Influence of Benomyl and Methyl 2-Benzimidazolecarbamate on Development of *Penicillium digitatum* in the Pericarp of Orange Fruit

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ABSTRACT

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Sporulation of *Penicillium digitatum* on the surface of decaying oranges was inhibited by superficial residues of benomyl and methyl 2-benzimidazolecarbamate (MBC) resulting from postharvest treatments with wax formulations of the fungicides. Benomyl residues prevented emergence of hyphae from the fruit surface during 2 wk of storage at 20 C, whereas MBC primarily inhibited sporulation of the mycelial mat that formed on fruit after treatment. Penetration of the two fungicides was investigated by (i) microscopic observations of *P. digitatum* in sections of the peel (pericarp) of diseased oranges, (ii) bioassay of serial sections of peel cylinders from treated oranges for fungistatic activity against *P. digitatum*, and (iii) measurement of the cross-sectional distribution of radioactivity in the peel of fruit treated with tritium-labeled fungicides. All techniques indicated that benomyl penetrated the peel more efficiently than MBC, forming a fungistatic barrier in the flavedo (exocarp) that prevented eruption of the hyphae of *P. digitatum* through the epidermis.

Additional key words: carbendazim, citrus fruit, postharvest decay control, sporulation inhibition.

Green mold incited by *Penicillium digitatum* Sacc. is a limiting factor in long-distance marketing of citrus fruits, especially those produced in arid areas (9). The disease is initiated by germination of airborne conidia in harvest-related injuries in the peel (pericarp) of the fruit. Fungicides inhibitory to the pathogen must be applied within 48 hr when ambient temperatures are 20–27 C in order to abort the infection process. Prompt treatment is not always possible owing to the vicissitudes of commercial handling practices. A percentage of fruit in most crops become infected by *P. digitatum* after harvest. Practical losses due to green mold arise from two sources: individual diseased fruit and sound fruit devalued ("soiled") by superficial contamination with spores of *P. digitatum*.

Benomyl (methyl-1-[butylcarbamoyl]-2-benzimidazolecarbamate) has been used extensively since the early 1970s to prevent infection of citrus fruits after harvest by *P. digitatum* and other fungi (4–6,8–10,15) and in more recent years to control sporulation of *P. digitatum* on diseased fruit (8–10). Application of benomyl to fruit under commercial conditions is difficult because the compound is unstable in most wax formulations, decomposing to MBC (carbendazim) to a significant degree within several hours after mixing (M. J. Kolbezen and J. W. Eckert, *unpublished*). MBC is as active as benomyl against *P. digitatum* in vitro and also has been reported to equal benomyl in preventing fruit infection (14,17). We reported recently that MBC was less effective than benomyl in suppressing sporulation of *P. digitatum* on diseased fruit (10). The present investigation was undertaken to examine the behavior of the two fungicides on oranges in an effort to explain their differential effect on sporulation of *P. digitatum*. Portions of this investigation have been reported in abstract form (11).

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MATERIALS AND METHODS

Treatment of oranges with benomyl and MBC. Benomyl (50% W.P., Benlate®) and MBC (carbendazim, 50% W.P., BAS 3460F, Badische Anilin-& Soda-Fabrik AG, Limburgerhof, West Germany) were suspended in a water-based wax formulation (Sta-Fresh 200, FMC Corp., Riverside, CA 92502) diluted with water to contain 7% (w/w) shellac. The fungicide suspensions in the wax formulation were passed through a 75-μm screen to eliminate undispersed solid material.

Washed navel oranges (size 113, 6.60 cm diam) with a closed blossom end were selected at a local packinghouse. The fruit were dipped individually in the wax formulation or in one of the fungicide-wax formulations for 1 sec, then dried on a wire rack placed in

front of a fan. Three fruit from each treatment were analyzed for deposit of benzimidazole fungicides. Some of the fruit treated with each fungicide were inoculated with *P. digitatum* by injection of 0.2 ml of a suspension of 10^7 conidia per milliliter into the pulp. All fruit were stored at 20 C. After 4 days, hyphae of *P. digitatum* were just visible on the surface of inoculated fruit treated with wax only or MBC-wax. After 14 days, no hyphae were visible on fruit treated with benomyl-wax.

