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ALLELOCHEMICALS IN SOIL FROM NO-TILLAGE VERSUS CONVENTIONAL-TILLAGE WHEAT (*Triticum aestivum*) FIELDS[1]

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Abstract—Putative allelochemicals found in the soil of no-tillage and conventional-tillage wheat plots near Stillwater, Oklahoma, were obtained by a mild alkaline aqueous extraction procedure, bioassayed to determine their biological activity, purified, and analyzed with a capillary gas chromatography–mass spectrometry–data analysis system. The most significant inhibition was found in bioassays of extracts from soil collected immediately after harvest in June, July, and August. No-tillage soils produced significant inhibition during the rest of the year also. Mass spectrometry showed fatty acids as the most abundant compounds. However, when bioassayed authentic samples of the five free fatty acids showed no significant biological activity toward wheat.

Key Words—Allelochemicals, no-tillage, conventional-tillage, soils, wheat, *Triticum aestivum*, mass spectrometry, Petri-dish bioassay, fatty acids.

INTRODUCTION

Growing wheat without conventional tillage of the soil has become common in the last 25 years. There are various versions of "conservation tillage" practice, but the simplest and most common is no-tillage in which the wheat is planted directly into the residue of the past crop. Soil erosion is reduced and there are

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other benefits, but the residues may also harm the next crop. A summary of the benefits and difficulties of this practice is given by Waller et al. (1987).

Several other workers have studied allelopathy in wheat and other small grains (McCalla and Duley, 1949; Guenzi and McCalla, 1962; Norstadt and McCalla, 1963; McCalla and Haskins, 1964; Kimber, 1973; Wallace and Elliott, 1979; Wallace and Whitehand, 1980). The literature on allelopathy, including substantial amounts of work on crops and their residues, has recently been reviewed by Rice (1984) and by Putnam and Tang (1986). Elliott et al. (1984) reviewed the extensive literature on various aspects of the rhizosphere soil; allelochemicals can be released into soil as a result of root and microbial exudates and/or metabolism. They can accumulate in the rhizosphere and probably can combine with humus. Samtsevitch (1965) estimated that the dry weight of root mucilage caps deposited in the soil of wheat was similar to the dry weight of grain produced. Although the allelopathic properties of wheat have been studied at several times and places, little research has been done on accumulated allelochemicals in untilled wheat soil, and apart from our own studies (Waller et al., 1987; Thorne et al., 1990), we know of none in the southern Great Plains region of the United States.

Numerous compounds have been isolated and identified from plant residues, including those of small grains (Rice, 1984; Thompson, 1985; Putnam and Tang, 1986; Waller, 1987; Waller et al., 1987; Thorne et al., 1990). The presence of an allelochemical in plant residue does not necessarily mean that it can escape into the environment where it can affect recipient plants. Fuerst and Putman (1983) suggested that, as one of the four guidelines in the proof of allelopathy, suspected compounds must be detected in the environment around the recipient.

Using relatively gentle alkaline–aqueous methods, soils were leached from conventional-tillage (CT) and no-tillage (NT) plots. Bioassay techniques were used to survey the resulting extracts for allelopathic activity, and mass spectrometry to identify several compounds, although not ones active toward wheat.

METHODS AND MATERIALS

Sample Characteristics. Soils were taken from plots that had been under tillage treatment (CT or NT) for five years at the beginning of the study. These plots belong to the Department of Agronomy, Oklahoma State University, and are part of a long-term study of tillage practices. The soil was Easpur fine sandy loam, fine-loamy, mixed Thermic Fluventic Haplustoll (Oklahoma Department of Agriculture, 1987). The CT plots were moldboard-plowed 15–20 cm deep, and disked, shortly after harvest. Thus, essentially all residue was buried for about four months. NT plots were replanted with no disturbance other than the

planting itself, so that both standing stubble and fallen straw, leaves, and chaff were present. Soil samples were collected monthly by bulking 8–10 cores per plot from two plots of each type. Cores were of the upper 3 cm of soil, plant residue having been brushed aside. Samples were taken from January 1986 through February 1987 and were placed in jars, frozen in the field with Dry Ice, and stored frozen at −18°C until used. The pH of in situ soil in 1986 was 5.0 in NT and 5.8 in CT plots. Rainfall for the period of February 1986 through January 1987 was 82 cm.

