Rhizobacteria Mediate the Phytotoxicity of a Range of Biorefinery-Relevant Compounds

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Abstract: Advances in engineering biology have expanded the list of renewable compounds that can be produced at scale via biological routes from plant biomass. In most cases, these chemical products have not been evaluated for effects on biological systems, defined in the present study as bioactivity, that may be relevant to their manufacture. For sustainable chemical and fuel production, the industry needs to transition from fossil to renewable carbon sources, resulting in unprecedented expansion in the production and environmental distribution of chemicals used in biomanufacturing. Further, although some chemicals have been assessed for mammalian toxicity, environmental and agricultural hazards are largely unknown. We assessed 6 compounds that are representative of the emerging biofuel and bioproduct manufacturing process for their effect on model plants (Arabidopsis thaliana, Sorghum bicolor) and show that several alter plant seedling physiology at submillimolar concentrations. However, these responses change in the presence of individual bacterial species from the A. thaliana root microbiome. We identified 2 individual microbes that change the effect of chemical treatment on root architecture and a pooled microbial community with different effects relative to its constituents individually. The present study indicates that screening industrial chemicals for bioactivity on model organisms in the presence of their microbiomes is important for biologically and ecologically relevant risk analyses.

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INTRODUCTION

Concerns over oil prices as well as the harm caused by fossil fuel consumption have accelerated bioeconomy-related research (Mortimer 2019). This includes plants genetically engineered to synthesize valuable compounds (Zhang et al. 2015; Eudes et al. 2016a, 2016b), methods developed to isolate those chemicals or intermediates and a maximum amount of accessible carbon from plant biomass (Neupane et al. 2017; Pérez-Pimenta et al. 2017; Yuan et al. 2017), as well as microorganisms engineered to efficiently convert plant-derived carbon into desirable products (Phelan et al. 2015; Goh et al. 2018; Sasaki et al. 2019; Figure 1).

The development and deployment of these technologies will greatly expand the range of accessible bioderived chemicals, many of which have not been evaluated for effects on the environment. This gap in knowledge is problematic considering the potential scale of accidental exposure. Biofuel production alone is projected to expand approximately 200 million tonnes of oil equivalent by 2040 (International Energy Agency 2018) and hence will come under regulation by the Frank R. Lautenberg Chemical Safety for the 21st Century Act in the United States and by the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) Regulation in the European Union. In addition, these chemicals may have valuable secondary applications yet to be discovered. For example, the promising biofuel candidates
α-pinene and α-limonene have been used or proposed as insecticides (Hollingsworth 2005; Chiu et al. 2017), antimicrobials (Rivas da Silva et al. 2012; Zahi et al. 2017), and therapeutics for several conditions (Lappas and Lappas 2012; Nam et al. 2014). Despite their long history of use and toxicity testing, there are still major gaps in our understanding (e.g., Ravichandran et al. 2018). Bioactivity screening of candidate chemicals may therefore inform both their risk management as well as their industrial value and utilities.

To date, chemical safety evaluation pipelines have focused on a handful of model organisms (Campos et al. 2018), whereas chemicals released into the environment affect functioning ecosystems. Ecotoxicological studies have attempted to predict multitrophic effects by examining a representative primary producer (an algae), a planktonic crustacean, and a fish but are nonetheless conducted in isolation (Campos et al. 2018). Microorganisms are present in these systems in that the animals or plants are not usually gnotobiotic, but the activity of the microbiome is not assessed. Chemical exposures may affect host cells and associated microbial communities—and only a handful of studies have assessed the importance of the microbiome in assessing risks, and these are predominantly in animal systems (Wilson and Nicholson 2017). Plants have a wide range of associated microbes, many of which are known to alter plant physiology (Finkel et al. 2017; Liu et al. 2018), making these interactions critical to understanding how plants transduce and respond to signals in their environment, including the effects of chemical treatment. Progress has been hampered in part by the fact that only a small number of nonpathogenic plant-associated microbes have been well characterized. The recent resolution of a core root microbiome (rhizobiome) of the dicotyledonous model plant Arabidopsis thaliana (Lundberg et al. 2012; Levy et al. 2018), however, provides an opportunity to explore the effect of relevant plant-associated microorganisms on the bioactivity of industrially relevant chemicals.

