Effect of Heating or Drying on Armillaria mellea or Trichoderma viride and the Relation to Survival of A. mellea in Soil

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ABSTRACT

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Sublethal heating of infested roots in soil reduced survival of *Armillaria mellea*. Similar heat treatments did not produce measurable secondary effects on living peach or citrus trees. Drying of infested root segments followed by burial in soil likewise decreased the survival of this fungus. On agar medium, *A. mellea* was more sensitive to increased temperature than was its antagonist, *Trichoderma viride*. In orchards, indirect control of *A. mellea* by heat treatment followed by action of fungal antagonists on the weakened pathogen may be possible.

Additional key words: control of *Armillaria mellea*, ecological relations.

Results of experiments designed to measure the in vitro response of *Armillaria mellea* (Vahl) Quél. and *Trichoderma viride* Pers. ex S. F. Gray to toxicants, primarily methyl bromide (MB) and carbon disulfide (CS_2), have been published (3, 4, 6). The present paper reports similar studies on the effects of physical stresses, heat, and drying on the two organisms.

MATERIALS AND METHODS

Fungi.—Clones of *A. mellea* were obtained from naturally infected roots of various hosts in California. All clones were maintained on potato-dextrose agar (PDA). Clones of *Trichoderma* spp. were isolated from roots infected with *A. mellea*. The *Trichoderma* isolates originally identified as *T. viride* Pers. ex Fr. were no longer available when Rifai's (8) revision of the genus came to our attention.

Growth-rate studies on agar.—Growth rate on citrus agar (3) was measured either by determining increases of colony diameter of cultures in 100-mm diameter petri dishes, or by noting linear growth in glass tubes (25 × 350 mm).

Colonization of wood.—*Citrus* spp. roots naturally or artificially infested with *A. mellea* by methods described previously (4) were used throughout the experiments. Peach [*Prunus persica* (L.) Batsch] limb wood and birch (*Betula* spp.) wood veneer occasionally were used to supplement citrus roots. Wood segments were used 9-24 months after they were inoculated with the fungus.

Application of heat.—Heat was applied to *A. mellea* by holding infested wood pieces in incubators or in sealed containers immersed in water baths, usually at temperatures less than 38 C for periods up to several days. In a second system, a continuous flow of a steam-air mix was passed over wood pieces buried in sand in an apparatus of the type designed by Baker et al. (1) at temperatures greater than 38 C for periods up to 8 hours. Temperatures, sensed by butt-welded iron-constantan thermocouples in the sand and in infested roots, were automatically recorded on continuous or intermittent recorders. Thermocouples in the roots were seated at the bottom of small holes drilled to the center of the root and the holes were plugged with cork doweling. For a given heat treatment, the sand was heated to the stated temperature and then the roots were quickly buried in the sand. Time of treatment started with placing the roots in the sand.

Determination of the effects of heat on Armillaria mellea and on living plants.—Wood chips were taken aseptically from heated and nontreated wood pieces and placed on agar. The *direct* effect of treatment on *A. mellea* was determined by isolations immediately following the treatment period. The *indirect* effect was determined by placing root pieces in nonsterile orchard soil for at least 15 days at 22-25 C and subsequently plating-out chips on agar.

Heat was applied to bare- or balled-roots of trees buried in sand in the steam-air apparatus noted above. Noninfected trees were used to determine heat effects on plants alone. Temperatures of 40-49 C for periods of 0.5 - 72 hours were maintained at ± 0.5 C. After treatment, plants were potted and observed for signs of wilting, stunting, or other adverse effects for periods up to 12 months.

Infected trees from the field were used to determine heat effects on *A. mellea* on living plants. Three to 12 months after inoculation, the trees were dug, roots were freed of soil, and plants with symptoms were heat-treated at 40 C for 2-3 hours. After treatment, plants were replanted in field soil and observed 1-3 months to determine the indirect effects of heat on viability of the fungus. Tree rootstocks included: Trifoliate orange, *Poncirus trifoliata* (L.) Raf.; Troyer citrange, *P. trifoliata* × navel orange, *Citrus sinensis* (L.) Osb.; Pummelo, *C. grandis* (L.) Osb.; and Lovell seedling peach, *Prunus persica* (L.) Batsch.

