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Interactions Involved in

Fig. 1. *Armillaria mellea* on citrus, with bark exposed to show mycelia. Mushroom gills are evident because tree was lying horizontally on the ground during sporulation.

The *Armillaria* story is an interesting one. *A. mellea* is not a soil inhabitant and must survive as mycelia (Fig. 1) or rhizomorphs (Fig. 2) on large root and stem pieces. The fungus may remain alive for years—even decades—on these roots and stems. The history of many orchards and plantations worldwide includes *A. mellea* surviving for long periods in the absence of living host plants, then taking a toll of new plantings of susceptible crops, such as citrus (Fig. 3), peach, and grape. For biocontrol of the fungus, certain factors must interact in a relatively short time under rather specialized conditions.

The California Project

H. S. Fawcett and R. Weindling, working in the Department of Plant Pathology, Citrus Experiment Station, now the Department of Plant Pathology, University of California, Riverside (UCR), showed in the 1930s that substances produced by one organism were toxic to other organisms. Some of their ideas concerning the ecological relationships of soilborne organisms were put into practice when Fawcett and D. E. Bliss initiated a project on *A. mellea* at the California Agricultural Experiment Station in 1939. Bliss (1) showed that *A. mellea* was killed in some soils treated with carbon disulfide (CS_2), even though the initial concentrations were not sufficient to kill the fungus directly. He postulated that *Trichoderma* spp. were responsible for the direct kill. After Bliss's death, the project was continued by E. F. Darley until 1963, when leadership was assumed by D. E. Munnecke. W. D. Wilbur has worked continuously on the project since 1947.

Fig. 2. *Armillaria mellea* on citrus roots. (Left) Rhizomorphs on surface; (right) bark removed to show pseudosclerotia zone lines.

The thrust of the research since 1963 has been to answer the question: "Why is *A. mellea* often killed in soil after exposure to treatments with fumigants, heat, or drying that are known to be sublethal under other conditions?" The UCR group of J. L. Bricker, Darley, N. T. Keen, M. J. Kolbezen, Munnecke, K. A. Oduro, H. D. Ohr, J. J. Sims, and Wilbur has discovered some answers to this question, and enough data are at hand to support the following ideas about the phenomenon.

Obviously, at some very high concentrations of a fumigant in soil, *A. mellea* will be killed directly. At sublethal concentrations of the fumigant, however, biocontrol mechanisms kill *A. mellea*, usually 15–30 days after fumigation. *Trichoderma* spp., the most common antagonists under these situations, are potent because they may produce antibiotic substances and because they are parasitic on other fungi (11). Also, *Trichoderma* spp. are more resistant than *A. mellea* to the fumigant, have fewer competitors, and can reproduce rapidly. Thus, a potent antagonist may build up very rapidly in fumigated soil. The antagonist cannot effectively attack *A. mellea*, however, until the defense mechanisms of the fungus are weakened or perhaps eliminated. We refer to this as the "stressing effect." The stressing effect follows exposure of *A. mellea* to sublethal concentrations of a fumigant and is manifested by a lack of measurable growth of the fungus in vitro for up to 4 weeks after fumigation. The stressing effect is induced not only by fumigants but also by heating and drying.

The nature of the stressing effect is not known. Presumably, metabolism is affected (bioluminescence ceases) and repair of ruptures in, or formation of, pseudosclerotial walls of the fungus is hindered. *A. mellea* produces antibiotic substances in vitro but not during the lag period of growth after fumigation. Thus, the subsequent breach in the defense mechanisms combined with an increase in numbers of the antagonist can be lethal to *A. mellea*. When these factors interact within a relatively short time, *A. mellea* may be killed after exposure to sublethal concentrations of a fumigant, such as methyl bromide (MB) or CS_2, or to heating or drying.

Methyl Bromide's Effectiveness in Soil and on Agar Disks

We have used an apparatus designed by Kolbezen and Abu-El-Haj (4) and modified by Ohr, Munnecke, and Millhouse (12) to fumigate propagules of organisms with accurately controlled concentrations of gases in a moving

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Controlling *Armillaria mellea*

airstream (Fig. 4); the concentration of a toxic gas is held constant and the dosage is regulated by varying the exposure time. Two preparations of *A. mellea* were studied: 1) mycelia growing on agar disks and 2) mycelia and rhizomorphs growing on steam-sterilized citrus roots; naturally infected citrus roots were used in some experiments. The agar pieces were fumigated, then plated-out on potato-dextrose agar (PDA) containing 15 μg ml (ppm) of benomyl. The root pieces were placed in containers and buried for 21 days in unsterilized orchard soil. Then, pieces of wood cut from the roots were plated on PDA-benomyl agar; benomyl inhibited growth of *Trichoderma* spp. but not of *A. mellea*, so the slow-growing *A. mellea* was not obscured by *Trichoderma* spp.

