Collection and Identification of Allelopathic Compounds from the Undisturbed Root System of Bigalta Limpograss (Hemarthria altissima)

CHUNG-SHIH TANG and CHIU-CHUNG YOUNG
Department of Agricultural Biochemistry, University of Hawaii, Honolulu, Hawaii 96822

ABSTRACT

Collection of allelopathic chemicals from the undisturbed root system is difficult because of their low concentrations and the high level of contaminants in growth media such as soil. A new approach for the continuous trapping of quantities of extracellular chemicals from donor plants is described. Bigalta limograss (Hemarthria altissima), a tropical forage with allelopathic activities, was established in sand culture. Nutrient solution was circulated continuously through the root system and a column containing XAD-4 resin. Extracellular hydrophobic metabolites were selectively adsorbed by the resin, while inorganic nutrients were recycled to sustain plant growth. Columns were eluted with methanol and the eluate separated into neutral, acidic, and basic fractions. Bioassays of trapped root exudates using lettuce seed combined with paper and thin layer chromatography showed that the inhibitors were mainly phenolic compounds. The active neutral fraction was methylated and analyzed by gas chromatography-mass spectrometry. Twelve compounds were identified, with two additional compounds tentatively identified. 3-Hydroxyhydrocinnamic, benzoic, phenylacetic, and hydrocinnamic acids were the major rhizospheric compounds with known growth regulatory activities.

Inasmuch as the root system was undisturbed and the recovery of exudates was highly efficient compared to conventional solvent extraction methods, this trapping system should be useful for a wide range of studies that relate to the chemistry of the rhizosphere.

In a recent review updating research on allelopathy, Rice (13) cited more than 400 references, with the majority published after 1970. This large volume of research activity reflects the growing awareness of the implications of allelopathic interactions in agricultural and ecological systems. Numerous observations have been recorded on the harmful effects of one plant species upon another grown in the same community (14). To prove that the effects were allelopathic, careful experimental design was used to differentiate them from the influences of competition for light, water, and nutrients. More detailed work (13, 14) provided evidence that organic compounds isolated from the dominant plants or their environment were phytotoxic. Unfortunately, chemical approaches in allelopathy were often performed under arbitrary conditions. Rather than collecting the responsible extracellular inhibitors from the intact, living donor plants, tissue extracts from either fresh or dried plant materials were commonly used (8, 14). Compounds identified in this manner, however, were not necessarily responsible for the observed allelopathic interaction. Efforts were made in the past to collect inhibitors from the donor root systems under undisturbed conditions. Samples were obtained by solvent extraction of the medium of hydroponic cultures (3, 5) or by the washings of sand (1) or gravel cultures (6). These methods were often tedious, and their success in the isolation of the allelochemics limited (14). Consequently, our knowledge of allelopathic chemistry remains inadequate, despite the increasing capability of modern instrumental analysis for trace organic molecules.

To overcome difficulties in sample collection, we have developed a continuous root exudate trapping system, which effectively extracts bioactive metabolites from the rhizosphere of bigalta limograss, a tetraploid selection of Hemarthria altissima (Poir.) Stapf. and Hubb. This tropical forage grass had been observed to inhibit the growth of a legume, Desmodium intortum (Mill.) Urb., in a mixed pasture in Hawaii. Greenhouse experiments established that the inhibition was due to allelopathic interaction caused by root exudation (21). This report describes the collection, bioassay, isolation, and identification of these root exudates.

MATERIALS AND METHODS

Establishment of Grass Culture. Stolons of bigalta limograss were collected from the Hawaii Agricultural Experiment Station at Kula on the island of Maui, Hawaii. Stolon cuttings were washed and treated with 5% (v/v) Clorox for 15 min prior to planting. The containers used were made from 1-gallon brown glass solvent bottles with the bottoms removed. The pots were filled with a 4-cm layer of crushed basaltic rock (about 2-cm size), followed by a 2:1 (v/v) sand:rock mixture up to 3 cm from the edge (Fig. 1). Containers were wrapped with aluminum foil and heat-sterilized for 2 days at 100°C. Eight cuttings were planted in each pot in a greenhouse and irrigated with 0.1-strength Hoagland solution at a rate of 100 ml/day. Additional distilled H2O was supplied as needed. Pot controls without grass were treated identically. The grass was well established and had a full-grown root system after 40 days.

