential candidates responsible for paper deterioration (Coughlan *et al.*, 1993).

It is well known that fungi are also involved in biodeterioration, in plant pathology and in human diseases. Chemicals that specifically inhibit chitin (main component of fungal wall) and ergosterol (main sterol of fungal membranes) are used to prevent fungal infections.

Paper deterioration is caused by different 'internal' and 'external' factors (Lanting, 1990). These factors affect the so-called "paper ageing" phenomenon related to the yellowing and embrittlement of papers that is associated with changes in its properties with time (Havermans, 1994). Internal factors are related to manufacturing processes such as the use of modern high yield chemithermomechanical pulp (Priest and Stanley, 1994). External factors depend on light, temperature, oxygen, humidity changes, air pollutants and, not less important, microorganisms and insects.

In the present work, the effect of some antifungals in prevention or control fungal growth on different papers, is reported.

MATERIALS AND METHODS

The fungi tested were *Penicillium chrysogenum* Thom, *Aspergillus terreus* Thom, *Stachybotrys atra* Corda and *Chaetomium elatum* Kunze. All strains were isolated from biodeteriorated papers plated onto Mycological agar medium (Difco) at 23°C for 7 days.

The isolated strains were kept on PDA (Potato Dextrose Agar) at 25°C for 15 days.

In vitro experiments

The effect of all chemicals tested on conidia/spore germination (% germination) were carried out in 2 ml saline Czapek Medium (without sucrose) with 1% (w/v) of carboxymethylcellulose as carbon source (SCC). The inoculum (1×10^6 conidia/spores) were prepared as suspension in distilled water with 0·01% (w/v) of Triton X-100.

The compounds tested were: (A) Azole inhibitors (AI): Ketoconazole (Keto), Miconazole (Mico), Econazole (Eco) at concentration of 10^{-5} M; (B) Chitin Synthase Inhibitors (CSI): Uridine, 2-Deoxyuridine (2-DOU) and 5-Fluorouridine (5-FUR) at concentration of 10^{-5} M; (C) Antimicrobials (AM): Butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) at a concentration of 0·1% (w/v) alone or in associations.

The experiments were carried out at a temperature of 27°C over 30 days.

In vivo experiments

The same fungi used for the *in vitro* experiments were tested on different kinds of papers (*in vivo* experiments). The papers used were: Whatman

Table 1. Composition of Different Paper Used *in vivo* Experiment

Whatman (WT):	100% Pure cellulose
Giornale(GIOR):	25% Conifer chemical/pulp
	75% Hardwood and softwood mechanical pulp
Mezzofino (MF):	35.7 Bleached straw cellulose
	19.8 Sulfite raw fir pulp
	19.8 Loading materials
	15.8 Poplar pulp
	4.7 Aluminium sulfite
	4.2 Sizing agents

(WT), Giornale (GIOR) and Mezzo Fino (MF) kindly obtained from dr.ssa Fausta Gallo of Istituto Centrale Patologia del Libro of Rome, Italy. The composition of the three kinds of paper is given in Table 1.

The compounds assayed were Eco, Mico and 5-FUR at concentrations from 10^{-5} to 10^{-3} M, BHA 0·1 and 0·2% (w/v) and BHA + BHT 0·1 + 0·1% and 0·2% + 0.2% (w/v).

The inoculum (1000 spores or conidia/50 μl of potato dextrose broth) was placed on 50 mg paper strips previously supplemented with antifungals. The strips were kept in Petri dishes at the relative humidity (RH) of 85% and incubated at 27°C up to 30 days.

Fungal growth was tested as ergosterol content as previously reported by Padgett and Posey (1994).

Recovery of tested compounds

The stability of some azole antifungals (Mico and Eco 10^{-3} M) and antimicrobials (BHA 0·2% w/v and BHA + BHT 0·2 + 0·2% w/v) were also determined in the absence of the fungus after 30 days of incubation at 27°C and relative humidity of 85% on different kinds of paper.

The paper strips were extracted in methanol (2 × 20 ml); the solvent was filtered and concentrated with N_2. Aliquots of methanol were injected into a liquid chromatograph (HPLC) Perkin Elmer LC75.

The analyses of azoles and CSI was obtained on a reverse phase RP-18 column (25 × 4 mm i.d.), RP-18 × 5 um Supelco operating at 30°C.