LD_{90} or LD_{95} values were obtained by plotting probit percent kill of the fungus vs. log time (T) of exposure. Steep linear dosage response (DR) curves were obtained. At least six concentrations (C) of MB were used to obtain LD values for each concentration. Concentration × time (CT) curves were obtained by plotting individual values of C and T for each LD value using log C as the ordinate and log T as the abscissa. Resulting plots were linear.

A. mellea mycelium growing in roots and subsequently buried in unsterilized soil was very susceptible to MB. CT required for a LD_{95} dose was approximately 115,000 μl l. × h (ppm × h), even when very low concentrations of MB were used (8). In contrast, approximately 220,000 μl l. × h was required for a LD_{90} dose when agar disks containing the fungus were fumigated but not exposed to soil (5).

The fact that *A. mellea* is killed at lower CTs in soil than in agar indicates that some factors in soil enhance MB's effectiveness.

Evidence of Biological Activity in Soil After Fumigation

In early fumigation experiments to control *A. mellea*, direct fungicidal action of the chemical was assumed to kill the pathogen. Bliss (1) questioned this concept when he observed that the number of *Trichoderma* spp. isolations increased as those of *A. mellea* decreased in fumigated *A. mellea*-infected roots incubated in unsterilized soil. He observed that when *A. mellea*-infected root pieces were fumigated with CS_2 and incubated either without soil or in previously sterilized soil, *A. mellea* survived. When the infected root pieces were buried in unsterilized soil or in *Trichoderma*-infested soil after fumigation, however, *A. mellea* was killed and replaced by *Trichoderma* spp. Also, when unfumigated *A. mellea*-infected roots were buried in pure soil cultures of *Trichoderma* spp., the fungus did not survive. Bliss concluded that *A. mellea* was not killed by direct fungicidal action of CS_2, but by the increase in the *Trichoderma* spp. population. Garrett (3) confirmed some of Bliss's work but his major contribution was to demonstrate that fumigation of the soil did not stimulate *Trichoderma* spp. population increases to levels sufficient to kill unfumigated *A. mellea*. He reasoned that *A. mellea* must also be affected in some way.

Ohr et al (11) investigated these relationships in a quantitative manner. They treated naturally as well as artificially infected citrus root pieces with sublethal combinations of high concentrations of MB for short times, usually less than 5 hours. After fumigation, some pieces were stored in unsterilized soil and others in sterilized soil. Ohr et al made approximately 2,600 isolations per experiment, sampling the surface, the outer cortex, and the center of the root.

Almost 100% of MB-treated inoculum stored in sterilized field soil survived, but only a small fraction of that stored in unsterilized soil survived after 30 days. This confirmed Bliss's data that biological activity in soil may be responsible for death of *A. mellea* after fumigation. Most important, a decrease in *A. mellea* was positively correlated with an increase in *Trichoderma* spp. in fumigated root pieces incubated in field soil (Fig. 5). Similar responses were obtained when naturally infected roots were used. *Trichoderma* spp. were the only organisms directly correlated with the decrease in

Fig. 3. Citrus grove severely infected by *Armillaria mellea* (healthy tree in right background). Grower planted several times to replace killed trees, with no success.

