

## Defoliation-Induced Chemical Changes in Sugar Maple Roots Stimulate Growth of *Armillaria mellea*

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### ABSTRACT

Chemical changes induced in roots by artificially defoliating sugar maple trees in the field were examined for their effects on the growth of *Armillaria mellea*. Defoliation followed by production of new leaves caused a significant decrease in starch content of the roots and an increase in glucose and fructose in the outermost root wood but not in the root bark. Both total number and concentrations of some amino acids increased in the roots of defoliated trees, especially in the outer wood. The

fatty acid content of the roots was low, and was not affected by defoliation. Growth of *A. mellea* was greater in all media amended with extracts of outer wood from defoliated trees, but not in all media amended with bark extracts. Defoliation may predispose sugar maple trees to attack by *A. mellea* by initiating in roots chemical changes that are favorable for the growth of the fungus.

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*Additional key words:* predisposition, *Acer saccharum*, root-infecting fungi.

Defoliation by insects can initiate dieback and decline of trees, but mortality of defoliated trees is often associated with other organisms (9), principally the root-infecting fungus *Armillaria mellea* (Vahl.) Quel. (10, 21). This fungus was consistently isolated from dead and dying roots of red and scarlet oaks (*Quercus rubra* L. and *Q. coccinea* Muenchh., respectively) that were dying or undergoing decline subsequent to defoliation by leaf rollers (21). Mortality of sugar maple (*Acer saccharum* Marsh) in Wisconsin in the late 1950's after defoliation by a complex of insects was associated with attack by *A. mellea* (10). The fungus was recovered from 70% of the stumps of trees with decline symptoms; stumps of such trees were more extensively colonized than were stumps of trees free of decline symptoms (36 and 7%, respectively, of circumference invaded).

Artificially defoliating sugar maple may predispose the trees to attack by *A. mellea* by effecting chemical changes in the roots. Defoliated trees had high amounts of glucose and fructose in the roots in the autumn after defoliation (16). *A. mellea* grows better on glucose and fructose than on other sugars such as sucrose (24). In addition, amino acids (24) and fatty acids (13, 14) that stimulate growth of *A. mellea* also may be involved.

The study reported herein was undertaken, therefore, to determine (i) the sequence, magnitude, and duration of changes in carbohydrates (especially starch, glucose, fructose, and maltose), free amino acids, and fatty acids in roots of defoliated sugar maple; (ii) whether the bark or outer wood was the primary site of these changes; and (iii) the relationship of these changes to the growth in vitro of *A. mellea* on extracts from these root tissues.

**MATERIALS AND METHODS.**—Dominant and codominant sugar maples growing in southern Connecticut were used. The trees were 3- to 6-cm diam at 1.4 m aboveground and 5 to 8 m tall.

Twenty-four trees were designated as nondefoliated control trees. Groups of 12 trees were completely defoliated by hand on 17 June, on 1, 15, or 29 July 1970. This is the period when defoliation has the greatest adverse effect on sugar maple (10).

Roots of three defoliated and three control trees were harvested 2, 4, 6, and 8 weeks after each defoliation date. Roots were cut into 15-cm segments, put in polyethylene bags, and placed in insulated bags containing dry ice. In the laboratory, a portion of each of three roots was removed for starch analysis, and the remaining roots were stored at -30 C.

**Chemical analysis.**—Only the root wood was analyzed for starch. Histochemical tests indicated that the bark tissue contained little or no starch. Root segments were cut into 3- to 5-mm slices and dried in a forced-air oven for 24 hr at 80 C. Starch was extracted according to the procedure of Hassid & Neufeld (6), and quantified by the method of Siminovitch et al. (19). Standard solutions were prepared from starch extracted from sugar maple roots.

The bark and outer wood were analyzed separately to determine whether changes occurring in the bark, the tissue the fungus penetrates, were different from changes in the outer wood, the tissue over which the fungus grows rapidly in infected roots. Roots were removed from the freezer and washed in cold tap water. The bark was removed and cut into smaller sections. The outermost wood was scraped from the peeled roots with a scalpel. Sugars and amino acids were extracted with cold 80% ethanol and concentrated in an evaporator flask as previously described (23). Concentrated extracts were rinsed from the evaporator flask with 30% ethanol and diluted with deionized distilled water to a final volume of 2 ml/g of tissue. A 3-ml portion of each extract was prepared for chromatographic analysis by

treatment for 5 min in an electric desalter (Torbal). The remaining extract was bioassayed, using *A. mellea*. Extracted tissue was dried for 24 hr at 80 C to determine dry weight. All determinations were made on an oven-dry-weight basis (odw).