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Infected geranium (*Pelargonium hortorum* Bailey) plants also were used. Potted plants or bare-rooted plants were treated in hot water at 33 C for 7 days or 40 C for 2 hours, respectively.

RESULTS

Effect of temperature on growth of Armillaria mellea and Trichoderma viride.—Cultures of *A. mellea* were started at one end of long tubes (350 mm) containing citrus agar (4). After a constant growth rate was established at 23 C, the tubes were placed at constant temperatures of 6 to 39 C at 3-degree intervals. Three replicates were used for each treatment. After 7 days, the tubes were returned to 23 C. The cumulative linear growth was plotted as a function of time (Fig. 1).

At 6 and 9 C, growth of *A. mellea* ceased but resumed again at 23 C after a lag of 1 or 2 days. Growth diminished at 12 and 15 C and resumed at its former rate when cultures were returned to 23 C. As might be expected, treating at temperatures of 18, 21, 24, or 27 C did not adversely affect growth of *A. mellea* when cultures were restored to 23 C. Incubation at 30 to 36 C caused temporary cessation of growth and increasingly longer delays in the resumption of growth when cultures were restored to 23 C. Incubation at 30 to 36 C caused temporary cessation of growth and increasingly longer delays in the resumption of growth when cultures were returned to 23 C. When growth resumed, however, its rates were comparable to those of the pre-treatment growth period. Treatment at 39 C appeared to be lethal, since no growth occurred for 40 days following exposure.

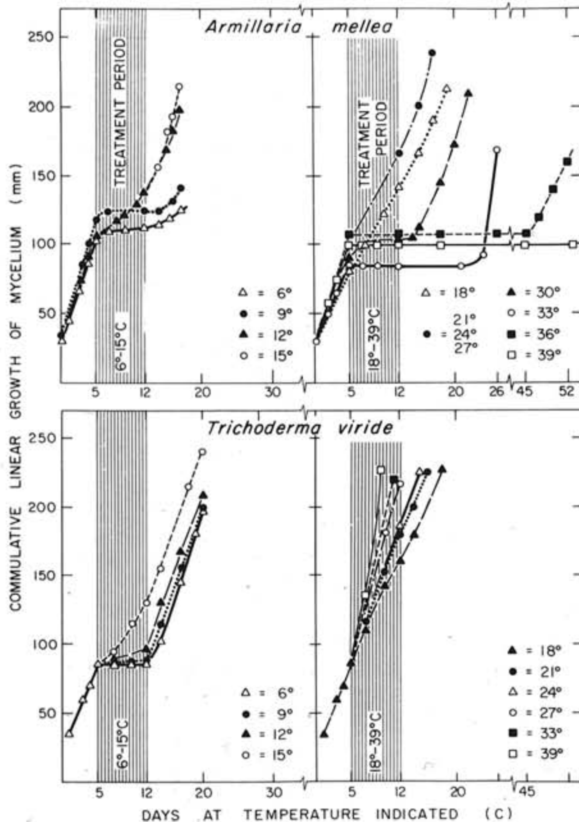

Fig. 1. Effect of temperature on linear growth of *Armillaria mellea* and *Trichoderma viride* on citrus agar. Fungi were grown at 23 C before and after the indicated treatment period. Points represent the mean of three replicates.

In another experiment, six clones of *A. mellea*, two from northern California and four from southern California, were grown on citrus agar in glass tubes at 23 C with three replicates per clone. After 11 days, with growth averaging 8 mm per day (6.6 - 12.8 mm), the cultures were incubated at 33 C for 3 days. During this period, the growth rate was reduced to 0.7 mm per day (0.0 - 1.3 mm). Upon returning the cultures to 23 C, the growth rate increased to only 2 mm per day (0.3 - 5.6 mm) for the first 7 days, and resumed a slightly higher than normal growth rate for this temperature of 9 mm per day (3.5 - 12.7 mm) the next 4 days. The same cultures then were placed at 6 C for 7 days and the growth rate averaged 1 mm per day (0.2 - 5.7 mm). Upon again returning the cultures to 23 C, the growth rate was 4 mm per day (0.9 - 7.0 mm) for the first 3 days, after which the normal rate of 8 mm per day (4.3 - 9.7 mm) was attained. Exposure of the cultures to 33 C for 3 days had a greater inhibitory effect on subsequent growth at 23 C than did exposure to 6 C for 7 days.