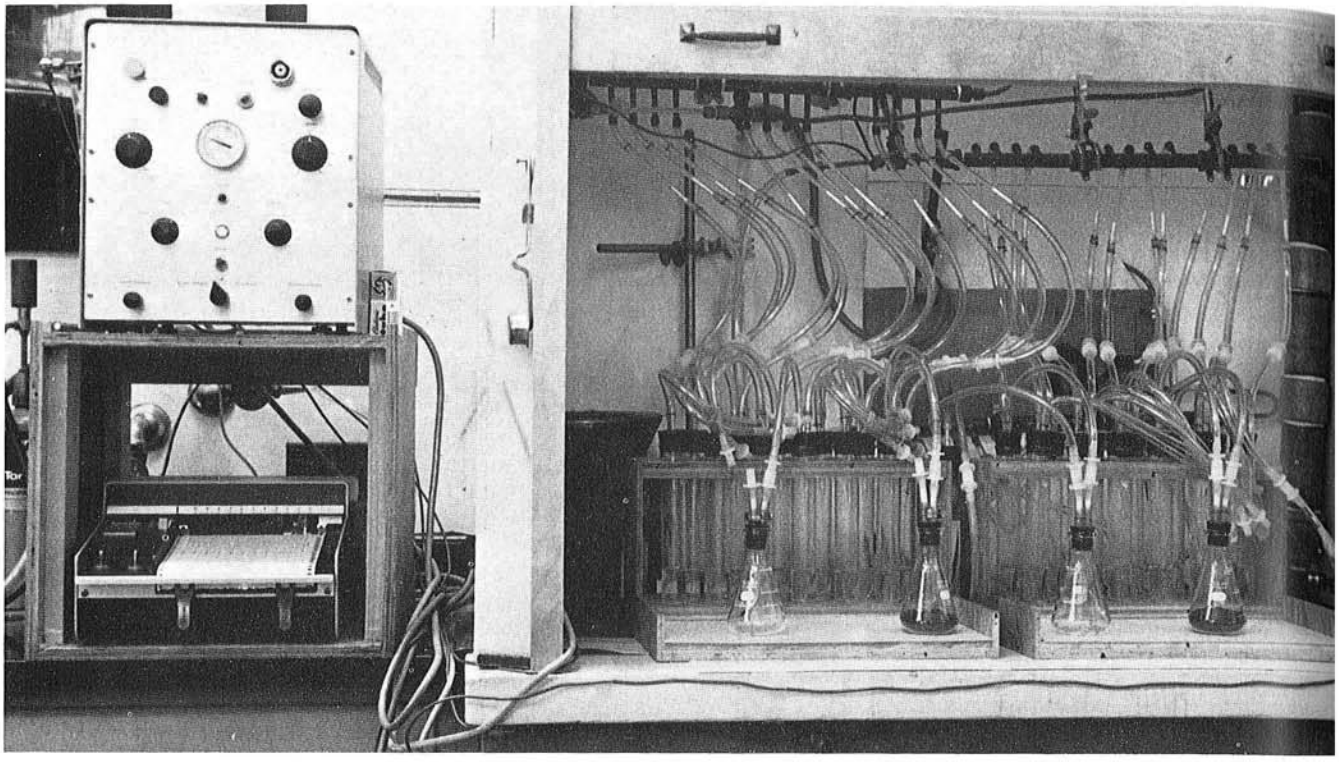

Fig. 4. With this apparatus for applying methyl bromide or carbon disulfide, the fumigant is mixed with air in a constant proportion, humidified, and swept over target fungi held in flasks (shown), soil, long tubes, or other containers. The concentration of gas is monitored by attaching inlet or outlet ports directly to a gas chromatograph (left). Safety features include installation in a fume hood, alarms that sound at current outages or exhaust fan breakage, and, in emergencies, automatic diversion of exhaust gases through a large column of activated charcoal (right).

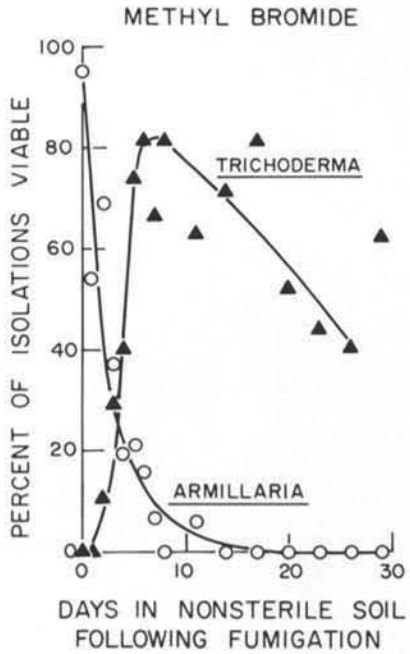

Fig. 5. Percent of isolations yielding *Armillaria* or *Trichoderma* from roots artificially infected with *A. mellea* and kept in unsterilized soil after treatment with methyl bromide.

viability of *A. mellea*, although species of *Fusarium*, *Rhizopus*, and *Mucor* and various unidentified bacteria also were frequently found.