Column Preparation. XAD-4 resin purchased from Rohm and Haas was heavily contaminated with aromatic impurities. It was cleaned with hot running tap water, followed by Sohujet extraction with acetone, acetonitrile, and diethyl ether, each for 24 h [9]. The cleaned resin was stored in methanol (glass distilled, Burdick and Jackson Laboratory) in a dark glass container until use. The column (18 × 150 mm) was packed with 12 g XAD-4 as an aqueous slurry, and the residual methanol was removed by wash-

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2 Present Address: Department of Soil Science, Chung-Hsin University, Taichung, Taiwan, R.O.C.
Acidic fraction
Neutral fraction
±21.1
resin
4
plants,
eluting
solution
TANG
Table
of Bigalta Limpograss
Exudates
30
50
11.5 ± 10.2
12.0 ± 12.0
24.7 ± 6.2
21.6 ± 4.5
16.9 ± 3.0
19.8 ± 2.3
18.4 ± 3.5
18.6 ± 4.9
50
30
10
5
Pot Control (Radi
circle Length)
Radicle length
Percent-
age of pot control
Neutral fraction
50
30
10
5
Acidic fraction
50
30
10
5
mm
mm
—
—
a
b
Note: Significance levels are not indicated in the text. The
significance levels are as follows:
Significant at P < 0.01.
Significant at P < 0.05.
Significantly less than resin control (data not shown).

The hydrophobic root exudate trapping system. The nutrient solution was continuously circulated through the root systems of the living plants, eluting extracellular organics from the sand culture. Hydrophobic or partially hydrophobic exudates were selectively retained by the XAD-4 resin column while the inorganic nutrients were unaffected.

Table 1. Effects of Neutral and Acidic Fractions from Control and Root Exudates of Bigalta Limpograss on Radicle Growth of Lettuce Seedlings
Mean of distilled H2O control was 20.6 ± 4.5 and of resin control was 21.1 ± 4.4. Three replications were done of 10 seeds, or an average of 30 seedlings, ±1 sd. Measurement was made after 72-h incubation.

<table>
<thead>
<tr>
<th>Amount</th>
<th>Pot Control (Radicle Length)</th>
<th>Radicle length</th>
<th>Percent- age of pot control</th>
</tr>
</thead>
<tbody>
<tr>
<td>µl/disc</td>
<td>mm</td>
<td>mm</td>
<td></td>
</tr>
<tr>
<td>Neutral fraction</td>
<td>11.5 ± 10.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0</td>
</tr>
<tr>
<td>30</td>
<td>12.0 ± 12.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>24.7 ± 6.2</td>
<td>4.3 ± 7.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>17.4</td>
</tr>
<tr>
<td>5</td>
<td>21.6 ± 4.5</td>
<td>15.9 ± 9.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>73.6</td>
</tr>
<tr>
<td>Acidic fraction</td>
<td>16.9 ± 3.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.1 ± 0.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.6</td>
</tr>
<tr>
<td>30</td>
<td>19.8 ± 2.3</td>
<td>0.7 ± 0.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.5</td>
</tr>
<tr>
<td>10</td>
<td>18.4 ± 3.5</td>
<td>16.0 ± 6.4</td>
<td>87.0</td>
</tr>
<tr>
<td>5</td>
<td>18.6 ± 4.9</td>
<td>20.8 ± 6.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>111.8</td>
</tr>
</tbody>
</table>

* Significance compared to pot control by Student's t test.
* Significant at P < 0.01.
* Significant at P < 0.05.
* Significantly less than resin control (data not shown).

Eluates from 15 columns were pooled, and methanol was evaporated under reduced pressure at 60°C. The concentrate was diluted with H2O to 50 ml (pH 5.5–6.0) and extracted three times with 100 ml CH2Cl2. The extracts (designated as the neutral fraction) were combined, dried over anhydrous MgSO4, and concentrated to 20 ml in a rotary evaporator at room temperature. Further concentration was carried out using a jet of N2 to a final volume of 3 ml.