Sugars were identified and quantified as previously described (23). Amino acids were separated two-dimensionally on thin-layer silica gel plates. Chloroform-methanol-ammonia (2:2:1, v/v) was used in the first direction; and butanol-acetic acid-water (4:1:1, v/v), in the second (20). Amino acids were detected with ninhydrin spray. A relative concentration was given to each ninhydrin-reacting spot based on size and the density of color in reference to a standard.

After ethanol extraction, the tissue residue was analyzed for fatty acid content. The tissue was dried, ground in a Wiley mill to pass a 60-mesh screen, soaked overnight in diethyl ether, and then refluxed 3 times, using fresh ether each time. Extracts were combined and evaporated at 24 C in a rotary evaporator to 1 ml solution/g of tissue (odw). Half of the condensed extract was bioassayed, using *A. mellea*; and half was saponified to free the fatty acids (5). The freed fatty acids were methylated with  $\text{BF}_3$ -methanol and extracted with petroleum ether. The acids were identified and quantified by gas chromatography.

**Bioassay.**—*A. mellea* was grown in liquid medium to bioassay the ethanol extracts, and on solid medium to bioassay the ether extracts. The fungus was grown in 4-oz prescription bottles containing 20 ml of ether medium plus materials extracted from 0.5 g of tissue (odw). The liquid medium consisted of extract added to deionized water. The final concentration of ethanol in the solution was adjusted to 2,500 ppm. Solutions were filtered through 0.5- $\mu$  disposable filters. The solid medium was prepared according to Weinhold & Garraway (24), and included 5 g glucose/liter. Ether extracts were spread evenly over the surface of the medium. After the ether evaporated, the bottles were aerated for 1 hr, then autoclaved in the horizontal position for 15 min at 15 psi. Both media were adjusted to pH 5.8 before filtering or autoclaving them.

Inoculum was obtained from 3- to 5-week-old colonies of *A. mellea* grown on water agar. Agar plugs, 10 mm in diameter and 6 to 7 mm high, were cut from the margins of the colony and were placed mycelial-side up in the liquid medium (height of the plug kept the inoculum above the liquid surface) and mycelial-side down on the solid medium. Five replicate cultures were prepared for each type of extract. The cultures were incubated in the dark at  $23 \pm 1$  C for 2 weeks. Growth was determined as mg dry weight after a drying of the fungal tissue for 24 hr at 80 C in a forced-air oven. Rhizomorph production was estimated visually and was rated: 0 = none produced; 1 = rhizomorph initials to 25% of total colony; 2 = 25-50%; 3 = 50-75%; and 4 = >75%.

A multivariate analysis was used to determine statistically significant differences among all data.

**RESULTS.**—*Chemical changes.*—All defoliated

trees produced new leaves 2 to 3 weeks after defoliation. All defoliation treatments lowered the starch content in the roots (Fig. 1). Minimum starch levels occurred 6 to 8 weeks after defoliation and were one-tenth to one-fifth of those in control trees. The rate of starch loss was approximately the same for defoliated trees, but amount of residual starch was affected by time of defoliation; early defoliations, 17 June or 1 July, resulted in lower residual starch in the roots than later defoliations, 15 or 29 July. This phenomenon is apparently related to the normal seasonal accumulation of starch, which begins sometime in June (23). Trees defoliated on 15 or 29 July probably had higher initial starch contents than trees defoliated on 17 June or 1 July.

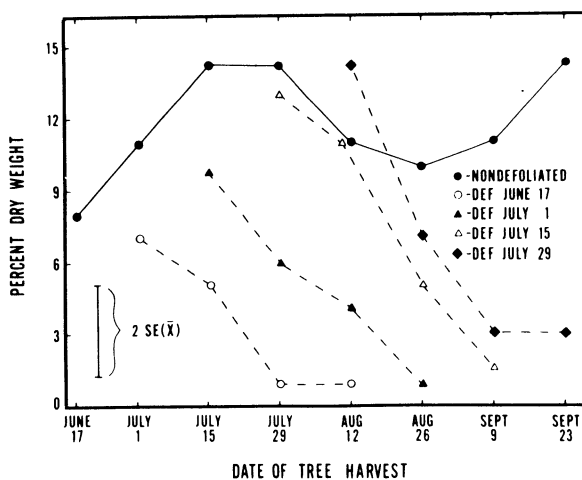


Fig. 1. Starch loss in roots of defoliated sugar maple trees. Each point represents average value of three trees.