The quantitative relationship between loss in viability of *A. mellea* and increase in numbers of *Trichoderma* spp. in soil is highly significant and further evidence of the biological activity in soil after fumigation.

Methyl Bromide's Effect on *Trichoderma* spp.

When responses of agar disks of *Trichoderma* spp. and *A. mellea* to MB were compared, the slopes of the DR curves were steep and almost parallel, but *Trichoderma* spp. were 1.9–2.3 times more resistant than *A. mellea* to MB. This was somewhat expected, since *Trichoderma* spp. are commonly found sporulating on fumigated soils and on *Armillaria*-infected roots. Thus, *Trichoderma* spp. are able to survive much higher concentrations of MB than *A. mellea* and populations presumably are increased by field fumigations with MB that are lethal to *A. mellea*.

Responses of Pathogen and Antagonist to Heat

Cultures of *A. mellea* or *Trichoderma* spp. were started at one end of long (35-cm) tubes containing citrus agar and held at 23 C until growth rate was constant. The tubes were then held at temperatures ranging from 6 to 39 C (with 3 C increments) for 7 days and returned to the 23 C incubator. The cumulative linear growth was plotted as a function of time (Fig. 6). The responses of the two fungi to temperatures of 30 to 36 C was particularly noteworthy. Growth of *A. mellea* ceased during the treatment and did not resume for approximately 10–30 days. Again, *Trichoderma* spp. were much more tolerant of environmental conditions than *A. mellea*. In fact, growth of *Trichoderma* spp. increased as temperature increased.

A. mellea-infected roots were exposed to various time and temperature combinations and tested for viability immediately after treatment and after storage in natural soil for 15 days (7). Some treatments killed the fungus immediately (direct kill), while others were not lethal unless the roots were subsequently stored in soil (indirect kill). The viability of *A. mellea* in unheated infected roots stored in soil was not affected by storage. Indirect kill in the soil required less time and lower temperatures than direct kill. The difference became apparent when temperature vs. exposure time curves were plotted. Exposure times required for indirect kill and direct kill, respectively, were 0.6 and 1.6–3.0 hours at 43 C, 0.5–1.0 and 4.8 hours at 41 C, 2–3 and 30–45 hours at 38 C, 23–30 and 120–250 hours at 35 C, and 70–170 and 350–700 hours at 33 C.

The fact that treatment with heat, a physical agent, elicits responses in the pathogen and its antagonist, both in vitro and in soil, similar to those elicited by treatment with fumigants indicates that

the stressing effect is not due to residual activity of the fumigants.

Effect of Fumigants on Linear Growth of Fungi on Agar Medium

Mycelia and rhizomorphs growing on agar in tubes (50 cm × 2.5 cm diam) were treated by continuously sweeping a mixture of MB or CS_2 in humidified air over the surface of cultures of *A. mellea* and *Trichoderma* spp. (6). The concentration was constant and exposure lasted 6–10 days. After treatment, the gases were swept away by flushing with moist air. The propagules were then grown 20–30 days longer under a continuously flowing stream of air. The effect of the toxicants was determined by comparing the linear growth of fungi on gas-treated cultures with that of fungi on cultures treated only with moving air.

Visible growth of *A. mellea* stopped almost immediately on exposure to MB at 600, 1,200, or 2,400 μl l. (ppm MB in air) for up to 24 days and did not start again for varying times after the gas was removed (Fig. 7). With treatments above 1,000 μl l. and exposures of 1–12 days, the lag period (duration of exposure to the toxicant plus period after treatment before growth resumed) was approximately 20 days with few exceptions. *Trichoderma* spp. were more resistant than *A. mellea* to MB and were capable of growing during treatment with 600 or 1,200 μl l. of the gas; growth ceased at 2,400 μl l. but resumed almost immediately when the gas was removed.

Responses of the two fungi to CS_2 treatment were similar to those to MB treatment; both fungi were more tolerant of CS_2 than of MB. Again, it is very significant that *Trichoderma* spp. were relatively unaffected by the concentrations of MB or CS_2 that were toxic to *A. mellea*.

