ELSEVIER

Agriculture, Ecosystems and Environment 78 (2000) 187–192

Agriculture Ecosystems & Environment

www.elsevier.com/locate/agee

Short communication

Phytotoxic effects of red clover amended soils on wild mustard seedling growth

Tsutomu Ohno [a,*], Kristan Doolan [a], Larry M. Zibilske [b], Matt Liebman [c], Eric R. Gallandt [d], Christi Berube [a]

[a] *Department of Plant, Soil and Environmental Sciences, 5722 Deering Hall, University of Maine, Orono, ME 04469-5722, USA*

[b] *U.S.D.A. – A.R.S., Subtropical Agricultural Research Center, Weslaco, TX 78596, USA*

[c] *Department of Agronomy, Iowa State University, Ames, IA 50011-1010, USA*

[d] *Department of Crop and Soil Sciences, Washington State University, Pullman, WA 99164-6420, USA*

Received 8 April 1999; received in revised form 17 August 1999; accepted 20 August 1999

Abstract

Previous studies have suggested that phenolics from legume green manures may contribute to weed control through allelopathy. The objective was to determine if red clover (*Trifolium pratense* L.) residue amended field soils expressed phytotoxicity to a weed species, wild mustard (*Sinapis arvensis* L.). Field plots involving incorporation treatments of wheat (*Triticum aestivum* L.) stubble or wheat stubble plus 2530 kg ha^{-1} red clover residue, were sampled at −12, 8, 21, 30, 41, 63, and 100 days after residue incorporation (DAI). Soil–water extracts (1 : 1, m : v) were analyzed for plant nutrients and phenolic content. Phytotoxicity of the extracts was measured using a laboratory wild mustard bioassay. There was a 20% reduction of radicle growth in the green manure treatment in comparison with the wheat stubble treatment, but only at the first sample date after residue incorporation (8 DAI). The radicle growth reduction had the highest correlation with the concentration of soluble phenolics in the soil : water extracts. Bioassays using aqueous extracts of the clover shoots and roots alone predicted a radicle growth reduction of 18% for the quantity of clover amendment rate used in the field plots. The close agreement of the predicted and observed root growth reduction at 8 DAI further supports clover residue as the source of the phytotoxicity. This study demonstrates that the potential exists for using legume green manures to reduce the amounts of synthetic herbicides needed for weed control. ©2000 Elsevier Science B.V. All rights reserved.

Keywords: Allelopathy; Weed management; Green manure; Wild mustard; Red clover; USA

1. Introduction

Agricultural production has become heavily reliant over the past 50 years on the use of synthetic fertilizers and pesticides. This reliance on agrochemicals has resulted in the contamination of surface and ground water resources by nutrients and pesticides in a number

* Corresponding author. Tel.: +001-207-581-2975; fax: +001-207-581-2999.
E-mail address: ohno@maine.edu (T. Ohno).

0167-8809/00/$ – see front matter ©2000 Elsevier Science B.V. All rights reserved.
PII: S0167-8809(99)00120-6

of different environments (National Research Council, 1993). Management methods that decrease requirements for agricultural chemicals are needed to reduce adverse environmental impacts.

Many reduced-input cropping systems include a legume green manure component that often has positive effects on the yield and nutrient use efficiency of subsequent crops (Karlen et al., 1994) as well as contributing to weed management (Liebman and Dyck, 1993; Weston, 1996; Liebman and Ohno, 1997). A legume green manure may suppress weed growth in a subsequent crop through allelopathy arising from chemical constituents of the legume residue (Dyck and Liebman, 1994). White et al. (1989) found that morning-glory (*Ipomoea lacunosa* L.) grown in soil containing either crimson clover (*Trifolium incaratum* L.) or hairy vetch (*Vicia villosa* Roth.) debris showed a substantial decrease in both emergence and dry weight relative to growth in soil without legume debris. Lehman and Blum (1997) reported that crimson clover and subterranean clover (*T. subterraneum* L.) amended soils decreased pigweed (*Amaranthus retroflexus* L.) and morning-glory emergence.

