Nature of Phytotoxic Substances Produced during Plant Residue Decomposition in Soil

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ABSTRACT

Production of water-soluble phytotoxins during decomposition of barley residue in soil in the laboratory was unaffected by temperature between 16 and 24 C, but required soil moisture content above 30% of the soil and residue dry weight. Laboratory production of toxins was similar to production under field conditions in that extracts were toxic 7-10 days after incorporation into soil. Phytotoxic activity reached a maximum in 3 weeks and then declined after 6-7 weeks. Water extracts of plant material decomposing in the field and laboratory contained high concentrations of electrolytes. Phytotoxic substances accounting for approximately 60% of the total toxicity were extractable in ether at pH 2.0. Ether extraction did not appreciably alter the conductivity of the water phase. On the basis of tobacco seedling bioassays, paper chromatography, and gas chromatography, the ether-soluble phytotoxins were found to be benzoic acid, phenylacetic acid, 3-phenyl propionic (hydrocinnamic) acid, and 4-phenylbutyric acid. Benzoic acid and phenylacetic acid were found to be the major components of the ether-soluble phytotoxins present in samples from barley decomposing in the field and from cotton, cowpea, and soybean decomposing in soil in the laboratory. This indicates that these and similar compounds may be prevalent in nature.

Decomposition of plant organic matter in soil is often accompanied by formation of substances with phytotoxic properties (2, 7, 8, 10, 11, 14). Our previous studies showed that the most severe phytotoxicity occurred in fields where decomposition of plant organic matter had taken place in cold, wet soil and during early stages of decomposition. When conditions were optimum for phytotoxin formation, type of plant material and soil type appeared to have little effect on over-all phytotoxicity (12).

Some of the phytotoxic effects noted were an inhibition or delay of seed germination, seed killing, necrosis of roots, and inhibition of root growth and root hair development of tobacco, lettuce, and bean seedlings. Plant roots and especially the root meristematic region appeared to be particularly sensitive to such compounds. Other effects were stunting of plants and a marked increase in bean foot rot and tobacco root rot susceptibility (9, 15).

The widespread occurrence of such phytotoxins in nature and their apparent involvement in root rots (1, 4, 5, 13) suggests their over-all importance in the etiology of root disease. Information on their production, activity, and chemical nature is therefore highly desirable. The purpose of this paper is to report on the identification of phytotoxic compounds formed during decomposition of plant residue in soil.

METHODS AND RESULTS.—*Characterization of phytotoxins.*—Most of the methods used to obtain and assay phytotoxic extracts have been described previously (12). Under conditions optimum for their formation, phytotoxins could be detected in the field within 5-10 days after the green cover crop had been turned under. Maximum toxicity was reached within approximately 25 days, after which phytotoxicity declined. This pattern, however, varied from field to field and even within the same field. To obviate some of these fluctuations and to ensure a constant supply, the major source of the toxic extracts in the present work was from plant material decomposing in the laboratory. However, extracts from material decomposing in the field were used to verify the laboratory findings.

The laboratory decomposition procedure employed was as follows: 100 g (fresh weight) of approximately 1-month-old, field-grown green barley plants or 1-month-old, greenhouse-grown cotton, cowpea, or soybean were mixed with 300 g of air-dried field soil and placed in beakers or earthenware crocks; 150 ml of water were added and the mixtures were incubated for periods up to 2 months. Extracts were obtained at weekly intervals. To obtain the extracts, 150 ml of water were added to the mixture and stirred; after 1 hr the supernatant of the mixture was decanted, centrifuged to remove soil and plant debris, filtered, concentrated to one-tenth its volume under reduced pressure, and centrifuged at 7,000 rpm. The supernatant was then decanted and frozen until needed.

Experiments revealed that temperatures from 16 to 24 C had little effect on phytotoxin production. Therefore, the decomposition experiments were conducted at room temperature (approximately 23 C). Moisture content of the soil-residue mixture, on the other hand, had a pronounced effect on phytotoxin production. At a moisture content of 30% (of the dry weight of the soil-residue mixture) and below (moisture retention of the soil at one-third atmosphere was 20.2%), extracts had stimulatory properties. Above this moisture content, highly phytotoxic extracts were obtained (Fig. 1).

