The Interaction of Armillaria mellea and Trichoderma spp. as Modified by Methyl Bromide

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ABSTRACT

Investigations were made of the early sequence of events occurring after methyl bromide fumigation of citrus roots infested with *Armillaria mellea* and their storage in nonfumigated, nonsterile soil. When infested roots were treated with sublethal concentrations of methyl bromide and stored in nonsterile soil, *Armillaria* died, but when stored in sterile soil the fungus survived.

Isolations of *Trichoderma* from the roots reached a maximum after storage in nonsterile soil for 7-8 days, and then declined as *Armillaria* populations approached zero. The population changes of the two fungi were directly correlated; a regression of viable *Armillaria* isolations on viable *Trichoderma* isolations had a linear correlation coefficient of –0.888 and a significance level of 99.9%. Bacteria and *Fusarium* spp. also increased after fumigation but were not correlated with the decline of *Armillaria*.

In vitro fumigation of mycelium growing in water agar using carefully controlled concentrations of methyl bromide in a moving air stream, demonstrated that *Trichoderma* was approximately two times more resistant to the chemical than *Armillaria*. The mean LD_{50} value for two *Armillaria* isolates was 128 CT (concentrations of methyl bromide times hours of treatment); whereas, the mean for four *Trichoderma* isolates was 261 CT. In this case concentration was 35 ml methyl bromide/liter air.

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In early fumigation experiments on the control of *Armillaria mellea*, it was assumed that the pathogen was killed by a direct fungicidal action of the chemical. Bliss (1) questioned this concept when he observed that the number of *Trichoderma* isolations increased as those of *Armillaria* decreased in fumigated, *Armillaria*-infested roots incubated in nonsterile soil. Later, he observed that when *Armillaria*-infested root pieces were fumigated with carbon disulfide (CS_2) and incubated either without soil or in previously sterilized soil, *Armillaria* survived. However, if the infested root pieces were buried in nonsterile soil or in *Trichoderma*-infested soil after fumigation, *Armillaria* was killed and replaced by *Trichoderma*. Also, when nonfumigated *Armillaria*-infested roots were buried in pure soil cultures of *Trichoderma, Armillaria* did not survive. He concluded that CS_2 did not kill *Armillaria* by direct fungicidal action, but that *Trichoderma* increased in number, due to the elimination of competitors and overcame *Armillaria*.

Garrett (4) tested Bliss' hypothesis. In one experiment, he demonstrated that fumigation with CS_2 was directly lethal to *Armillaria* and so did not implicate *Trichoderma*. The direct fungicidal action of CS_2 in his experiment is understandable because of the high concentration used. This statement is made even though the concentration expressed by him was only 207 ppm (liquid volume of CS_2 to gas volume of air in the containers used). When calculated as the volume of CS_2 as a gas to the volume of air in the containers, the concentration is about 77,000 ppm. This concentration is even greater if consideration is made for the decreased volume of air due to the presence of soil. By the same calculations, Bliss' (1) concentration is close to 86,000 ppm. With such high concentrations it is surprising that *Armillaria* survived at all in Bliss' experiments.

Garrett's (4) major contribution was to demonstrate that fumigation of the soil alone did not stimulate *Trichoderma* populations to increase to levels sufficient to kill nonfumigated *Armillaria*, but that *Armillaria* must also be affected in some way. He achieved only a 30% kill when nonfumigated *Armillaria*-inoculum was placed in fumigated or nonfumigated soil. He noted that this was probably due to the presence of other organisms that compete with *Trichoderma*, and that Bliss obtained a 100% kill because he used pure cultures of *Trichoderma* in the soil.

Trichoderma has been further implicated by Mughogho (9) who tested the effect of soil fumigants on populations of *Trichoderma* species groups, sensu Rifai (11), and the effect of these groups on *Armillaria*. He found that in nonfumigated *Armillaria*-infested willow pieces in soils previously fumigated with chloropicrin or allyl alcohol, *Armillaria* grew better than in nonfumigated soils, and that four *Trichoderma* species, previously selected on the basis of their tolerance to fumigation, varied in their ability to replace *Armillaria* in its substrate. He proposed that successful soil fumigation

and resultant biological control would depend on chance development of strains of *Trichoderma* spp. which are antagonistic to pathogens, such as *Armillaria*. Finally, he surmised that practical success, using biological controls must await discovery of a fumigant that will stimulate development of a *Trichoderma* population consisting predominantly of strains strongly antagonistic to a pathogen.

Although there may be evidence to support some of the statements made by Mughogho, his conclusions are open to criticism. Firstly, he based his conclusions on data obtained using chloropicrin or allyl alcohol, chemicals that are not used commercially for control of *Armillaria*. Secondly, *Armillaria* was not fumigated before being placed in fumigated soils or in *Trichoderma* cultures, so the system is atypical of fumigated soils. Finally, practical control of *Armillaria* has been achieved using methyl bromide or CS_2, so it may be misleading to believe that it is necessary to await development of new fumigants before utilization of biologic controls is a reality.

Evans (3), Saksena (12), and Moubasher (8) have conducted studies of the tolerance of *Trichoderma* compared to other fungi in soils fumigated with carbon disulfide. Their results demonstrated that *Trichoderma* is not particularly tolerant of the fumigant; but, as Saksena (12) pointed out, it is more tolerant than the faster growing fungi, and faster growing than the more tolerant fungi, normally found in soil. Thus, it is able to recolonize fumigated soils faster than other organisms can from outside the fumigated area.