The similarity between the 21-day lag period before growth resumes after fumigation and the approximately 3-week period of storage in unsterilized soil after fumigation before *A. mellea* is killed by its antagonists may be coincidental. We prefer to think the latter is an indication of the stressing effect that occurs when *A. mellea* is subjected to sublethal concentrations of MB or CS_2. We think that the antagonists act during this lag period to eliminate *A. mellea*.

Production of Antibiotic Substances by *A. mellea*

Antibiotic production by *A. mellea* appears to be quite widespread and could be a generalized phenomenon. It was reported first by Oppermann (13) in 1951 and later by Richard (14) in 1971. Ohr and Munnecke (10) found it occurring in some of the UCR cultures as well as in four of six isolates supplied by J. Rishbeth of Cambridge University, United Kingdom. In our studies, two isolates of *A. mellea* produced in vitro substances inhibitory to *Streptococcus*, *Mucor*, *Trichoderma longibrachiatum* Rifai aggr., *T. harzianum* Rifai aggr., *Rhizopus stolonifer* (Ehrenb. ex Fr.) Lind, *Fusarium solani* (Mart.) Sacc., *F. roseum* Link ex Fr., and one of two strains of *Gliocladium virens* Miller, Giddens and Foster; these were the first reports of *A. mellea* producing antibiotics inhibitory to *Trichoderma* spp. The fact that many species of fungi and bacteria are susceptible to the antibiotics produced by *A. mellea* may be the reason this organism manages to survive in host tissues in soil for long periods.

Oduro et al (9) found that four chloroform-soluble antibiotic substances were produced in cultures by 17 clones of *A. mellea*. Attempts to purify and identify these substances are continuing.

A significant finding providing further evidence of the importance of the stressing effect was that antibiotic production was reduced by fumigation of *A. mellea* inocula with sublethal concentrations of MB (10). Water agar disks of *A. mellea* mycelia were fumigated with MB and incubated for 3 days at 6 C on PDA seeded with a sensitive bacterium. Bacterial growth was inhibited around the disks, and the diameter of the areas of inhibited growth decreased as the length of fumigation time increased.