The chromatography conditions were: isocratic elution; mobile phase CH_3OH:H_2O (75:25 v/v); UV detector 272 nm; flow rate 1·0 $cm^3 m^{-1}$. The same methodology was used for the detection of CSI compounds. The antioxidants (BHA, BHT) were analysed as previously described (Passi and Nazzaro-Porro, 1981).

RESULTS

The effect of different chemicals on the germination (%) of conidia/spores of *P. chrysogenum, A. terreus, S. atra* and *C. elatum* inoculated in SCC is shown in Fig. 1A–C.

BHA and BHA + BHT at the concentration tested seem to be more effective compared with BHT at the same concentration on the conidia/spore germination for all tested fungi (Fig. 1A). All azoles assayed (Mico, Eco and Keto at 10^{-5} M) inhibit germination, although to a different extent, in comparison to controls. All AI, however, showed no inhibiting effect on *S. atra* conidia germination. Among the azoles, Eco and Mico were the most inhibitory. The inhibiting effect ranges from more than 90% in *P. chrysogenum* in the presence of 10^{-5} M Eco to 80% in *C. elatum* in the presence of the same concentration of Mico (Fig. 1B).

Among the CSI assayed 5-FUR at 10^{-5} M was more effective compared to controls and the other CSIs tested (Fig. 1C).

CSI compound had no effect on conidial germination of *S. atra* at the concentrations tested.

Figure 2A–D show the inhibiting effect of AI, CSI and AM on fungal growth (as ergosterol content) on different kinds of paper. Eco at 10^{-3} M shows the best inhibiting effect on *P. chrysogenum* (Fig. 2A) and *A. terreus* (Fig. 2B). BHA (0·2% w/v) shows a strong inhibiting effect on *P. chrysogenum* growth, but only on the experiments carried out on Whatman paper (Fig. 2A).

In Fig. 2C, the inhibiting effect of chemicals tested on *S. atra* growth is shown. This fungus is affected by all the compounds and in particular by Mico and Eco (10^{-3} M).

The 5-FUR at 10^{-3} M is the most inhibiting compound on *C. elatum* fungal growth as shown in Fig. 2D.

Each fungus tested showed a different amount of growth measured as ergosterol content; *P. chrysogenum* and *A. terreus* showed higher fungal growth (up to 14 μg of ergosterol/100 mg of paper) in comparison with *S. atra* and *C. elatum* (up to 3 μg ergosterol/100 mg of paper).

It is also evident that the different chemical composition of the three papers tested can heavily

