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Uncovering Plant Growth-Mediating Allelochemicals Produced by Soil Microorganisms

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Coevolving interactions between a plant population and its microbiota can potentially yield a rhizosphere enriched in metagenomes containing the blueprints for a vast array of natural products. We describe a method of isolating those metabolites through activity-based screening of soil metagenomic libraries. The method allows for the isolation of small molecules produced in vector–host expression systems containing large-insert DNA fragments extracted from the target plant rhizospheres. Allelopathic activities derived from selected clones were screened against a series of controls. Nonmetric multidimensional scaling (NMS) showed similar effects of the set of controls on lettuce growth, whereas annual bluegrass had a broader range of growth responses. Methanol extracts from clones indicating activity showed distinct patterns in grass seedling growth from the empty vector control, but the same extracts showed no effect on lettuce. The results indicate that the metagenomics method and bioassay screen of clone extracts are tools that can be used for initial determination of allelopathic activity from noncultured soil microbiota.

Nomenclature: Annual bluegrass, *Poa annua* L.; lettuce, *Lactuca sativa* L.

Key words: Allelochemical, bioherbicide, metagenomics, natural product, phytotoxic.

The accumulation of deleterious microbiota in the rhizosphere of plants performs an important role in structuring plant communities. Negative feedback responses involving deleterious microorganisms help maintain plant diversity by keeping dominant species in check and by facilitating the establishment of rare or immigrating plant species in a community (Petermann et al. 2008; Reynolds et al. 2003). In turn, plants have coevolved strategies to resist attacks by pathogens, primarily through innate immunity, but studies on disease suppression indicate that plants can accumulate beneficial microbiota that antagonize pathogens (Hadar and Papadopoulou 2012; Mazzola and Gu 2002; Raaijmakers et al. 2002). The composition of deleterious and beneficial microorganisms is due to a variety of factors including plant species or genotype, land–crop management, soil type, and other microorganisms and macrofauna present in the soil (Berendsen et al. 2012; Weller et al. 2002). Accumulation of beneficial microbiota can have an equally important role in structuring plant communities by partitioning offensive and defensive strategies to microbiota in the root zone, without relying on plant immunity defenses. For example, antibiotic production by beneficial microbiota is one of the effective strategies for protecting host plants from pathogens (Haas and Keel 2003; Raaijmakers and Mazzola 2012). Altogether, the collection of microorganisms coevolving with their plant hosts can serve as genetic reservoirs for biosynthetically produced allelochemicals.

Isolating these products of coevolutionary interactions could be valuable to plant production and land management, but most soil microorganisms remain elusive for research. It is estimated that $< 1\%$ of microorganisms imaged on a microscope have been cultured for isolation (Torsvik et al 1990). Although most soil microorganisms are unable to grow under laboratory conditions using standard solid or liquid media, microbial secondary metabolites, which include antibiotics and growth-promoting compounds, can be isolated using metagenomic methods combined with activity screening. Several cultivated bacteria known to produce allelopathic compounds have been useful to herbicide discovery; these include members of the Actinobacteria: *Streptomyces hygroscopicus*, *Streptomyces saganonensis*, *Streptomyces* sp. 8E-12, *Streptomyces viridochromogenes*, and *Streptomyces acidiscabies* (Barazani and Friedman 2001); many more uncultivated microorganisms are likely to produce natural products relevant to industry.

Metagenomic techniques have advanced greatly in the past two decades, allowing for natural product discovery of enzymes, antibiotics, and other important compounds (Lorenz and Eck 2005; Schloss and Handelsman 2003). Similar techniques can be developed to isolate allelopathic compounds produced by soil microorganisms (Kao-Kniffin et al.

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2013). Isolation of allelopathic compounds produced by soil microorganisms requires obtaining the complete set of biosynthetic genes coding for production of the compound (Rutledge and Challis 2015). Metagenomic approaches allow the capture of DNA fragments coding for biosynthetic pathways into large-insert vectors that range from 20 kb to > 100 kb in size. The DNA regions are subsequently cloned into vector-host systems that are typically modified *Escherichia coli* or *Streptomyces lividans* strains. The production of several allelopathic compounds synthesized by bacteria is coded in genes or gene clusters that are in the appropriate size range for cloning. L-phosphinothricin (one component of the ammonium salt herbicide, glufosinate) biosynthetic genes total approximately 40 kb (accession number AY632421.1, GenBank), whereas a known herbicidal antibiotic, nigericin, has biosynthetic genes, totaling 95.7 kb (accession number DQ354110, GenBank).

If a clone contains the complete biosynthetic pathway of an allelochemical, and the host is able to recognize the genetic mechanisms, the clone will produce the allelochemical and either secrete the compound or present it on the cell surface. The next step is to select potentially allelopathic clones from among thousands. Previous research has shown that host organisms expressing small, bioactive molecules, such as antibiotics, will change color, colony morphology, or start to exhibit antibiosis activity (Brady 2007). An initial screen for clones indicating small, bioactive molecule production can be used, followed by screening the activity of selected clones' compounds with plant bioassays.

We outline here a metagenomics-based method and bioassay to examine rhizosphere soil for allelochemicals produced by cultivation-independent microbiota. First, DNA is extracted directly from rhizosphere soil (without prior cultivation), purified, and cut into large fragments using restriction enzymes. The fragments of DNA are then ligated into vectors that are taken up by competent *Escherichia coli* hosts through transformation. Colonies of transformed *E. coli* that show phenotypic changes in pigmentation, morphology, texture, or activity (antibiosis) are selected in this initial screen for compound isolation. The selected clones are regrown and compounds are extracted using methanol. The methanol extracts are screened for growth responses affecting plants using a seedling bioassay. We include in this article a series of controls to determine allelopathic activity of compounds isolated from selected clones and to validate the method.

