An appraisal of Impregon as a deterrent of domestic fungus growth

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Impregon* brand of tetrachlorsalicylanilide (TCSA) has been proffered widely as a household fungistatic agent, although its value remains unproved. To assess its effects, this agent was used as a laundry and paint additive and as a treatment for burlap rug backing; after recommended applications of Impregon, coded replicate materials were inoculated with mixed suspensions of fungus particles. No difference was evident in fungus growth points on fabric swatches washed in tap water with and without Impregon. However, growth on both of these was significantly greater than on samples laundered in tap water using only a commercial soap or liquid detergent. Fungus soiling of burlap was not clearly diminished by prescribed applications of Impregon solution 3 mo previously. Similarly, the addition of this agent to paint did not suppress fungus growth on Masonite plaques to which it had been applied. However, comparable levels of Impregon incorporated into agar media substantially inhibited spore germination. These findings suggest that the bioavailability of TCSA is insufficient to provide desired household antifungal effects when Impregon is used in accord with current recommendations.

Persistent soiling of man-made surfaces by fungi and actinomycetes has been associated increasingly with immunologically mediated, adverse health effects. Allergic rhinitis and asthma as well as hypersensitivity pneumonitis (extrinsic allergic alveolitis) may result from colonization of indoor niches; appropriate exposures also may foster allergic bronchopulmonary aspergillosis in some cases. These associations have led to heightened interest in analyzing indoor microfloras and have prompted consideration of many compounds to decontaminate domestic interiors. Among the antifungal agents available, Impregon, a formulation containing 3,3',4',5-tetrachlorsalicylanilide (TCSA), has been employed especially widely. TCSA has well-established antibacterial properties and was used briefly in soaps before its potency as a photocontact sensitizer was evident. A variety of other aerobic organisms also are inhibited by this agent in suitable concentrations.1 Prolonged suppression of fungus growth is said to follow use of Impregon, in extreme dilution, as a laundry and paint additive and as a spray treatment for household furnishings. However, since claims for this product remain anecdotal, we undertook an evaluation of its action under simulated conditions of domestic use.

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Received for publication Dec. 30, 1975.
Accepted for publication April 21, 1976.
*Marketed by Fleming & Co., St. Louis, Mo.

Vol. 58, No. 4, pp. 491-499
MATERIALS AND METHODS

Common fungi were isolated from air with an Andersen* viable particle sampler containing one plate of Difco malt agar beneath the sixth stage; the colonies that developed were largely forms of Cladosporium, Penicillium, and Alternaria. After 7 days of incubation at room temperature, the plates were flooded with sterile distilled water. The water was agitated with a flamed platinum loop and decanted. Two suspensions, differing at least 10-fold in spore concentration, were prepared for subsequent inoculation. Each 10 ml of the mixed spore suspensions obtained was mixed with 5 ml Tween 20 to wet floating spores to prevent clumping.

Fabric treatments

White or gingham printed cotton/polyester fabric was cut into 7 x 10 cm swatches (gingham print squares were 0.42 cm²). Groups of 8 swatches were washed for about 3 min in each of the following: tap water + 1 tablespoon of Ivory Flakes, tap water + Ivory Flakes + chlorine bleach (1 tablespoon/gallon), tap water + Ivory Flakes + Impregon (1 teaspoon/gallon), tap water + 1 teaspoonful of a liquid household detergent (Dynamo), tap water + Dynamo + chlorine bleach (1 tablespoon/gallon), tap water + Dynamo + Impregon (1 teaspoon/gallon), and tap water alone.

The swatches were then rinsed in tap water, coded, arranged randomly, sprayed with the spore suspension (using a DeVilbiss No. 251 atomizer), and incubated in a moist chamber at 20° C for 7 days. An additional set of 8 swatches was washed in tap water and incubated without inoculation. Fungus colonies were counted, by a person not familiar with the code, using a dissecting microscope. Alternate square centimeters were counted on the white fabric; all white squares, constituting one fourth of total surface area, were counted on gingham fabric. All colony counts are expressed as growth points/cm² (± SD).

This procedure also was adapted to determine the effects of Impregon on spores present on fabric before washing. Eight swatches (7 x 10 cm) of white fabric were sprayed with concentrated fungus spore suspension and dried; 4 of these were washed in tap water + Dynamo, and 4 in tap water + Dynamo + Impregon (1 teaspoon/gallon). After a tap water rinse, the swatches were incubated as above, dried, and photographed for qualitative evaluation.

