Rhizomorph Initiation and Growth in Armillaria mellea Promoted by o-Aminobenzoic and p-Aminobenzoic Acids

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ABSTRACT

o-Aminobenzoic acid (anthranilic acid or AA) and *p*-aminobenzoic acid (PABA) promoted rhizomorph initiation and growth in *Armillaria mellea* after 5 weeks on a glucose-L-asparagine medium containing 10 ppm of either aromatic. Several other biosynthetically related aromatic compounds promoted only mycelial growth. Cultures of *A. mellea* grown for 7 days on a medium supplemented with 500 ppm ethanol continued to produce rhizomorphs after transfer to a fresh medium containing AA or PABA or both. After 21 days, however, there was more rhizomorph growth on media containing 5, 10, or 20 ppm AA than on media containing comparable concentrations of PABA. Rhizomorph growth of cultures transferred from an ethanol-supplemented medium was enhanced by the addition of 0.1 or 1.0 ppm AA to media containing 5 ppm PABA, but not by the addition of 0.1 or 1.0 ppm PABA to media containing 5 ppm AA.

Indole-3-acetic acid (IAA) promoted rhizomorph initiation and growth of *A. mellea* after 5 weeks on a glucose-L-asparagine medium containing 10 or 20 ppm but not 1 ppm. Comparable concentrations of folic acid (FA) were ineffective. Similarly, IAA promoted rhizomorph growth of cultures that were transferred from an ethanol-supplemented medium to one containing 10 or 20 ppm. At pH 4.8 comparable concentrations of FA were ineffective, but at pH 6.0, 20 ppm FA promoted a small amount of rhizomorph growth. These data indicate that IAA is more effective in promoting rhizomorph growth than is FA, and suggest that *A. mellea* may be deficient in IAA when grown on a glucose-L-asparagine medium. Thus AA may possibly function as a precursor for IAA. Phytopathology 60:861-865.

Empirical tests revealed that two aromatic compounds, *o*-aminobenzoic acid and *p*-aminobenzoic acid, promoted rhizomorph initiation and growth in *Armillaria mellea* (Vahl) Quél. (5). Low concentrations (10 ppm) of either were stimulatory when added to a glucose-L-asparagine medium. Many other aromatic compounds tested did not promote rhizomorph initiation and growth (5).

The observation that oils and fatty acids, including oleic and linoleic acids, promoted rhizomorph production only when supplied to a medium containing glucose (15), provided the impetus for an investigation of the role of aromatic compounds on growth. The effect of fatty acids suggested that lipids or products of lipid metabolism might be required for rhizomorph production by *A. mellea*. Since the presence of glucose was necessary for lipids to be effective, one or more compounds associated with glucose metabolism may also have been needed. Aromatic compounds were a logical choice for an empirical study because experiments with specifically labeled glucose-^{14}C revealed that the pentose phosphate pathway may be a major alternate glycolytic pathway in *A. mellea* (6, 7). This pathway provides precursors for the biosynthesis of aromatic compounds of biological significance (1, 12, 16).

Because rhizomorphs are important in infection (10, 17) and in spread (13) and survival (2) of *A. mellea*, a study of the effects of *o*-aminobenzoic acid (anthranilic acid or AA) and *p*-aminobenzoic acid (PABA) on rhizomorph formation is of interest. The purpose of this study was (i) to make a critical comparison of the growth promoting effects of AA and PABA; and (ii) to test the possibility that AA and PABA might function as precursors for growth factors.