The effect of temperature on growth of *T. viride* (D 34) was determined in four replicated treatments as was done with *A. mellea*. Growth of *T. viride* ceased or was retarded at temperatures below 15 C, but immediately resumed at its former rate when the cultures were returned to 23 C (Fig. 1). At 18-24 C, growth was unaffected. At temperatures above 24 C, however, growth rate was so greatly stimulated that the cultures approached the ends of the tubes and measurements had to be discontinued during the treatment period. Thus, at these temperatures, it was impossible to determine what subsequent growth at 23 C would have been.

The effect of heat treatment on *T. viride* was investigated in more detail by measuring the radial growth (in quadruplicate) of four clones of the fungus collected from roots of plants infected with *A. mellea*, one clone derived from a single-spore isolation from a similar collection, and seven clones which were single-spore isolations from this latter single-spore clone. Temperatures ranged from 3-33 C at intervals of 3 degrees. Cultures were started from 6-mm diameter inoculum plugs cut from colonies on agar in petri dishes and placed on PDA in petri dishes. Diameters of colonies were measured after 48 hours. There was considerable diversity in the response to temperature among the four wild-type clones. Three of them (D 34, D 72, and D 75) grew most rapidly at temperatures of 30-33 C. One clone (D 21) grew less rapidly and appeared not yet to have reached its optimum at 33 C. The single-spore clone (D 25) and the seven single-spore isolates derived from it all were similar in their responses, they grew slower than the other clones and exhibited a marked reduction in growth at 30-33 C. In this respect, clone D 25 and its derivatives differed from the other clones.

Effect of temperature on Armillaria mellea growing on birch wood veneer.—Cultures of *A. mellea* (D 73) 13-15 months old on previously autoclaved pieces of birch wood veneer (2 cm × 5 cm × 2 mm) were subjected to temperatures ranging from 33 C for 1-7 days to 49 C for 0.5 - 2.0 minutes. Two tests of three replicates each were

made. After the cultures were cooled on ice, they were transferred to agar.

Growth of most cultures subjected to heat was inhibited when transferred to agar. The lag in growth was difficult to measure quantitatively, but the effect was obvious qualitatively as shown in a typical response of inoculum pieces treated at 41 C (Fig. 2). The control was held at 23 C. The photograph was taken 12 days after the cultures were plated following heating. Growth was suppressed by treatment for as little as 10 minutes; the greatest inhibition was caused by treatments lasting 50 minutes or more. Except for the 33-C treatment, increasing or decreasing the heat either shortened or lengthened the time for comparable effects. At 46 and 49 C, subsequent growth was suppressed by treatments for as little as 2.0 and 1.5 minutes, respectively, whereas at 35 C about 20 hours of exposure were required. At 33 C, there was no lag period and the fungus was growing as vigorously as the control 12 days after the 7-day treatment. Thus, the effect of the 33-C treatment on subsequent growth of *A. mellea* in small wood pieces varied from that on agar (Fig. 1). In the latter, there was about a 10-day lag before growth resumed.

Direct vs. indirect effect of heat on Armillaria mellea.—Numerous experiments with *A. mellea* growing on sterilized roots or stems were made to compare the effect of heat on viability of *A. mellea* immediately after treatment (direct effect) vs. the effect after storage in nonsterile soil (indirect effect). Temperatures ranged from 30-49 C and storage in soil was usually for 15 days at 23 C. Treatments included those combinations of time and temperature which had no obvious direct effects on *A. mellea,* but which had pronounced indirect effects. In the latter case, *Trichoderma* spp. were the dominant colonizers of roots in which *A. mellea* was no longer viable.