The interaction of allelochemicals with soil components upon release from the plant is important in determining whether inhibition of the target plant is likely to occur in the field (Blum, 1995). Phenolic compounds are ubiquitous constituents of plant tissues and have been the focus of many allelopathic studies (Inderjit, 1996). Phenolic acids have been shown to be rapidly sorbed and/or oxidized by soil (Dalton et al., 1989; Lehmann et al., 1987; Makino et al., 1996). To fully exploit phytotoxicity after legume incorporation for weed management requires additional understanding of the soil chemical reactions of plant-derived phenolics. The specific objective was to identify when phytotoxicity was evident in the field soil and to develop an hypothesis for the mechanisms of field toxicity.

2. Materials and methods

This study was conducted on plots at the University of Maine's Rogers Farm in Stillwater, ME, USA. The plots were established to determine whether legume green manure may be exploited for weed control. The experimental design was a completely randomized design replicated four times. Treatments consisted of two soil management regimes: wheat stubble and wheat stubble with red clover green manure. Plots containing bean (*Phaseolus vulgaris* L. cv. 'Marafax') and wild mustard (*Sinapis arvensis* L.) grown together were sampled.

Soil was a Nicholville fine sandy loam (coarse-silty, mixed, frigid, Aquic Haplorthods) with a pH of 6.5 (1 : 1, soil : water) and organic carbon content of 18.9 g kg^{-1} soil. The experimental site had previously been planted with wheat and clover in 1993 and 1995, and with bean and mustard in 1994. Residue with above-ground dry matter biomass estimates of 3 g m^{-2} for the wheat and 253 g m^{-2} for clover treatments was incorporated with a tractor powered rotary tiller on 28 May 1996. Clover tissue were sampled just before incorporation and aqueous extracts of the clover shoot and root tissue yielded a water soluble Folin–Ciocalteu phenolic carbon (PhC) content of 8.6 g kg^{-1}. Field planting of beans and mustard took place on 17 DAI (days after incorporation).

Soil samples were collected at −12, 8, 21, 31, 41, 63, and 100 DAI of residue. Approximately 25 soil cores from each plot were collected from 0 to 10 cm depth, bulked and sieved (<4 mm) on site. Soil equivalent to 125 g dry weight was transferred to 250 ml plastic bottles. Deionized water (DI-H_2O) was added to a total water content (native soil water plus added DI-H_2O) of 125 g. The bottles were shaken gently for 15 s and allowed to stand for 5 min. The bottles were then centrifuged at 900 × g for 15 min and vacuum filtered through 0.2 μm Nylaflo filters (Gelman Science, Ann Arbor, MI) for chemical analysis and bioassay. The filtered extracts were analyzed for K, Ca, Mg and Al by inductively coupled plasma-atomic emission spectrometry.

The Folin–Ciocalteu method was used to determine the PhC concentration of the soil : water extract (Box, 1983). Briefly, 0.75 ml of 1.9 M Na_2CO_3 and 0.25 ml of Folin–Ciocalteu reagent (Sigma Chemical, St. Louis, MO) were added to a 5 ml sample or standard solution and absorbance read at 750 nm after development in the dark for 60 min. Ferulic acid was used to generate the standard curve and the concentrations were expressed in ferulic acid equivalent units (Blum et al., 1991). Reversibly bound soil phenolic substances were determined using citrate extraction (Blum, 1997). A 10 g dry weight equivalent soil sample was extracted with 40 ml of pH 7, 0.25 M sodium citrate and shaken for 2.5 h. The citrate extract samples were then centrifuged for 15 min at 900 × g and filtered through 0.2 μm Nylaflo filters under vacuum. Analysis of phenolic carbon was conducted using the Folin–Ciocalteu method as described above.

Phytotoxicity bioassay of the soil : water extracts used wild mustard seeds that were surface sterilized with commercial 50 g kg^{-1} sodium hypochlorite solution for 10 min and germinated for 24 h on autoclaved Whatman 1 (Whatman International; Maidstone, England) filter paper. Twenty pre-germinated seeds with radicles of uniform length were placed between two pieces of Whatman 1 filter paper moistened with 5 ml of the soil : water extract in a Petri dish. The dishes were placed in an incubator at 20°C and radicle lengths were measured after 72 h. The incubation temperature corresponded to the soil temperature at the time of field planting.