Coincident with the starch decrease was an increase in the glucose and fructose concentration in the outer wood of roots of defoliated trees (Fig. 2-A). The glucose-fructose concentration (glucose and fructose concentrations were usually equal) in defoliated trees was higher than in nondefoliated trees 2 weeks after treatment, and remained higher for all observations. The amount of glucose-fructose in trees defoliated 17 June or 1 July fluctuated, but in trees defoliated 15 or 29 July, these sugars increased with each succeeding harvest. Only the glucose-fructose content in trees defoliated 15 or 29 July was significantly greater ( $P = .01$ ) than in nondefoliated trees.

In contrast to the outer wood, the bark's glucose-fructose content was not affected by defoliation (Fig. 2-B). But the amount of sucrose in the root bark was significantly lowered ( $P = .01$ ) by all defoliation treatments (Fig. 3-A). In the outer wood, only the 17 June or 1 July defoliation treatments resulted in lower sucrose concentrations (Fig. 3-B).

Defoliation had no effect on the maltose concentration in the roots. Maltose was detected in trace amounts in the outer wood of both defoliated

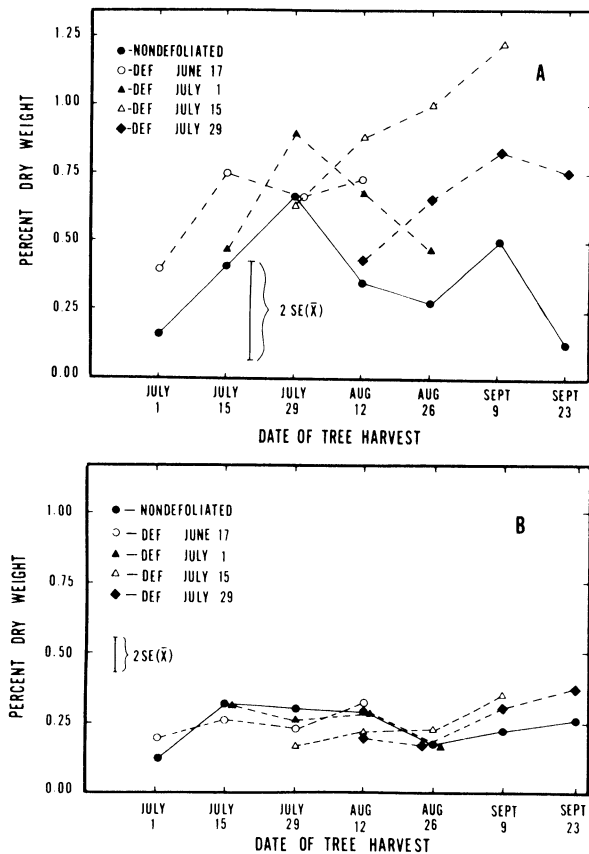


Fig. 2. Glucose-fructose concentrations in roots of defoliated and nondefoliated sugar maple trees. A) Outer wood. B) Bark. Each point represents average value for three trees.

and nondefoliated trees harvested 1 July; it was not detected again until the 23 September final harvest.

The relative amounts of certain amino acids in both bark and outer wood, and the number of amino acids in the outer wood, increased in the roots of defoliated trees. Concentrations of compounds having the correct  $R_F$  values for threonine, cysteine, tyrosine, histidine, and proline were higher in the outer wood of defoliated trees. In the bark, compounds corresponding to tyrosine and proline were higher in defoliated trees. Compounds with  $R_F$  values for leucine and isoleucine occurred in outer wood extracts of defoliated but not in nondefoliated trees.

Palmitic, stearic, oleic, linoleic, and linolenic acids were the dominant fatty acids identified in the ether extracts. All five were present in both bark and outer wood. No differences in fatty acid concentration were detected between outer wood and bark or between defoliated and nondefoliated trees. The average concentrations of the above acids were 4, 2, 2, 4, and 2  $\mu\text{g/g}$  of tissue (odw), respectively.