Isolate D 73 of *A. mellea* growing on citrus roots (2.5× 130 cm) with intact bark was heat-treated using one to four tests for each temperature and three or four roots per test. In tests of direct effect, the fungus was not viable after exposures to 49 C for 0.5 hour, 41 C for 4-7 hours, 38 C for 24-38 hours, or 33 C for 360-720 hours (15-30 days) (Fig. 3). That the "stress" imposed by exposure to 33 C might eventually kill *A. mellea* was indicated in Fig. 1, wherein growth was stopped when cultures were exposed for 7 days and did not resume until about 12 days after the cultures were returned to 23 C.

Less time and lower temperatures were required for indirect- than for direct kill. The difference became apparent at about 43 C where curves representing kill of the fungus under the two conditions diverged. Thereafter, the curves were nearly parallel with indirect kill of *A. mellea* occurring after exposure to 43 C for 0.6 hour, 41 C for 0.5 - 1.0 hour, 38 C for 2-3 hours, 35 C for 23-30 hours, and 33 C for 70-170 hours (3-7 days).

One of the problems in evaluating the effect of heat on

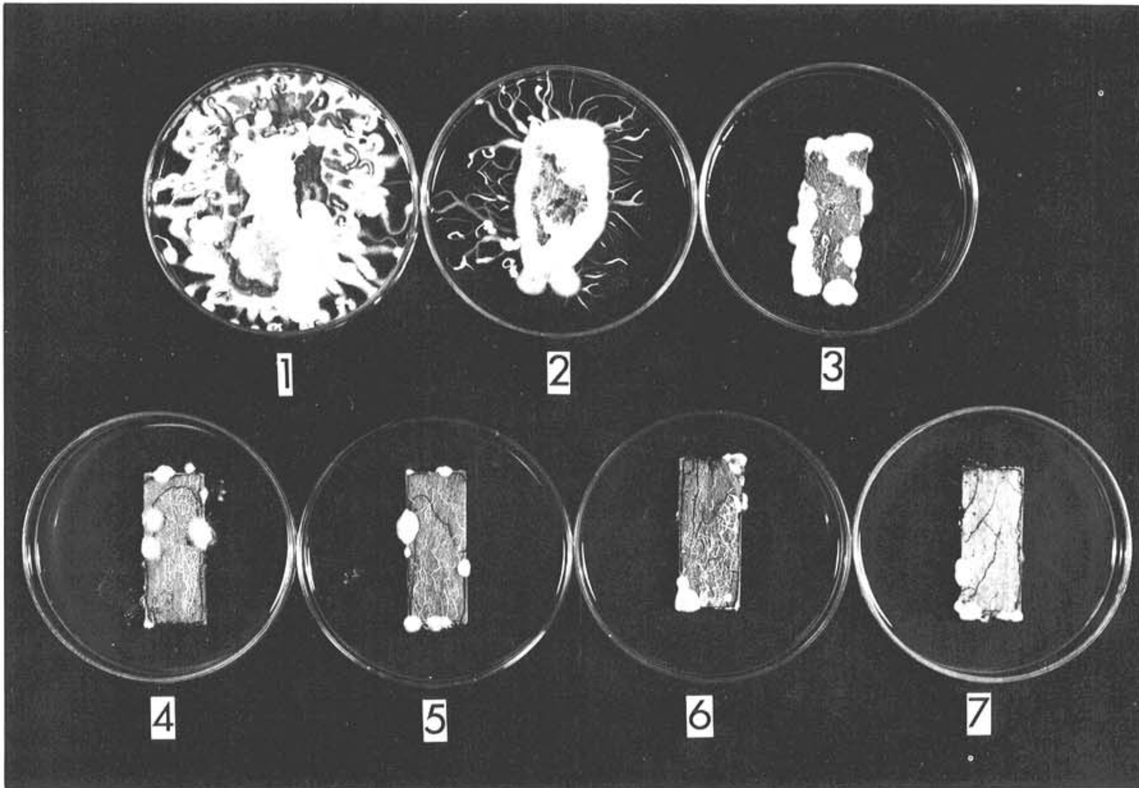

Fig. 2. Effect of treatment with steam-air on *Armillaria mellea* growing on birch veneer. Cultures were heated in steam-air at 41 C as follows: 1, no heat applied (23 C); 2, 10 minutes; 3, 30 minutes; 4, 50 minutes; 5, 70 minutes; 6, 90 minutes; and 7, 110 minutes. The cultures were transferred to citrus agar at 23 C 21 days before being photographed.