Red clover phytotoxicity was evaluated using clover tissues collected three days before residue incorporation from the field study described above. Three replicates of 1.25 g of clover (0.90 g shoot and 0.35 g root) were extracted in 50 ml DI-H_2O with shaking for 15 min. The extracts were filtered through 0.2 μm Nylaflo membrane filter. An aliquot was left either undiluted (25 g kg^{-1}) or diluted with DI-H_2O to 5 and 1 g kg^{-1} clover concentrations. Wild mustard seeds were germinated for 24–36 h on sterile petri plates with autoclaved Whatman 1 filter paper moistened with 50 μg ml^{-1} streptomycin solution. Agar plates were prepared in a laminar flow hood with autoclaved agar (12 g kg^{-1}) kept molten at 40°C and mixed with equal volume of clover extract solutions. The final clover concentrations were 12.5, 2.5 and 0.5 g kg^{-1}. Deionized water was used as a control treatment. Fifteen germinated mustard seeds were lined on plates, inclined at 45°, and incubated at 20°C for 48 h. Root length was measured at the end of incubation.

3. Results and discussion

The phytotoxicity of red clover shoot and root tissue was evaluated by an aqueous extract bioassay. There was a linear reduction in root growth with increasing extract concentration (Fig. 1). The concentration of PhC were 480 and 100 μM ferulic acid equivalent in the 12.5 and 2.5 g kg^{-1} extracts, respectively (data not shown). The observed phytotoxicity agrees with previous studies which have shown that solution phenolic acid concentrations in the 100–1000 μM range are toxic to seedlings (Guenzi and McCalla, 1962; Whitehead et al., 1981).

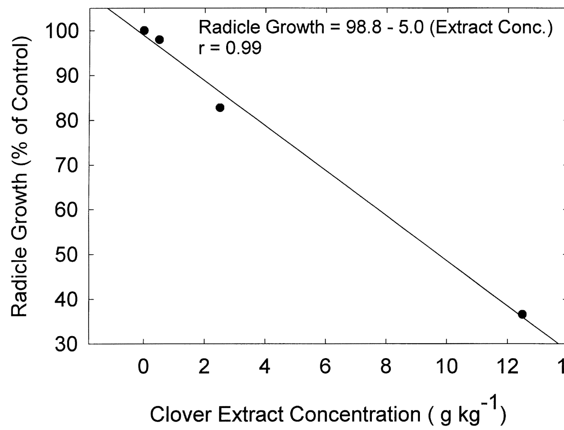

Fig. 1. Effects of red clover extract concentration on wild mustard radicle growth bioassay.

A wild mustard seedling radicle growth bioassay utilizing the soil : water extract was used to determine the presence of phytotoxicity in the field soil samples. The comparison of ratios of radicle growth of the green manure to the control treatments were used as the indicator of phytotoxicity from incorporation of red clover. The clover treatment significantly decreased radicle growth by 20% only at the first sampling after red clover incorporation (8 DAI) (Fig. 2). Correlation analysis of bioassay radicle growth with soil chemical properties from the soils collected at 8 DAI (Doolan, 1997) was used to identify factors involved in the observed phytotoxicity for the 8 DAI samples. The parameter with the highest correlation coefficient ($r = -0.74$, $p = 0.05$) for the radicle growth parameter was the phenolic concentration of the soil : water extract (Fig. 3). This negative correlation suggests that phenolic compounds were involved in the reduction of root growth at the sampling closest to the green manure incorporation date.

The red clover shoot and root biomass that was incorporated into 0–10 cm soil zone and sampled in the field study corresponds to a clover concentration of 3.1 g kg^{-1} assuming a bulk density of 1.2 g cm^{-3}. The linear regression equation shown in Fig. 1 predicts a

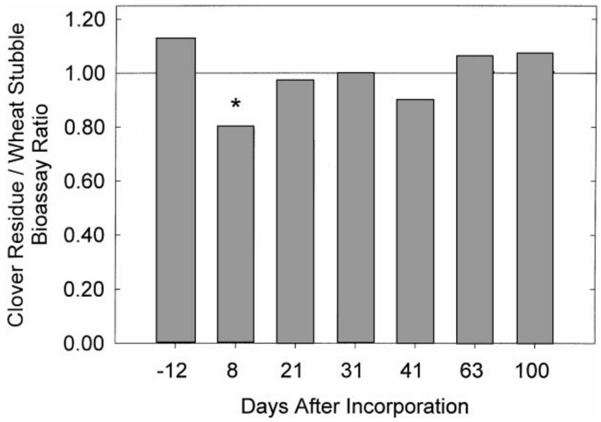

Fig. 2. Determination of phytotoxicity in the clover amended plots during the growing season using wild mustard radicle growth bioassay of 1 : 1 soil–water extracts from the field soils expressed as the ratio of clover residue plot bioassay to wheat stubble bioassay. The * indicates significant difference of the clover residue and wheat stubble bioassay at the $p=0.05$ level.