The Stressing Effect

In 1934, Campbell (2) proposed that the pseudosclerotial envelope often

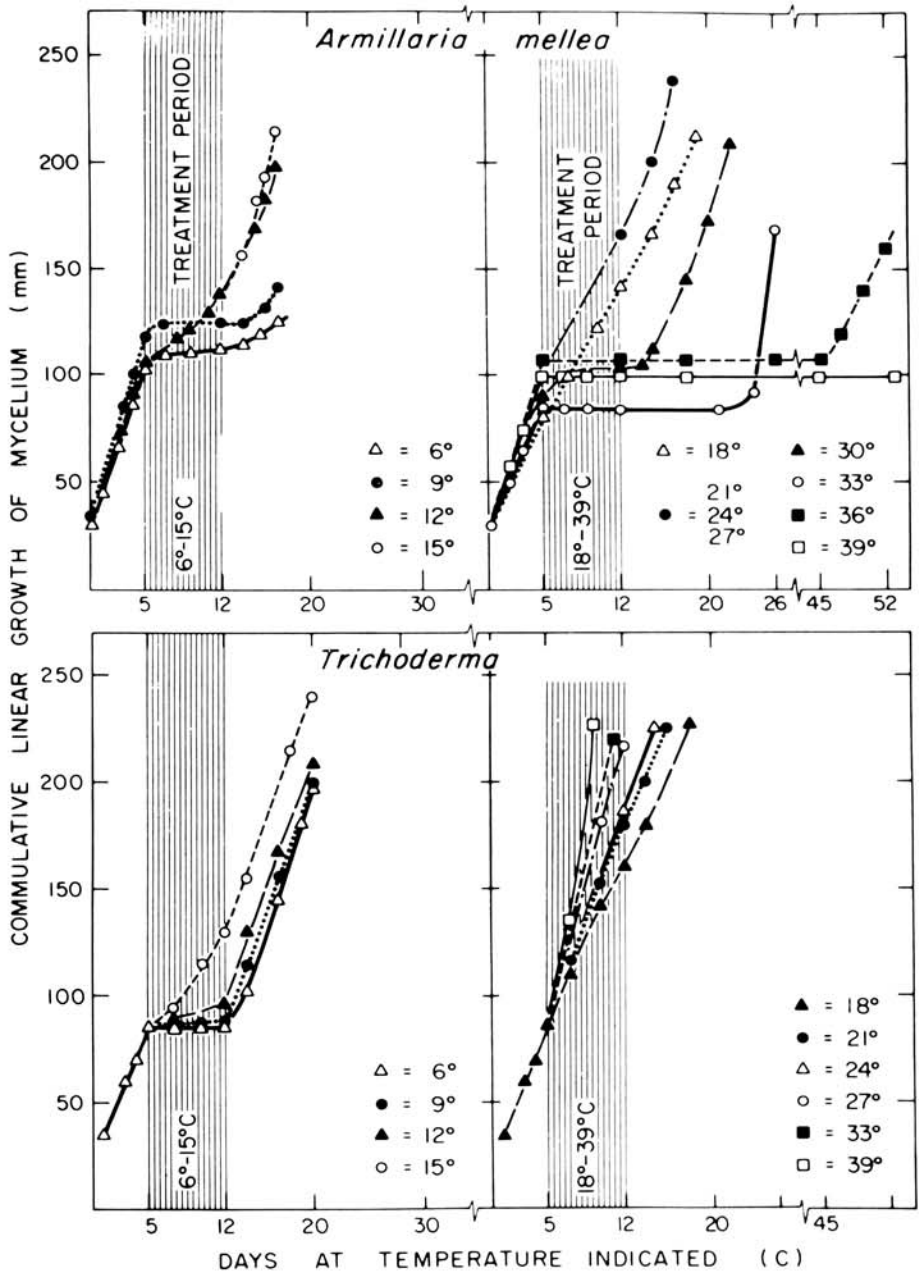

Fig. 6. Effect of temperature on linear growth of *Armillaria mellea* and *Trichoderma* spp. on citrus agar. Fungi were grown at 23 C before and after the treatment period. Points represent the mean of three replicates.

formed by *A. mellea* in its woody host tissues may act as a protective mechanism. Bliss apparently assumed this was the case and proposed that one of the effects of CS_2 was either to disrupt the pseudosclerotium or to prevent its formation, which allowed the antagonistic *Trichoderma* spp. to parasitize *A. mellea*. This is an extremely difficult system to manipulate experimentally, and presumably that is why so little is actually known about the process.

The stressing effect could be nutritional, since we have found stressed cells to leak substances that may be attractive or stimulatory to *Trichoderma* spp. and other antagonists of *A. mellea*. We have been unable to isolate such substances; perhaps detection is so difficult because the concentrations produced on root cell surfaces are very low. After exposures to sublethal fumigant concentrations (sublethal to the organism as a whole), stressed and perhaps even dead cells might leak nutrients that are attractive substrates for the antagonists.

The production of antibiotic substances by *A. mellea* decreases or stops during stress. The ability of the fungus to regenerate mycelia or be bioluminescent is likewise affected. It seems reasonable that these are very important factors in the survival of *A. mellea* in soil.

Our concept is that the metabolism of surviving cells of *A. mellea* is greatly affected by exposure to sublethal concentrations of fumigants. Experiments have shown that: 1) growth ceases for extended periods, 2) antibiotic production is reduced or eliminated, and 3) nutrients probably leak out of the tissues. Presumably, pseudosclerotia production does not occur. All these factors probably are responsible for the stressing effect.

With the stressing effect, biocontrol mechanisms are operative; without the stressing effect, biocontrol mechanisms cannot function. The complex interactions