Preparation of peel samples for microscopic observation. Samples of peel of inoculated fruit were collected from control and MBC-treated fruit after 4 days of incubation and from benomyl-treated fruit after 11 days. The 4–5 mm^2 squares of peel were fixed in formalin-alcohol-acetic acid-water (13:100:8:90, v/v) (FAA) and embedded with paraffin. Sections 20 μm thick were cut perpendicular to the surface of the fruit on a rotary microtome. The hyphae were stained with 1% (w/v) safranin O in 50% (v/v) ethanol, and the host tissue was differentiated with 10% (w/v) ferric chloride and 2% (w/v) potassium ferrocyanide.

Bioassay of fungicide residues in orange peel. Cylinders (4 mm diam) were cut from the peel of oranges stored for 14 days at 20 C after fungicide treatment. Serial cross sections 40 μm thick were cut from the cylinders, from the epidermis inward, on a freezing microtome. Each section was placed in a microbeaker (5 mm deep, 10 mm diam) and 5 μl of a suspension of conidia of *P. digitatum* (about 10^4 conidia) was spread on the surface of the section. Each microbeaker with a section adhering to the bottom was inverted on the surface of 1% (w/v) water agar in a petri dish and incubated at 25 C for 40 hr. The sections were then fixed with FAA. The fixative was removed from the microbeaker with a microsyringe and replaced with saturated aqueous chloral hydrate (about 50%, w/v). The peel tissue was transparent after 15 min; the chloral hydrate solution was then removed, and the hyphae were stained with 1% (w/v) acid fuchsin in lactophenol (phenol-lactic acid-glycerol-water, 20:20:40:20, w/w). Excess stain was removed by repeatedly rinsing the sections in clear lactophenol.

Analysis of fungicide residues on oranges. The deposit of benomyl and MBC on treated fruit was determined by the general procedure described elsewhere (10). Individual oranges were tumbled gently for 10 min in four consecutive 200-ml portions of ethyl acetate. The extracts were combined and heated to convert benomyl to MBC. The ethyl acetate solution of MBC was extracted with four 10-ml portions of 0.5 *N* HCl. The combined HCl extract was adjusted to pH 8 by addition of 5 *N* KOH, 12 g of NaCl was added, and the solution was buffered at pH 8 by addition of 5 ml of saturated Na_2HPO_4. This aqueous solution of MBC was extracted with four 10-ml portions of chloroform. Each of the chloroform solutions was then extracted with 0.1 *N* HCl. The concentrations of MBC in each of the HCl extracts was determined spectrophotometrically at 281 nm. The residues are reported as benomyl (MBC × 1.519).

Synthesis of ^{3}H-MBC and ^{3}H-benomyl. Methyl 2-benzimidazolecarbamate (MBC) was synthesized by reaction of *o*-phenylenediamine with dimethyl 2-methylthiopseudourea-1,3-dicarboxylate (16). The identity and purity of the product were confirmed by infrared and mass spectra and by thin-layer chromotography. A sample of authentic MBC was tritiated by New England Nuclear Corp., Boston, MA 02118, and the resultant crude trifluoroacetate salt of ^{3}H-MBC was dissolved in water and precipitated with NH_4OH (specific activity, 4.45 mCi/mg). For final purification, 1mCi of ^{3}H-MBC was dissolved in chloroform-methanol azeotrope (87:13, w/w) and the solution was streaked on a 20 × 20 cm thin-layer chromatogram coated with a 2-mm thick layer of silica gel (EM Laboratories Inc., 500 Executive Blvd., Elmsford, NY 10523). The chromatogram was developed with benzene-ethyl acetate-acetic acid (25:25:1, v/v). After the chromatogram was dry, ^{3}H-MBC was located by autoradiography and the band was scraped from the plate. ^{3}H-MBC was eluted from the silica powder with water-saturated chloroform-methanol azeotrope. This purification procedure was repeated during the course of the investigation when analytical radiochromatograms revealed decomposition of ^{3}H-MBC. The tritium label, which presumably was in the benzene ring of MBC, was quite stable. Heating the sample of ^{3}H-MBC in 5% (w/w) trichloroacetic acid at 80 C did not release a significant amount of radioactivity to the aqueous phase after precipitation of MBC.