root growth reduction of 18% for the 3.1 g kg^{-1} quantity of red clover incorporated which agrees well with the 20% reduction observed in the field soil extract bioassay (Fig. 2). This excellent agreement between predicted and observed radicle growth reduction further supports the role of red clover amendment in the phytotoxicity of the soils being studied.

The results from this study demonstrate that phenolic substances released from crop residue are highly reactive in the soil environment. The red clover PhC

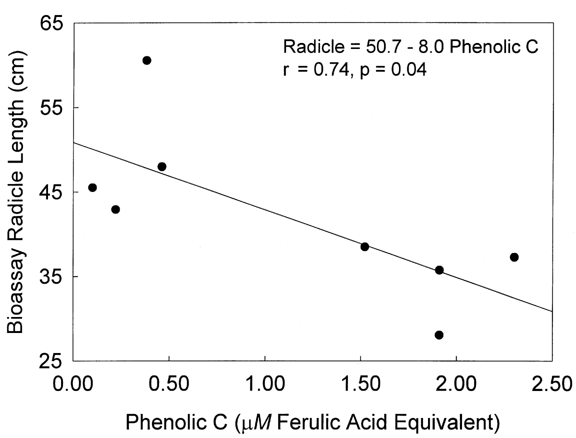

Fig. 3. Relationship between wild mustard bioassay radicle length and water soluble phenolic carbon concentration at 8 DAI.

concentration data combined with the incorporation of 2520 kg ha^{-1} above-ground biomass plus the 970 kg ha^{-1} below-ground biomass yields an estimate of 30 kg ha^{-1} of phenolic C added to the green manure-amended plots in excess of what was added in the control plots. The PhC content was low in the soil : water extract with a range between 0.2 and 3.8 μmol kg^{-1} soil ferulic acid equivalent (Doolan, 1997). The predicted concentration of PhC would be 330 μmol kg^{-1} soil ferulic acid equivalent assuming that all of the PhC was dissolved in the soil water present at 20% soil moisture level. The much lower levels of PhC found indicates that phenolic compounds released by the decomposing clover residue were sorbed and/or oxidized within eight days of incorporation by the soil. Reversibly sorbed phenolics determined by the citrate extraction (Blum, 1997) was much greater than the soluble soil : water extract phenolics with a range between 300 and 550 μmol kg^{-1} soil (Doolan, 1997).

Recently, Ohno and First (1998) have shown that the citrate extraction of soils used in conjunction with the Folin–Ciocalteu methodology for PhC determinations suffers from extensive positive interference because of the presence of dissolved organic matter in soil solutions. The use of the Folin–Ciocalteu method for PhC determination in aqueous extracts were much less problematic. Although it is difficult to determine unambiguously both the soil : water extract and reversibly sorbed PhC content in these soils, the increase in PhC content of the clover amended soils relative to the control values should be an estimate of the quantity of phenolics released by the residue (Ohno and First, 1998). Both the corrected soluble and sorbed phenolic concentrations are elevated after incorporation (Fig. 4). The estimated increases in PhC in the clover soils are similar to the concentrations of 130 μmol kg^{-1} soil of 4-hydroxy-cinnamic acid which decreased the biomass of morning-glory seedlings (Blum, 1996).

Both the water soluble and citrate extractable PhC contents showed dual peaks in concentration (Fig. 4). It is believed that the initial peak is directly related to the incorporation of the red clover resulting in the release of phenolic compounds to the soil. The lag in the citrate extractable PhC peak in the first half of the growing season suggests that the clover-derived PhC may remain in the soil solution for up to several weeks. However, the phytotoxicity was present only