Extraction and Purification. Allelochemicals were extracted by making a slurry of 400 g freshly thawed soil and 800 ml distilled water and adjusting the pH to 8.0 ± 0.2 with 1 M NaOH (approximately 8 ml for CT and 12 ml for NT soils). This mixture was shaken for 2 hr at 5–10°C, allowed to stand for 2 hr at 5–10°C, and then filtered through glass wool. Material trapped by the filter was discarded and the filtrate centrifuged for 25 min at 15×10^3 rpm (Sorvall Superspeed RC2-B). The supernatant fluid was microfiltered through a 0.45-μm glass fiber filter (MSI MAGNA, Millipore Corp.) and divided into three portions; 10 ml were used for an immediate bioassay, 10 ml for a final pH test (usually 7.4–7.6 for CT and 7.1–7.3 for NT) and 400 ml was lyophilized, weighed, and reserved for later use. The procedure was conducted at least twice for each monthly set of soil samples.

In using the lyophilized residue for bioassay, an amount of material equivalent to 5 g of soil was extracted with three 10-ml portions of hot methanol (MeOH extract). A second extract was made in the same manner using material from 25 g soil (concentrated MeOH extract). The methanol solutions were vacuum-filtered through Whatman No. 1 filter paper, evaporated to dryness in a hood at room temperature, and redissolved in methanol. Frequently it was necessary to microfilter the extract again due to presence of insoluble material in the dried residue, possibly caused by oxidation and polymerization during the process.

Analysis was begun by extracting 150 mg of each month's lyophilized residue three times with 25-ml portions of hot methanol. The combined extracts were suction-filtered through Whatman No. 1, the methanol evaporated at room temperature, and the remaining residue reextracted three times with 1-ml portions of hot methanol. The extracts were combined, further purified by microfiltering through a 0.45-μm glass fiber or nylon membrane filter, then evaporated to dryness under nitrogen.

Bioassay. Each bioassay test or control used four, 100 × 15-mm glass Petri dishes containing two sheets of 7.5-cm Whatman No. 1 filter paper. Ten seeds per dish of Pioneer brand 2157 wheat were placed between the filter paper disks. Two milliliters of the aqueous test solution (adjusted to pH 7.0 ± 0.2) was added to the dish and 2 ml distilled water was used in the control dishes. For solutions with organic solvent and solvent control, they were applied to the

filter paper and allowed to dry before seeds were placed in the dishes. Two milliliters of distilled water were placed in each dish, a sheet of plastic film stretched over it to retard evaporation, and the cover replaced. After a 72-hr incubation at 20°C in darkness, the length of each seedling root and shoot was measured.

Bioassays also were carried out on commercial (>99% purity) fatty acids. Ether solutions (1.0 mM) of myristic, palmitic, heptadecanoic, stearic, and eicosanoic acids were prepared and tested separately, and in all cases the total concentration was held at 1.0 mM. In addition, a bioassay of all five fatty acids together was performed at a total concentration of 1.0 and 5.0 mM.

Statistical Analaysis of Data. The year's bioassay data were subject to a two-way analysis of variance (ANOVA) to determine the statistical significance of differences attributable to the two tillage treatments versus the distilled water control, attributable to the date of soil collection, and attributable to treatment × date interactions. Separate one-way ANOVAs were performed on each month's data to determine significance of tillage treatment effects, and Tukey's studentized range test was employed to determine which treatments (CT, NT, and control) differed significantly from which others. Moreover, the correlation coefficient between the weight of the lyophilized solids from an extract and the growth of bioassay seedlings in that extract was calculated, to indicate whether the effects of the extracts were due to qualitative or quantitative chemical differences.