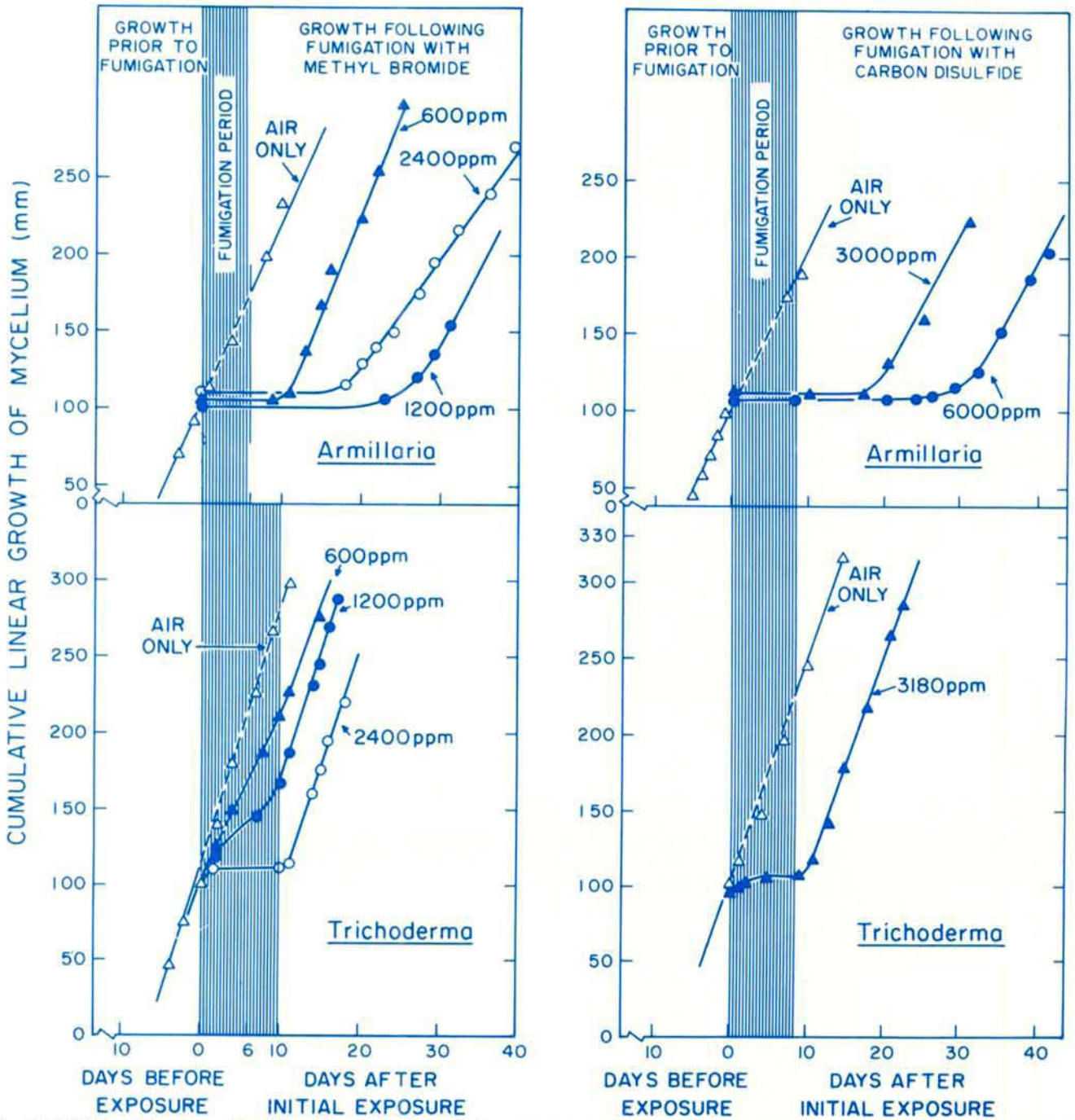

Fig. 7. Inhibition of linear growth of fungi treated with methyl bromide or carbon disulfide, applied in an airstream passing continuously over the colony. (Left) Methyl bromide applied at 20 ml/min for 6 days to *A. mellea* and 10 days to *Trichoderma* spp. (Right) Carbon disulfide applied at 20 ml/min for 8 days to both fungi.

involved explain why biological measures for practical field control of this important pathogen of crop plants remain so elusive.

Acknowledgments

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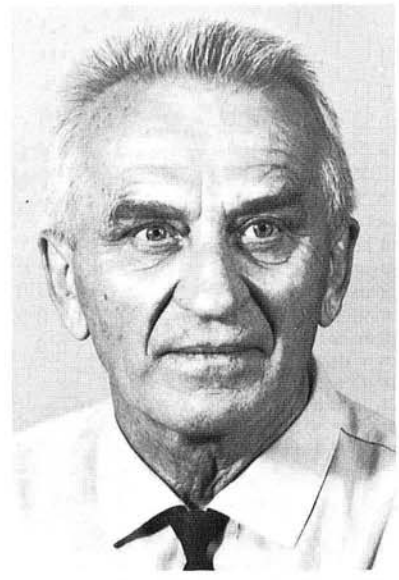

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