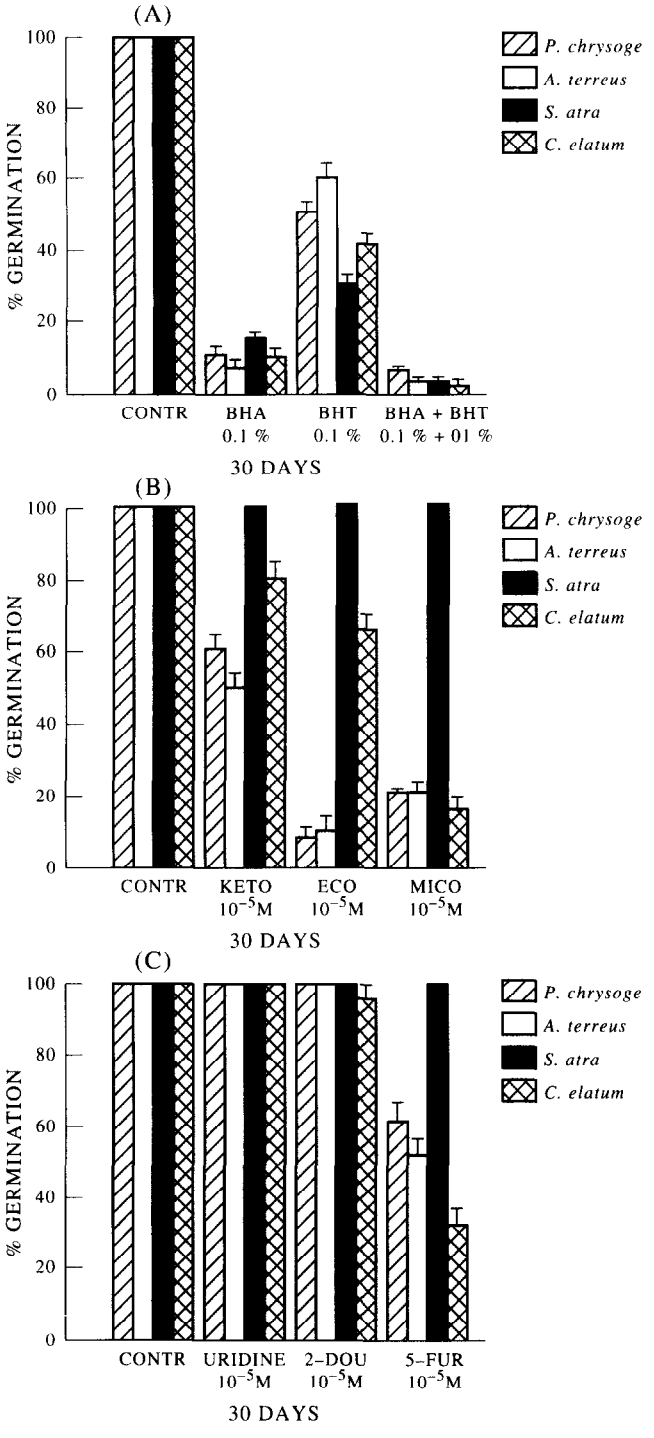

Fig. 1. Effect of antimicrobials (A), azole inhibitors (B) and chitin-synthase inhibitors (C) on conidia/spore germination(%) of *P. chrysogenum, A. terreus, S. atra* and *C. elatum* inoculated on SCC and incubated at 27°C for 30 days (germination 100% = hyphal growth).

affect fungal growth. In all cases MF appears the more resistent to fungal growth. The lower concentrations of tested AI and CSI (10^{-5} and 10^{-4}M) and AM (0·1% w/v and 0·1+0·1% w/v) have no inhibiting effect on *in vivo* experiments (data not shown).

We have also analysed the recovery (%) of the compounds tested on different kinds of papers without fungal inoculum. As shown in Table 2, after 30 days of incubation at 27°C and RH of 85%, all the analysed compounds were recovered at very high percentage. Only when BHT was placed on MF did its recovery appear lower.

Table 2. Recovery (%) of MICO and ECO 10^{-3}M , BHA 0.2% w/v and BHA + BHT 0.2+0.2% w/v from Different Kinds of Papers (MF, GIOR, WT) After 30 days of Incubation at 27°C and RH 85% , Without Fungal Inoculum

	Recovery %		
	MF	GIOR	WT
MICO 10^{-3}M	85.1±8.0	90.3±8.1	75.8±6.6
ECO 10^{-3}M	83.1±7.4	75.5±6.0	70.1±6.0
BHA 0.2%	76.2±6.3	95.0±8.4	91.3±7.3
BHA + 0.2 + 0.2%	71.2±5.8	95.0±8.0	91.0±8.3
BHT	38.4±3.5	74.1±6.6	86.0±7.6

Each value represents the mean±S.E.M. of three determinations.

The recovery of 5-FUR from different kinds of paper was not reproducible and for this reason the data are not reported. Work is in progress to improve the methodology both for the extraction and the analysis of 5-FUR.

DISCUSSION

The application of antimicrobials to control fungal biodeterioration is well known (Deacon, 1984; Griffin, 1994). BHA and BHT are antioxidants known to limit microbial deterioration of foods and to reduce peroxidation of lipids due to environmental factors such as oxygen, humidity and metals (Fanelli *et al.*, 1987; Passi *et al.*, 1987).

The azole antifungals are compounds used in dermatology as antifungal drugs (Harvey, 1985) and in plant pathology against fungal infections. They act by selectively impairing the cytochrome P-450 dependent 14a-demethylase, a key enzyme of ergosterol biosynthesis (Vanden Bossche, 1990).

The inhibitors of chitin synthase impair fungal growth by inhibiting the synthesis of chitin, one of the main components present in fungal walls (Gooday, 1990, 1994).

Our results show that the different biocides tested can affect the studied strains in different ways (Fig. 2A–D). Nevertheless, the well-known inhibitors of ergosterol synthesis (Vanden Bossche, 1990), Mico and Eco, at concentrations of 10^{-3}M show a strong inhibiting effect on all