Rug backing treatment

Eight by twelve cm burlap swatches were sprayed with Impregon (2 teaspoons/gallon tap water) using a household “spray gun,” air-dried, coded, and stored 3 mo in the dark. A spore suspension was then brushed on 4 treated and 4 untreated burlap swatches. After 7 days of incubation in a moist chamber at 20° C, the swatches were qualitatively evaluated and photographed.

Paint surface treatment

Eight Masonite rectangles (7.5 x 12 x 0.5 cm), painted on one face with latex paint (Meijer’s Best) or latex paint + Impregon (2 teaspoons/gallon), were allowed to dry, and, after 18 to 24 hr., inoculated with a spore suspension using a small, clean paint brush. The plaques were then soaked overnight in tap water that did not cover the uppermost (painted) surfaces. After incubation at 20° C in a moist chamber for 7 days, fungus growth was qualitatively evaluated and the rectangles were photographed.

Germination study

Twenty-four microscope slides were cleaned with ethanol, air-dried, covered with a thin layer of 1% Difco Malt Agar with (12 slides) or without (12 slides) Impregon (1 tea...
FIG. 1. Fabric swatches sprayed with spore suspension and then washed in Dynamo (left), Dynamo + bleach (center), and Dynamo + Impregon (right).

TABLE 1. Effects on colony counts of pretreatment with water, detergent, soap, bleach, and Impregon with subsequent spore inoculation

<table>
<thead>
<tr>
<th>Preinoculation treatment</th>
<th>Colonies/cm² ± SD (n=8)</th>
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<tbody>
<tr>
<td></td>
<td></td>
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<tr>
<td>1 (low spore conc.)</td>
<td></td>
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<tr>
<td>Dynamo</td>
<td>4.06 ± 2.52 p &lt; 0.05</td>
</tr>
<tr>
<td>Dynamo + Impregon</td>
<td>6.66 ± 2.20 p &lt; 0.05</td>
</tr>
<tr>
<td>Dynamo + bleach</td>
<td>3.99 ± 2.27</td>
</tr>
<tr>
<td>2 (high spore conc.)</td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>130.37 ± 85.03 p &lt; 0.05</td>
</tr>
<tr>
<td>Ivory Flakes</td>
<td>60.80 ± 15.58 p &lt; 0.05</td>
</tr>
<tr>
<td>Ivory + bleach</td>
<td>68.33 ± 38.54</td>
</tr>
<tr>
<td>Ivory + Impregon</td>
<td>100.77 ± 46.76</td>
</tr>
<tr>
<td>Water (no spores)</td>
<td>0.76 ± 0.49</td>
</tr>
</tbody>
</table>

spoon/gallon), and coded. While the agar was cooling but still fluid, a loop of dry spores of Cladosporium cladosporioides was transferred to each slide and spread through the agar to achieve a uniform suspension. After incubation in a moist chamber at 20°C overnight, the slides were examined at a magnification of 250× by a person not familiar with the code. Germination percentages were obtained by scoring approximately 700 spores on each slide either + (germinated) or – (not germinated). Lengths of ten germ tubes from each slide were measured using an ocular micrometer.

Growth of germinated spores in the presence of Impregon

Spores of C. cladosporioides and Aspergillus fumigatus were streaked onto replicate culture plates of 2% malt agar, with and without 1 teaspoon/gallon of Impregon, and incubated at 20°C. After 24 hr, single spores were isolated from the streaked plates and transferred to additional plates of malt agar with and without Impregon. Both the original and recipient plates were then incubated for 5 days at 20°C and photographed.

RESULTS

Fungus growth on sets of differentially treated fabric samples was evaluated grossly at first using inoculation mixtures with and without Tween 20. Fig. 1 shows a group of swatches that was sprayed with spores and then washed using either Dynamo (swatch No. 4), Dynamo + bleach (No. 5), or Dynamo + Impregon (No. 6); no evident difference in density of colonies was suggested. In Fig. 2, swatches 1 and 2 had been prewashed with detergent alone, while, for swatches 3 and 4, detergent + Impregon had been used; all samples were then rinsed and
inoculated. After several days, colonies had developed abundantly on all swatches (Fig. 2) regardless of pretreatment.