Measurement of penetration of ^{3}H-MBC and ^{3}H-benomyl into orange peel. ^{3}H-MBC and ^{3}H-benomyl were dispersed in 50% methanol-water (v/v) containing wax solids of Sta-Fresh 200 so that application of a 25–50 μl portion of the mixture to a 0.636-cm^2 circular area (9 mm diam) on the surface of an orange resulted in a deposit similar to that produced when the whole fruit was treated with the fungicide-wax formulation. Specifically, 2.62 μg of ^{3}H-MBC (2.6×10^7 dpm) and 44 μg of wax solids (mostly shellac) in 25 μl of 50% methanol was applied to an inked circle on the surface of the fruit. An identical quantity of ^{3}H-MBC was reacted with excess *n*-butylisocyanate for 2 hr at room temperature, and the reaction mixture was dried under vacuum. The dry residue, benomyl, was suspended in methanol-water containing wax and applied to fruit in the same manner as the wax formulation of ^{3}H-MBC. In practice, a multiple of the specified quantities of the fungicide-wax formulations was prepared to provide (i) three 25-μl aliquots each of ^{3}H-MBC-wax and ^{3}H-benomyl-wax to be placed on the surface of a single orange, (ii) two aliquots of each formulation to determine the radioactivity in the final formulation, and (iii) two aliquots of the benomyl formulation to establish the benomyl-MBC ratio in the formulation applied to the fruit. The fruit with three 0.636-cm^2 areas of the surface covered with benomyl-wax and three identical areas covered with MBC-wax was "painted" with the wax-only formulation except for the areas with the ^{3}H-labeled fungicides.

Samples of both fungicide-wax formulations were added to the scintillation fluid (toluene-Triton X-100, 2:1, v/v) containing 3.33 g of 2,5-diphenyloxazole per liter of the mixture, and radioactivity was measured in a Beckman CPM-100 liquid scintillation spectrometer. The relative amount of benomyl and MBC in the benomyl-wax formulation was determined by heating an aliquot of the formulation under alkaline conditions to convert benomyl to 1-(2-benzimidazolyl)-3-n-butyl urea (BBU) and MBC to 2-aminobenzimidazole (2-ABZ) (1,10,23) (Fig. 1). Duplicate samples of the benomyl-wax formulation were added to 1-ml portions of 0.5 *N* NaOH and heated at 100 C for 30 min. The reaction mixture was cooled, adjusted to pH 6 with NaH_2PO_4, and extracted five times with fresh volumes of ethyl acetate, each equal in volume to the aqueous phase. The BBU was extracted into the ethyl acetate and the 2-ABZ remained in the aqueous phase, pH 6. The radioactivity in both phases was measured, and the benomyl-MBC ratio in the formulation was calculated.

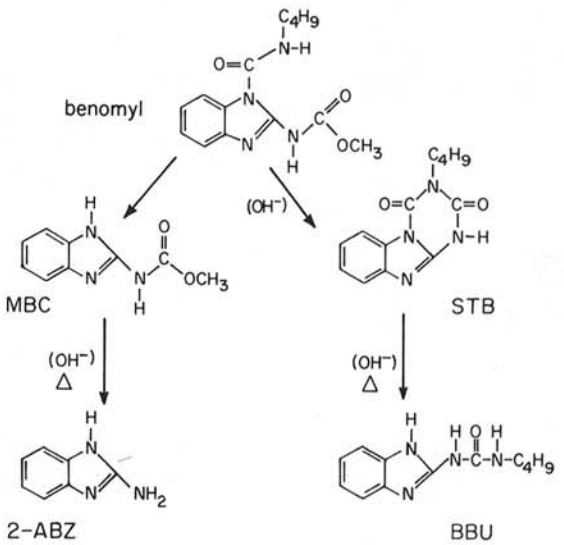

Fig. 1. Reactions of benomyl relevant to its behavior in formulations and to characterization of its residues on fruit.