Low-Resolution Mass Spectrometry. To improve resolution and separation of the compounds of interest, acids were converted to the methyl esters. Accordingly, 1 ml of diazomethane was added to the dried residue and the container was shaken for 5 min or until the yellow color disappeared, indicating completion of the reaction. As an internal standard, 1.0 mg of caffeine was added to each sample. Any excess diazomethane and its solvent, ethyl ether, were evaporated under nitrogen. The diazomethane was synthesized by the method of Ruehle et al. (1979).

Low-resolution mass spectra of the diazomethane-treated samples were obtained using an LKB-2091 (LKB Produckter, Bromma, Sweden) capillary gas chromatograph—mass spectrometer–data analysis system (CGC-MS-DAS) (McGown and Waller, 1986). These devices were linked so that the chromatographic column was directly connected to the ion source of the mass spectrometer and data output went directly to the data-analysis computer. The column was 30 m × 0.32 mm DW-5 (J & W Scientific, Folsom, California), and a flow rate of 2–3 ml He/min was used. Up to 2.0 μl of sample in methanol was injected with a 1:4 split at 65°C. The temperature was programmed to rise (after 4 min) 10°C/min until 300°C was reached, and then to remain there for 10 min.

The mass spectrometer conditions were: injector temperature 275°C, separator temperature 275°C, initial eV 21, scan eV 70, box current 15–20 μA,

accelerating voltage 3.2–3.4 V, filament current 3.3 A, trap current 80–100 A, multiplier voltage 500 mV, and source temperature 265°C.

Identification of spectra was done both visually and by automated [IBM AT microcomputer with Technivent (St. Louis, Missouri) programming system] means. Comparison was with known spectra given in the Eight Peak Index of Mass Spectra (1983), by Waller (1972), and by Waller and Dermer (1980).

High Resolution Mass Spectrometry. Confirmation of the identity (by formula: C, H, O, N) of each ester mentioned in this paper was done using a model 70-SE spectrometer (CGC-MS-DAS) (VG Instruments, Manchester, United Kingdom).

RESULTS

Yield of Extracts. No-till extracts generally yielded more lyophylized residue (802 ± 58 mg) than CT (481 ± 54 mg) from 400 ml of the crude soil extracts. They ranged in ratio from 1.0 to 3.6 and averaged 1.67 (NT:CT).

Bioasssay of Aqueous Extracts. Analysis of variance on bioassays of direct aqueous extracts revealed highly significant variation ($p < 0.0001$) in root lengths attributable to the main effects of treatment and date, and also the treatment × date interaction. On an annual basis, seedling roots were significantly longer ($p < 0.01$, Tukey's test) in the CT extracts than in the distilled water control and significantly shorter in the NT extracts than in the control (Table 1). Trends were the same for shoot lengths, but not statistically significant. Because of the significant treatment × date interaction, these annual results are less revealing than the month-by-month comparisons.

TABLE 1. ANNUAL MEAN ROOT AND SHOOT LENGTHS (mm)[a]

Treatment[b]	Aqueous extract	MeOH extract	Conc. MeOH extract
Root			
DW	27.56a	26.29a	26.29a
CT	28.83b	25.19b	24.49b
NT	26.04c	24.46c	23.09c
Shoot			
DW	7.42a	7.28a	7.29a
CT	7.47a	7.35a	7.54a
NT	6.97a	6.85b	6.84a

[a] Means with the same letter in a given column are not significantly different ($P > 0.05$) by Tukey's studentized range test.
[b] DW = distilled water; CT = conventional-tillage; NT = no tillage.

Figure 1 shows monthly means for roots and shoots, expressed as a percentage of the distilled water control. This is a useful method of presentation because both stimulation (values > 100%) and inhibition (< 100%) are clearly expressed; however, it is not amenable to presentations of statistical significance levels because each point on the graph represents a quotient of two means.