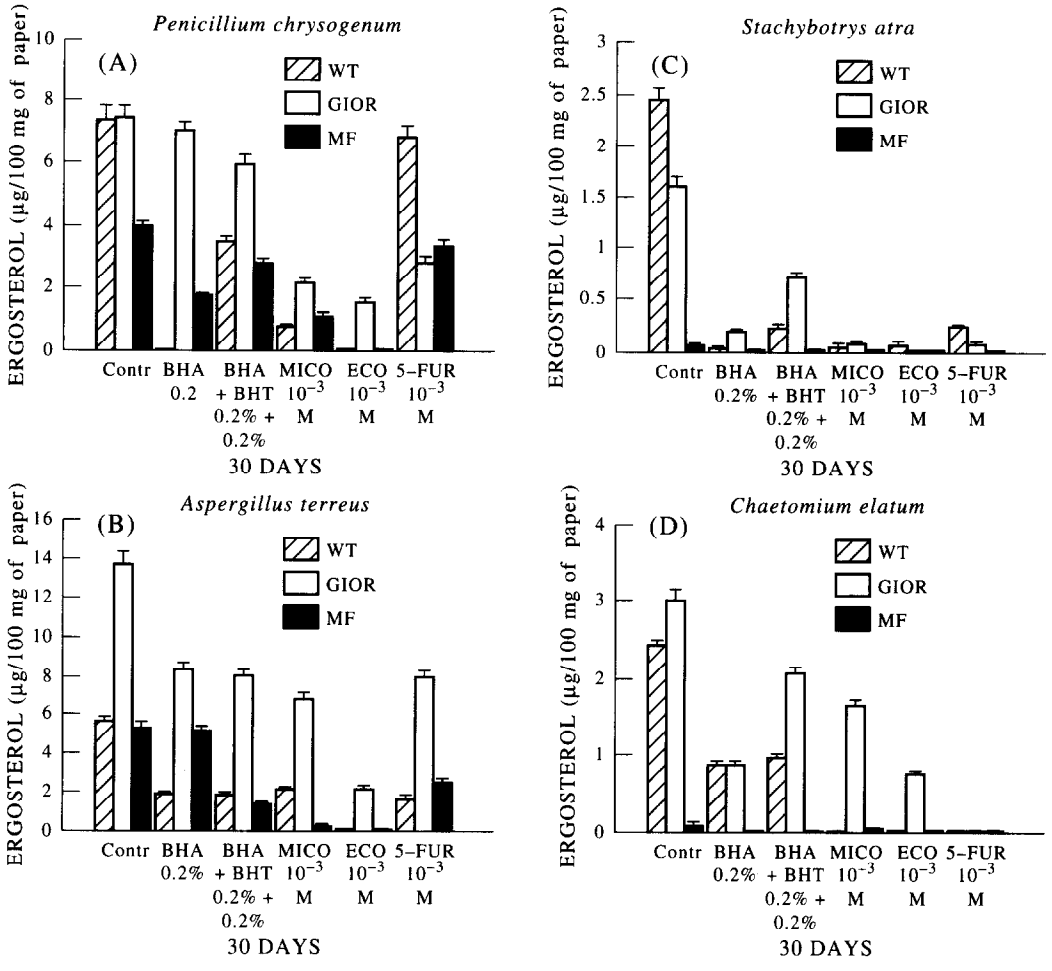

Fig. 2. Fungal growth (ergosterol content) of *P. chrysogenum* (A), *A. terreus* (B), *S. atra* (C) and *C. elatum* (D) on paper stripes Whatman (WT), Giornale (GIOR), Mezzo Fino (MF) supplemented with AM, AI, CSI incubated at 27°C for 30 days.

assayed fungi. Paper composition can affect fungal growth, when MF is used, all fungi tested grew less than on WT and GIOR and *S. atra* and *C. elatum* showed significant differences. These strains were also significantly affected by 5-FUR 10^{-3} M, an inhibitor of the chitin-synthase enzyme (Gooday, 1989, 1990) (Fig. 2C–D). The antimicrobials and antioxidants BHA and BHT showed variable inhibition effects between *in vitro* (Fig. 1) and *in vivo* experiments (Fig. 2A–D). This may have been due to different distributions of the compounds leading to a non homogeneous contact with fungal spore/conidia.

However, all compounds were readily recovered (Table 2) with the exception of BHT on MF. This was probably due to the complex composition of this paper (Table 1).

Nevertheless, the compounds showed different efficiencies against the tested fungi. Similar results were obtained with different azoles showing variable inhibition of *A. parasiticus* (Fanelli *et al.*, 1995).

In conclusion, the azole inhibitors Mico and Eco, the chitin-synthase inhibitor 5-FUR and, to a lesser extent, the antimicrobials BHA and BHT give promising results for the control of paper biodeterioration by fungi.

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