Munnecke et al. (10) have published the dosage response of *A. mellea* in infested roots to methyl bromide, but studies of the in vitro (growing on agar medium) dosage responses of *Armillaria* and *Trichoderma* using the same fumigation system have not been made. To compare the effects of the fumigant on the two fungi they must be in similar growth stages. Since *Armillaria* does not exist in soil as mycelium or spores, and *Trichoderma* does not infest large roots, as does *Armillaria*, they were compared as mycelium growing in water agar.

Our objectives were to determine the behavior of *A. mellea* in sterile and nonsterile soil after fumigation with methyl bromide (MB), placing particular emphasis on the sequence of colonization of *Armillaria*-infested roots by other microorganisms and their interrlations during the first 4 weeks after fumigation, and to determine whether the mycelia of *Trichoderma* sp. and *A. mellea* differed in resistance to the chemical.

MATERIALS AND METHODS.—*Apparatus*.—The laboratory fumigation system used was essentially as described by Kolbezen & Abu-El-Haj (6) with the following modifications. MB was led from a supply tank in an incubator held at 17.5 ± 0.5 C via 3.2-mm (outside diam) copper tubing through a micrometering valve into 6.4-mm (outside diam) copper tubing packed with 2.5 cm of diatomaceous earth to restrict passage of the gas. From the restrictor, MB was mixed with air and delivered to a manifold with 12 outlets and applied as described later. With this system the concentration of MB could be quickly changed, reaching a new equilibrium within 20 min. Once adjusted, the concentration of MB did not vary more than 3% for periods up to 12 hr. As cautioned by Kolbezen & Abu-El-Haj (6), it is extremely important to maintain the MB supply at a temperature lower than that of the rest of the system. Failure to do so can result in fluctuating concentrations and deposition of liquid MB in the manifold.

As a safety measure, effluent from fumigation vessels was collected by a manifold similar to the delivery manifold. MB flowed from this manifold through a solenoid-controlled valve (Robert Shaw Control Systems Division Model R 427-20) into a fume hood exhaust vent. If the exhaust fan stopped due to a power failure, the solenoid would deactivate and the MB would be routed into a galvanized pipe (5.1 cm internal diam × 1 m) filled with activated charcoal. The charcoal trap could remove MB from the air stream as follows: 7 days at a concentration of 1.2 ml MB/liter of air or 6 hr at concentration of 35 ml MB/liter of air where total flow rate was 240 ml/min. MB was used at a concentration of 35 ml MB/liter of air delivered from the manifold at the rate of 20 ml/min via plastic tubing through two 2.5 × 20 cm test tubes from each of 12 outlets from the manifold. The gas entered by glass tubing to the bottom of the first tube, flowed upward through glass wool and into the second tube, which was the humidifying chamber, where it was released 2.5 cm below water level through a Pasteur pipette. The humidified gas was conducted via plastic tubing to the fumigation chambers. The fumigation chambers were 2-liter jars capped with a lid punched with a hole to accommodate a rubber stopper through which two pieces of glass tubing were inserted. One tube, extending 6 cm below the stopper with a 2-cm right angle bend at the end, was used to introduce the gas. Gas escaped through the other tube which extended 1 cm below the stopper. Plastic tubing was secured with small hose clamps to the external portions of the glass tubes. Glass wool was inserted into the plastic tubing to serve as filters to prevent entry of foreign matter. Thus, the system could be easily disassembled for sterilization.

Measurement of MB.—Methyl bromide concentrations were measured using a Varian 600 D flame ionization gas chromatograph fitted with a six-port gas sampling valve with a sample loop of about 0.7 ml capacity. The column was 20% Dow Corning high vacuum grease on 80-100 mesh Chromosorb W in a 6.4 mm × 61 cm aluminum tubing. The carrier gas was purified N_2 flowing at the rate of 25 ml/min. Oven temperature was 150 C; this gave a retention time of 32 sec. Concentration measurements were made by comparison to a standard of 24 ml MB/liter air prepared as described by Kolbezen & Abu-El-Haj (7).

Definition of CT units.—The dosage was measured as concentration-time (CT) units obtained by multiplying the concentration in ml MB per liter air by the duration of exposure to that concentration in

hours. Since the size of the chambers required long periods for equilibration, the concentration of the effluent was determined every 15 min, multiplied by 0.25, and added to a cumulative total until a desired CT was obtained.

Sources of A. mellea and preparation of inoculum.—*Armillaria* inoculum consisted of roots artificially inoculated in the laboratory or naturally infected in the field. The naturally infected roots were collected from citrus trees infected by *A. mellea*.

Isolate A-2, *A. mellea*, was collected from an infected orange tree in the Hellebrecht grove, San Pasquel Valley, San Diego County, California. Isolate D 73, *A. mellea*, was isolated by W. Wilbur from an infected orange tree on the Curtis ranch in Bryn Mawr, California. *Armillaria* cultures were maintained in the laboratory on potato-dextrose agar (PDA) in tubes or petri dishes.

Artificial inoculum was produced as follows. Citrus roots with a diameter of 2.5 to 4 cm collected from freshly uprooted trees were sawed into pieces 7.5 or 15 cm long. These were sorted according to diameter and placed in 2-liter jars which were half-filled with distilled water, capped with a lid having a 1-cm hole plugged with cotton, and autoclaved 1 hr at 121 C. After cooling, the root pieces were inoculated with a 3- to 4-week-old culture of *Armillaria* growing on PDA. The jars were kept at room temperature in the dark until used.