The relative phytotoxicity of these extracts was determined by means of bioassays, usually with tobacco seeds, as described previously (12). For the bioassays, extracts were diluted with water to their original concentration. Toxin production with time under labo-

ratory conditions was similar to that obtained from barley residues decomposing in the field. Extracts were usually first toxic 7-10 days after incorporation of the barley into the soil. Maximum toxicity occurred in about 3 weeks, remained high until 6 or 7 weeks, and then declined (Fig. 2).

As previously indicated (12), extracts had high electrical conductivity, a characteristic of high salt concentrations. Such a high concentration of electrolytes interfered with the bioassays. The extracts, therefore, were dialyzed against running tap water for 1 hr or until their conductivity was reduced to less than 2.0 millimhos/cm. In general, the activity of the dialyzed extracts was reduced by 5-10%. Phytotoxicity was lost, however, after 5 hr of dialysis.

The crude aqueous extracts of the decomposing plant organic matter were first adjusted to pH 2.0 with 1 N HCl and then extracted three times with peroxide-free diethyl ether (1:1, v/v). The ether-soluble fraction was washed three times with 5% aqueous $NaHCO_3$; the aqueous layer was retained and acidified with 1 N HCl to pH 2.0 and subjected to extraction three times with ether. The ether-soluble fraction was dried over $CaCl_2$ and evaporated. The product obtained was a brown viscous liquid with a pungent odor. Little or no phytotoxic materials were obtained when the crude aqueous solution was extracted with ether at pH 10.0. The brown residue, when diluted with water to the volume of the original crude extracts, was found to be highly phytotoxic. The phytotoxicities of ether extracts and the parent crude nondialyzed extracts were compared by means of a dilution series (Fig. 3). These experiments revealed that the phytotoxicity of the ether-soluble fraction was approximately 60% of that of the crude parent extracts. The remaining phytotoxicity may represent salts not extractable in ether.

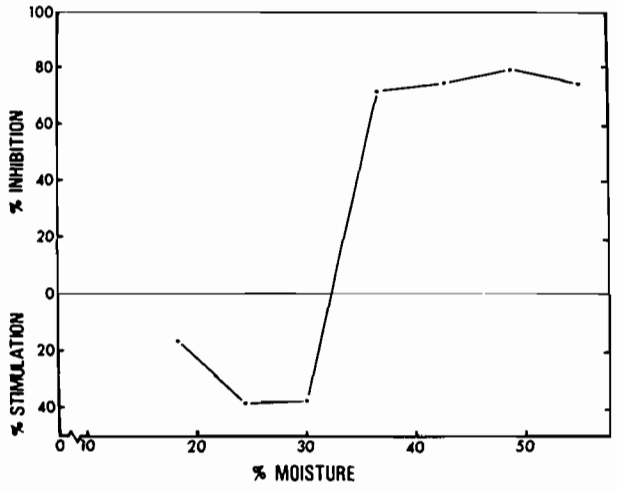

Fig. 1. Effect of soil moisture on phytotoxin production by barley residue after 10 days of decomposition in the laboratory, as indicated by tobacco seed bioassays.

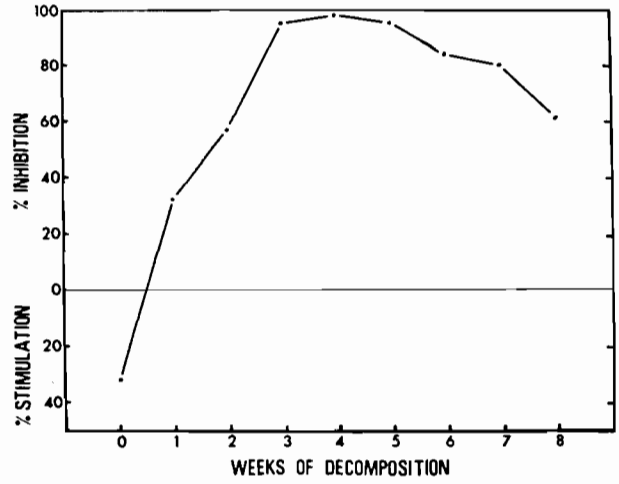

Fig. 2. Effect of decomposition time of barley residue in the laboratory on phytotoxin production, as indicated by tobacco seed bioassays made at weekly intervals. Similar curves were obtained from field-decomposed residues.