The acidic fraction was obtained in a similar manner by first acidifying the remaining aqueous fraction to pH 2 with 1 N HCl and extracting with CH2Cl2. The basic fraction was obtained by adjusting the acidified residue to pH 11 with 1 N NaOH and extracting with CH2Cl2. Both fractions were concentrated to a final volume of 3 ml.

Preliminary Separation and Characterization of Allelopathic Substances. Two TLC systems were used: (a) microcrystalline cellulose plates (Avicel precoated plates, Analtech) using 2% (v/v) acetic acid as the developing solvent; and (b) silica gel plates (Polygram Sil G, Brinkmann Instruments) using toluene:methyl formate:formic acid (5:4:1, v/v/v) as the developing solvent. Spots were detected under UV (365 and 254 nm) or by spraying with DNAP<sub>3</sub> followed by 10% (v/v) sodium carbonate for phenolic compounds (4).

Bioassay. Aliquots (5, 10, 30, and 50 µl) of neutral, acidic, and basic fractions were applied separately on 3.5-cm<sup>2</sup> discs of Whatman No. 3 MM filter paper with a micropipet. The solvent was evaporated, and the paper discs were placed in 5.5-cm diameter Petri dishes. The discs were wetted with 0.2 ml distilled H2O. Lettuce seeds (Lactuca sativa L., var. Auenume) were obtained from the Department of Horticulture, University of Hawaii. Ten seeds, presoaked for 2 h, were placed on each paper disc. Germination was carried out in a moisture-saturated dark chamber for 48 h at 24°C. Results (Table I) were taken by measuring the length of the radicle. All treatments were performed in triplicate, and the effects of root exudates were expressed as percentage of inhibition relative to the pot control. Both distilled H2O control and resin control using methanol eluate from unused XAD-4 resin columns were also performed at the same time. Data were analyzed statistically by the Student's t test.

Bioassays combined with paper chromatography were carried out by a method similar to that of McPherson et al. (10). Each 100-µl aliquot of neutral fraction was spotted on a Whatman No. 3 MM paper strip (2 × 56 cm) and developed in the descending mode with 2% glacial acetic acid. Control strips, including pot, resin, and H2O controls, were developed simultaneously. The

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<sup>3</sup>Abbreviations: DNAP, diazotized p-nitroaniline; SCOT, support coated open tubular.
strips were dried under room conditions and then cut into segments according to UV absorption and DPNA color reaction of the sample chromatograms (Tables II and III). Since spot sizes varied, the segments were wetted with water at 60 μl per cm², and lettuce seeds were placed 3 per cm². The rest of the procedure was the same as that of the bioassay described above.

**Isolation and Identification of Compounds in Root Exudates.** Chemicals used as standards in GC-MS were purchased from commercial suppliers. Benzoic, 3,4-dimethylbenzoic, phenylactic, 2-hydroxy-phenylactic, cinnamic, 2,5-dimethoxycinnamic, 3,5-dimethoxycinnamic, 4-hydroxy-3-methoxycinnamic (or ferulic), 4-hydroxy-3,5-dimethoxycinnamic (or sinapic), and hydrocinnamic acids were from Aldrich Chemical Company. 4-Hydroxy-3,5-dimethoxybenzoic (or syringic) acid was from Eastman Kodak, and 3-hydroxyhydrocinnamic acid was obtained from ICN/K and K Laboratories. Ethereal diazomethane was prepared from Diaklold. Deuterated diazomethane (CD₃N₂) was prepared from the Deuterodiazal Prep Kit. Both were supplied by Aldrich. The standards were methyliated with an excess of diazomethane for 2 h prior to GC-MS analysis.