Natural inoculum pieces (7.5 cm × 2-4 cm) were selected from roots apparently thoroughly colonized by *Armillaria*.

Soil used.—The soil used was from a young citrus grove at the University of California, Riverside. It was collected from within the row between the trees, screened to remove large clumps and debris, and stored in a container with water added to equilibrate at 36% of the soil moisture holding capacity (MHC). It was a sandy loam with a MHC of 41.7% and a pH of 8.0. Sterile soil was obtained by autoclaving for 1 hr at 121 C on each of two consecutive days.

Isolation of A. mellea and other microorganisms following treatments.—*Armillaria* and other microorganisms were detected by plating-out on agar media, chips of wood taken from external and internal portions of the treated pieces. At each time period, three root pieces from each treatment were sampled to determine the viability of *Armillaria* as well as the sequence of colonization by other microorganisms. In initial experiments, roots were split lengthwise and isolations were made from diagonal corners 1 cm from the edges and from the middle of one of the exposed surfaces. In the more extensive experiments three chips of wood were taken from different locations just under the bark surface; three from the wood surface, and three from the internal wood after splitting the root. Each wood chip was divided into three pieces and plated-out separately. Thus, 27 isolations were made from each root (making a total of 81 per treatment and 2,592 per experiment).

Prior to isolation, the root pieces were washed thoroughly, soaked for 5 min in 0.6% sodium hypochlorite, and drained on paper towels. Bark isolations were made by removing the surface layer with a sterilized scalpel and sampling from the remaining bark. Wood surface isolations were made after removing the bark and scraping away the exposed mycelium with a sterile scalpel. Internal isolations were made by splitting the root with a hatchet, and after aseptically removing a wood layer, cutting out the desired pieces. Size of isolation chips was approximately 2 × 3 mm.

RESULTS.—*Effect of storage in soil following fumigation with MB on survival of Armillaria.*—In an early experiment in which liquid MB was applied to *Armillaria*-infested citrus roots in one dose and held in sealed jars for four days before burial in soil, the biological action of natural soil on the survival of *Armillaria* was clearly indicated (Table 1). When viability of *Armillaria* was tested immediately following exposure of inoculum to MB for 4 days,the

TABLE 1. The survival of *Armillaria mellea* in infested citrus roots fumigated with methyl bromide and incubated in sterile or nonsterile soil

MB applied in one dose and kept in closed containers for 4 days

Treatment (ml liquid MB/2 liter)	Percent survival of *A. mellea* in root pieces[a]: No soil immediately after fumigation	After incubation in sterile field soil for 30 days	After incubation in nonsterile field soil for 30 days
0	96	100	100
5	100	100	0
9	100	100	0
17	66	(Contaminated)	0
34	63	66	0
67	0	0	0

MB applied in continuous doses in open containers

Treatment[b]	Percent survival of *A. mellea* in root pieces[a]: Immediately after fumigation	After incubation in sterile field soil for 30 days	After incubation in nonsterile field soil for 30 days
None	100	100	100
Air only 2 hr	100	100	78
Air only 4.5 hr	100	100	100
Air only 6.75 hr	100	100	100
38 CT MB	100	100	33
100 CT MB	100	100	0
176 CT MB	100	11	0

[a] Percent survival is based on number of isolations with viable *Armillaria* out of 24 possible for one-dose applications, nine for continuous application.

[b] CT is the concentration of MB times the exposure in hours. (35 ml MB/liter air × hours).

responses varied from complete kill (67 ml) to no kill (5 and 9 ml) showing that the range of concentrations included sub-lethal doses. When inoculum was stored in sterile soil for 30 days and then tested for viability, there was no significant change in percent viability, indicating that such storage did not affect *Armillaria*. However, when stored in nonsterile soil, all inoculum exposed to MB was killed after 30 days, whereas 100% of untreated inoculum was viable. In a later experiment, citrus roots infested for 17-19 months with *Armillaria* isolate A-2 were removed from the culture jars and the external mycelium was removed with a sterile spatula. The infested roots were placed randomly in sterile 2-liter jars and subjected to a continuous flow of either air only, 2 hr; air only, 4.5 hr; air only, 6.75 hr; MB, 38 CT; MB, 100 CT; or MB, 176 CT. Nine pieces were used for each treatment. After treatment with MB, the jars were aerated by passing air through them for 1 hr. Three pieces from each treatment were used for isolation immediately following aeration; three were stored in 800 g sterile soil, and three were stored in 800 g nonsterile field soil for 30 days. The materials were kept in the dark during the storage period.

A wood chip from each location was plated-out on each of three isolation media as follows: Difco PDA containing 2 g/liter Difco yeast extract; the same medium containing benomyl (15 μg/ml) to suppress *Trichoderma*; the same medium containing benomyl and PCNB (400 μg/ml) to suppress *Trichoderma* and Mucorales fungi. Antibacterial agents were not used in the media, since bacteria isolations were desired. This omission did not affect the growth of *Armillaria*, since it grew well in the presence of bacteria of various kinds. Only one unidentified species of bacterium was capable of inhibiting *Armillaria*; fortunately, it was observed infrequently. As the various fungi grew out from the chips and onto the agar, mycelial tip isolations were made and transferred to test tubes containing PDA-yeast extract medium and later identified. The most rapid growing fungi were removed from the agar medium to prevent them from obscuring the slower-growing organisms.