Results of Tukey's test will be described below; all differences noted were statistically significant with $P < 0.05$ (for full results see Cast, 1987). Roots of seedlings exposed to NT soil extracts were significantly inhibited relative to the control in February, June, July, and August. NT extracts stimulated root growth relative to the control in May, but in no cases were NT roots significantly longer than CT. Root growth in CT extracts was stimulated relative to control in March, April, May, and June but inhibited relative to control in July and August. Shoot growth showed similar patterns, although fewer comparisons

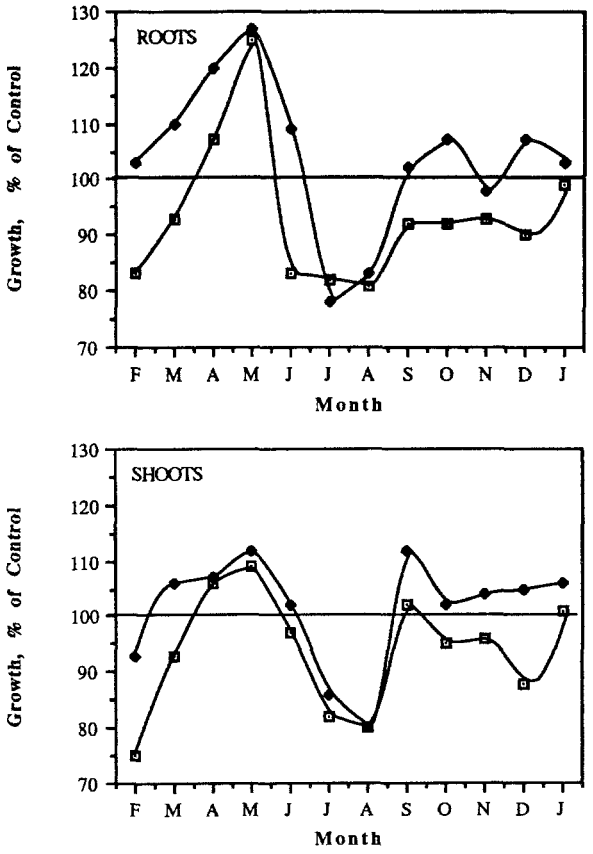

FIG. 1. Growth as a percentage of control from bioassay of direct aqueous extract of NT (⊡) and CT(♦) soils from February 1986 to January 1987.

were statistically significant. NT treatments were inhibitory relative to control in February, August, and December. CT was stimulatory in May and September, but inhibitory in July and August.

Bioassay of Methanol Extracts. An important point of difference between the methanol and aqueous bioassays was that significant stimulation of root or shoot growth, while common in aqueous extracts, was observed only once in methanol extracts (shoots assayed with December MeOH extract). On an annual basis (Table 1), the distilled water control had significantly longer roots than either extract. In general, NT extracts were broadly inhibitory; CT extracts were inhibitory when prepared from soils collected in the late summer months.

Figure 2 shows the monthly means (as percentage of control) for the bioassays of methanol-soluble fractions of the lyophilized aqueous extracts, at