After the fruit was stored for 2 wk at 20 C, cylinders of peel corresponding to the inked circular areas covered with the fungicide-wax deposits were removed with a cork borer. Each cylinder was cut into a series of 1-mm cross sections, starting at the epidermis end of the cylinder. One cylinder was cut in the reverse direction to assess the possibility of anomalous measurements caused by contamination of the samples in the sectioning process. The first (epidermal) section of each cylinder from the benomyl-treated locations was heated in NaOH, and the extract was partitioned between ethyl acetate and pH 6 buffer to determine the benomyl-MBC ratio in the residue after storage. The first section cut from cylinders from the MBC-treated area was extracted with 1 ml of ethyl acetate-benzene (5:1, v/v) in a capped tube at 100 C for 30 min. All other sections (cut from the albedo portion of the peel) were extracted with 5% (w/w) aqueous trichloroacetic acid at 80 C for 30 min. Radioactivity in all samples was measured in the toluene-Triton X-100 scintillation fluid.

RESULTS

Effect of benomyl and MBC residues on distribution and sporulation of *P. digitatum*. Figure 2 shows extensive mycelial growth and sporulation on the surface of inoculated navel oranges treated with wax only or MBC-wax formulation (1.25 g of MBC per liter) and stored for 14 days at 20 C but virtually no hyphae on the surface of fruit treated with 1.90 g of benomyl per liter. The peel of the benomyl-treated fruit developed a light brown color but retained the leathery consistency characteristic of sound fruit. Concentrations of the two fungicides were equimolar. The mean residue of MBC-benomyl (expressed as benomyl) and standard deviation on fruit treated with benomyl or MBC was 4.86 ± 0.54 and 4.72 ± 0.31 mg/kg, respectively. The average surface concentration was 4.94 $\mu g/cm^2$.

Figure 3A shows hyphae in the peel (pericarp) of MBC-treated oranges and eruption of sporophores through the epidermis after incubation of the fruit for 4 days at 20 C. Figure 3B shows hyphae in the exocarp (flavedo) of benomyl-treated fruit but an intact epidermis 11 days after inoculation, suggesting a fungistatic barrier in the subepidermal tissue of the peel.

Determination of penetration of benomyl and MBC by bioassay of peel sections. Cylinders (4 mm diam) were removed from the peel at the equator of fruit from the lot described in the previous section and cut into serial cross sections 40 μm thick. Sections 1–5 consisted of epidermis and flavedo (exocarp) tissue, and the remaining sections were of albedo (mesocarp) tissue. Conidia of *P. digitatum* germinated, and germ tubes developed vigorously on all sections from fruit treated with wax only. Germination of conidia was greatly inhibited on sections 1–10 from MBC-treated fruit, and germ tubes were stunted, gnarled, and excessively branched. Conidia on sections 11–15 germinated normally, but germ tube growth was slightly less than that of controls. Development of conidia on sections 16–30 was similar to that on corresponding sections from the peel of fruit treated with wax only. In benomyl-treated fruit, conidial germination was severely inhibited on sections 1–5 and germ tube growth was strongly reduced on sections 6–50. Thus, the MBC-wax treatment resulted in strong inhibition of hyphal growth to a depth of 0.4 mm in the peel and some reduction in germ tube length to 0.6 mm. In fruit treated with an equivalent dosage of benomyl, strong fungistatic activity was detected in all tissue from the epidermis to 2 mm below the surface.

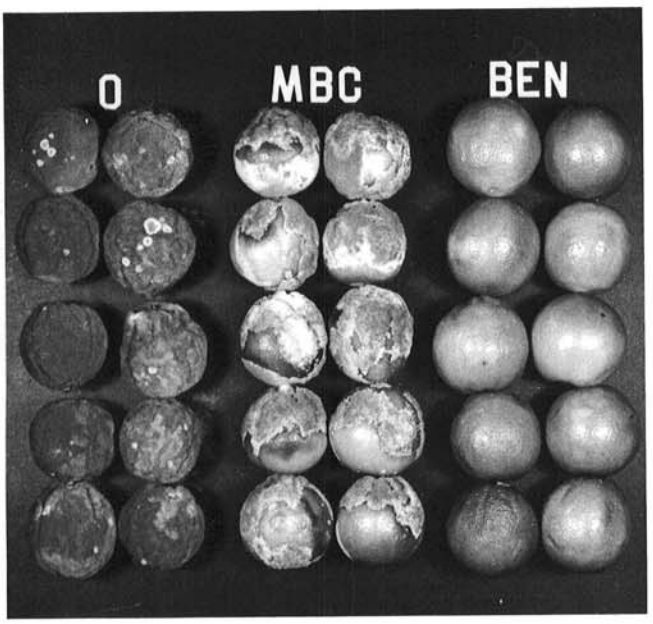

Fig. 2. Growth of *Penicillium digitatum* on navel oranges treated with wax formulations containing equimolar concentrations of benomyl and MBC. 0 = wax only; MBC = 1.25 g of MBC per liter; BEN = 1.90 g of benomyl per liter.