Materials and Methods

Buffers and Media. All buffers and media were prepared with MQ water and kept sterile. Z buffer contained 0.1 M Tris–HCl pH 8, 25 mM NaH_2PO_4, 25 mM Na_2HPO_4, 0.1 M ethylenediaminetetraacetic acid (EDTA) pH 8, 1.5 M NaCl, 1% cetyltrimethylammonium bromide (CTAB). Buffer $T_{10}E_{10}$ consisted of 10 mM Tris–HCl pH 8, 10 mM EDTA pH 8. Buffer $T_{10}E_1$ contained 10 mM Tris–HCl pH 7.5, 1 mM EDTA pH 8.0. Phage dilution buffer contained 10 mM Tris–HCl pH 8.3, 100 mM NaCl, 10 mM $MgCl_2$. Medium Luria-Bertani broth (LB) + chloramphenicol (cm) + induction solution (IS) (Epicenter, Madison, WI) contained per L: 10 g tryptone, 10 g NaCl, 5 g yeast extract, 15 g agar, chloramphenicol (cm; 20 μg ml^{-1}), and induction solution (IS, CopyControl Fosmid Kit, Epicenter; 1 ×). Soy flour mannitol (SFM), contained per L: 20 g mannitol, 20 g organic soy flour, 20 g agar.

Soil Preparation. Soil was collected from a population of a single common ragweed plant (*Ambrosia artemisiifolia* L.) growing in an organic cropping system in Freeville, NY, August 2011. The plant was pulled with root mass intact, the aboveground biomass was removed, and the loose soil was shaken from the roots three times before storage of the root mass and rhizosphere soil at −20 C. To harvest rhizosphere soil, the root mass was thawed at room temperature (22 C ± 2 C), and rhizosphere soil was gently dislodged from the roots. The soil was sieved through a sterile 2 mm mesh sieve. Roots > 0.5 mm in diameter were removed with sterile forceps.

Ethanol and Isopropanol Precipitation. Ethanol or isopropanol precipitation was used during library construction to clean and concentrate DNA aliquots. Ethanol precipitation began with the addition of one-tenth volume of 3 M sodium acetate and 25 times the volume of ambient 100% ethanol. After mixing via inversion, the sample was placed at −80 C for 30 min. The sample was then centrifuged at 4 C for 20 min at 12,000 × *g* (n = 5). The supernatant was removed, and the pellet was washed with 70% cold ethanol and inverted to mix. The sample was centrifuged at 4 C for 5 min at 12,000 × *g* (n = 5). The supernatant was removed, the pellet dried and resuspended in $T_{10}E_1$ buffer by incubation for 5 min at room temperature.

The first step of isopropanol precipitation was the addition of 7/10th volume of 100% isopropanol and incubation at room temperature for 1 h. The sample

was centrifuged at 12,000 $\times g$ (n = 5) for 20 min at 4 C, and the supernatant was removed. The pellet was gently washed with 70% cold ethanol and centrifuged for 5 min at 12,000 $\times g$ (n = 5). The supernatant was removed and the pellet was air dried and resuspended in $T_{10}E_1$ buffer in a similar way as ethanol precipitation.

Freeze–Thaw DNA extraction. DNA extraction was a combination of a freeze–thaw method and CTAB–sodium dodecyl sulfate (SDS) lysis (Zhou et al. 1996). In an autoclaved, 250 ml centrifuge tube, the 50-g soil sample was combined with 75 ml Z buffer. The soil slurry was frozen in an ethanol–dry ice bath and then in a 65 C water bath for 40 min at each step. Lysozyme was added (1 mg ml^{-1}) and incubated at 37 C for 15 min. Then the soil slurry was put through another freeze–thaw set. Next, each sample got 9 ml of 20% SDS and 4.5 ml of 5M guanidinium thiocyanate before 2 h at 65 C with inversion every 20 min. Samples were centrifuged at 10 C for 20 min at 12,000 $\times g$ (n = 5), and the supernatant was collected.

Chloroform : isoamyl alcohol (25 ml; 24 : 1) was added to each sample's supernatant and mixed gently for 10 min. The mixture was centrifuged for 10 C for 20 min at 12,000 $\times g$ (n = 3), and the aqueous phase was moved to a clean tube. Isopropanol (100%) was added at 70% of the aqueous phase volume. Samples were shaken for 5 min and incubated, undisturbed, for 20 min at room temperature. Following centrifugation at 10 C for 40 min at 12,000 $\times g$ (n = 3), the supernatant was removed, and the DNA pellet was resuspended with a wide-bore pipette tip in a minimum amount of $T_{10}E_{10}$ pH 8 solution.

An equal volume of Tris-buffered phenol-chloroform was added to the extracted DNA. Following inversion, centrifugation occurred at 12,000 $\times g$ (n = 3) for 10 min. The aqueous layer was moved with a wide-bore tip, placed into a clean tube, and combined with an equal volume of chloroform : isoamyl alcohol (24 : 1). Following inversion and centrifugation for another 10 min, the DNA-containing aqueous layer was removed with a wide-bore tip and stored for short-term analysis at 4 C or for long-term analysis at −80 C.

Size Selection of DNA with Pulsed Field Gel Electrophoresis and Electroelution. Pulsed field gel electrophoresis was completed using a 1% low melting point agarose in 0.5× Tris-acetate-EDTA (TAE) buffer. Samples were prepared with loading dye, and the complete sample was loaded into the gel. A pulsed feed gel (PFG) ladder (MidRange PFG Marker II, New England BioLabs), 40 kb control (Epicentre kit), and 1 kb ladder (Promega) were used as standards. The gel was run for 4 h at 8 V, 120°, 3- to 10-s switch, linear ramping factor. Using a ruler and scalpel, the control lanes and the edge of sample lanes were cut out. These sections were stained with ethidium bromide and visualized next to a ruler. By using the stained edges of sample lanes, the location of DNA in the 20 to 60 kb area of the unstained sample lanes was found and cut out. The gel piece was placed in 0.5× TAE on ice for 20 min until electroelution.