MATERIALS AND METHODS.—The isolate of *A. mellea* was the same as that used previously (19). The basal medium consisted of 5 g of D-glucose, 2 g of L-asparagine, 1.75 g of KH_2PO_4, 0.75 g of $MgSO_4 \cdot 7H_2O$, and 1 mg thiamine/liter of distilled water. The medium was adjusted to pH 6.0 with 1 N NaOH, siphoned into 8-oz medicine bottles (50 ml/bottle), then sterilized by autoclaving. Standard solutions (1 mg/ml) of the aromatic compounds were prepared by placing 50 mg of each chemical into separate dry 125 ml Erlenmeyer flasks, autoclaving for 20 min, allowing to cool, and adding 50 ml of sterile basal medium. The sterile standard solutions were added aseptically to the above medium to give the concentrations desired. The aromatic and related compounds used were, unless indicated otherwise, *o*-aminobenzoic acid (AA), *p*-aminobenzoic acid (PABA), *m*-aminobenzoic acid, *p*-hydroxybenzoic acid, quinic acid, protocatachualdehyde, L-phenylalanine, L-tyrosine, L-tryptophan, shikimic acid (purchased from Nutritional Biochemicals Corp., Cleveland, Ohio, or Aldrich Chemical Co., Inc., Milwaukee, Wisconsin). The foregoing method for preparation of sterile standard solutions of aromatic compounds was used instead of filtration because it was more convenient. Moreover, preliminary tests indicated that standard solutions of aromatics prepared as above produced comparable effects on growth of *A. mellea* to identical solutions sterilized by filtration.

The techniques for preparation of the inoculum, incubation of the cultures, and determination of dry wt have been described (19). A 5-week incubation period was used when the inoculum was from water agar plates because of the long lag (2 weeks or more) preceding rhizomorph initiation. A 3-week incubation period was used when the inoculum consisted of colonies grown for 7 days on a basal medium supplemented with ethanol (500 ppm). Colonies from the ethanol medium already showed rhizomorph primordia, which appeared

as dark spots on the under side at this time. For either incubation period, growth (dry wt) on a basal medium supplemented with aromatics was compared with that on a nonsupplemented medium and on one supplemented with ethanol (500 ppm). The cultures were incubated in the dark at 22 C. The pH of the medium dropped to 5.0 by the end of either incubation period. The results reported are one of at least two experiments, and the values reported are the mean of at least five replications.

RESULTS.—*Influence of selected aromatic compounds on rhizomorph initiation and growth.*—Aromatic and related compounds whose synthesis is linked to carbohydrate metabolism via the pentose phosphate and shikimic acid pathways (12, 16) were selected for this study. AA stimulated rhizomorph initiation and promoted rhizomorph growth (Table 1). PABA also stimulated rhizomorph initiation and growth, but was less effective than was AA. On AA- and PABA-supplemented media, rhizomorphs comprised 65.2% and 58.0%, respectively, of the total growth. On the AA-supplemented medium, rhizomorph production was almost comparable to that on a medium supplemented with ethanol (500 ppm). On the ethanol-supplemented medium, however, maximum growth was attained after 3 weeks, whereas cultures incubated on media supplemented with aromatics required 5 weeks for maximum growth.

Shikimic acid, quinic acid, protocatachualdehyde, *p*-hydroxybenzoic acid, L-phenylalanine, and L-tryptophan stimulated mycelial growth, but no rhizomorphs developed. In contrast, mycelial growth on *m*-aminobenzoic acid- and on L-tyrosine-supplemented media were comparable to that on the nonsupplemented glucose medium.

Comparison of AA and PABA as growth factors for rhizomorph production.—To compare and critically evaluate the relative effectiveness of AA and PABA as growth factors for rhizomorph production in *A. mellea*, (i) the time course of growth on media supplemented with AA and PABA, (ii) the influence of various concentrations of AA and PABA, and (iii) the effect of combining various concentrations of AA and PABA were studied. For these studies, the inoculum consisted of colonies grown on an ethanol-supplemented medium for 7 days. This inoculum required a shorter incubation time for maximum growth than inoculum from water agar. Also, replicates of each treatment were less variable and, consequently, the data were more reproducible.

The time course study (Fig. 1) was done to determine whether growth (dry wt/culture) was consistently greater with AA than with PABA after different incubation times. The dry wt per colony doubled after the first 6 days of incubation on media supplemented with AA or AA + PABA. In contrast, PABA caused no significant dry wt increase after 6 days. After 20 days, i.e., at termination of the experiment, there was more growth on media containing AA or AA + PABA than on media containing PABA.

On media containing ethanol (500 ppm), there was more growth after 6 days of incubation than there was on media containing AA (Fig. 1), but after 20 days there was more growth on media containing AA or AA + PABA. On media containing either ethanol or aromatics, rhizomorphs comprised over 60% of the total growth after 20 days. On a nonsupplemented medium (Fig. 1), growth was less than on a supplemented medium, and no rhizomorphs developed.