Armillaria survived in 100% of the isolations made immediately after fumigation, indicating that the doses were sub-lethal (Table 1). This was substantiated by the results where treated inoculum stored in sterile field soil survived 100% except for those pieces treated with 176 CT MB, in which only 11% survived. When the inoculum was stored for 30 days in nonsterile soil, however, the effect of antagonism by soil organisms became very pronounced and only 33% survived the MB treatment of 38 CT, and none survived treatment with the two higher doses of MB.

The data indicate that biological activity in the soil is responsible for the death of *Armillaria* following fumigation.

Sequence of microorganisms colonizing Armillaria-infested roots and relationship to survival of Armillaria.—The sequence of microorganisms that colonized *Armillaria* inoculum pieces in soil following treatment with sublethal concentrations of MB was accurately determined. This was accomplished by exposing *Armillaria* (D73) in naturally or artificially infested citrus roots to a continuous flow of MB for 105 CT or to air for 4.25 hr. After treatment the pieces were stored in nonsterile field soil until isolations were made. Three root pieces for each treatment were removed from storage at 24-hr intervals for 8 days then at 72-hr intervals until a total of 29 days had elapsed; subsamples were taken from the root pieces and plated out as described previously. Since two of the three media were selective against *Trichoderma*, data for *Trichoderma* are expressed as a percentage of 27 isolations. Data for all other organisms are expressed as a percentage of 81 isolations.

One of the most important results was the correlation between the abrupt decrease of viable *Armillaria* and the equally abrupt increase of *Trichoderma* (Fig. 1-A, B). These rapid changes suggested a possible causal relationship between *Trichoderma* and the decline of *Armillaria*. The regression of the percent of viable isolations of *Armillaria* on those of *Trichoderma* for the first 11 days was a straight line with a significance level greater than 99.9% giving strong support to the causal relationship (Fig. 1-C). The data were from artificial inoculum. When natural inoculum was used, a similar response was observed, except that all the *Armillaria* was not killed and the *Trichoderma* population did not decline as quickly (Fig. 1-B) as the population did in artificial inoculum when *Armillaria* died (Fig. 1-A). This decline was observed in a total of three experiments and is interpreted as circumstantial evidence for a loss of *Trichoderma* substrate due to the decrease of *Armillaria*.

The colonization of artificial inoculum pieces, treated with air only and buried in soil, is presented in Fig. 1-D. For unknown reasons, in contrast to data reported in Table 1, as well as from other experiments using artificial inoculum, the viability of *Armillaria* decreased rapidly for seven days before stabilizing at approximately 65% viability. The incidence of *Trichoderma* increased slowly and was not correlated with the decrease in viability of *Armillaria*.

Microorganisms other than *Trichoderma* may affect the survival of *Armillaria* in citrus roots following MB treatment. The two most frequently isolated, after *Trichoderma*, were *Fusarium* spp. and various unidentified bacteria (Fig. 2-A). Of these, perhaps the incidence of *Fusarium* spp. is the more significant. The increase in incidence of *Fusarium* spp. in Fig. 2-A was not correlated with the decrease in incidence of *Armillaria* as shown in Fig. 1-C, for *Trichoderma*, but the fact that it increased at a steady rate may be important, particularly since a similar increase did not occur in the air-only treatments (Fig. 2-B).

In one experiment, a *Rhizopus* sp. and several *Fusarium* spp. rapidly penetrated the bark of the fumigated inoculum, reaching peak populations within four days and then declining before