Electroelution was used to collect the DNA from the gel piece in a dialysis membrane (Strong et al. 1997). After preparing the dialysis membrane (Spectra/Por MWCO 12-14,000, 45 mm in flat width, 29 mm in diameter), according to manufacturer's instructions, the membrane was folded and closed at one end. The gel slice was placed into the bag, and bubbles were minimized. Excess buffer and bubbles were removed before closing the dialysis membrane. The dialysis bag was positioned in the gel box containing 0.5× TAE at 4 C, so that DNA would move out of the gel and remain in the bag. The system was run overnight at 3 V cm^{-1} between electrodes. Before removing the dialysis membrane, the current was run in reverse for 5 min to ensure that any DNA attached to the inside of the bag was removed.

Using a wide-bore pipette tip, the DNA solution in the dialysis membrane was removed and placed in a microcentrifuge tube. Cold 0.5× TAE was used to rinse the inside of the membrane, removed with a wide-bore tip, and combined with the rest of the DNA. Ethanol precipitation was then performed, and the DNA resuspended in $T_{10}E_1$ buffer.

Library Construction. Blunt-end repair of sample DNA was completed according to the fosmid kit (CopyControl Fosmid Library Production Kit, Epicentre) or End-It DNA end-repair kit (Epicentre). Several modifications from the manufacturer's instruction were performed. Enzyme inactivation was held at 70 C for 30 min. Then, the DNA was cleaned using isopropanol precipitation. Ligation was completed with the pCC1FOS vector in a 20-µl reaction. Packaging of ligated DNA was completed with MaxPlax Lambda packaging extracts and the *E. coli* EPI300-T1 plating strain. The packaged DNA was stored at 4 C for up to 7 d.

To test the infection efficiency of *E. coli* EPI300-T1, a fresh EPI300 culture, same-day, was grown in LB + 10 mM $MgSO_4$ + 2% maltose and incubated at 37 C, 200 rpm (n = 2) until optical density 600 nm (OD_{600}) was 1.4 to 2.0. Undiluted phage and 1 : 10 phage diluted in phage dilution buffer were tested on LB + cm. Packaged DNA was combined with EPI300 at a 1/10th concentration, incubated at 37 C for 1 h, and plated on LB + cm (20 μg ml^{-1}). Inoculated plates were incubated overnight at 37 C before calculating packaging efficiency.

To ensure insert diversity, cultures of 10 to 20 clones were incubated in LB + cm + IS at 37 C at 200 rpm (n = 2) overnight. Plasmids were harvested using a modified protocol for a plasmid miniprep kit (Qiagen, Venlo, the Netherlands). The clones were pelleted with a centrifuge at 3,000 $\times g$ (n = 1) for 20 min at 4 C. The pellet was resuspended with 250 μl of P1 buffer and 250 μl of P2 lysis buffer before inverting. After 2 min, N3 neutralization buffer (350 μl) was added, inverted, and allowed to sit for 5 min. Next, chloroform (300 μl) was added, shaken well, and centrifuged at 12,000 $\times g$ (n = 1) for 10 min. The aqueous layer was transferred to a fresh tube, and isopropanol precipitation was used to clean and collect the DNA. The clones' DNA was digested using *PstI* (New England BioLabs) at 37 C for 2 h. Each digest was run on a 1% agarose gel to check for a variety of banding patterns. If only two or three patterns occurred, ligation was considered unsuccessful.

Library Storage. A larger phage infection was run based on optimal dilution from test infection. Using a same-day, fresh EPI300 culture, the packaged DNA was combined with EPI300 in small aliquots and incubated for 1 h at 37 C. All infected *E. coli* cells were plated on LB + cm at 37 C for 24 to 48 h. Based on the number of clones per plate, LB + cm with glycerol (25%) was added to plates of clones. A sterile glass spreader was used to scrape colonies and mix into the LB + cm and glycerol solution. The clone suspension was mixed well with a pipette and aliquoted into subpools containing approximately 10,000 clones. All plates and clones were collected into 26 subpools, and an aliquot of all subpools was mixed to form a mastermix of the library for storage at −80 C.

Screening. An aliquot of one −80 C subpool stock was added to 2 ml LB broth and incubated for approximately 2 h at 37 C, 200 rpm (n = 2), or until $OD_{630} > 0.075$. The culture was then diluted aseptically in 1× phosphate-buffered saline to achieve approximately 50 to 200 colony-forming units $plate^{-1}$ on LB + cm medium with incubation at 37 C overnight. Plates were then incubated at room temperature (22 ± 2 C) for 7 d and observed for unusual morphology, pigmentation, or antibiosis activity (Brady 2007). To screen for antibiosis activity, plates were overlaid with molten LB containing *Escherichia coli* (empty pCC2FOS vector), *Bacillus subtilis* BR151 pTMH33 (Henkin and Sonenshein 1987), or spores from *Trichoderma harzianum* strain T22. *E. coli* and *B. subtilis* overlays were incubated for 24 h at 37 C and monitored for 7 d at room temperature. *T. harzianum* overlays were incubated at 30 C for 2 to 3 d, and monitored for 7 d at room temperature. Any clones showing changes in pigmentation, colony morphology, or antibiosis activity were isolated and stored as −80 C glycerol stocks.