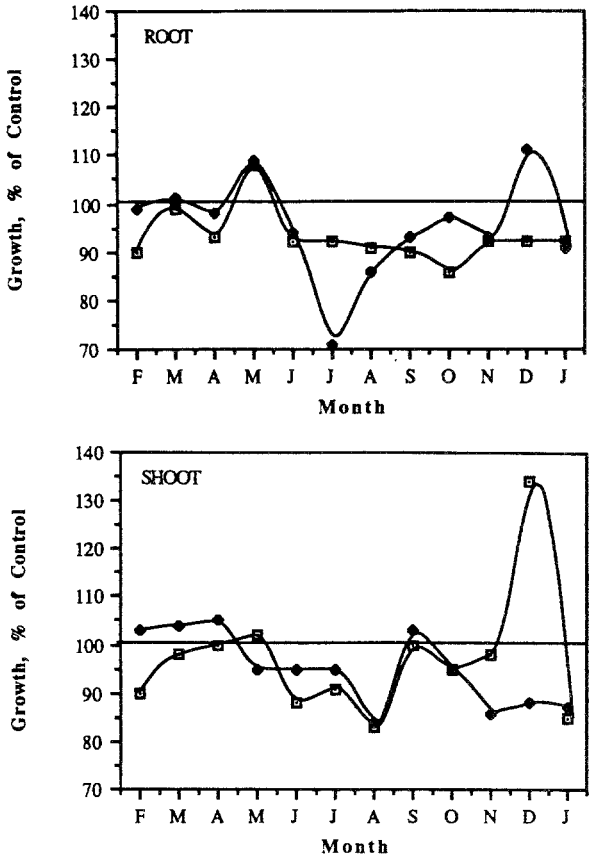

FIG. 2. Growth as a percentage of control from bioassay of methanol extract of lyophilized residue at approximately the same concentration as the aqueous extract of NT (⊡) and CT (♦) soils from February 1986 to January 1987.

approximately the same concentrations as the aqueous. Figure 3 shows the same information for the concentrated MeOH extract. For the MeOH extracts of NT soils, roots were significantly inhibited relative to control by soils collected in every month from June through November, and shoots were inhibited by extracts from January, June, July, and August. CT extracts were inhibitory to roots in July, August, and September soils only, and to shoots only in January and August. Inhibition was stronger, as might be expected, in the concentrated MeOH extracts. NT soils from February, April, June, and August through December were all inhibitory to roots, and those from June, August, September, and October were inhibitory to shoots. Significant inhibition by CT extracts

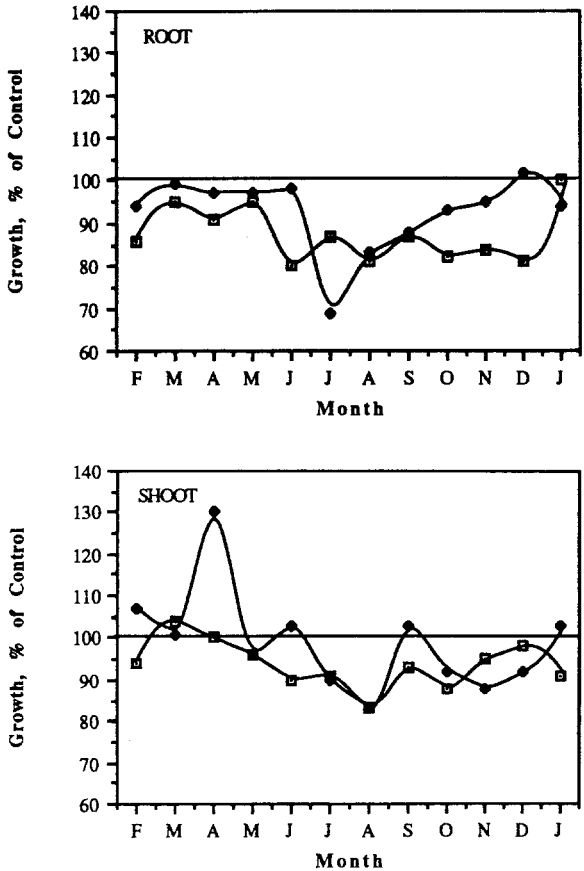

FIG. 3. Growth as a percentage of control from bioassay of methanol extract of lyophilized residue at approximately three times the concentration as the aqueous extract of NT (⊡) and CT (♦) soils from February 1985 to January 1986.

was seen in July–October for roots, and in July, August, October, and December for shoots.

A puzzling feature of the methanol bioassay was that July CT extracts produced less root growth than NT extracts, and November CT extracts produced less shoot growth than NT extracts. These statistically significant differences were observed in both the MeOH and concentrated MeOH preparations. This result is unexpected and unexplained.

Correlation Analysis. In no cases were significant correlations observed between the quantity of lyophilized residue recovered from aqueous extracts and the growth of seedlings in bioassay. This implies that inhibition or stimulation of root growth was caused by qualitative differences among the extracts rather than quantitative dosage effects. These data must be interpreted with caution, however, because the lyophilized residues consisted primarily of inorganic materials, and it is still possible that organic constituents present in small quantities could act in a dosage-dependent manner.