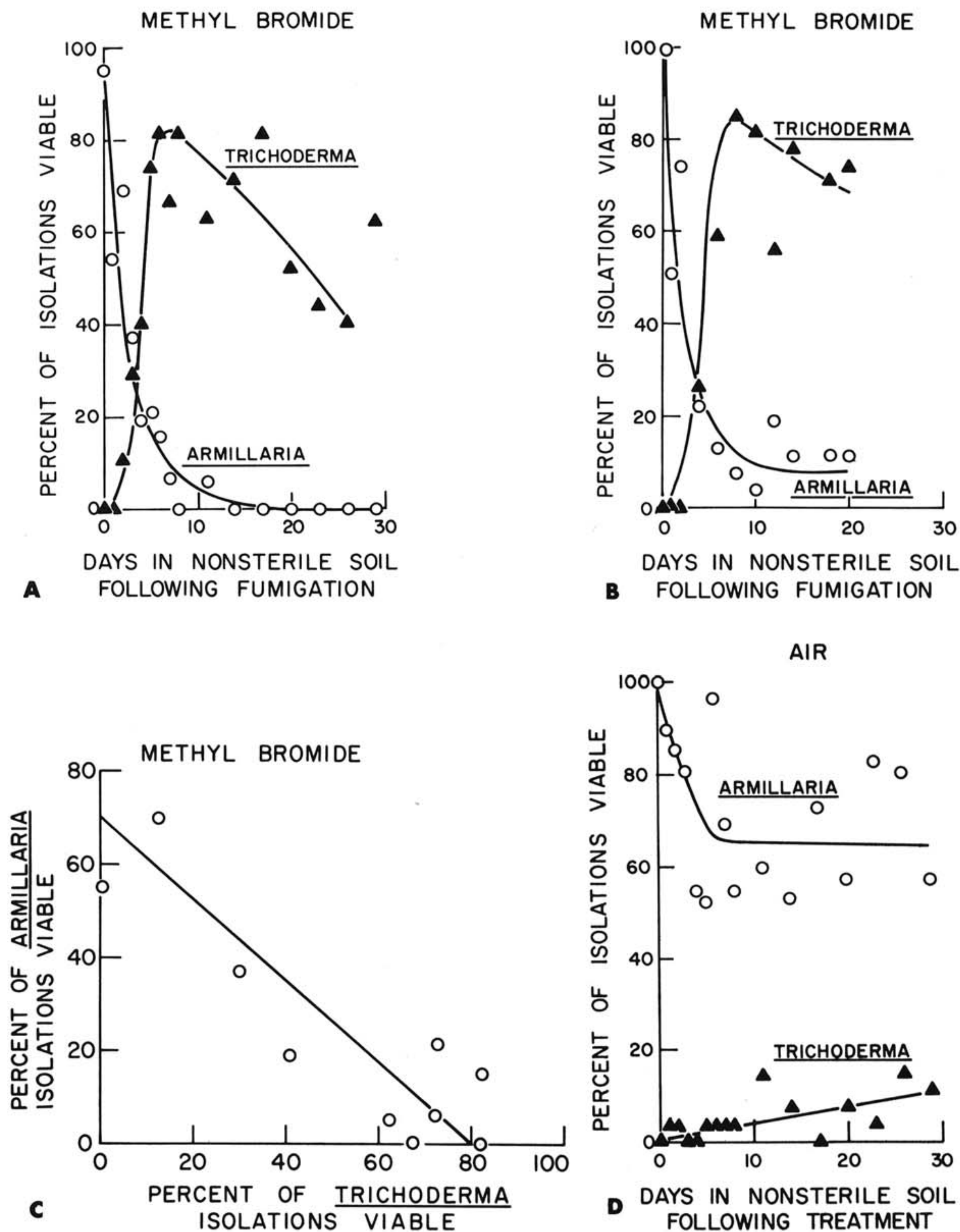

Fig. 1. Percent of isolations yielding *Armillaria* or *Trichoderma* from roots infested with *A. mellea* kept in non-sterile soil after treatment with methyl bromide [35 ml gaseous methyl bromide/liter of air for 3 hours, a total of 3 × 35 = 105 CT (concn.-time) units: **A)** artificial inoculum; **B)** natural inoculum; **C)** regression of viable *Armillaria* isolations on viable *Trichoderma* isolations for the first 11 days of incubation. Data are from Fig. 1-A (linear correlation coefficient = –0.888, with a significance level >99.9%, and a regression coefficient = 0.324); **D)** controls–artificial inoculum treated with air only.