Crude Extract. Clones were grown from glycerol stocks as lawns on plates of LB + cm + IS overnight at 37 C and then incubated at room temperature for 7 d. Plates were then placed at −20 C overnight. Then, the frozen lawn was chopped into pieces of approximately 5 by 5 mm and placed into a sterile glass bottle with pure methanol (approximate 100 ml) and incubated at room temperature for 2 d at 200 rpm (n = 2). The slurry was then filtered through a coarse-glass filter unit (40 to 60 μm pore size) to remove agar, and the resulting supernatant was transferred to a preweighed beaker. The methanol was allowed to completely evaporate in a fume hood. The remaining solids, i.e., crude extract, were diluted to 5 mg ml^{-1} in pure methanol and sonicated for 30 min. The resulting extract was then aliquoted into glass vials, sealed, and stored at −80 C until testing.

Allelopathic Activity. Crude extracts were added to the bottom of a sterile petri plate (1 mg extract ml^{-1} agar), the methanol was allowed to evaporate, and then 20 ml of sterile molten agar (1.5%). Once the plate cooled, surface-sterilized seeds of pregerminated lettuce and annual bluegrass were placed on the surface. Seeds were surface-sterilized by placement into 10% bleach (Clorox, Oakland, CA) for 5 min, followed by three rinses with sterile MQ water for 5 min each, and the seeds were air dried. Germination was initiated by incubating surface-sterilized seeds in sterile MQ water for 1 or 3 d for lettuce and bluegrass, respectively. Germinated seeds were placed on the agar containing crude extract, the plates were stored vertically under light at room

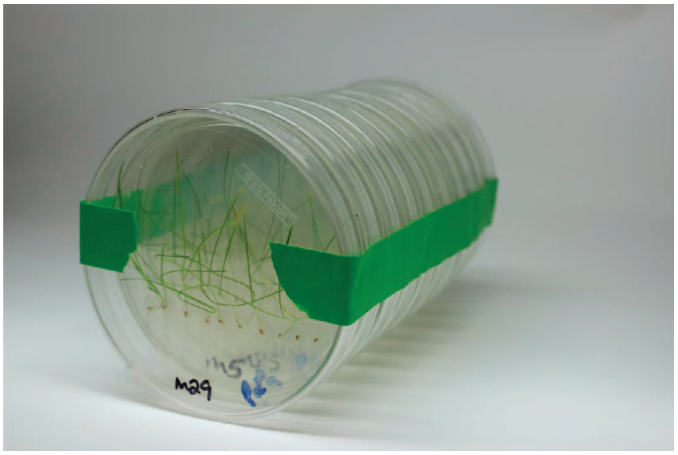

Figure 1. Allelopathic activity bioassay plates. Crude extracts were prepared for screening by adding 10 mg of the extract to the bottom of a sterile petri plate, allowing the methanol to evaporate completely, and then layering 20 ml of sterile molten agar a final concentration of 1 mg extract/ml agar. Surface-sterilized seeds of pregerminated lettuce and annual bluegrass were screened for growth development. The plates are arranged vertically to facilitate seedling contact with compounds on agar. (Color for this figure is available in the online version of this article.)

temperature with 14/10 h light/dark days for 2 wk (Figure 1).

At 2 wk, lettuce and annual bluegrass seedlings were measured by several parameters: longest root or primary root length, shoot height, number of leaves, number of roots, and dry biomass (lettuce only). As described in Table 1, a series of controls were used to determine the effectiveness of the allelopathic bioassay from crude extracts produced by clones of interest. Negative controls included agar only, methanol only, extract of LB medium only, and *E. coli* with an empty vector grown on LB + cm + IS. To test for methanol extraction efficiency, glufosinate was added to LB medium at 4 kg ai ha^{-1} (157 μg ml^{-1}) before methanol extraction, and for a diffusion control, glufosinate was added to the surface of the agar plate (4 kg ai ha^{-1}). This amount of Finale was used to simulate application rates in the field. Positive controls for the culturing process consisted of *Streptomyces albus* subsp. *chlorinus* (B-24108) (producer of albucidin) grown on SFM medium, using a paired negative control of SFM medium only. Another positive control included a *Streptomyces lividians* clone containing the L-phosphinothricin (part of synthetic glufosinate) biosynthetic pathway and grown under conditions known to elicit production (Blodgett et al. 2005).

Dimethyl Chloride Extraction and Analysis. *E. coli* clones empty and m10 were chosen for further extraction and detailed analysis because of the unique set of bioassay responses detailed in Figure 2 and Table 2. Large volumes of cultures were grown overnight at LB + cm 37 C on 150 rpm (n = 1). The cultures were then centrifuged at 860 $\times g$ (n = 1) for 15 min at 4 C (Beckman Coulter Avanti j20xp centrifuge). The cell pellet was extracted with methanol, sonicated for 5 min, centrifuged, and the supernatant dried down. The culture supernatant was combined with dimethyl chloride in excess of a 1 : 2 ratio. The extraction was shaken several times, and the excess gas was released in a fume hood. Then, the organic layer was collected, the extraction was repeated two more times, and the organic fractions were combined. Water was removed from the organic fraction, using excess sodium sulfate, and filtered. A rotary evaporator was used to dry the organic fraction, and the extract was resuspended in pure methanol and stored at −80 C until analysis.