Penetration of ^{3}H-benomyl and ^{3}H-MBC into orange peel. Demonstration that a substantial proportion of the radioactivity in the benomyl-wax formulation was indeed in the form of benomyl was essential because (i) the yield of the microsynthesis of about 40 μg of benomyl could not be determined directly and (ii) benomyl is known to be unstable in the presence of methanol and the Sta-Fresh 200 wax formulation. Analysis of the benomyl-wax formulation after the samples had been spotted on the surface of fruit showed that 85% of the radioactivity was in the form of benomyl and 15% was in the form of MBC. After 2 wk of storage of the fruit, areas with the benomyl deposits revealed that 71% of the radioactivity was still in the form of benomyl and 29% was in the form of MBC.

Figure 4 shows the distribution of radioactivity in serial 1-mm cross sections cut from cylinders removed from the peel of a Valencia orange treated with benomyl-wax or MBC-wax formulation and stored for 2 wk at 20 C. The peel section 0–1 mm included the surface wax, cuticle, epidermis, and outermost part of the flavedo (exocarp). Section 1–2 mm included the major part of the flavedo and the outermost portion of the albedo (mesocarp). Sections 3–4 mm, 4–5 mm, and 5–6 mm consisted entirely of albedo tissue. As expected, the major portion of the applied fungicides (70–80%) remained associated with the outermost layer of the peel, probably external to the epidermis, for the experimental period. Subepidermal sections at all depths from benomyl-treated fruit contained slightly more than twice the radioactivity found in corresponding sections from MBC-treated fruit. Although the distribution of radioactivity does not provide information on the intensity of antifungal activity, the data do support the evidence obtained by microscopic observation and bioassay that benomyl moved into the peel more readily than MBC and inhibited the growth of *P. digitatum* in subepidermal tissues.

DISCUSSION

Numerous investigations have shown that postharvest application of benomyl can prevent development of certain latent infections on tropical fruits, including citrus, that are not controlled by conventional protective fungicides (6,8). These observations indicate that an effective dosage of benomyl-MBC can penetrate surface barriers of fruits that exclude nonsystemic fungicides. Brown and Albrigo (3,5) showed that preharvest and postharvest applications of benomyl to oranges resulted in fungistatic concentrations of MBC in the peel tissues, and they suggested that these residues might prevent later development of *P. digitatum* in the peel. Additional evidence for penetration of benomyl into subepidermal layers of the peel is provided by our earlier observation (10) that hyphae of *P. digitatum* develop weakly, if at all, on the surface of the fruit with substantial residues of benomyl (eg, 3.80 mg of benomyl per kilogram), despite extensive colonization of the interior of the fruit by the fungus. Penetration of benomyl-MBC to a depth of 4–6 mm or greater into the flesh of peaches (19,20) and pears (2) has been shown by bioassay of subepidermal tissues of these fruits after storage at 20 C for several days following postharvest treatment with benomyl.