**Bioassay of extracts using *A. mellea*.**—*Ethanol extracts*.—Extracts from the outer wood of roots of defoliated trees stimulated the growth of *A. mellea*

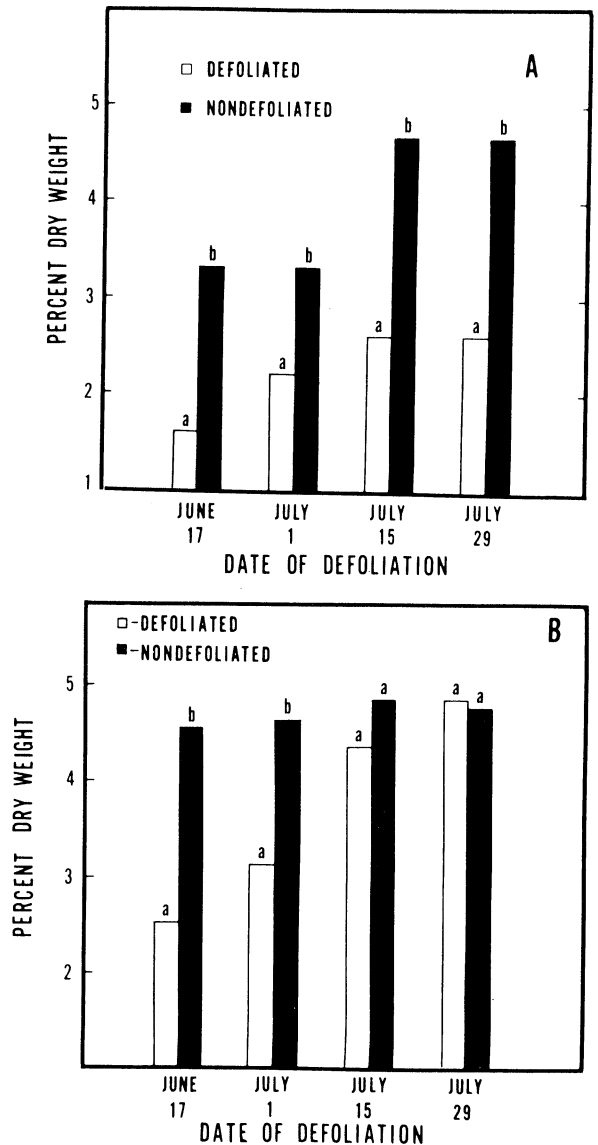


Fig. 3. Sucrose concentrations of roots of defoliated and nondefoliated sugar maple trees. A) Bark. B) Outer wood. Each bar represents average value for 12 trees. Unlike letters above bars indicate a significant difference at  $P = .01$ .

(Fig. 4-A). Growth of the fungus in extracts from trees of all defoliation treatments for all harvests was significantly better ( $P = .01$ ) than on extracts from nondefoliated trees. And growth on extracts from defoliated trees usually was better than on extracts from nondefoliated trees for each successive harvest date. Growth of *A. mellea* on bark extracts from defoliated trees was significantly better ( $P = .05$ ) only for trees defoliated 17 June or 1 July (Fig. 4-B). Fungal growth on bark and outer wood extracts from nondefoliated trees was not significantly different, but on extracts from defoliated trees, growth on the wood extracts was superior to that on bark extracts.

Although growth of *A. mellea* was not highly

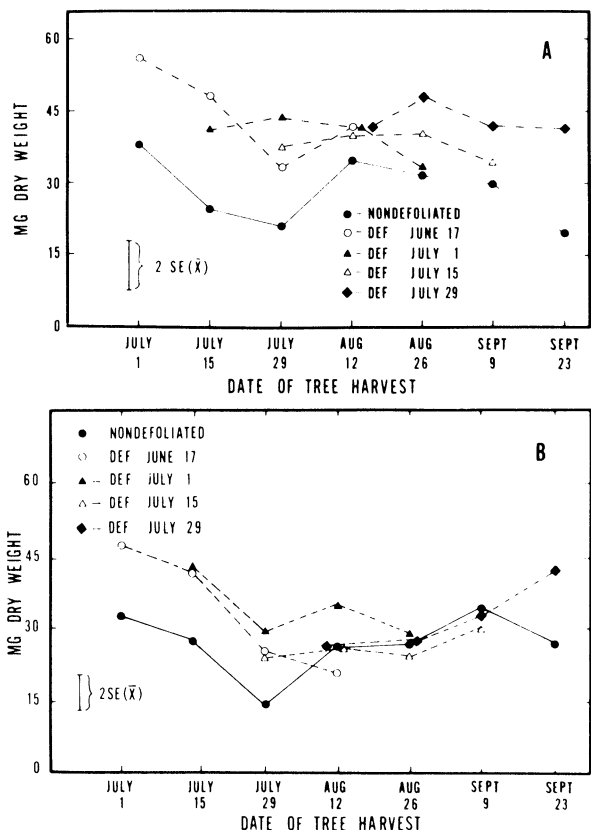


Fig. 4. Growth in vitro of *Armillaria mellea* on extracts from roots of defoliated and nondefoliated sugar maple trees. A) Outer wood. B) Bark. Each point represents average value for three extracts and five cultures per extract.

correlated with any specific carbohydrate, greater growth of the fungus always occurred on the outer wood extracts with the higher glucose-fructose level (Fig. 5-A). This relationship was not observed in the bark extracts (Fig. 5-B).