survival of *A. mellea* is that the direct kill effectiveness is inversely proportional to the diameter of the root. For example, with roots 19 or 23 mm in diameter exposed to 46 C for 30 minutes, the center of the root reached maximum temperature during the treatment and the fungus was killed. For roots 25 mm in diameter, if the maximum temperature was reached, the fungus also was killed. In some cases, the maximum was not reached and the fungus survived, although a lag period of 30 days was required before fungus growth resumed. When roots 30-32 mm in diameter were used, temperature did not reach maximum and the fungus survived; the subsequent lag period for growth was 17 days.

The extremes in heat transfer for the heating and cooling cycle for roots 25 and 60 mm in diameter are shown in Fig. 4. Heat penetration in the 25-mm diameter root was rapid, reaching ambient temperature in 20 minutes, and cooling also was rapid. In contrast, ambient temperature in the large root was not attained until 80 minutes and cooling was much slower. It is obvious that the ambient temperature, which is more quickly attained in smaller roots than in larger roots, would affect the kill of *A. mellea*.

Effect of heat on living plants and relation to survival of Armillaria mellea.—Citrus or peach seedlings of the rootstock cultivars noted earlier were used in these experiments. Two types of data were obtained; injury to living plants and indirect kill of *A. mellea* after treated plants were replanted in nonsterile soil. Temperatures used varied from 41-49 C with times ranging from 0.5 - 72 hours, using from four to 10 replications per treatment.

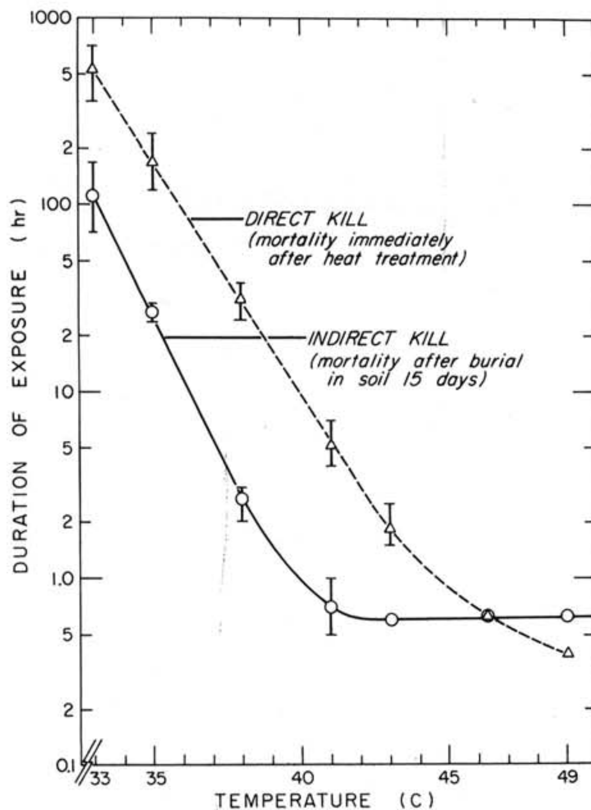

Fig. 3. Direct and indirect effect of heat on killing of *Armillaria mellea* growing in citrus root segments (2.5 × 13 cm). Chips from treated roots were plated on citrus agar immediately after treatment to determine the direct effect, and after storage in nonsterile orchard soil for 15 days, chips were taken from other roots to determine the indirect effect.