TABLE 1. Mycelial growth and rhizomorph production by *Armillaria mellea* on a glucose-L-asparagine medium supplemented with various aromatic compounds, shikimic acid or ethanol

Compound[b]	Growth: mg dry wt/culture[a]		
	Rhizomorphs	Mycelium	Total
o-Aminobenzoic acid	38.8	10.6	49.4 ± 10.1
p-Aminobenzoic acid	23.5	9.8	33.3 ± 2.8
m-Aminobenzoic acid	0.0	17.3	17.3 ± 2.4
p-Hydroxybenzoic acid	0.0	34.8	34.8 ± 5.2
Quinic acid	0.0	36.5	36.5 ± 4.8
Protocatachualdehyde	0.0	44.1	44.1 ± 9.2
L-Phenylalanine	0.0	24.8	24.8 ± 8.0
L-Tyrosine	0.0	19.1	19.1 ± 7.1
L-Tryptophan	0.0	31.4	31.4 ± 4.7
Shikimic acid	0.0	41.9	41.9 ± 6.5
Ethanol (500 ppm)	49.7	23.8	73.5 ± 9.1
Control	0.0	13.8	13.8 ± 0.4

[a] Each figure is the mean of five replications. Based on the total growth following 5 weeks of incubation. Mean deviation is indicated.

[b] 10 ppm of each aromatic or related compound was added to the medium.

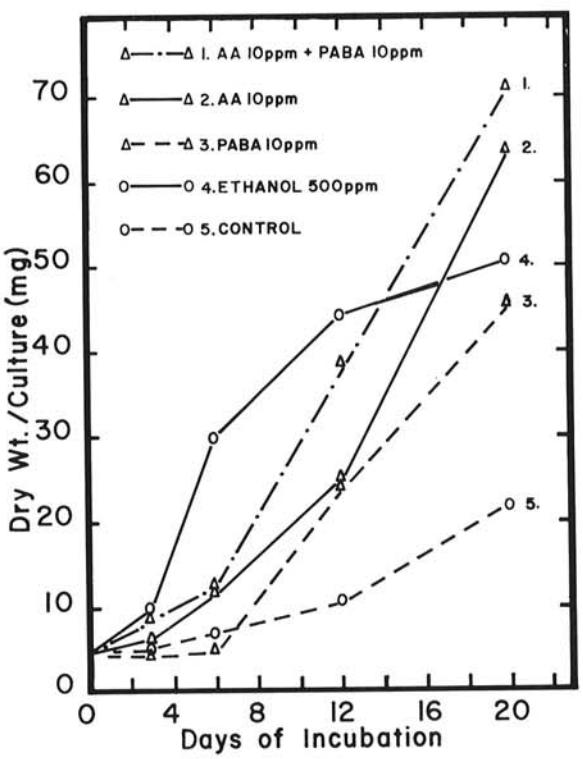

Fig. 1. Growth of *Armillaria mellea* after different incubation times on a glucose-L-asparagine medium supplemented with 10 ppm anthranilic acid or AA + 10 ppm *p*-aminobenzoic acid or PABA (1), 10 ppm AA (2), 10 ppm PABA (3), 500 ppm ethanol (4), and nonsupplemented (5).