Chemical Analysis. The two instruments yielded similar results except that the VG 70-SE provided high resolution of the individual compounds. The methyl esters of fatty acids were found to dominate the methanol fraction of soil throughout the year. Figures 4 and 5 show the reconstructed total ion current chromatogram with some identification of individual compounds for the NT (Figure 4) and CT (Figure 5) samples for June 1986. There were more peaks in the NT (26 compounds) than in the CT chromatograms (20), indicating that some of the unidentified compounds were allelochemicals that would require

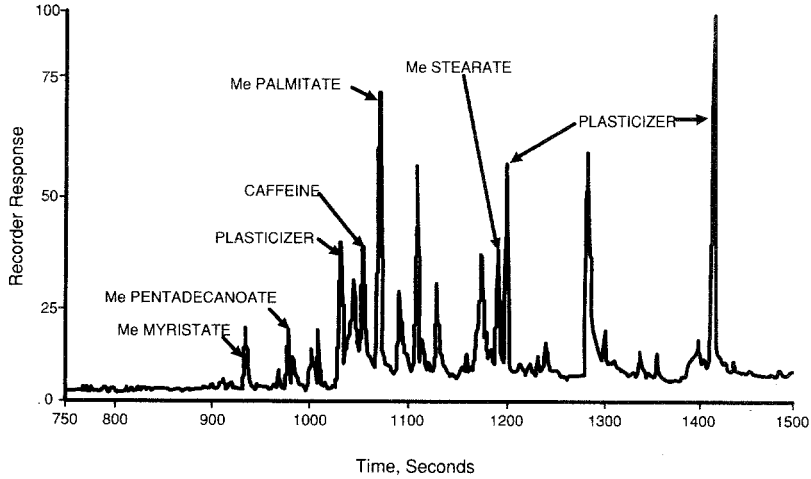

FIG. 4. Reconstruction total ion current chromatogram of the methanolic fraction from lyophylization of NT soil extract (LKB-2091 CGC-MS-DA): Peaks represent compounds; identified peaks are labeled. Soil sample: June 1986.

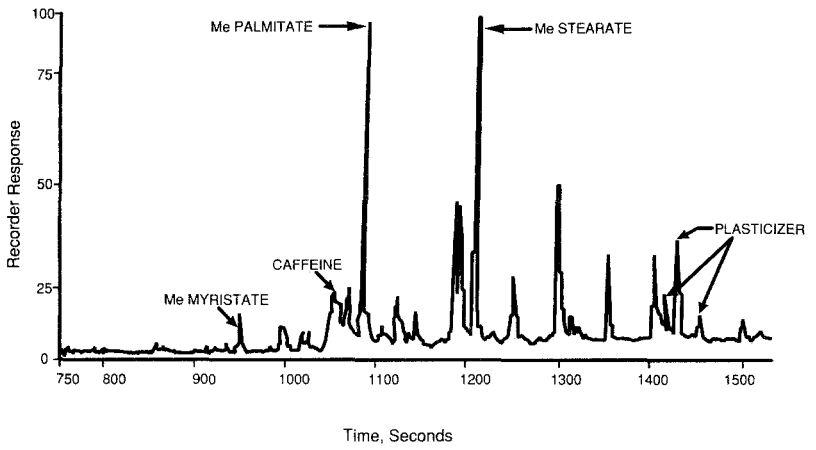

FIG. 5. Reconstructed total ion current chromatogram of the methanolic fraction from lyophylization of CT soil extract (LKB-2091 GCC-MS-DA): Peaks represent compounds, and identified peaks are labeled. Soil sample: June 1986.

further derivatization before they could be identified. Caffeine peaks represent the internal standard, and phthalate plasticizers constituted artifactual compounds obtained from the soil (Waller et al., 1987). In all months and treatments, the most prevalent fatty acids (seen as the methyl esters) were palmitic and stearic acids; shorter-chain fatty acids such as myristic acid and pentadecanoic acid were present in much lower concentrations.