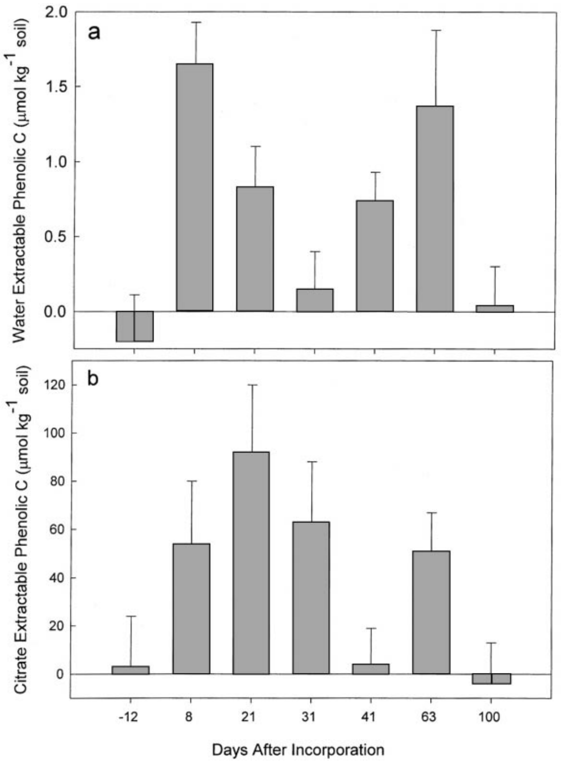

Fig. 4. Adjusted phenolic carbon content of the red clover amended soils over the growing season: (a) 1 : 1 soil–water extract and (b) 0.25 M sodium citrate extract. The error bars are standard deviations.

in the immediate sampling after red clover incorporation (Fig. 1). This is probably because of the close relationship between phytotoxicity of phenolic acids (Yu and Matsui, 1997) and oxidation reactivity with soils (Lehmann et al., 1987).

Table 1
Quantile distribution of soil pH and 1 : 1 soil–water extract concentrations of Ca, Mg and Al during the growing season in the wheat stubble and clover plots

Parameter	Quantile distribution (%)				
	100	75	50	25	0
Ca (mM)	2.32	0.45	0.16	0.12	0.06
Mg (mM)	0.65	0.11	0.05	0.04	0.02
Al (mM)	12.6	4.4	3.0	2.6	1.8
pH[a]	5.3	6.2	6.4	6.5	6.7

[a] Soil pH values were converted to molar H^+ concentrations prior to distribution analysis and reconverted to pH values.

The multiple sampling during the growing season allowed determination of the range of the major cation concentrations present in the soil : water extract. The dynamic nature of the soil solution cationic concentration can be seen in Table 1 which gives the quantile distribution of the dominant cation concentrations during the growing season. Increased concentrations in the divalent (Ca^{2+} and Mg^{2+}) and trivalent Al^{3+} cations may be ecologically significant because of formation of ion-pairs between these cations and phenolic acids (Martell and Smith, 1976; Hue and Licudine, 1999). Ionic complexation reactions are known to be sensitive to pH of the system (Martell and Smith, 1976). There is growing evidence for the theory that plant responses to elements that are essential to growth or phytotoxic are related to the concentrations of free, uncomplexed ions in solution (Parker et al., 1995). Elevation of cation concentration in soil solutions containing phenolic acids would decrease equilibrium concentrations of the uncomplexed form of the phenolic acid, and possibly, decrease its toxicity. The results suggest that legume cover crop incorporation could be used as a component of a cropping system that exploits allelopathy as one stress factor in weed control, but for allelopathy to be fully exploited as a method of weed control, the mechanisms by which it operates must be better understood.

Acknowledgements

Support for this work was provided to T.O. by agreement 96-35107-3274 of the USDA NRI Competitive Grants Program and Hatch funds from the Maine Agricultural and Forest Experiment Station. Partial support for the field work was provided to M.L. by agreement 93-37303-8909 of the USDA NRI Competitive Grants Program. Maine Agricultural and Forest Experiment Station Journal Publication 2357.

References

Blum, U., 1995. The value of model plant-microbe-soil systems for understanding processes associated with allelopathic interactions. In: Inderjit, Dakshini, K.M.M., Einhellig, F.A. (Eds.), Allelopathy: Organisms, Processes and Applications. American Chemical Society, Washington, DC, pp. 127–131.

Blum, U., 1996. Allelopathic interactions involving phenolic acids. J. Nematol. 28, 259–267.

Blum, U., 1997. Benefits of citrate over EDTA for extracting phenolic acids from soils and plant debris. J. Chem. Ecol. 23, 347–362.