Rhizomorph production was stimulated by extracts from both bark and outer wood from roots of defoliated trees (Table 1). Over all, rhizomorph production was greater in outer wood extracts.

TABLE 1. Average rhizomorph production by *Armillaria mellea* growing on ethanol extracts from bark and outer wood of roots of defoliated and nondefoliated sugar maple trees

Tissue	Defoliation date				
	Control	17 June	1 July	15 July	29 July
Bark	1.0 y <sup>a,b</sup>	1.5 z	1.5 z	1.0 y	1.5 z
Outer wood	2.0 y	3.0 z	2.5 z	2.5 z	2.5 z

<sup>a</sup>Rhizomorph production: 0 = none produced; 1 = initials to 25% of total colony; 2 = 25 to 50%; 3 = 50 to 75%; and 4 = >75% of colony was rhizomorphic.

<sup>b</sup>Mean value not followed by the same letter indicates a significant difference at the 1% level.

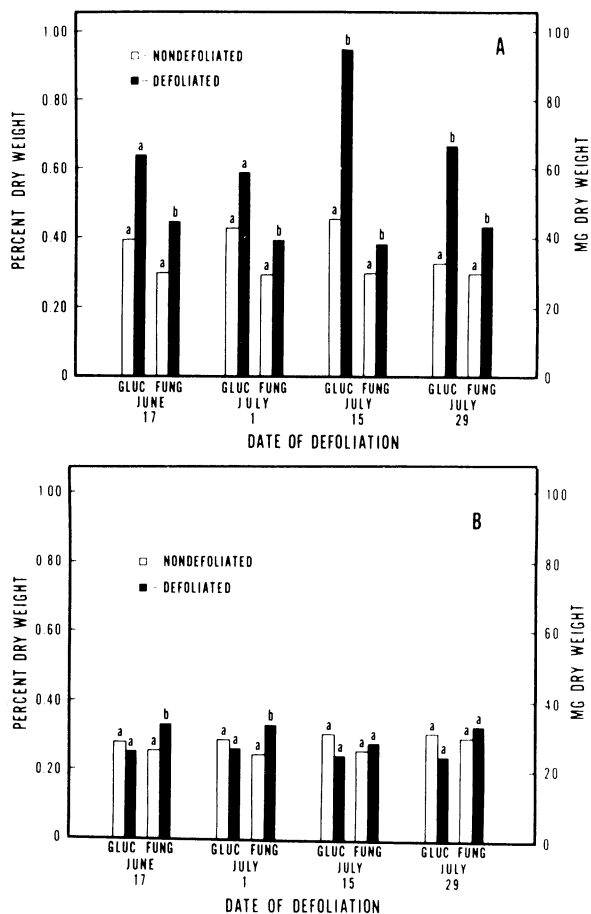


Fig. 5. Glucose-fructose concentrations in extracts from roots of defoliated and nondefoliated sugar maple trees and growth of *Armillaria mellea* on the extracts. A) Outer wood. B) Bark. Each bar represents average glucose-fructose (Gluc) level for 12 trees or average fungal growth (Fung) on 12 extracts, five cultures/extract. Unlike letters above bars indicate a significant difference at  $P = .01$  for outer wood and  $P = .05$  for bark.

*Ether extracts.*—*A. mellea* grew well on all ether extracts. No differences were observed between bark and outer wood or between defoliated and nondefoliated treatments. Growth on the medium amended with ether extracts appeared comparable to that on media amended with known unsaturated fatty acids at concentrations of 50  $\mu\text{g/ml}$ .

DISCUSSION.—The common association of *A. mellea* with roots and root collars of dead and dying trees in areas where severe defoliation by insects has occurred suggests that defoliation predisposes the roots of trees to attack by the fungus.