The major ether-soluble phytotoxins were separated using descending paper chromatography. Materials were spotted on Whatman No. 3MM paper and the chromatograms developed with isopropanol:14% NH_4OH (4:1, v/v). The chromatograms were cut into 1-inch strips and bioassayed. A single highly toxic area with an R_F value of 0.5-0.6 was repeatedly obtained. Field-decomposed barley residue was extracted and run in the isopropanol:NH_4OH solvent. The results were the same as those obtained with laboratory-decomposed barley residues. Laboratory-decomposed cotton, cowpea, and soybean residues also gave a single toxic area on chromatograms.

Other solvent systems were used in attempts to increase resolution. The substances in the toxic area of the chromatogram obtained in the isopropanol:NH_4OH solvent were eluted, concentrated, and chromatographed using the following solvents: *n*-butanol:acetic acid:water 8:2:2 (v/v); ethanol:NH_4OH:water 80:5:15 (v/v); acetone:urea:water 60:0.5:40 (v/v). In each case, only one area proved to be highly toxic. The area was most concentrated in the *n*-butanol:acetic acid:water solvent where it had an R_F value of 0.82-0.95.

Paper chromatography was also used to follow the production of phytotoxins in the laboratory over a 3-month period. At weekly intervals, water extracts were made and bioassayed for phytotoxicity. They were then

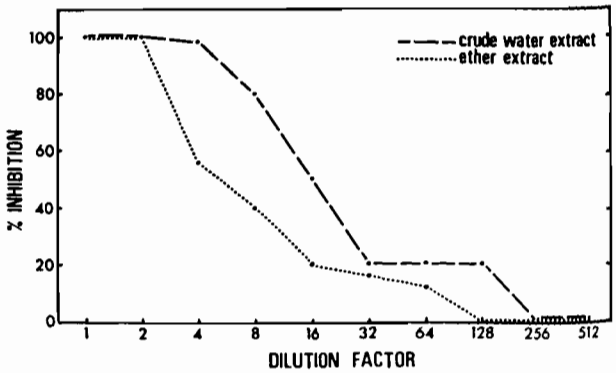

Fig. 3. Dilution series showing comparative phytotoxicity of ether extract and parent crude water extract of barley residue that had decomposed 4 weeks in the laboratory, as indicated by tobacco seed bioassay. Phytotoxins soluble in ether accounted for approximately 60% of the total phytotoxicity of the parent crude water extract.

extracted with ether, concentrated, and chromatographed in the isopropanol:NH_4OH solvent. Only the one highly toxic area was present on the chromatogram. The presence or absence of this area coincided with the presence or absence of phytotoxicity of the original crude water extracts.

Identification of phytotoxins.—The ether-soluble phytotoxins were chromatographed in the isopropanol: NH_4OH solvent and the paper was sprayed with pH indicators bromthymol blue and bromcresol green. Three acidic spots appeared in the toxic area (Fig. 4). These three spots were bioassayed separately and substances from each spot inhibited seed germination and/or root elongation. The materials in these three spots also were ninhydrin positive, giving a reddish-brown color, but only when the solvent system contained NH_3. Thus, when *n*-butanol:acetic acid:water was used, the ninhydrin-positive area was not evident, although an acidic reaction in the three areas could be demonstrated by means of pH indicators. These three areas, in addition, did not show any reaction with diazotized sulfanilic acid (DSA).

The clear-cut relationship between acidic spots and phytotoxicity indicated that organic acids were involved. Recently, Guenzi and McCalla (6) identified vanillic acid, *p*-coumaric acid, *p*-hydroxybenzoic acid, syringic acid, protocatechuic acid, and ferulic acid in toxic extracts from stubble mulch fields. These compounds were cochromatographed with the unknowns in the isopropanol:NH_4OH solvent. No similarities in R_F values were obtained. Furthermore, the known compounds gave positive reactions with DSA.