Fatty Acid Bioassay: Fatty acids were regarded as possible allelochemicals in the soil of wheat systems. Not only were these the main organic compounds in the extracts, but other researchers have considered fatty acids as possible allelochemicals. Much of the previous research focused upon volatile, short-chain fatty acids (Stevenson, 1967; Lynch 1977; Tang and Waiss, 1978; Wallace and Elliott, 1979; Wallace and Whitehand, 1982), but some long-chain ones have been considered (Spoehr et al., 1949). Alsaadawi et al. (1983) investigated allelopathic interaction of nine fatty acids in decomposing residues of *Polygonum aviculare* (myristic, palmitic, linolelaidic, oleic, stearic, arachidic, 11,14-eicosadienoic, heneicosanoic, and behenic acids), and they found all except myristic and heneicosanoic acids in soil under such residues. Sodium salts of all of these fatty acids inhibited seedling growth of Bermuda grass and also some test strains of *Azotobacter* and *Rhizobium* at a concentration of 5 ppm. Waller et al. (1984, 1985, 1987) found exhaustive steam distillation of wheat soil (Tillman, clayloam fine, mixed, Thermic Typic Haplustolls) where wheat had been grown for the past decade to yield *n*-saturated fatty acids from C_4 to C_{18} as well as other compounds; the Petri-dish bioassay results showed different amounts of inhibition in the fractions (neutral, acidic, and alkaline)

TABLE 2. RESULT OF BIOASSAY WITH WHEAT OF PURE FATTY ACIDS AT 1.0 mM

Fatty acid	Growth (% of control)	
	Root	Shoot
Myristic	107.1	94.4
Palmitic	89.9	81.3
Heptadecanoic	112.9	98.1
Stearic	97.6	85.0
Eicosanoic	100.9	96.7

with neutral and acidic being less so that the basic fraction. As expected, most fatty acids were found in the acidic fraction of steam distillation.

Since the predominant compounds in the methanol fraction were free fatty acids, the bioassay results of authentic ones are shown in Table 2. For those fatty acids assayed at 1.0 mM, no statistically significant inhibition or stimulation was noted. When five fatty acids were mixed (1 mM and 5 mM concentration) and bioassayed to test for synergistic effects, no significant effect was seen. Based on these results, as well as those from exhaustive steam distillation of wheat soil, it was our finding that the long-chain fatty acids do not have a significant allelochemical action toward wheat.

DISCUSSION

Stimulation of seedling growth in bioassays was found in aqueous extracts but seldom in methanol extracts. This suggests that stimulation may have been a consequence of water-soluble compounds leached out of the soil samples.

NT extracts were broadly inhibitory, with roots showing this inhibition most clearly. Significant inhibition by at least one of the NT extracts was found in every month except March and May. CT extracts were most commonly observed to be inhibitory in soils collected from July through September. This is the period of time following wheat harvest when straw and stubble are still standing or have been recently plowed under, and conditions for both NT and CT would be similar. This observation leads further support to the hypothesis that allelochemicals in wheat soils arise from wheat residue.

Wheat field soils collected at monthly intervals for one year and analyzed by MS-CGC-DAS showed that stearic and palmitic acids were the major compounds present in each month. Other data (Waller et al., 1987) support the consistent presence of stearic and palmitic acids in wheat field soils in Oklahoma. There was a definite tendency toward greater autotoxicity in the no-tillage soil compared to conventional tillage; however, it was clear that the long-

chain fatty acids did not produce this effect because they had little effect on germination and early growth of wheat. Our data suggest that there are one or more organic allelochemicals present in wheat soils, especially untilled ones, but we have not identified them.

Microbial breakdown of wheat residue is slowed by no-till practice (Lynch, 1987; Elliott and Cheng, 1987). It seems likely that the allelochemicals responsible for the inhibition are microbial products that accumulate more in no-till than in conventionally-tilled soil because it is not disturbed and dispersed by cultivation. We recognize that environmental conditions, especially temperature, moisture, and soil texture, greatly influence microbial activity. Thus, it further seems likely that there will be large variations in allelochemical content of the soil from place to place and year to year. Under some conditions, this could be a substantial problem to wheat growers. Research is continuing on the nature and source of the allelochemicals and the conditions influencing their production.

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