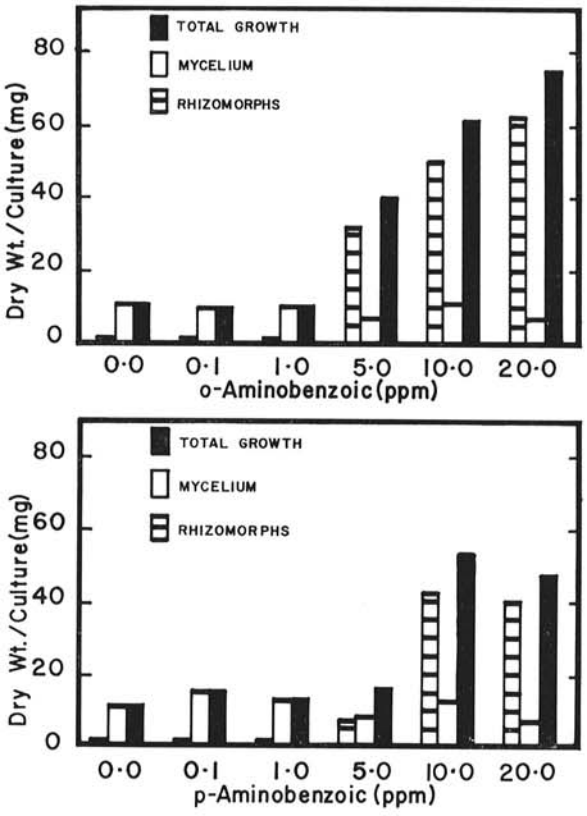

Fig. 2. Growth and rhizomorph production by *Armillaria mellea* on a glucose-L-asparagine medium supplemented with different concentrations of *o*-aminobenzoic acid or anthranilic acid (above) and *p*-aminobenzoic acid or PABA (below).

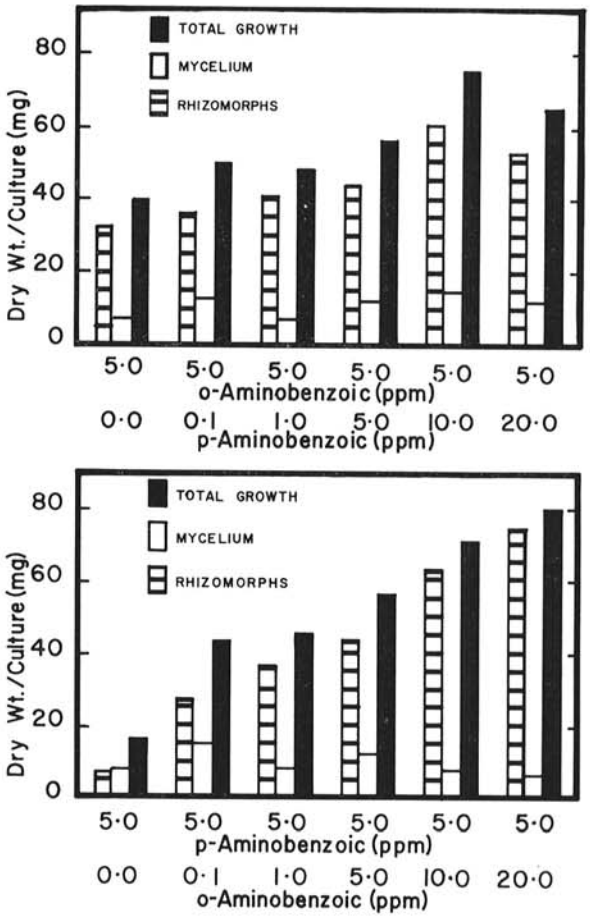

Fig. 3. Growth and rhizomorph production by *Armillaria mellea* on a glucose-L-asparagine medium supplemented with 5 ppm *o*-aminobenzoic acid or anthranilic acid and various concentrations of *p*-aminobenzoic acid or PABA (above), or 5 ppm PABA and various concentrations of anthranilic acid (below).

A comparison of the effect of various concentrations of AA or PABA on rhizomorph production (Fig. 2) revealed that at 1.0 ppm or less, neither AA nor PABA promoted rhizomorph growth, but at 5 ppm, AA (Fig. 2, above) induced about 4 times more rhizomorph growth than did a corresponding concentration of PABA (Fig. 2, below). At 10 and 20 ppm, AA also promoted more rhizomorph growth than did corresponding concentrations of PABA.