Blum, U., Wentworth, T.R., Klein, K., Worsham, A.D., King, L.D., Gerig, T.M., Lyu, S.W., 1991. Phenolic acid content of soils from wheat-no till soils: development of soil extract bioassay. J. Chem. Ecol. 18, 2191–2221.

Box, J.D., 1983. Investigation of the Folin–Ciocalteu phenol reagent for the determination of phenolic substances in natural waters. Water. Res. 17, 511–525.

Dalton, B.R., Blum, U., Weed, S.B., 1989. Differential sorption of exogenously applied ferulic p-courmaric p-hydroxybenzoic and vanillic acids in soil. Soil Sci. Soc. Am. J. 53, 757–762.

Doolan, K.L., 1997. The allelopathic potential of red clover residue. M.S. Thesis. The Graduate School, University of Maine.

Dyck, E., Liebman, M., 1994. Soil fertility management as a factor in weed control: the effect of crimson clover residue, synthetic nitrogen fertilizer, and their interactions on emergence and early growth of lambsquarters and sweet corn. Plant Soil 167, 227–237.

Guenzi, W.D., McCalla, T.M., 1962. Inhibition of germination and seedling development by crop residues. Soil Sci. Soc. Am. Proc. 26, 456–458.

Hue, N.V., Licudine, D.L., 1999. Amelioration of subsoil acidity through surface application of organic manures. J. Environ. Qual. 28, 623–632.

Inderjit, 1996. Plant phenolics in allelopathy. Bot. Rev. 62, 186–202

Karlen, D.L., Varvel, G.E., Bullock, D.G., Cruse, R.M., 1994. Crop rotations for the 21st century. Adv. Agron. 53, 1–45.

Lehman, M.E., Blum, U., 1997. Cover crop debris effects on weed emergence as modified by environmental factors. Allelopathy J. 4, 69–88.

Lehmann, R.G., Cheng, H.H., Harsh, J.B., 1987. Oxidation of phenolic acids by soil iron and manganese oxides. Soil Sci. Soc. Am. J. 51, 352–356.

Liebman, M., Dyck, E., 1993. Crop rotation and intercropping strategies for weed management. Ecol. Appl. 3, 92–122.

Liebman, M., Ohno, T., 1997. Crop rotation and legume residue effects on weed emergence and growth: application for weed management. In: Hatfield, J.L., Buhler, D.D., Stewart, B.A. (Eds.), Integrated Weed and Soil Management. Ann Arbor Press, Chelsea, MI, pp. 181–221.

Makino, T., Takahashi, Y., Sakurai, Y., Nanzyo, M., 1996. Influence of soil chemical properties on adsorption and oxidation phenolic acids in soil suspension. Soil Sci. Plant Nutr. 42 867–879.

Martell, A.E., Smith, R.M., 1976. Critical Stability Constants, vol. 3, Other Organic Ligands. Plenum Press, New York.

National Research Council, 1993. Soil and Water Quality: An Agenda for Agriculture. National Academy Press, Washington, DC.

Ohno, T., First, P.R., 1998. Assessment of the Folin and Ciocalteu's method for determining soil phenolic carbon. J. Environ. Qual. 27, 776–782.

Parker, D.R., Norvell, W.A., Chaney, R.L., 1995. GEOCHEM-PC: a chemical speciation program for IBM and compatible person computers. In: Loeppert, R.H., Schwab, A.P., Goldberg, S. (Eds.), Chemical Equilibrium and Reaction Models. Soil Sci. Soc. Am., Madison, WI, pp. 253–269.

Weston, L.A., 1996. Utilization of allelopathy for weed management in agroecosystems. Agron. J. 88, 860–866.

White, R.H., Worsham, A.D., Blum, U., 1989. Allelopathic potential of legume debris and aqueous extracts. Weed Sci. 37, 674–679.

Whitehead, D.C., Dibb, H., Hartley, R.D., 1981. Extractant pH and the release of phenolic compounds from soils, plant roots, and leaf litter. Soil Biol. Biochem. 13, 343–348.

Yu, J.Q., Matsui, Y., 1997. Effects of root exudates of cucumber (*Cucumis sativus*) and allelochemicals on ion uptake by cucumber seedlings. J. Chem. Ecol. 23, 817–827.