The methanol pellet extract and dimethyl chloride supernatant extract were analyzed using gas

Table 1. List of controls used to test the metagenomic method's effectiveness for herbicide discovery.[a]

	Before extraction				
Name	Medium	Added to medium	Present with seeds	Herbicide	Microorganism
No treatment					
Methanol			Methanol		
LB medium	LB + cm + IS		Extract		
Empty vector	LB + cm + IS	*E. coli* with empty vector	Extract		*E. coli*
E. coli + PTT pathway	LB	*E. coli* with PTT pathway	Extract	PTT	*E. coli*
LB + glufosinate	LB	Glufosinate, 4 kg ai ha^{-1}	Extract	Glufosinate	
Glufosinate without extraction			Glufosinate, 4 kg ai ha^{-1}	Glufosinate	
Streptomyces albus	SFM	*Streptomyces albus* subsp. *chlorinus*	Extract	Albucidin	*Streptomyces albus* subsp. *chlorinus*
SFM medium	SFM		Extract		

[a] Abbreviations: LB, Luria-Bertani broth; cm, chloramphenicol; IS, induction solution, w/, with; *E. coli*, *Escherichia coli*; PTT, phenylalanine, tyrosine, and tryptophan; SFM, soy flour mannitol.

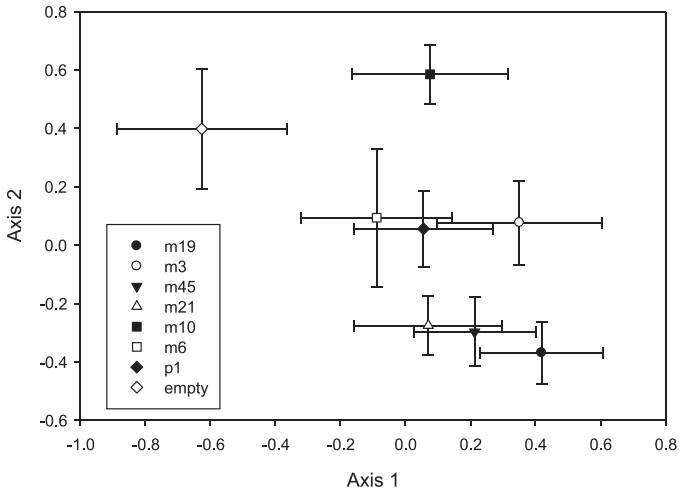

Figure 2. Similarities and dissimilarities in seedling responses to bioactive clone compounds. Nonmetric multidimensional scaling (NMDS) analysis of clone extracts having significant effects on annual bluegrass seedlings, in reference to the *Escherichia coli* empty vector control. Bars are ± 1 SE for replicates $n = 9$ to 10.

chromatography (GC) and liquid chromatography (LC), both coupled with mass spectrometry (MS). Aliquots of samples were prepared for GC-MS by first drying the extract in a vacuum concentrator within a fume hood. Derivatization was completed by resuspending samples in methoxyamine hydrochloride (20 mg ml^{-1} in pyridine), incubating for 2 h at 37 °C with 950 rpm (n = 1), adding *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide, and incubating again at 37 C for 30 min at 300 rpm (n = 1). Samples were analyzed on GC-MS (Agilent 7890A GC/5975 MS) with a capillary column (DB-5MS, 20 m by 0.18 mm by 0.18 μm) with a Duraguard column (5 m, Agilent). Injection of the sample (1 μl) was at 230 C in splitless mode using a helium carrier gas at a flow rate of 1 ml min^{-1}. The temperature program was isothermal for 5 min, 70 C for 2.471 min followed by 10.119 C min^{-1} ramp to 330 C with a final hold at 33 C for 2.471 min and further cooling before the next run. Mass spectrometry was run with an electron ionization detector at 230 C. Data were collected at 5.6 scan s^{-1} over a mass-to-charge ratio of 50 to 600 scan range. The LC-MS analysis was run on an Accela-TSQ Quantum Access (Thermo Fisher Scientific, Waltham, MA) with a Gemini-NX (150 mm by 2.1 mm, 3 μm; Phenomenex, Torrance, CA) column. The mobile phase was 0.1% formic acid in acetonitrile (A) and 0.1% formic acid in MQ water (B). The sample (10 μl) was injected over a gradient of 10% A for 3 min, a ramp to 100% A over 30 min, and then back to 10% A in 5 min. The mass spectrometry was run in two ways: positive and negative electrospray ionization with runs over 150 to 1,050 units with a scan time of 0.65 s.

Statistics. Data were analyzed in JMP Pro 10 (SAS Institute, Cary, NC) and PC-ORD (MjM Software Design, Gleneden Beach, OR). Data were first tested for outliers using Cook's D, and the data transformed as necessary to achieve normal distribution. Values of zero for log transformed data were handled as previously described (McCune and Grace 2002). In PC-Ord, data were converted to z scores to allow equal statistical comparisons across different units and magnitudes (Kenkel 2006). Outliers were identified as seedling samples that fell outside of 2 SD from the average Euclidean distance and were removed from the data set. Because of the samples' lack of normality, nonmetric multidimensional

Table 2. Effect of clone extracts on annual bluegrass and lettuce physical properties. Means ± shown with Tukey's analysis. Different letters indicate significance at P value of ≤ 0.05 within columns. Only clones which showed significance with multivariate analysis are presented, $n = 9$ to 10.[a]

Plant	Clone	Leaves	Height	Root length	Roots	
		No.	mm		No.	
Annual bluegrass	Empty	1.4 ± 0.16 b	31.8 ± 2.52 c	33.1 ± 2.40 a	3.4 ± 0.27 ab	
	M19	2.0 ± 0.00 a	44.3 ± 1.58 a	38.0 ± 2.56 a	4.1 ± 0.23 a	
	M3	1.9 ± 0.10 a	45.4 ± 2.31 a	38.7 ± 2.41 a	2.9 ± 0.28 bc	
	M45	2.0 ± 0.00 a	37.8 ± 1.91 abc	36.7 ± 2.78 a	4.2 ± 0.13 a	
	M21	1.9 ± 0.10 a	41.9 ± 1.82 ab	34.9 ± 2.22 a	3.8 ± 0.25 ab	
	M10	1.9 ± 0.11 ab	34.3 ± 2.72 bc	38.2 ± 2.39 a	2.3 ± 0.17 c	
	M6	1.9 ± 0.13 ab	44.5 ± 3.18 ab	32.4 ± 2.25 a	2.4 ± 0.32 c	
	P1	1.6 ± 0.16 ab	46.2 ± 1.81 a	37.1 ± 1.59 a	3.4 ± 0.22 ab	
			Stem length	Primary root length		Biomass
			mm			g
Lettuce	Empty	3.0 ± 0.00 a	8.7 ± 0.7 a	59.4 ± 5.57 a	3.4 ± 0.50 a	1.239 ± 0.095 a
	M10	2.6 ± 0.16 b	11.6 ± 1.17 a	44.3 ± 4.94 a	2.9 ± 0.62 a	0.932 ± 0.080 b