The effects of heat were difficult to assess because symptoms varied from no obvious effects to death of the plants; therefore, treated plants were observed for 9-12 months. Initial symptoms were wilting, which often developed further, leading to death of the plant or to stunted growth compared to that of the untreated controls. The temperature-time differential between treatments that caused no injury to living plants and those that killed *Armillaria* directly (see Fig. 3, direct effect) were rather substantial. For example, at 41 C citrus trees were not injured after a treatment in excess of 72 hours, whereas at this temperature, 4-7 hours of exposure was sufficient to kill the fungus. Similarly, at 43 and 46 C, citrus roots were not injured after 6 and 2 hours, respectively, whereas the fungus was killed after exposures of approximately 2 and 1 hours, respectively. At 49 C the temperature differential was less obvious. Citrus seedlings appeared to be normal after 0.5 hour of treatment, but were stunted 1 year later. This same treatment directly killed *A. mellea*.

The results of heat treatment on the survival of *A. mellea* in the roots of living citrus and peach seedlings and geranium plants were similar to those for the fungus in artificially inoculated root pieces (see Fig. 3, indirect effect). At 40 C for 2-3 hours, the fungus was killed in the roots of five citrus seedlings and on three of the four peach seedlings treated; the fungus survived on one of the latter trees. At the same treatment, *A. mellea* was not viable in 67 of the 77 geranium plants treated. *Armillaria mellea* was no longer viable on all nine of the geraniums treated at 33 C for 7 days. *Trichoderma viride* consistently was isolated from roots in all cases where *A. mellea* had not survived.

In summary, heat applied to living plants by steam-air mixtures could be adjusted to a combination of time and temperatures that were sufficient to kill *A. mellea* directly or indirectly without damage to the host plant.

Effect of drying conditions on survival of Armillaria mellea.—*Artificial cultures in peach wood.*—The direct effect of drying on survival of *A. mellea,* culture D 73, growing in peach limb wood was determined. For each treatment, four pieces of wood 20-25 cm in length and 25-28 mm in diameter were held in an incubator set at 15, 18, 21, or 24 C. Controls were held in a moist condition in polyethylene bags at the same temperatures. Wood pieces were weighed after 7, 13, 20, 30, 60, and 90 days and loss of water was expressed as a percentage of the total water content determined by oven-dry weights at the conclusion of the experiment. Viability of the fungus was verified after 30, 60, and 90 days by plating wood chips on agar. In general, the smaller the root diameter, the shorter was the drying time; the higher the temperature the more rapid the drying rate. Since the results of all treatments were similar, only those at 24 C will be described. At 24 C initial water loss was slow, reaching 90% in 13 days and 95% in 20 days. Thereafter, water loss was slower, reaching 98% in 90 days. The direct effect of drying was as follows: After 30 days, 4/4; after 60 days, 1/4; and after 90

days, 0/4 root pieces yielded viable *A. mellea*. The fungus was viable after 108 days in all of the root pieces used in the control.

Inoculum from naturally infected citrus roots.—The direct and indirect effects of drying on survival of *A. mellea* in naturally infected citrus roots was tested. If water loss reached 95-99%, *A. mellea* was killed directly, but if drying was not so complete, a difference between direct and indirect killing of *A. mellea* was observed. Four root pieces per treatment were used in an experiment in which root pieces were held at 9 C or 24 C for varying times, and the effects were tested for the direct and indirect (burial in soil for 15 days) kill of the fungus as follows: At 24 C moisture loss for exposures of 1, 3, 5, or 12 days averaged 22% (20-26), 32% (29-35), 77% (only one root was used), and 60% (56-65), respectively. All of the cultures were viable after exposure, but those exposed for 5 or 12 days were indirectly killed. At 9 C, moisture loss for exposures of 12 or 35 days was 40% (29-50) and 62% (56-64), respectively. All cultures were viable after exposure, but those that had dried for 35 days were indirectly killed. Thus, if root pieces lost approximately 60% of their total moisture, they were vulnerable to indirect killing; if relatively moist (less than 40% moisture loss), the fungus was unaffected by drying.