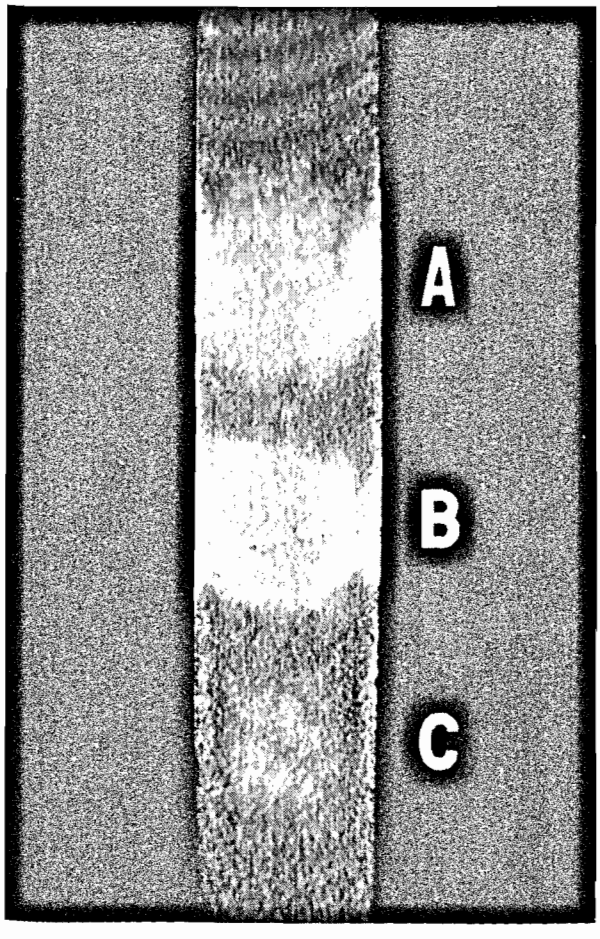

Fig. 4. Paper chromatogram treated with ferric cyanide and showing the three acid areas (A, B, C) coinciding with the phytotoxic areas. Substances in these spots were eluted separately and analyzed by gas chromatography (see Fig. 5). Similar results were obtained with pH indicators bromthymol blue and bromcresol green.

Of organic acids with R_F values similar to the unknown phytotoxins, four aromatic acids, benzoic acid (BA), phenylacetic acid (PA), 3-phenylpropionic (PP), and 4-phenylbutyric acid (PB), corresponded most closely with the phytotoxins when compared by cochromatography and the color reactions on chromatograms obtained with ferric cyanide and silver nitrate reagents prepared according to Carles et al. (3). These acids gave positive ninhydrin reactions only when chromatographed in a solvent system containing NH_3. Similarities were also noted in short-wave ultraviolet absorption, and in a negative reaction with DSA. The pungent odor of PA was similar to that of the ether extract of the unknown. Finally, bioassays of chromatographed and eluted authentic samples of PA, PP, and PB at 50 ppm and of BA at 100 ppm showed inhibition of tobacco seedling root elongation similar to that caused by the unknowns.

The paper chromatographic comparison and bioassay data strongly suggested that one or more of the aromatic acids mentioned above were present in the ether extracts from decomposing residue. Samples for gas chromatographic analysis were obtained by eluting substances from each of the three areas exhibiting phytotoxicity on paper chromatograms developed with isopropanol:NH_4OH. The materials were eluted with water and the eluate was extracted with ether at pH 2.0 and concentrated to a standard volume. Samples of each of the four aromatic acids were carried through the same procedure and known and unknown materials were sent to the Stanford Research Institute, Palo Alto, Calif., to be analyzed by gas chromatography. The column used was glass (5 ft × ¼ inch) packed with 20% diethylene glycol adipate (DEGA) + 3% H_3PO_4 on a 60/80 Gas-Chrome Q substrate. The column was conditioned for 1 hr at 275-300 C. A Micro-Tek 220 dual flame detector at a temperature of 225 C was used. The column temperature was maintained at 190 C and the inlet temperature was 250 C. N_2 gas at 45 ml/min was used as a carrier. A comparison of retention times indicated that unknown area A contained primarily PA with a lesser amount of BA; area B contained PP with a small amount of PA; and area C contained BA, PA, PP, and PB, in small amounts. The appearance of single peaks when a sample of the appropriate authentic acid was added to the unknown further confirmed the identification (Fig. 5).