Because the neutral fraction contained most of the inhibitory activity, this fraction was chosen for chemical analysis. One-half ml of the sample, roughly equivalent to a 1,500-h collection from one plant, was methyliated by adding 5 ml ethereal CH₃N₂ or CD₃N₂. The solution was kept at room temperature for 10 h and then concentrated to 0.1 ml for GC-MS analysis. The neutral fraction for the pot control was analyzed under similar conditions.

GC-MS. A Finnigan Model 3000 mass spectrometer (Finnegan Corp., Sunnyvale, CA) interfaced with a Varian Aerograph 1400 Gas Chromatograph was used for the separation and identification of methylated exudates. A 30 m OV-17 SCOT glass capillary column (Scientific Glass Engineering, Inc., Austin, TX) was connected directly to the ionizer without using a jet separator normally used for the packed columns. The carrier gas (helium) flow rate was 15 cm/s. The injector temperature was 220°C. The column temperature was programmed as indicated in Figure 2. Conditions used for the mass spectrometer were as follows: sensitivity, 10⁻⁶ amp/v; electron multiplier high voltage, 2.00 kv; and electron energy, 69.5 ev.

**RESULTS**

**Bioassay.** Results from the lettuce seed bioassays (Table I) indicated that inhibitory substances were effectively trapped by the XAD-4 columns. Toxicities were observed in both neutral and acidic, but not in basic, fraction of the eluate. Since the inhibition is expressed as percentage of radicle growth of the sample against that of the pot control, in which the only difference is the absence of the bigalda limopgrass, data in Table I suggest that the inhibitors originated from the root system. The XAD-4 resin control did not show any toxicity when compared with the distilled H₂O control (data not shown), indicating that the resin cleaning process was adequate. Both the neutral and the acidic fraction of the pot control exhibited toxicity when tested at high concentrations (e.g. 30 and 50 μl). We attribute this inhibition to toxicants of microbial origin in the control pots.

Based on the total number of plants involved, time of collection, and the final volume of the sample concentrate, it was estimated that 1 μl of the sample concentrate was roughly equivalent to a 3-h collection of exudate from one bigalda limopgrass. According to Table I, in 30 h, a single plant produced enough neutral inhibitors to reduce the radicle growth of lettuce seedling by 83%. The acidic fraction was less toxic; only 13% reduction was observed. High concentration of salts and extremes of pH were not the causative factors in the observed inhibition. Osmotic pressure of the exudates at concentrations used for bioassays was found to be similar to that of the distilled H₂O when determined by a Wescor vapor pressure osmometer, and the pH was near 6.

Paper chromatography using 2% acetic acid led to similar separation when compared with results from microcrystalline cellulose TLC. Lettuce seed bioassays on the segments cut from paper chromatograms showed that, in the neutral fraction, toxicities were strongest at regions positive to DPNA (Table II), suggesting that phenolic compounds were responsible for the inhibitory activities. In the acidic fraction (Table III), only one segment showed any significant inhibitory effect, and most spots

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**Table II. Lettuce Seed Bioassay on Segments of Paper Chromatograms, Neutral Fractions of Pot Control, and Root Exudates from Bigalda Limopgrass**