Table 3. The effect of various controls on lettuce characteristics. Data are presented untransformed, whereas statistics, including Tukey's analysis, were performed on transformed data. The effect of phenylalanine, tyrosine, and tryptophan (PTT) was performed at a separate time, and a corresponding empty vector set of results are shown. These results were analyzed with a Student's *t* test.[a,b]

	Leaves	Stem length	Secondary roots	Longest root	Biomass
	No.	Mm	No.	mm	Mg
No treatment	2.9 ± 0.10 a	8.0 ± 0.77 bc	1.40 ± 0.37 a	61.40 ± 6.29 a	0.88 ± 0.08 b
Methanol	3.0 ± 0.00 a	7.7 ± 0.63 bc	2.50 ± 0.54 a	63.00 ± 6.32 a	0.89 ± 0.06 ab
LB medium	3.0 ± 0.00 a	12.0 ± 1.23 a	3.30 ± 0.56 a	65.30 ± 2.96 a	1.24 ± 0.08 a
LB w/ glufosinate	3.0 ± 0.00 a	12.8 ± 0.88 a	3.20 ± 0.42 a	72.50 ± 5.56 a	1.25 ± 0.13 a
Glufosinate	2.0 ± 0.00 b	2.7 ± 0.37 d		8.80 ± 0.87 c	0.41 ± 0.04 c
SFM medium	3.0 ± 0.00 a	9.6 ± 1.05 abc	3.00 ± 0.52 a	77.10 ± 5.21 a	1.16 ± 0.06 ab
Streptomyces albus	3.0 ± 0.00 a	7.1 ± 0.55 c	0.20 ± 0.13 a	23.20 ± 1.46 b	1.00 ± 0.06 ab
Empty vector	3.0 ± 0.00 a	10.8 ± 0.63 ab	1.40 ± 0.45 a	64.80 ± 3.03 a	1.16 ± 0.10 ab
E. coli w/ PTT	3.2 ± 0.13 a	6.2 ± 0.59 a	5.1 ± 0.72 a	53.6 ± 8.39 a	1.65 ± 0.14 a
E. coli w/ empty vector	3.3 ± 0.15 a	6.0 ± 0.33 a	4.5 ± 0.48 a	60.1 ± 6.32 a	1.73 ± 0.18 a

[a] Abbreviations: LB, Luria-Bertani broth; w/, with; E. coli, Escherichia coli; SFM, soy flour mannitol.
[b] Different lowercase letters indicate significant differences within columns ($P < 0.05$).

scaling (NMDS) with a Euclidean distance metric and 500 iterations, 250 runs with data, and 250 runs with randomized data, was used to analyze each data set. Data sets were extracts and controls run at the exact time to prevent any effect on analysis from environmental differences. To compare extracts to the empty vector control, a multiresponse permutation procedure (MRPP) was used with Euclidean distance. Significance was set at $P < 0.05$ but corrected for multiple hypotheses by the Benjamin-Hochberg correction (Benjamini and Hochberg 1995; Waite and Campbell 2006). Extracts with significant effects during the multivariate analysis were then reanalyzed using univariate ANOVA and post hoc Tukey analysis to identify the positive or negative effects of the clone's extract on the seedlings.

Results and Discussion

Growth Response Patterns in Monocot vs. Dicot Using a Suite of Controls. Two plant species were selected for analyzing growth response patterns: a monocot, annual bluegrass, and a dicot, lettuce. These plants were chosen for their high germination rate and ease of use in the allelopathic assay. For lettuce, the NMDS and individual tests showed significant differences between controls ($P < 0.05$). Whole model and most of the individual measurements showed that *S. albus* subsp. *chlorinus* and glufosinate on agar (no extraction) showed the greatest difference in decreased stem length, primary root length, and number of secondary roots (Table 3). Only glufosinate on agar showed a decrease in the number of leaves and biomass. There was a slight nutrient effect on stem length with LB medium, empty vector, SFM, and LB with glufosinate, which is likely due to the growing medium because all controls hold this in common. Glufosinate medium that went through the methanol extraction protocol showed no allelopathic activity, which indicated a limitation with using methanol as a solvent. Methanol cannot capture all compounds; the compound's chemical characteristics could prevent efficient extraction resulting in a lack of plant growth response shown in the seedling bioassay. Extraction from a phosphinothricin- (glufosinate)-producing clone showed no effect on lettuce. Again, this was likely due to extraction techniques that were not optimal for the compound.

Similar results were seen when controls were tested with annual bluegrass. NMDS showed stronger significance by including leaf number, root number, longest root length, and shoot length. Significance at $P < 0.05$ was seen with NMDS and all the individual variables. The *S. albus* subsp. *chlorinus* and glufosinate on agar (no extraction) showed decreases in all variables but especially with root numbers and root length (Table 4). LB medium with glufosinate showed a slight decrease in root length but was not significantly different from most negative controls. Extract from a phosphinothricin (glufosinate)-producing clone significantly decreased the height and number of roots for annual bluegrass but increased root length in comparison to the empty vector control. When testing clones, the empty vector should always be included to determine clones with significant differences.