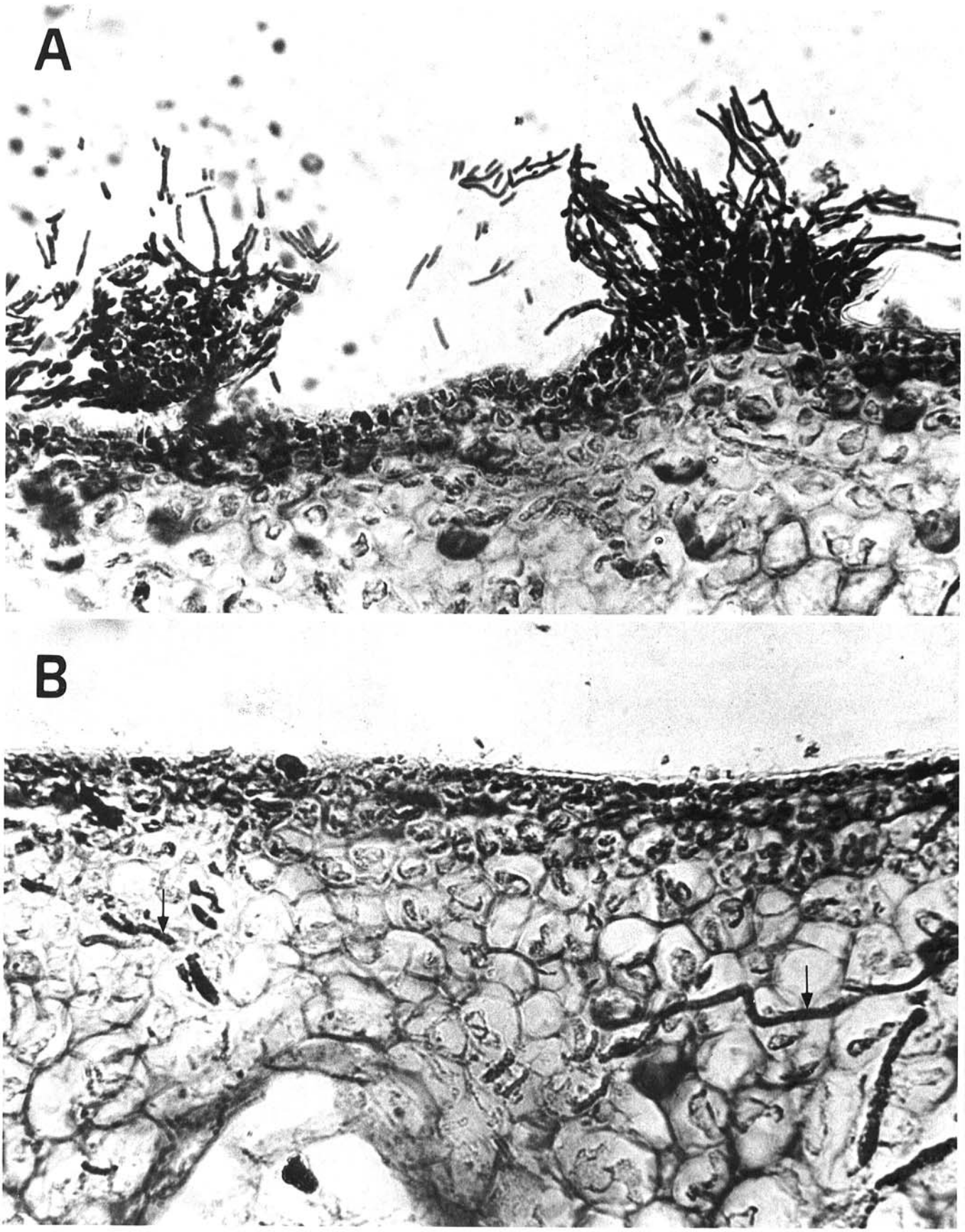

Fig. 3. Cross sections of outer peel (exocarp) of navel oranges inoculated internally with *Penicillium digitatum*. **A,** Fruit treated with 1.25 g of MBC per liter showing eruption of fungus sporophores through the epidermis after 4 days of incubation. **B,** Fruit treated with 1.90 g of benomyl per liter showing distribution of hyphae (arrows) beneath the surface after 11 days of incubation.

Benomyl has been used extensively to control postharvest diseases of several fruits (eg, citrus, apples, pears, and peaches) and often is formulated with substances that accelerate decomposition of benomyl to MBC (M. J. Kolbezen and J. W. Eckert, *unpublished*). However, no information is available on penetration of MBC into fruit relative to benomyl. Solel and Edgington (21) reported that benomyl moved more efficiently than MBC through the isolated cuticle of apple leaves. Upham and Delp (22) applied ^{14}C-benomyl and ^{14}C-MBC to bean and cucumber leaves and measured a greater accumulation of radioactivity in benomyl-treated leaves. Young and Hammett (24) reported smaller differences in uptake of the two fungicides when measured by accumulation at the margin of cucumber leaves after application at the leaf base.