<table>
<thead>
<tr>
<th>Segment Range</th>
<th>Pot Control (Radicle Length) mm</th>
<th>Radicle length*</th>
<th>Percent- age of pot control UV*</th>
<th>DPNA†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rₚ</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.00 to 0.14</td>
<td>13.2 ± 2.9</td>
<td>12.4 ± 5.6</td>
<td>94</td>
<td></td>
</tr>
<tr>
<td>0.14 to 0.28</td>
<td>11.8 ± 3.0</td>
<td>13.9 ± 1.6</td>
<td>118</td>
<td></td>
</tr>
<tr>
<td>0.28 to 0.41</td>
<td>11.7 ± 2.6</td>
<td>11.5 ± 3.7</td>
<td>98 F</td>
<td></td>
</tr>
<tr>
<td>0.41 to 0.53</td>
<td>12.6 ± 3.1</td>
<td>13.4 ± 4.0</td>
<td>106 WF</td>
<td></td>
</tr>
<tr>
<td>0.53 to 0.65</td>
<td>14.3 ± 3.1</td>
<td>14.5 ± 3.6</td>
<td>101 BF</td>
<td></td>
</tr>
<tr>
<td>0.63 to 0.75</td>
<td>13.8 ± 3.4</td>
<td>13.6 ± 2.5</td>
<td>99 A</td>
<td></td>
</tr>
<tr>
<td>0.72 to 0.83</td>
<td>12.0 ± 2.8</td>
<td>8.3 ± 2.4</td>
<td>69 LF Blue</td>
<td></td>
</tr>
<tr>
<td>0.83 to 0.94</td>
<td>13.5 ± 3.4</td>
<td>8.5 ± 4.0</td>
<td>63 SF Pink</td>
<td></td>
</tr>
<tr>
<td>0.93 to 1.00</td>
<td>13.4 ± 4.7</td>
<td>0.1 ± 0.5</td>
<td>1 Yellow</td>
<td></td>
</tr>
<tr>
<td>0.93 to 1.00</td>
<td>10.1 ± 2.3b</td>
<td>8.0 ± 1.4</td>
<td>79 A Pink</td>
<td></td>
</tr>
</tbody>
</table>

* LF, Fluorescence; A, absorption; B, blue; W, white; L, light; S, strong.
† Chromogenic reagent = DPNA followed by 10% sodium carbonate (4).

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**Table III. Lettuce Seed Bioassay on Segments of Paper Chromatograms, Acidic Fractions of the Pot Control, and Root Exudates from Bigalda Limopgrass**

<table>
<thead>
<tr>
<th>Segment Range</th>
<th>Pot Control (Radicle Length) mm</th>
<th>Radicle length*</th>
<th>Percent- age of pot control UV*</th>
<th>DPNA†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rₚ</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.00 to 0.09</td>
<td>12.2 ± 2.4</td>
<td>17.9 ± 3.6</td>
<td>147 F</td>
<td></td>
</tr>
<tr>
<td>0.09 to 0.22</td>
<td>12.9 ± 2.6</td>
<td>15.0 ± 2.9</td>
<td>116 F</td>
<td></td>
</tr>
<tr>
<td>0.22 to 0.35</td>
<td>13.2 ± 2.2</td>
<td>15.9 ± 3.8</td>
<td>128 B Blue</td>
<td></td>
</tr>
<tr>
<td>0.35 to 0.43</td>
<td>12.7 ± 2.3</td>
<td>16.3 ± 3.5</td>
<td>107 F</td>
<td></td>
</tr>
<tr>
<td>0.43 to 0.51</td>
<td>12.9 ± 3.7</td>
<td>18.9 ± 4.0</td>
<td>147</td>
<td></td>
</tr>
<tr>
<td>0.49 to 0.64</td>
<td>14.0 ± 3.1</td>
<td>18.3 ± 3.8</td>
<td>131</td>
<td></td>
</tr>
<tr>
<td>0.64 to 0.79</td>
<td>11.3 ± 2.3</td>
<td>17.4 ± 2.1</td>
<td>154</td>
<td></td>
</tr>
<tr>
<td>0.79 to 0.87</td>
<td>15.3 ± 2.2</td>
<td>17.8 ± 2.1</td>
<td>51 LF</td>
<td></td>
</tr>
<tr>
<td>0.76 to 0.87</td>
<td>14.7 ± 2.6</td>
<td>14.2 ± 3.3</td>
<td>97</td>
<td></td>
</tr>
<tr>
<td>0.87 to 1.00</td>
<td>12.9 ± 2.5</td>
<td>12.8 ± 2.4</td>
<td>99</td>
<td></td>
</tr>
</tbody>
</table>

* LF, Fluorescence; A, absorption; B, blue; W, white; L, light; S, strong.
detected under UV were stimulatory to radicle elongation. This observation coincided with the results in Table I: at a low dosage of 5 μl per paper disc, the acidic fraction enhanced the radicle elongation by more than 10% compared to that of the pot control.