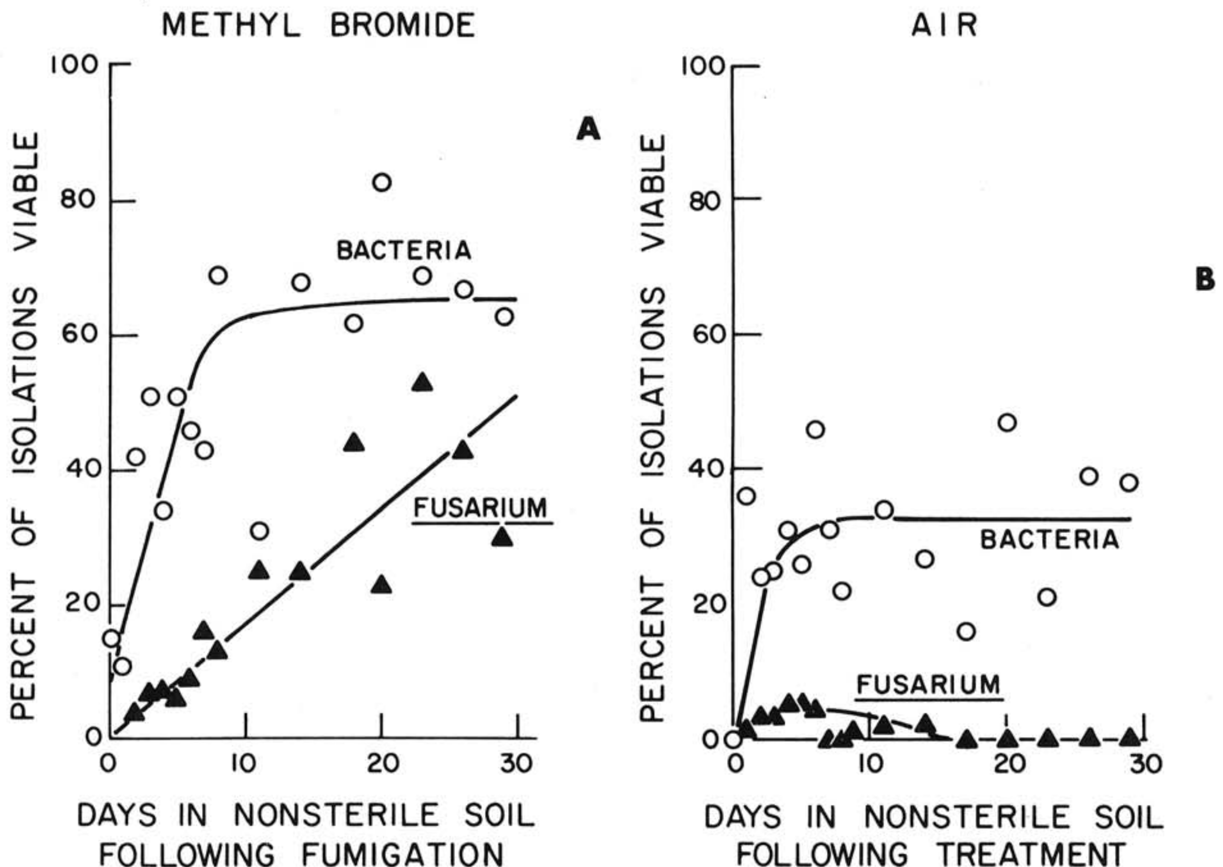

Fig. 2. Percent of isolations yielding *Fusarium* spp. or various unidentified bacteria from roots artificially infested with *Armillaria mellea* and held in nonsterile soil after treatment: **A)** Treated with methyl bromide [35 ml gaseous methyl bromide/liter of air for 3 hours, a total of 3 × 35 = 105 CT (concn.-time) units; **B)** Control–treated with air only.

Trichoderma had reached its peak. *Mucor* sp. reached its peak population at about the same time as *Trichoderma*. Penetration into the interior of the root pieces was not made in significant number except by *Trichoderma. Rhizopus* and *Mucor* were infrequently isolated in two subsequent experiments, whereas *Fusarium* spp. were present in all experiments. However, in the two later experiments, *Fusarium* did not precede *Trichoderma* or was not correlated with the decline of *Armillaria*.

Parasitism of Armillaria and Rhizopus by Trichoderma.–Frequently parasitism of *Rhizopus* by *Trichoderma* was observed in the mixed cultures obtained during isolation procedures. Usually *Rhizopus* would grow out from wood chips on to the medium and later the effects of parasitism by *Trichoderma* could be noted visually. The method of parasitization was by coiling of *Trichoderma* hyphae around the host. The coiling frequently was seen as simple coils or as an extensive network of mycelium over the sporangiophore stalk (Fig. 3-A). The network of *Trichoderma* hyphae sometimes became so thick that it would form a mantle obscuring the sporangiophore (Fig. 3-B). Penetration as described by Durrell (2) was not observed, but obviously must have occurred, since *Trichoderma* hyphae and chlamydospores were observed within *Rhozopus* hyphae, sporangiophores and columellas (Fig. 3-C, D, E). Coiling of *Trichoderma* hyphae around the hyphae of *Armillaria* in culture (Fig. 3-F) was observed infrequently and only in combinations of *Armillaria* isolate A-2 and *Trichoderma viride* (No. 45553 ii obtained from Commonwealth Mycological Institute).

Appearance of mycelium and formation of pseudosclerotial zone lines as indicators of biological action following exposure to MB.–The decline in recovery of *Armillaria* from fumigated pieces was not the only indication of biological action. The appearance of mycelia and formation of pseudosclerotial zone lines gave indications also. The following descriptions are valid for both artificial and natural inoculum. The main difference between the two was that the artificial inoculum was more vigorous. By day 2 of the incubation period in nonsterile soil, pseudosclerotial zone lines had formed on the control (air only) but not on the MB-treated pieces. At day 4, growth of some species of Phycomycetes was noted on the treated pieces and in the soil but not on the control. On day 6, the

Phycomycete growth was more profuse in the MB-treated jars. The control pieces were forming pseudosclerotial plates over the cut ends and aerial tufts of *Armillaria* mycelium were appearing. By day 8, the Phycomycete growth was still evident on the pieces treated with MB and *Trichoderma* was sporulating heavily on them. Neither fungus was evident on the control pieces. On days 10 and 12, rhizomorphs were produced on all three control pieces, the mycelial fans were white and looked healthy, and a pseudosclerotium had formed around the pieces just under the bark surface. There were no rhizomorphs on the MB-treated pieces, the mycelial fans were yellow and disintegrating, and pseudosclerotia were not present. Also, on the controls, the bark adhered tightly to the wood and it was difficult to remove; whereas, on the treated pieces, it was very loose and slipped off easily. On day 16, a slight growth of *Trichoderma* and of the Phycomycetes was evident on the soil surface of the control. No other changes occurred before the experiment was terminated.

On natural inoculum, wherever the xylostroma penetrated through the bark on fumigated pieces, *Trichoderma* grew profusely on it. This was not evident in control pieces. Although there were not great differences between natural and artificial inoculum, the natural inoculum seemed to be slightly more resistant to MB effects. In some instances, longer incubation periods were required to kill *Armillaria* after fumigation, even though growth from artificial inoculum was more vigorous.