Analysis by gas chromatography was repeated in our laboratory with a sample obtained from barley residue that had decomposed in the field. This sample contained BA and PA. Cotton, soybean, and cowpea residues were obtained from plants grown in the greenhouse and decomposed in the laboratory for 3 weeks. Gas chromatographic analysis of their ether extracts indicated

that BA and PA were present. Quantitative analysis indicated that the concentration of these aromatic acids present in the residue extracts was sufficient to account for the toxicity of these extracts.

DISCUSSION.—The effects of organic matter decomposition on plants have been extensively studied during the past 50 years. This work, which has been the subject of several recent reviews (2, 7, 10, 11), has shown that such decomposition can lead to the formation and liberation of numerous products which may be phytotoxic. Although many of the phytotoxins have been identified, conclusive evidence for their existence and activity in nature is, in most cases, lacking. Our previous work (12) showed that green barley residue decomposing in fields of the Salinas Valley, Calif., liberated water-soluble materials that were highly phytotoxic. The objective of this research, therefore, was to determine the chemical nature of these phytotoxic materials. To facilitate production and analysis of phytotoxins, field-grown barley was decomposed in field soil in containers in the laboratory. The results of these laboratory studies were confirmed by parallel studies with barley decomposed under field conditions in the Salinas Valley.

The present work shows that when green barley residues are decomposed in soils with high moisture, two major water-soluble phytotoxic fractions are present. The main fraction, soluble in ether at pH 2.0, contains phytotoxins accounting for approximately 60% of the total toxicity of the original water extract. The remaining ether-insoluble fraction contains a high concentration of electrolytes whose toxicity has been discussed previously (12). Efforts to identify phytotoxic components were directed primarily to the ether-soluble fraction. It was found to contain four aromatic acids in concentrations sufficient to account for the observed phytotoxicity. These structurally similar acids were benzoic acid, phenylacetic acid, 3-phenylpropionic (hydrocinnamic) acid, and 4-phenylbutyric acid. These compounds, with the exception of benzoic acid, have not heretofore been reported as products of crop residue decomposition, nor have their phytotoxic properties been previously demonstrated.

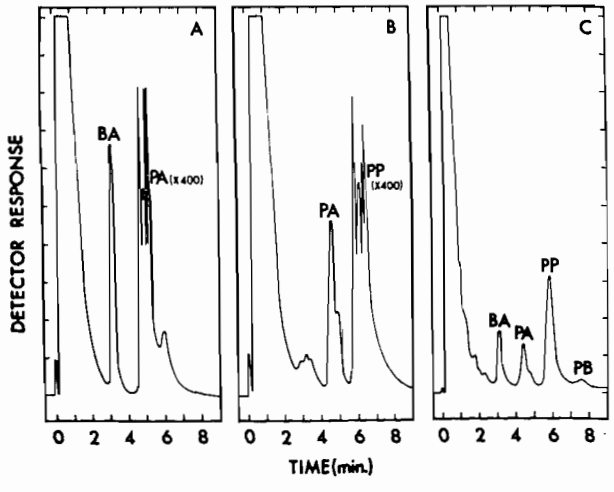

Fig. 5. Gas chromatographic analysis of areas A, B, and C (see Fig. 4) as obtained by Stanford Research Institute, Palo Alto, Calif. Baseline attenuation was 100 times for A, B, and C. Area A contains mainly phenylacetic acid (PA) and some benzoic acid (BA). Area B contains mainly 3-phenylpropionic acid (PP) with residue of phenylacetic acid. Area C contains small amounts of benzoic, phenylacetic, 3-phenylpropionic, and 4-phenylbutyric acids (PB). Ether solvent peak occurs during first 2 min in each case.

Since the parallel studies using extracts of green barley decomposing in the field also showed the presence of benzoic acid and phenylacetic acid, and since these compounds were also present as phytotoxic decomposition products of such diverse species as cotton, cowpea, and soybean, it would appear that they are of common occurrence in nature when environmental factors are favorable. The presence or absence of one or more of the compounds in an extract may be a result of the environment during decomposition or the length of time the material had been decomposing. It is hoped that further work will yield both quantitative and qualitative information regarding phytotoxic compounds produced in nature under varying conditions of residue, soil, and environment.

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