Chemical analyses. Preliminary examination by TLC showed that the neutral fraction contained a complex mixture of phenolic compounds. Using DPNA as a chromogenic reagent, the chromatograms indicated that neither of the two TLC systems was adequate to resolve this mixture, although it was possible to speculate that ferulic acid and 3-hydroxyhydrocinnamic acid were present based on their Rf values and color reactions.

A GC-MS equipped with a 30 m OV-17 SCOT column showed that the methylated neutral fraction contained more than 40 compounds (Fig. 2); among them, 16 peaks were resolved well enough for identification (Table IV), based on the comparison of their mass spectra and retention times with those of the respective standard compounds. The MS data were also verified by comparing with the EPA/NIH Mass Spectral Data Base (19) whenever possible.

Table IV. Compounds Identified from the Root Exudates of Bigalta Limpograss

<table>
<thead>
<tr>
<th>Peak No.</th>
<th>M* (m/e)</th>
<th>Compounds Identified in Methylated Exudates</th>
<th>Number of Methylation</th>
<th>Compounds in Original Exudates</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>136</td>
<td>Methyl benzoate</td>
<td>1</td>
<td>Benzoic acid</td>
</tr>
<tr>
<td>2</td>
<td>150</td>
<td>Methyl phenylacetate</td>
<td>1</td>
<td>Phenylacetic acid</td>
</tr>
<tr>
<td>3</td>
<td>124</td>
<td>2-Methoxyphenol</td>
<td>0</td>
<td>2-Methoxyphenol</td>
</tr>
<tr>
<td>4</td>
<td>164</td>
<td>Methyl hydrocinnamate</td>
<td>1</td>
<td>Hydrocinnamic acid</td>
</tr>
<tr>
<td>5</td>
<td>164</td>
<td>Methyl 3,4-dimethyl benzoate</td>
<td>1</td>
<td>3,4-Dimethyl benzoic acid</td>
</tr>
<tr>
<td>6</td>
<td>162</td>
<td>Methyl cinnamate</td>
<td>1</td>
<td>Cinnamic acid</td>
</tr>
<tr>
<td>7</td>
<td>182</td>
<td>Methyl 2-methoxy phenylacetate</td>
<td>1</td>
<td>2-Methoxy phenylacetic acid</td>
</tr>
<tr>
<td>8</td>
<td>194</td>
<td>Methyl 3-methoxy hydrocinnamate</td>
<td>1</td>
<td>3-Hydroxy hydrocinnamic acid</td>
</tr>
<tr>
<td>9</td>
<td>180</td>
<td>Methyl 3-hydroxy hydrocinnamate</td>
<td>1</td>
<td>3-Hydroxy hydrocinnamic acid</td>
</tr>
<tr>
<td>10</td>
<td>256</td>
<td>Methyl pentadecanoate</td>
<td>1</td>
<td>Pentadecanoic acid</td>
</tr>
<tr>
<td>11</td>
<td>210</td>
<td>Methyl 4-hydroxy-3-methoxy hydrocinnamate</td>
<td>1</td>
<td>4-Hydroxy-3-methoxy hydrocinnamic acid</td>
</tr>
<tr>
<td>12</td>
<td>224</td>
<td>Methyl 3,4-dimethoxy hydrocinnamate</td>
<td>2</td>
<td>4-Hydroxy-3-methoxy hydrocinnamic acid</td>
</tr>
<tr>
<td>13</td>
<td>226</td>
<td>Methyl 3,4,5-trimethoxy benzoate</td>
<td>2</td>
<td>Syringic acid</td>
</tr>
<tr>
<td>14</td>
<td>222</td>
<td>Methyl 2,4-dimethoxy cinnamate</td>
<td>2</td>
<td>4-Hydroxy-2-methoxy cinnamic acid</td>
</tr>
<tr>
<td>15</td>
<td>222</td>
<td>Methyl 3,4-dimethoxy cinnamate</td>
<td>2</td>
<td>Ferulic acid</td>
</tr>
<tr>
<td>16</td>
<td>252</td>
<td>Methyl 2,4,5-trimethoxy cinnamate</td>
<td>2</td>
<td>Sinapic acid</td>
</tr>
</tbody>
</table>

* See Figure 2 for peak numbers and retention times.
† Numbers of methylation were determined by deuterated diazomethane. Each deuterated methylation increased the m/e of the molecular ion (M*) by 2 to 3 units over its nondeuterated counterpart.
‡ Insufficient data.
§ Tentative identification.
available.