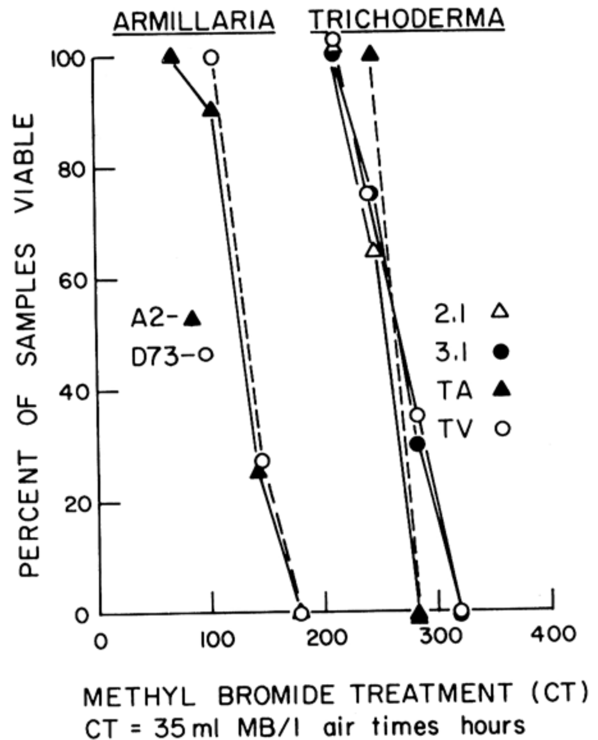

Fig. 4. Dosage response to methyl bromide of *Armillaria mellea* A-2 and D-73, *Trichoderma koningi* I (2.1), *T. koningi* II (TA), *T. glaucum* (3.1), and *T. lignorum* I (TV) when growing in water agar.

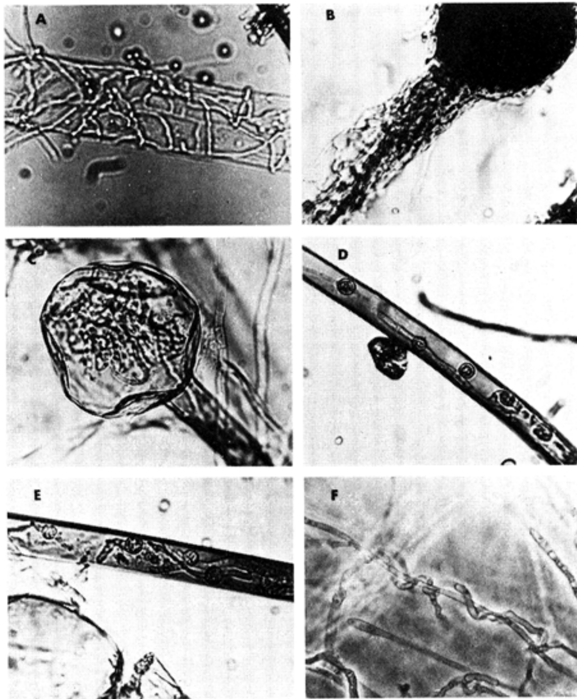

Fig. 3. Parasitism of *Rhizopus stolonifer* and *Armillaria mellea* by *Trichoderma* spp: **A, B)** Network of *Trichoderma* hyphae on outside surface of *Rhizopus* sporangiophore; **C)** Hyphae of *Trichoderma* within *Rhizopus* columella; **D, E)** Chlamydospores and hyphae of *Trichoderma* sp. within sporangiophore of *Rhizopus* sp.; **F)** *Trichoderma viride* coiling around hyphae of *A. mellea*.

In vitro response of A. mellea and Trichoderma mycelium to methyl bromide.—To determine the in vitro resistance of *A. mellea* and *Trichoderma* mycelium to methyl bromide, *A. mellea* isolates D-73 and A-2 were used. Water agar cultures were prepared by transferring three 2 X 2 cm squares, of a 2- to 4-week-old culture of the appropriate isolate growing on PDA, to 9-cm petri dishes each containing 20 ml water agar. Cultures were incubated at room temperature in the dark 4-6 weeks before use.

Water agar disks containing *Armillaria* mycelium were used because they produced the most uniform inoculum. Nutrient media were not used since they stimulated rhizomorph production, making it impossible to obtain uniform sample disks.

The *Trichoderma* isolates were grown in the same manner as *A. mellea*, except that the water agar medium was inoculated in the center with spores 2-3 days prior to fumigation. With both fungi, twenty 5-mm disks were cut from the colony margin and placed in sterile 125-ml Erlenmeyer flasks. A treatment consisted of the fumigation of one flask containing 20 samples. The flasks were attached to the MB or air manifolds using the apparatus described

previously. Exposure times were determined by previous experiments and were in 35 CT steps (35 ml MB/liter air × hours). The range of *Armillaria* fumigation was 35 CT to 175 CT and for *Trichoderma* it was 175 CT to 315 CT. At the end of each MB treatment, the cultures were aerated 30 min. The disks were placed on PDA plates, 10 per plate, and incubated at room temperature. *Armillaria* cultures were incubated at least 30 days, since some grew very slowly. *Trichoderma* disks were cut out as growth became apparent to prevent overgrowing of the plate and the plates were retained seven days to ensure that all possible growth occurred. The experiments were repeated for each *Trichoderma* isolate three times and for each *Armillaria* isolate four times.

Four isolates of *Trichoderma* were used in the experiments. Identification of the *Trichoderma* species, using Rifai's key (11), proved unsatisfactory. Instead, identification was based upon Gilman's (5) methods. The *Trichoderma* species were as follows: *T. lignorum* I, obtained from the Commonwealth Mycological Institute, No. 45553 ii, labeled by them, as *T. viride* (in Gilman's system *T. lignorum* is a synonym for *T. viride*); *T. koningi* I, isolated from soil (D. E. Munnecke 1959); *T. koningi* II and *T. glaucum*, isolated from *Armillaria*-infested roots fumigated in the field (Ohr 1970). All cultures were derived from single spores and maintained on PDA in tubes.