To provide a more quantitative assessment of Impregon, a dissecting microscope was used subsequently to count foci of growth. Table I displays direct counts of fungus growth points on fabric laundered with and without Impregon and inoculated, after treatment, with a fungus spore suspension. Similar results were obtained in both sets of experiments despite an approximately 10-fold difference in the concentrations of the inocula.

Experiments with burlap were done to simulate treatment of carpet backing material with Impregon to reduce mold growth. Fig. 3 shows the similar effects obtained with spraying Impregon solution (bottom row) and distilled water (top row) on burlap swatches and then inoculating with a fungus spore suspension.
Three out of four swatches in both Impregon and distilled water-treated groups were completely covered with hyphae.

Fig. 4 demonstrates the effects of adding Impregon as directed (1 teaspoon/gallon latex paint) to interior wall paint. Again, no discernible difference exists between the two Impregon-treated (left) and paint only (right) samples. A second set of Masonite blocks treated similarly (4 blocks/set) produced entirely similar results.

Data describing the effects of Impregon (1 teaspoon/gallon) on spore germination by C. cladosporioides are presented in Table II. Impregon, when present in the agar medium, strongly inhibited spore germination in spite of the high spore concentration employed. In addition, germ tubes were significantly longer in the Impregon-treated group.

To evaluate the possibility that this level of Impregon might have divergent effects on germination and hyphal growth, a series of transfer studies was undertaken using discrete, isolated spores and derived growth. However, such single-spore colonies of C. cladosporioides from malt agar uniformly ceased growing when transferred to malt agar with Impregon. By contrast, the use of a large number of spores for inoculation resulted in abundant growth and sporulation despite the presence of Impregon (Fig. 5). When single spore colonies from such plates were transferred to fresh malt agar with Impregon, growth was arrested, suggesting that these were not Impregon-resistant variants. However, growth proceeded normally when malt + Impregon-germinated spores were transferred to unmodified malt agar (Fig. 6). Essentially similar results were obtained when Aspergillus fumigatus isolates were employed.
FIG. 5. Aspergillus fumigatus after 5 days' incubation on malt agar (left) and malt-Impreggon agar (right). Inhibition is not evident in these generously inoculated plates.

FIG. 6. Single-spore colonies of C. cladosporioides after 5 days on malt agar (left) or malt-Impreggon agar (right).

DISCUSSION

Impreggon, when used as directed, did not inhibit mixed mold growth on cotton/polyester fabric, burlap rug backing, or painted wood; however, in apparently identical concentrations, it drastically inhibited spore germination by two of the component taxa. Possible explanations for this apparent contradiction include: (1) TCSA stimulates hyphal growth or sporulation while suppressing spore germination, (2) certain of the fungus taxa or strains employed are resistant to the effects of Impreggon, or secrete Impreggon-inhibitory factors, (3) TCSA dose levels are inadequate to effect inhibition when Impreggon is used as directed for household surfaces.

The possibility that TCSA might stimulate hyphal growth is consonant with observations that Impreggon-treated fabric samples often supported a slightly greater mold growth than controls, and that the germ tubes in Impreggon-treated
spores were much longer than those of control spores. However, single-spore studies clearly indicated that, when present in sufficient concentration, TCSA inhibits both spore germination and hyphal growth. It remains possible that one or both of these processes may be stimulated at doses too low to inhibit spore germination. Such enhancement was noted by Slifkin\(^2\) in a study of the mutagenic effects of common fungicides.

Doubt is cast on the second hypothesis by our experiments in which large numbers of *C. cladosporioides* and *A. fumigatus* spores were inoculated on malt-Impregon agar. Although a large number of colonies did appear, subsequent transfer of these apparently resistant colonies to fresh malt-Impregon agar completely inhibited their growth. It is most likely, in these experiments, that the amount of TCSA in the original malt-Impregon agar was inadequate to inhibit the large number of spores inoculated. A direct correlation between minimum inhibitory concentration and inoculum size for three bacterial taxa is reported by Woodroffe and Wilkinson\(^3\) using this compound. Strains of *C. cladosporioides* isolated from air comprised the bulk of growth in surface treatment studies. Sporulation and germ tube growth by this same fungus were readily inhibited by Impregon, however, essentially eliminating specific resistance as a factor. The possibility that other organisms present in the inoculation mixtures were appreciably less susceptible to inhibition by TCSA remains to be evaluated.