Extracts from Clones that Modify Plant-Growth Responses in Annual Bluegrass. A metagenomic clone library originating from common ragweed rhizosphere soil DNA was screened for promising

Table 4. The effect of various controls on annual bluegrass characteristics. Data are presented untransformed, whereas statistics, including Tukey's analysis, were performed on transformed data. The effect of phenylalanine, tyrosine, and tryptophan (PTT) was performed at a separate time and a corresponding empty vector set of results are shown. These results were analyzed with a Student's *t* test.[a,b]

Control	Leaves	Height	Roots	Longest root
	No.	mm	No.	mm
No treatment	1.22 ± 0.15 bc	26.11 ± 1.85 ab	3.11 ± 0.35 a	41.56 ± 3.17 a
Methanol	1.44 ± 0.18 bc	26.33 ± 2.76 ab	3.56 ± 0.38 a	37.67 ± 2.97 ab
LB medium	2.0 ± 0.0 a	39.40 ± 0.92 a	3.10 ± 0.23 a	37.70 ± 2.42 ab
LB w/ glufosinate	2.0 ± 0.0 a	40.78 ± 2.23 a	2.67 ± 0.29 a	30.22 ± 1.32 b
Glufosinate	1.0 ± 0.0 c	7.90 ± 0.96 c	1.10 ± 0.10 b	11.60 ± 1.60 c
SFM medium	1.7 ± 0.15 ab	33.60 ± 4.26 a	4.40 ± 0.31 a	39.10 ± 2.75 ab
Streptomyces albus	1.0 ± 0.0 c	15.50 ± 2.13 b	1.40 ± 0.40 b	11.00 ± 1.54 c
Empty vector	1.4 ± 0.16 bc	31.80 ± 2.52 a	3.40 ± 0.27 a	33.10 ± 2.40 ab
E. coli w/ PTT	2.0 ± 0.0 a	32.88 ± 2.80 b	4.13 ± 0.34 b	29.25 ± 2.38 a
E. coli w/ empty vector	2.0 ± 0.0 a	50.22 ± 3.52 a	5.44 ± 0.47 a	16.44 ± 1.77 a

[a] Abbreviations: LB, Luria-Bertani broth; w/, with; E. coli, Escherichia coli; SFM, soy flour mannitol.
[b] Different lowercase letters indicate significant differences within columns ($P < 0.05$).

extracts. Of the approximately 8,000 clones screened to date, 28 clones were confirmed to have indications of small-molecule production using the initial screen (0.38% positive clones). These clone extracts were screened for annual bluegrass and lettuce seedling growth effects. Following multivariate statistical analysis, seven clones showed a significant effect on annual bluegrass as shown in Figure 2. NMDS analyses takes into account all of the seedling physiological traits measured and plots points based on these metrics. Samples that aggregate closely indicate similarity in the full suite of seedling physiological responses affected by compounds from the bioactive clones. Once the effects of the clone compounds were checked using univariate and post hoc Tukey analyses, we found that the clones positively affected seedling leaf number and height, but mixed results were obtained with the number of roots (Table 2). Lettuce was affected by a single clone, m10 (Table 2). Although m10 had a positive effect on annual bluegrass; the clone's extract had a negative effect on lettuce leaf number and biomass.

Compounds Produced from Clones. Two cultures were prepared for further analysis with GC-MS and LC-MS: *E. coli* containing an empty vector and the clone identified as m10. Dimethyl chloride extraction was carried out on the two culture supernatants, and methanol extraction was performed on cell pellets. GC-MS results only showed differences in compounds able to be volatized in the supernatant with no differences seen in the pellet samples. Increases in peaks that were found in m10 preparation occurred at 8.41 and 21.40 min (Figure 3). LC-MS was expected to illustrate more of the compounds present in the liquid phase that would affect lettuce and annual bluegrass growth. LC with MS using negative electrospray ionization yielded more unique peaks in the clone m10 pellet extracted with methanol than in the empty vector control (Figure 4). This indicates that it is likely that the compound of interest was exposed on the surface or was unable to transition across the *E. coli* host membrane. None of the unique peaks matched known compounds. The differences indicate this set of subtle changes synergistically affected the model plant species. More research would be necessary to identify the compounds in greater detail.

The overall set of techniques we describe in this article have several concerns that should be noted and possibly alleviated in future research. Building the metagenomic library has caveats covered in previous reviews (Ekkers et al. 2012; Kao-Kniffin et al. 2013). Also, the initial screen might not be the best method for narrowing clones for further studies on allelopathic activity. The methanol extraction of

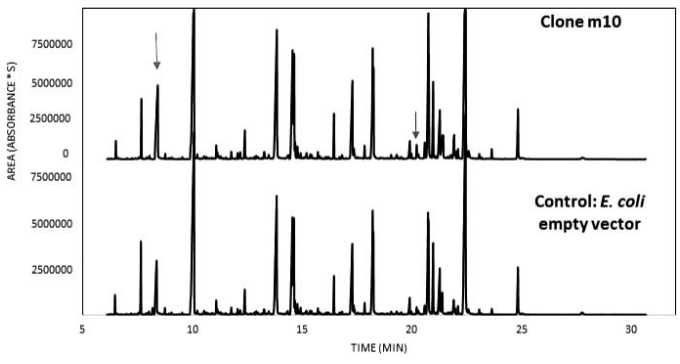

Figure 3. Analysis of compounds from a bioactive clone (m10) using gas chromatography–mass spectrometry. The top spectra show results for clone m10, and the bottom shows the empty vector control. The samples were extracted with dimethyl chloride. The arrows indicate increases in peak area for m10.