The dosage response curves for *Armillaria* and *Trichoderma* isolates were strikingly similar. The curves in Fig. 4 are of the mean values of four experiments for each *Armillaria* isolate and three experiments for each *Trichoderma* isolate. Experimental ranges and means of the LD_{50} values appear in Table 2. All controls were 100% viable. Viability of all isolates dropped sharply from 100% to zero within a range of 105 CT. Neither fungus was adversely affected by a treatment of 70 CT, but both isolates of *Armillaria* were killed by 175 CT. The *Trichoderma* isolates were more resistant than *Armillaria*, since they were unaffected until subjected to 210 CT, and were all killed by 315 CT. When compared at the LD_{50} level, all three species of *Trichoderma* were 1.9 to 2.3 times more resistant than *A. mellea* to MB.

TABLE 2. Range and mean LD_{50} values of two *Armillaria* isolates and four *Trichoderma* isolates in water agar when fumigated with methyl bromide

Isolate	LD_{50}	
	Range (CT)[a]	Mean (CT)
Armillaria mellea A-2	119-152	126
A. mellea D-73	122-152	130
	Range = 119-152	Mean = 128
Trichoderma koningi I	259-262	261
T. lignorum I	245-292	268
T. koningi II	224-266	252
T. glaucum	233-294	264
	Range = 224-294	Mean = 261

[a] CT = 35 ml MB/liter air × hours.

DISCUSSION.–The effect of microorganisms on *Armillaria* in infested roots following fumigation has been amply demonstrated. These microorganisms are directly involved in the death of sublethally fumigated *Armillaria* when it is incubated in nonsterile soil; since it survives when incubated in sterile soil. These results agree with those of Bliss (1) who used carbon disulfide.

Since the soil was not fumigated and therefore did not have a selected population of microorganisms, it must be concluded that the stress placed on *Armillaria* by the fumigant is of major importance. This agrees with Garrett's (4) observations that the buildup of microorganisms in a fumigated soil was not capable of killing more than 30% of nonfumigated *Armillaria* inoculum.

Of the organisms isolated, it is evident that *Trichoderma* was primarily responsible for the decline of *Armillaria*, thus confirming the earlier reports of Bliss (1) and Garrett (4). Other organisms, although not directly involved in the decline of *Armillaria*, may be involved in the succession into the fumigated roots.

From the results, a hypothetical sequence of colonization can be described for fumigated *Armillaria*-infested roots placed in nonsterile soil. Mucorales members may be the first invaders into the dead outer tissues of *Armillaria* but they probably cannot rapidly invade the still-living *Armillaria*. *Trichoderma* follows, and in addition to invading living *Armillaria*, is capable of parasitizing the Mucorales members; decreasing their population. As *Armillaria* declines, *Trichoderma* also declines and is replaced by *Fusarium* and bacteria. The sequence would not necessarily be the same in a fumigated soil, since many of the Mucorales would be eliminated by the fumigant (3, 8, 12).

A possible reason for the increase of *Fusarium* in the MB-treated inoculum may be that *Fusarium* succeeds *Trichoderma*. The bacterial population, which increased rapidly (Fig. 2-A) with the decline of *Armillaria* (Fig. 1-A), presumably is not active in affecting survival of *Armillaria*. This statement is based on the fact that, for the most part, there was no in vitro antagonism shown between the bacteria and *Armillaria*. Only one infrequently isolated bacterial species inhibited *Armillaria*. Also, bacterial isolations increased rapidly in the air-only treatments (Fig. 2-B), indicating that stimulation of bacterial growth probably was a result of the presence of the artificial inoculum, rather than due to fumigation. Although the increase of the bacterial population in the fumigated pieces over that in the controls could conceivably be part of the reason for the decline of *Armillaria*, it is interpreted here as an effect, and not as a cause.

Greater variability was present in isolations of *Armillaria* from natural inoculum treated with air only and buried in soil. Although the lowest mean of viable isolations from artificial inoculum was approximately 65% with a range of 50 to 100%, the lowest mean from natural inoculum was approximately 30% with a range of 12.5 to 100%. The variability demonstrated by the two types of

inoculum may have several explanations. *Armillaria* is an extremely variable organism with isolations from the same strain acting differently. With artificial inoculum, variability may be a result of the isolate used, its age, and adverse effects of other organisms present in nonsterile soil. Natural inoculum from diseased trees is probably extremely variable due to the varying time the roots have been infested and the possible strains involved.

For the integrated chemical-biological control of a pathogen to be operable in fumigated soils, as in the case of the control of *Armillaria* by *Trichoderma*, it is necessary for the controlling organism to be more tolerant of the chemical used than the pathogen, or to have the ability to recover more rapidly from its effects. If not, the controlling organism would be affected or eliminated before the pathogen is damaged enough for the integrated control to be effective.

The results of the experiments reported in this paper confirm that *Trichoderma* meets this requirement. It was approximately twice as resistant in vitro, to methyl bromide as *Armillaria*. This resistance of *Trichoderma* to the chemical along with its well-known antagonistic abilities and the elimination of competitors in a fumigated soil make an effective combination for the control of *Armillaria*.

An interesting aspect of the experiments is the similarity of the dosage-response curves of *Armillaria* and *Trichoderma*. Such similarity may suggest that the two fungi are affected by the toxicant in the same way.

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