In this study, a dominant effect of defoliation was the lowering of starch content in roots. Girdling trees prior to felling to deplete root starch has been suggested as a way to reduce susceptibility of root systems to attack by *A. mellea* (11, 12, 25). But other girdling experiments have indicated that root starch content may have no direct role in

susceptibility to *A. mellea*, since stumps of girdled (ring-barked) and cut trees were attacked equally by the fungus (17). In studies of the colonization of stumps by basidiospores of *A. mellea*, no relationship was found between ability to colonize a tissue and the starch content (18).

Results of the present study indicate that in response to defoliation and growth of new leaves, the products of starch conversion and not the actual starch content may influence susceptibility to *A. mellea*. It is known that the fungus can utilize glucose and fructose better than sucrose (24). An immediate increase in these reducing sugars accompanied the decrease in starch content in defoliated trees, and their concentration remained high for at least 2 months. Both the reducing sugar increase and starch decrease were much greater than the normal seasonal fluctuations, even those fluctuations that occur in spring when buds break dormancy (23).

The bioassay data indicate that the increase in reducing sugars may have at least a partial role in increased susceptibility of the roots to *A. mellea*. *A. mellea* grew best in extracts from the outer wood, the tissue with the highest concentrations of glucose and fructose. It is interesting to note in view of the observed relationship of *A. mellea* and trees under stress that other stress factors such as drought (15), water logging (7), and lightning strike (8) resulted in increased reducing sugar concentrations in forest trees.

However, growth of the fungus was not proportional to the glucose-fructose concentration. In extracts of outer wood from trees defoliated 15 July, the glucose-fructose content was 50% more than in extracts from trees defoliated 1 July; but growth in each extract was comparable (Fig. 5-A). In bark extracts from trees defoliated 17 June or 1 July, growth of the fungus was greater than in the extracts from nondefoliated trees; and the glucose-fructose content was lower in the defoliated trees (Fig. 5-B). These findings suggest that components of the extract other than carbon source may have influenced fungal growth. Garraway (2) has shown that fungal growth, especially rhizomorph production, can be increased significantly by the concentration of indole-3-acetic acid. Since defoliation and growth of new leaves involve hormonal changes, the content of certain growth regulators in roots could have been affected.

The total free amino acid content of extracts from defoliated trees could also have influenced fungal growth. The carbon-nitrogen ratio affects growth and rhizomorph production in vitro (3). Of the amino acids affected by defoliation, leucine when tested as the sole source of nitrogen for growth of *A. mellea* was considered unsatisfactory (24). Histidine and cysteine, which increased in concentration in defoliated trees, have been related to disease resistance and susceptibility in other species. Increased histidine content was associated with increased susceptibility of blueberry fruits to *Glomerella cingulata* (22), and higher cysteine content enhanced resistance of four cucurbits to *Colletotrichum lagenarium* (4). A more precise

measure, qualitative and quantitative, of amino acids must be made before their role in pathogenicity of *A. mellea* can be determined.

Although not affected by defoliation, total fatty acid content of the roots was sufficient to maintain good growth of *A. mellea* in the presence of a suitable carbohydrate. The unsaturated fatty acid content of roots of several other tree species has been related to over-all growth and rhizomorph production by the fungus (13), and may be related to susceptibility of such trees to infection by *A. mellea*. However, since fatty acids appear to function as rhizomorph-promoting substances and not as carbon sources (14), they may be more important in the spread of *A. mellea* via rhizomorph development than in determining susceptibility of the tree to the fungus.

The ability of *A. mellea* to colonize freshly cut stems has also been related to the nutrient status of tissues (18). Stem sections of plum trees that had more nitrogen in the bark were on the average more readily colonized by *A. mellea*. Stem sections from several other tree species that were not readily colonized by *A. mellea* could be rendered susceptible by autoclaving or treating the sections with 2,4,5-trichlorophenoxyacetic acid (2,4,5-T). Autoclaving tissues can cause breakdown of complex sugars and proteins, which would increase the simple sugar and free nitrogen content; and one of the short term effects of 2,4,5-T is to increase the reducing sugar content of the treated tissue (1).

The primary site of chemical changes observed in this study was the outermost wood. Rishbeth (18) found that colonization of woody stems began in the cambial zone. In my study, most of the cambial tissue was analyzed as part of the bark tissues, and any increase in cambial sugars or amino acids could have been diluted and masked by the other bark tissues. It is also possible that the chemical changes observed do occur in the outermost wood, and the nutrients diffuse into the cambial region, initially stimulating the fungus to grow there. This undifferentiated tissue may offer the least physical resistance to mycelial ramification.

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