The effect of combining different concentrations of AA with PABA was studied to determine whether a small amount of one aromatic would enhance the effect of the other. Colonies were grown for 21 days on media containing 5 ppm PABA and various concentrations of AA (Fig. 3, below). Colonies also were grown on media containing 5 ppm AA and various concentrations of PABA (Fig. 3, above). Rhizomorph growth was poor on media containing 5 ppm PABA. The addition of 0.1 or 1.0 ppm AA to media containing 5 ppm PABA caused a considerable increase in rhizomorph growth, which increased with increasing increments of AA (Fig. 3, below). In contrast, the addition of 0.1 or 1.0 ppm PABA to media containing 5 ppm AA did not cause a significant increase in rhizomorph growth (Fig. 3, above). Also, the addition of 5, 10, or 20 ppm PABA to a medium containing 5 ppm AA (Fig. 3, above) did not cause as great an increase in rhizomorph production as did the addition of 5, 10, or 20 ppm AA to a medium containing 5 ppm PABA (Fig. 3, below). These data indicate that AA may be a limiting factor for growth on media supplemented with PABA alone, but not vice versa.

Comparison of indole-3-acetic acid (IAA) and folic acid (FA) as growth factors for rhizomorph production. —In view of the foregoing studies, the proposition that AA and derived compounds might be more closely related to rhizomorph growth than PABA and derived compounds seems feasible. I sought further experimental support for this hypothesis by comparing the relative effectiveness of IAA and FA as growth factors for rhizomorph initiation and growth. IAA was selected because it is a growth hormone (4, 8), and its synthesis involves anthranilic acid as a precursor (12). Moreover, preliminary tests showed that IAA promoted rhizomorph formation in *A. mellea* (20). Folic acid (FA)

TABLE 2. Mycelial growth and rhizomorph production by *Armillaria mellea* (inoculum from water agar) incubated for 35 days on a glucose-L-asparagine medium at an initial pH of 4.8 or 6.0 and supplemented with various concentrations of indole-3-acetic acid or folic acid

	Growth: dry wt/culture[a]					
	Initial pH, 4.8[b]			Initial pH, 6.0[b]		
Compound	Rhizomorphs	Mycelium	Total	Rhizomorphs	Mycelium	Total
Indole-3-acetic acid	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*
0 ppm	0.0	23.6	23.6 ± 6.1	0.0	19.6	19.6 ± 4.3
1 ppm	0.0	22.6	22.6 ± 6.0	0.0	18.0	18.0 ± 2.5
10 ppm	21.2	17.0	38.2 ± 7.5	41.7	12.9	54.6 ± 11.4
20 ppm	62.7	11.9	74.6 ± 10.4	46.6	12.5	59.1 ± 4.7
Folic acid						
0 ppm	0.0	14.9	14.9 ± 3.8	0.0	17.5	17.5 ± 4.4
1 ppm	0.0	12.5	12.5 ± 4.8	0.0	16.0	16.0 ± 4.2
10 ppm	0.0	14.5	14.5 ± 3.1	0.0	15.6	15.6 ± 5.3
20 ppm	0.0	19.9	19.9 ± 5.4	0.0	17.4	17.4 ± 5.2

[a] Each figure is the mean of five replications. Mean deviation is indicated for total wt.
[b] Final pH varied from 4.5 to 5.0.

was selected because it is a vitamin of universal importance in living organisms (3, 9, 12), and PABA is a constituent of FA (12).

To evaluate the effectiveness of IAA and FA as growth factors for rhizomorph production, I determined whether media supplemented with these compounds promoted rhizomorph initiation and growth when (i) seeded with inoculum from water agar; and (ii) seeded with inoculum from an ethanol-supplemented medium. For these studies, sterile standard solutions containing 0.1 mg/ml of either IAA or FA were prepared as described previously. The standard solutions were added aseptically to the basal media giving the concentrations indicated in Tables 2 and 3. The initial pH of the media was adjusted to 4.8 or 6.0.

IAA induced rhizomorph initiation and growth on media containing 10 or 20 ppm but not 1 ppm (Table 2). In contrast, all comparable concentrations of FA were ineffective. At an initial pH of 4.8, 20 ppm IAA induced significantly more rhizomorph growth than did 10 ppm. At an initial pH of 6.0, rhizomorph production was comparable at 10 and 20 ppm IAA. FA was ineffective at either pH.