TCSA is known to inhibit both prokaryotic and eukaryotic cells and uncouples oxidative phosphorylation in rat liver and fly mitochondria,\(^4\) indicating a wide range of effectiveness. However, species differences in susceptibility to fungicides appear to be well established.\(^5\) Furthermore, cell membrane lipid composition, which probably differs among fungus taxa, may affect the surface binding of TCSA and its related fungistatic effect.\(^3\)

The most likely source of these results is that Impregon, when used according to the manufacturer's directions, provides inadequate concentrations of TCSA to suppress germination or inhibit growth. The apparent relationship between inhibition by TCSA and inoculum size mentioned previously supports this hypothesis. Hamilton\(^6\) states that the antibacterial action of TCSA results from its binding to the cell membrane, and the amount of TCSA available to each bacterial cell is of critical importance to the inhibitory effect. Although documentation of TCSA effects on fungi is scanty and its mode of action conjectural, a similar effect of inoculum size would not be surprising.

The data in Table I and effects depicted in Figs. 1 to 4 suggest that Impregon pretreatment did not suppress fungus growth (and may have favored it, at times). Such results could be explained in part by assuming that inocula were frankly excessive. However, the presence of significant intergroup differences (Table I) implies that the spore suspensions were not too concentrated to permit desired comparisons.

The final concentration of TCSA attained in our surface studies was 26 \(\mu g/ml\) for wash solutions (fabric) and 52 \(\mu g/ml\) in the paint and the spray for rugs. These concentrations, when present in semisolid laboratory media, have been more than adequate to inhibit spore germination and hyphal growth of several of the test organisms. However, especially in the fabric studies, the TCSA
concentration in the wash water cannot be equated with the final concentration on the washed fabric or to the availability of the TCSA deposited on fabric, walls, or rug backing.

The Impregon package directions state that molds already present must be washed away before Impregon is applied, suggesting that Impregon has its effect on fungus particles deposited subsequent to treatment. The instructions for fabrics further state that after Impregon "does its job" in the wash water, bleach can be added. This implies, paradoxically, that Impregon acts on already present fungi and can subsequently be removed. Actually, for inhibition of bacteria, TCSA is effective only while in contact with and bound to the cell surface, and the bacteriostatic effect is reversible when TCSA is removed. Addition of bleach to rinses following Impregon treatment would inactivate the compound and prevent its further action on subsequently deposited spores. In addition, TCSA is soluble in detergents, rendering it likely that most of the TCSA (at least that not bound to living cells) is removed during rinsing. These effects leave little hope that Impregon might be effective when used in wash water. The reported contact-photosensitizing effects of halogenated salicylanilides render more permanent impregnation of fabrics with these compounds perilous.

In experiments involving surface spraying or painting with Impregon, one might assume that the TCSA concentration available on the surface would, at least initially, correspond to that applied. However, additional variables must be considered: (1) is the TCSA concentration per unit area of surface sufficient for inhibition; (2) is the full concentration of TCSA available to a spore landing on a previously treated surface?

The paint used is designed to cover 400 ft²/gallon or 97 cm²/ml, providing each square centimeter of wood surface with 0.01 ml paint and 0.52 µg TCSA. This level is above the minimum inhibitory concentration for bacteria reported by Woodroffe and Wilkinson. However, for antimicrobial activity to be realized, some suitable solvent in adequate amounts must be present. Considering that TCSA is almost insoluble in water, achieving anywhere near the mean inhibitory concentration reported for bacteria of 0.15 µg/ml in moisture condensed from air is unlikely. The ability of the vinyl copolymer in the paint to physically bind TCSA may further restrict the activity of this agent. Probably, only a thin micro-layer of TCSA is available for dissolution in surface water, and this may be present only at freshly painted surfaces.

Similar drawbacks appear to prevail at many surfaces sprayed with Impregon solutions. The fibrous nature of burlap makes the total surface to be covered enormous, and the resultant concentration of TCSA per unit area correspondingly small. This effect, combined with the insolubility of TCSA, suggests that a minimum inhibitory concentration would be difficult to attain. We did not study other surfaces for which Impregon has been proffered, but the present data give no indication of probable success. We have no information on Impregn's suitability as a fungistatic agent in plant substrates or in humidifiers, vaporizers, or dehumidifiers. Addition of Impregon to humidifiers or vaporizer reservoirs would promote aerosolization of TCSA with both skin and inhalant exposure. Dehumidifier reservoirs seem the logical areas for employment of
sparsely soluble antimicrobial agents. However, both the safety and the bioavailability of any agent proposed for this purpose must be scrutinized carefully.

REFERENCES