The GC chromatogram of the neutral fraction methylated by deuterated diazomethane closely resembled Figure 2. However, mass spectra of the corresponding peaks were different, particularly at the molecular ion region. For each methylation, a $-\text{CD}_3\text{H}$ or a $-\text{CD}_2\text{D}$ was introduced in place of the active hydrogen (e.g. carboxyl or phenoxyl hydrogen), and molecular ions both 2 and 3 m/e units greater than that of the nondeuterated counterpart were expected. The substitution of $-\text{CD}_3\text{H}$ was probably due to the exchange of active hydrogen with the residual carb处于-d in the ethereal diazomethane-d$_2$ preparation. This situation is demonstrated by peak 9, the most abundant methyl derivative in the mixture. Peak 9 has a molecular ion of 180 by regular methylation, but the deuteromethyl derivatives have prominent peaks at m/e 182 and 183, suggesting one methylation at the preferred carboxyl group. GC-MS data of the methyl ester derived from authentic 3-hydroxyhydrocinnamic acid matched those of peak 9. Based on this information, we identified 3-hydroxyhydrocinnamic acid as the major phenolic acid in the root exudates of bigalta limopgrass. Eleven other compounds were identified by a similar approach.

Two additional compounds were tentatively identified. Peak 10 did not provide clear data for deuterated methyl pentadecanoate, and positive identification of peak 14 was not possible for lack of a standard compound, although the possibility of its being a 2,5- or 3,5-substituted cinnamate was ruled out based on its retention times.

Seven peaks (A to G) in Figure 2 were also found in the methylated neutral fraction of the pot control. No effort was made to identify these peaks, since they were not root exudates of the bigalta limopgrass.

**DISCUSSION**

Root exudates are defined here as low-mol-wt compounds which are released into the surrounding medium by living and intact roots (15, 16). Under normal growth conditions, exudation probably represents a major mechanism of releasing organic chemicals into the rhizosphere. Our interest in the rhizospheric chemicals is confined to the biologically active secondary metabolites which may bear more relevance to allelopathy than do water soluble exudates, such as common sugars and amino acids (20).

The chemistry of the bioactive compounds in the rhizosphere is of fundamental importance to the understanding of interactions between the plant root systems and other living organisms. However, our present knowledge of rhizospheric chemistry remains extremely limited. The difficulties encountered, in general, by the researchers are as follows: (a) the chemicals of interest are usually present at extremely low concentrations, requiring large numbers of plants and prolonged collection periods; (b) the tedium of sample collection and preparation leads to chemical, as well as microbial, modification of less stable compounds; and (c) contaminants from the growth media, containers, nutrient solutions, water, and extraction solvents are serious problems when dealing with the recovery of trace organic compounds by conventional solvent extraction methods.

The continuous hydrophobic root exudate trapping system described in this report is designed to overcome these difficulties. The key to this success is the use of XAD-4 resin columns. Amberlite XAD-4 is a hydrophobic styrene-divinyl benzene co-polymer with a specific surface area of 750 m$^2$/g. Recovery of model organic compounds from water at 2 to 10 parts per billion levels is better than 99% for most of the tested alcohols, esters, ethers, phenols, ketones, aldehydes, and acids (9, 18). Inorganic ions and water soluble organic molecules such as sugars and most of the amino acids pass through the column without substantial retention. Description of the adsorbed molecules usually occurs readily upon elution with water-miscible solvents such as methanol or acetone. The physical and chemical characteristics of XAD-4 make it ideal for the continuous extraction of allelopathic compounds from the recirculating nutrient solution. According to Whitaker and Feeny (20), the known allelopathic agents are secondary plant metabolites including phenolic acids and flavonoids and other aromatics, terpenoids, steroids, alkaloids, and organic cyanides. Most of these compounds are sufficiently hydrophobic to be trapped by XAD-4.