The response to IAA and FA (Table 3) was somewhat different from above when the inoculum was from ethanol-supplemented medium rather than water agar. As with the previous tests (Table 2), IAA was a considerably more effective inducer of rhizomorph growth than was FA (Table 3). However, at an initial pH of either 4.8 or 6.0, 20 ppm IAA induced considerably more rhizomorph growth than did 10 ppm. FA was ineffective when supplied at 10 or 20 ppm to media at pH 4.8, or at 10 ppm to media at pH 6.0. However, at the pH 6.0, 20 ppm FA induced a small amount of rhizomorph growth (Table 3). These data indicate clearly that IAA is more effective than FA as a growth factor for rhizomorph production, and provide experimental support for the idea that AA may function as a precursor for auxin.

DISCUSSION.—The stimulation of rhizomorph production by AA and PABA and the lack of stimulation by other aromatics and related compounds suggest that the composition of the aromatics studied partly determines their effectiveness. A comparison of the effects of AA, PABA, and *m*-aminobenzoic acid, however, indicates that the structure of the aromatics involved

TABLE 3. Mycelial growth and rhizomorph production by *Armillaria mellea* (inoculum from an ethanol-supplemented medium) incubated for 21 days on a glucose-L-asparagine medium at pH 4.8 or 6.0 and supplemented with various concentrations of indole-3-acetic acid or folic acid

	Growth: dry wt/culture[a]					
	Initial pH, 4.8[b]			Initial pH, 6.0[b]		
Compound	Rhizomorphs	Mycelium	Total	Rhizomorphs	Mycelium	Total
Indole-3-acetic acid	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*
0 ppm	0.0	13.1	13.1 ± 3.8	0.0	8.8	8.8 ± 1.2
1 ppm	0.0	18.4	18.4 ± 3.1	0.0	7.9	7.9 ± 1.1
10 ppm	23.6	13.0	36.6 ± 4.4	18.1	11.0	29.1 ± 4.3
20 ppm	66.0	12.7	78.7 ± 15.7	70.8	20.4	91.2 ± 14.2
Folic acid						
0 ppm	0.0	12.6	12.6 ± 1.9	0.0	8.5	8.5 ± 0.6
1 ppm	0.0	12.5	12.5 ± 1.5	0.0	8.8	8.8 ± 1.0
10 ppm	0.0	13.2	13.2 ± 1.9	0.0	8.3	8.3 ± 1.2
20 ppm	0.0	17.8	17.8 ± 1.1	6.4	9.7	16.1 ± 4.2

[a] Each figure is the mean of three replications. Mean deviation is indicated for total wt.
[b] Final pH varied from 4.5 to 5.0.

may also be important. Thus, *m*-aminobenzoic acid (10 ppm) failed to stimulate rhizomorph initiation and growth at a concentration at which *o*-aminobenzoic acid (AA) and *p*-aminobenzoic acid (PABA) were effective. Although *m*-aminobenzoic acid is probably not synthesized naturally, the results obtained with this aminobenzoic acid isomer suggest that for aromatics of comparable composition, the structure may be critically important for promotion of rhizomorph initiation and growth.

The apparent relationship of the structure of AA to its effectiveness in promoting rhizomorph initiation and growth may mean that it influences specific physiological mechanisms that have direct significance for morphogenesis. Recent studies with *Neurospora crassa* mutants (11, 14, 18), which showed that AA is a precursor of substituted indoles (including IAA) and a probable inducer or de-repressor of tryptophan synthetase, a key enzyme associated with the biosynthesis of substituted indoles, could provide a precedent for this conclusion. In this connection, the observation that IAA induced rhizomorph initiation and growth may contribute to an explanation of the effect of AA on rhizomorph production. Thus, IAA may possibly be critically needed for rhizomorph production when *A. mellea* is grown on a basal medium containing glucose. By analogy with the *Neurospora* findings, AA may be effective as a promoter of rhizomorph initiation and growth because it is a readily convertible precursor of IAA or inducer of IAA synthesis. Possibly, PABA is less effective than AA because it is less readily converted to IAA. On the other hand, the other aromatics tested are ineffective because they are probably not converted at all to IAA. The foregoing simple explanation appears to be consistent with the data presented, and will be used as a working hypothesis to determine the direction of future research on the physiological basis of rhizomorph morphogenesis in *A. mellea*.

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