Effect of exposure of roots of orchard trees in relation to survival of Armillaria mellea.—For more than 50 years, a common practice in citrus and other fruit orchards for preventing tree-to-tree spread of *Armillaria*, and for prolonging the productive life of trees already infected, has been to remove the soil from the crown roots. It has been inferred that the fungus does not survive because of the subsequent exposure to heat and drying. We have observed orchards in which the fungus has been contained successfully by root exposure and one of these will be described. At the time an 8-year-old 65-hectare planting of citrus was surveyed, 35 sites were found in which one to 38 trees either were dead or had severe secondary symptoms. The crown root of trees surrounding all 35 sites were exposed. At the largest site where all 38 trees were dead, 35 of the surrounding 92 trees were infected. In the 11 years following root exposure, none of the 35 trees at this site has died. Furthermore, there is no evidence at the other 34 infection sites that the fungus has spread to other noninfected trees.

Numerous observations of root excavations showed that *A. mellea* mycelia were killed in exposed stems and roots, and *Trichoderma* spp. usually was observed sporulating on the infections. However, contiguous areas of roots remaining in soil invariably harbored viable *A. mellea*.

Thermocouples were affixed to the cambial areas of trunks and exposed crown roots of some infected trees. Cambial temperatures were lower than air temperatures

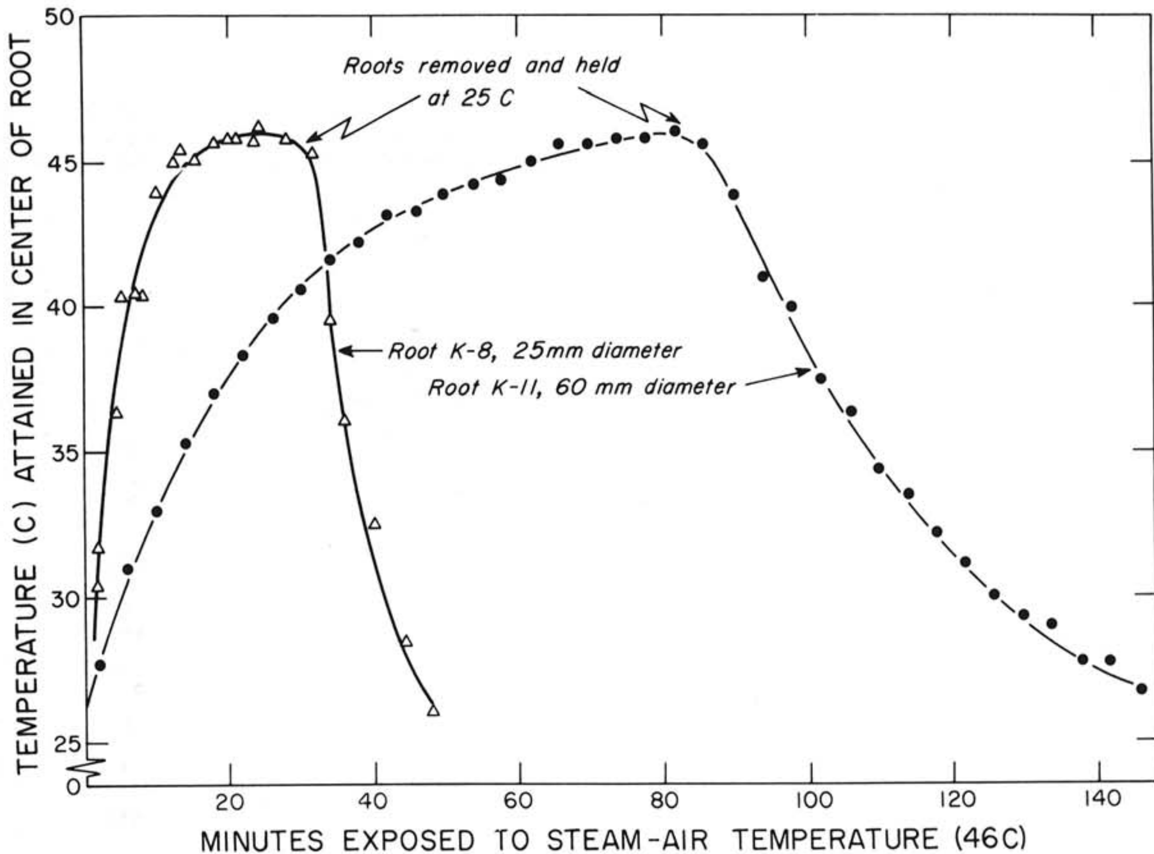

Fig. 4. Temperatures attained in citrus root segments of 25 and 60 mm diameter subjected to steam-air mixtures at 46 C. When 46 C was attained in the centers of the roots, they were removed and held at 25 C to measure post-heating effects.

under the tree. In one experiment, cambial temperatures did not exceed 24 C in the shade or 32 C in direct sunlight when the air temperature was 35 C. Other temperature measurements of 28 roots under six trees gave a minimum of 22 C when air was 30 C, and a maximum of 34 C when the air temperature was 35 C.

DISCUSSION

The response of *A. mellea* and *T. viride* to heating were similar to responses to CS_2 and to methyl bromide reported earlier (3, 6). That is, *A. mellea* is much more sensitive than its antagonist, *T. viride,* to the adverse effects of heating. These facts accentuate the paramount importance of the "stress" factor in the ecological relationship between *A. mellea* and *T. viride* in soil, as discussed previously (5), and it briefly will be reviewed here. When *A. mellea* is "stressed" by treatments with sublethal doses of chemicals, heat, or drying, it may be killed by subsequent attacks by soil microorganisms, primarily *Trichoderma* spp. Other organisms such as bacteria, *Fusarium* spp., and Mucorales presumably function in these relationships, but their roles have not been