Since the established sand culture of bigalta limopgrass was prewashed prior to the attachment of the resin column, any compounds collected immediately thereafter were likely to be the freshly released root exudates rather than residues accumulated in the medium. The entire trapping system was protected from light by aluminum foil, which eliminated possible photoconversions during the collection. Other types of chemical reactions, such as oxidation, polymerization, and microbial degradation, could still occur to the less stable compounds. However, since the columns were changed every 3 days and organic molecules, once adsorbed by the resin particles, may actually increase in chemical stability (2), the present technique provides a good possibility for collecting genuine root exudates.

The problem of contamination from water and solvents was drastically curtailed compared to that in conventional collection methods. Assuming the trapping of hydrophobic exudates by the XAD-4 column was nearly complete, the amount of compounds collected was equivalent to the washing of 120 plants with about 1,000 L nutrient solution for 3 days. Yet, in this experiment, only about 20 L distilled H$_2$O, 300 ml CH$_2$Cl$_2$, and 3 L high purity methanol were used. Despite these advantages, control experiments were carried out simultaneously for two reasons: (a) XAD-4 resin as a commercial product was heavily contaminated with aromatic monomers and required a rigorous cleaning procedure to eliminate the contaminants; and (b) some of the inhibitors may have been generated in the medium during sample collection. This was, indeed, the case, as extracts from the pot control exhibited some inhibition, probably caused by microbial toxins produced in the system. To reflect toxicity contributed by the donor plant, results are expressed as percentage of radicle growth in exudates against that of pot control.

TLC, reverse phase HPLC, and GLC using packed columns were evaluated as methods for separating root exudates. These methods provided limited information on the identification of unknowns due to the complexity of samples. Our present approach relies on the high resolution of the SCOT column in GC-MS and the combined use of methylation and deuteromethylation for structural elucidation. Peaks contributed by sources other than the living root system were recognized readily by the GC-MS analysis of the pot control. This capability is essential, especially when a more complicated medium, such as soil, is used in the trapping system.

Except for pentadecanoic acid, all the compounds identified are plant phenolics originating from the shikimic acid pathway. These secondary metabolites are often growth-regulating substances (7), and they have been most frequently associated with allelopathic interactions and toxic crop residue problems (8, 13, 14). The major compound, 3-hydroxyhydrocinnamic acid, was found to have a strong inhibitory effect on the elongation of radish root (11). Toxicities of 2-methoxyphenol, benzoic, cinnamic, hydrocinnamic, syringic, ferulic, and sinapic acids against lettuce seed germination have been evaluated by Reynolds (12). The first four compounds inhibited 50% germination at concentrations near 0.5 mM, while syringic, ferulic, and sinapic were less inhibitory. The richness of hydrocinnamic acids in the mixture is interesting, because they have synergistic effects on the gibberellic acid-induced hypocotyl elongation of lettuce seedlings (17).

To the best of our knowledge, the present work is the first successful attempt to isolate and identify multiple bioactive metabolites from the environment of undisturbed living plant roots.

**Plant Physiol. Vol. 69, 1982**  
**ROOT EXUDATE TRAPPING AND IDENTIFICATION**  
159

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We suggest that hydrophobic root exudates of other higher plants may also be collected by similar methods. Trapping of hydrophobic extracellular metabolites from fresh water or marine phytoplanktons would also be feasible using similar techniques. Other applications would include qualitative and quantitative studies of root exudates in relation to nutrients, various chemical and physiological stress factors, interactions between root systems and beneficial (e.g. mycorrhizae and N₂-fixing organisms) or harmful (e.g. pathogens) soil-borne microorganisms, autoinhibitions, systemic pesticides, and other agricultural and ecological problems pertinent to the rhizospheric chemistry. For more critical work, the system may be modified for axenic cultural conditions or for radiotracer studies. It is because of this versatility, we believe, that the hydrophobic exudate trapping system may become a useful tool in the understanding of the physiology and functions of extracellular bioactive molecules.

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LITERATURE CITED