Our investigation showed, by three criteria, that benomyl applied to the surface of oranges penetrates to a greater depth in the peel than an equimolar quantity of MBC. We considered the possibility that the ingredients of the commercial wettable powder formulations used may have contributed to the relative penetration of the two fungicides into the pericarp. We regard an interaction of this type to be unlikely because the fungicides were essentially reformulated by mixing with a single wax formulation containing a large amount of surface-active materials relative to the original wettable powder formulations. Furthermore, the increased penetration of ^{3}H-benomyl compared with that of ^{3}H-MBC after identical application correlated well with the biological assays of fungicide penetration into the pericarp of the orange.

Evidently, the properties favoring movement of benomyl through the cuticle and other surface barriers of the leaves of other plant species also increase penetration of benomyl into the peel of oranges. Molecules such as MBC are highly associated because of hydrogen bonding through the imino hydrogen atom (12). Replacement of this hydrogen atom with a butylcarbamoyl group, as in the case of benomyl, reduces molecular association and should be accompanied by an increase in hydrophobic character of the molecule. Evidence for this difference in the two molecules is provided by the greater solubility of benomyl in hydrocarbon solvents compared with that of MBC (7). This factor apparently enhances the movement of benomyl through the lipophilic barriers (eg, wax and cuticle) on the surface of citrus fruits. The well-known instability of benomyl in dilute solutions should lead to its conversion to MBC at an early stage of its migration into the peel. Further movement of benomyl-MBC in the peel tissues could take place through intercellular spaces, as with paraffin oil (13), or by diffusion through cells, as in slices of potato tuber (18).

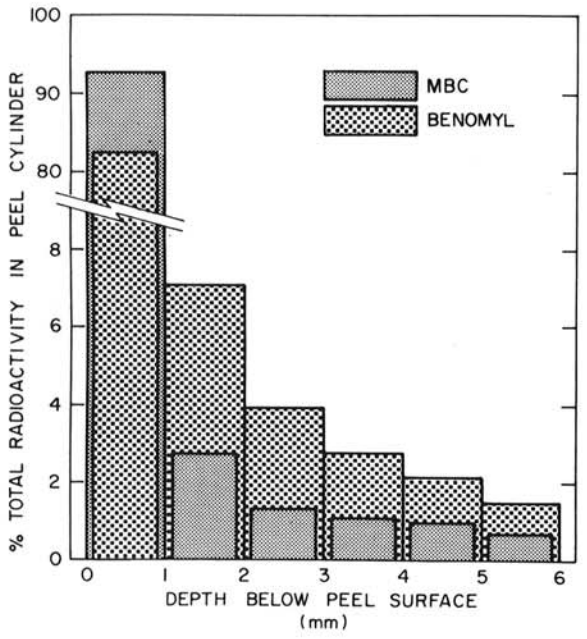

Fig. 4. Penetration of ^{3}H-MBC and ^{3}H-benomyl into the peel (pericarp) of a Valencia orange. An equimolar (2.6×10^{7} dpm) amount of each compound was applied in the wax formulation to 0.636-cm^{2} areas of peel.

Formulations of benomyl that minimize its conversion to MBC should provide better control of certain diseases of citrus fruits, such as stem end rot (*Diplodia natalensis* Pole-Evans) and anthracnose (*Colletotrichum gloeosporioides* Penz.), initiated by propagules apparently shielded from aqueous solutions of polar fungicides by lipophilic barriers of the fruit surface (6). Development of *P. digitatum* on the surface of citrus fruit is controlled more efficiently by benomyl than by MBC, but elimination of superficial hyphae from diseased fruit may permit serious secondary infection by other fungi. *Geotrichum candidum* Link ex Pers. and *P. italicum* Wehmer often produce a secondary rot on the surface of benomyl-treated fruit colonized internally by *P. digitatum*. These wound pathogens are more tolerant of benomyl than *P. digitatum* is and can transform the isolated leathery rot produced by the latter fungus into a soft rot capable of spreading to adjacent fruit by contact. In contrast, citrus fruits infected with *P. digitatum* and treated with biphenyl or high concentrations of thiabendazole characteristically are covered with a thick mat of nonsporulating hyphae. Such fruit rarely develop a secondary soft rot, either because of the competition of the established surface mycelium of *P. digitatum* or because the interwoven hyphae provide mechanical support for the surface of the decayed fruit.

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