elucidated. The stress on *A. mellea* is due to reduced growth, reduced production of antibiotic substances, and perhaps to increased exudation of substances from the fungus, all of which combine to make a favorable environment for the antagonists. At a dose which is sublethal to *A. mellea, Trichoderma* spp. actually may be stimulated to increase in numbers and parasitic activity on the weakened *A. mellea*. Under such conditions, *Trichoderma* spp. may become actively parasitic to *A. mellea,* whereas in untreated soil it would be unable to do so. Thus, the key to these relationships is the subjection of *A. mellea* to an unfavorable environmental stress which tips the biological equilibrium in favor of the omnipresent, but normally impotent, antagonists (notably *Trichoderma* spp.) and results in the destruction of *A. mellea*.

The control of *A. mellea* by exposure of crown roots has been used in Australia (2) and California (7) for many years. The success of the practice has presented a dilemma to us in that it is difficult to resolve the differences between our experimental data and the field observations. Temperatures recorded in cambial areas of exposed roots were not high enough, according to our experimental data, to result in a direct kill of *A. mellea*. Also, the roots do not dry out extensively, since they remain alive and functional. Nevertheless, *A. mellea* is killed. Perhaps root exposure is effective because the increased temperature and drying imposed stresses that were mild but *continuous,* whereas in our experiments the conditions were more severe but of shorter duration.

The use of heat therapy for infected plants has been demonstrated. Citrus and the other plants tested have a greater tolerance to high temperatures than *A. mellea*. It might be feasible to inject steam-air mixtures around living, infected trees, and either kill *A. mellea* directly, or more likely, weaken it sufficiently so that natural antagonists could attack the fungus. It is conceivable that valuable infected shrubs and small trees could be removed from an infested area, the root ball treated with heat (steam-air would be best, but hot air could possibly be used) the soil treated with methyl bromide, and the plant later replanted. With proper equipment, control by these means might be economically profitable.

All of the data from various researches on *A. mellea* indicate that the fungus is not capable of independent existence in soil; nevertheless it survives for many years on infected host tissues in soil. The reason probably is that it exists in its ecologic niche under the bark and in wood of roots in soil. The control strategy therefore is to upset the ecologic niche by using heat, drying, or chemicals to weaken the fungus and to stimulate its antagonists to action. Current research in this laboratory is being conducted along these lines.

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