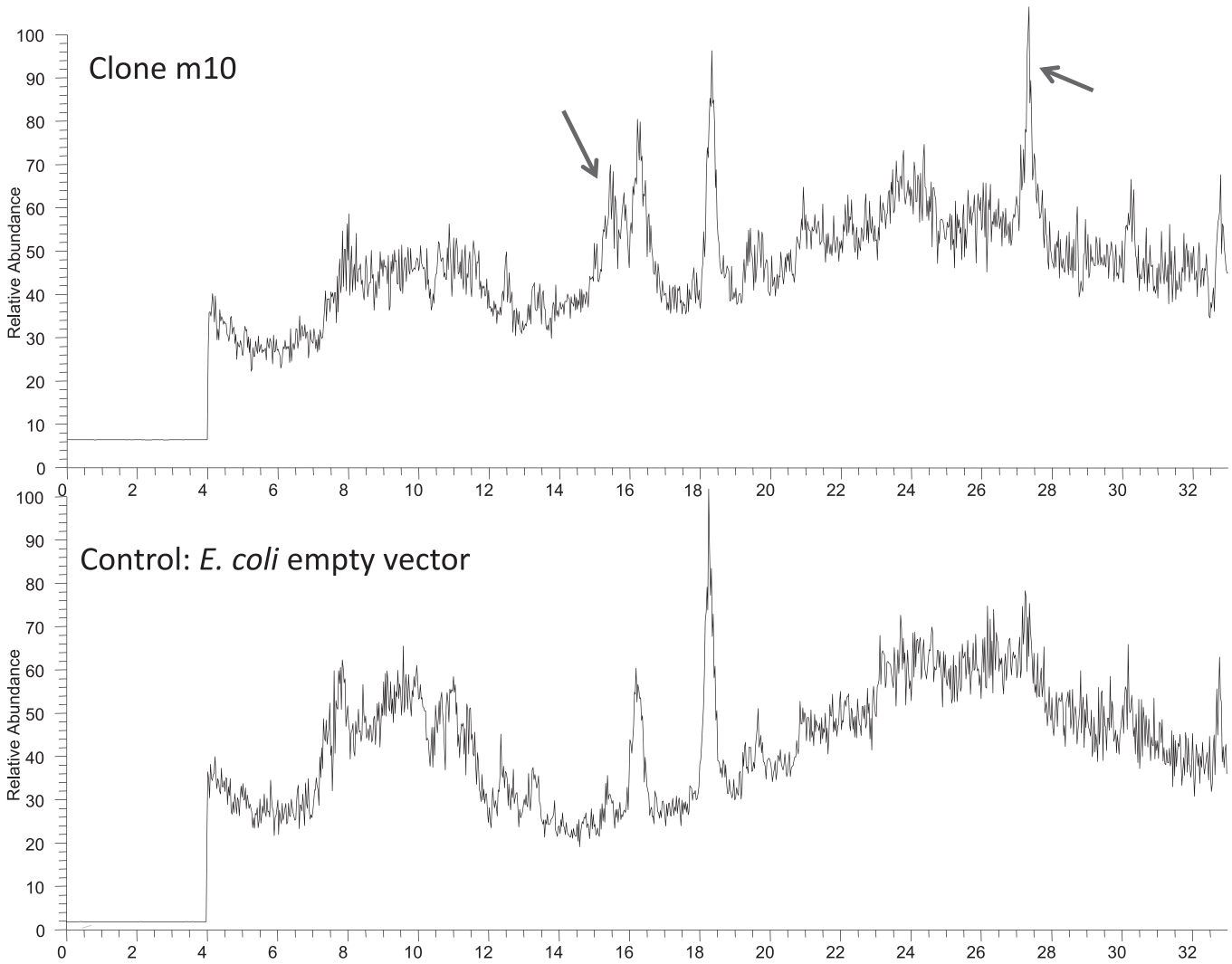

Figure 4. Analysis of compounds from a bioactive clone (m10) using liquid chromatography–mass spectrometry. The top spectra show results for clone m10, and the bottom shows the empty vector control. The samples were extracted with methanol. The arrows indicate increases in peak area for m10.

clones is meant to collect the broadest range of protein characteristics but could prevent discovery of very hydrophobic or hydrophilic herbicidal compounds. Future research could focus on collecting proteins with more extreme characteristics by changing the solvent. Attempts were made to test clones for allelopathic activity without any extraction but problems occurred with contamination, consistency, and efficiency. If allelopathic activity is found with a crude extract, optimized extraction techniques can be designed for the compound of interest. Extracts were screened at a final concentration of 5 mg ml^{-1} to be sure to capture all activity, even that of weaker compounds. The bioassay for allelopathic activity could fail to identify herbicide candidates with low yields in the extract or weak activity, which could be improved by optimizing protocols. Although our bioassay uses annual bluegrass and lettuce, the design allows for adaptability to many plant types. We used pregerminated seeds to minimize any variation between replicates, but the bioassay can be modified to test for the extracts' effect on germination as long as proper controls and replicate numbers are used.

Looking beyond the apparent constraints, the combination of metagenomics with a screen for allelopathic activity provides several benefits over the traditional culture techniques. The screen is designed to find plant growth-mediating activity and encompasses known and unknown modes of action. In addition, researchers can probe previously uncultured microorganisms for potential ability to produce allelopathic compounds. In our case, we have been screening metagenomes potentially enriched with the DNA of soil pathogens accumulating in the rhizosphere of a native, invasive weed with the intent of

increasing the chance for isolating biosynthetic genes coding for production of allechemicals. The technique has been designed to allow for many areas of modification to optimize or narrow the search. Lastly, the bioassay is relatively simple and can be easily modified to search for other compounds of interest, such as plant growth-regulating compounds or other pesticides.

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