In the present study we selected 6 chemicals relevant to the biofuel industry and characterized their effects on Arabidopsis seedling development. We identified novel, unanticipated bioactivities in several industrially relevant compounds, reinforcing the value of screening candidate chemicals for off-target effects. We then used members of the Arabidopsis core-rhizobiome (Lundberg et al. 2012) that represent phyla enriched in the endophytic compartment (i.e., within the root), to test whether the effects of chemical treatment are altered by microbial inoculation. We then compared the effects on Arabidopsis to Sorghum bicolor (sorghum), a grain and forage crop and a monocotyledon. These data indicate that root-associated microorganisms have a complex influence on the tolerance of a plant to a chemical stress; therefore, considering the native rhizobiome of crops will be important for ensuring accuracy in environmental chemical safety assessments.

**MATERIALS AND METHODS**

**Chemicals and reagents**

Protocatechuate, α-limonene, and α-pinene were purchased with >97% purity from Sigma-Aldrich and p-coumarate from TCI America. Stocks of the ionic liquids cholinium lysinate ([Ch][Lys]) and 1-ethyl-3-methylimidazolium acetate ([C2C1im][OAc]) were prepared at close to 100% concentration, as described (Sun et al. 2014). Each chemical was stored under ambient conditions in the dark in sealed containers, with the exception of α-limonene, which was stored under nitrogen gas at 4 °C to reduce spontaneous oxidation.

**Microbial growth conditions**

Microbial strains were maintained in glycerol stocks stored at −80 °C. Media used were lysogeny broth (LB; 10 g/L tryptone, 5 g/L yeast extract, and 5 g/L NaCl) or M9 minimal media (M9; 6.8 g/L Na2HPO4, 3 g/L KH2PO4, 1 g/L NH4Cl, 0.5 g/L NaCl, 0.12 g/L Na3P...
Phylogenetic trees were generated using NCBI taxonomy IDs and instruments) kept at 30 °C and shaking on the wavelength of 600 nm using a Synergy 4 plate reader (BioTek Instruments) kept at 30 °C and shaking on the “high” setting. Phylogenetic trees were generated using NCBI taxonomy IDs and visualized using iTOLv4 (Letunic and Bork 2011).

Plant growth conditions

Arabidopsis ecotype Col-0 seeds were obtained from the Arabidopsis Biological Resource Center. Seeds were surface-sterilized by a brief wash in 70% (v/v) ethanol, followed by incubation in 30% (v/v) sodium hypochlorite (commercial bleach) for 15 min. Seeds were subsequently rinsed 5 times in sterile water, and sown on one-half Murashige and Skoog agar plates immediately postinoculation and grown as before. Plants were transferred to fresh one-half Murashige and Skoog medium where indicated. Before their addition to growth media, 1.2-M stock solutions of p-coumarate and 1.5-M stock solutions of protocatechuate were prepared in dimethylsulfoxide (DMSO) and ethanol, respectively. Controls of DMSO alone or ethanol alone were performed and found to have no effect on plant growth (Supplemental Data, Figure S7).

Microscopy

Seedlings were inoculated or chemically treated as previously described. After the treatment period, roots were gently washed in sterile water and the cell walls stained for 1 min in 10 µM propidium iodide (PI; Sigma), then briefly rinsed in sterile water. Cellular morphology was determined using a Zeiss LSM 710 confocal microscope with PI fluorescence emission at 550 to 680 nm and 488 nm excitation.

Imaging and root tracing

Plants were lit from the sides and photographed using a Canon 550D camera; images were saved as JPEG files for further analysis. The contrast was adjusted to maximize root visibility, but any adjustments were applied uniformly. Root growth was quantified using ImageJ (Schneider et al. 2012) with the Smart-Root plugin using default parameters (Lobet et al. 2011).

RESULTS

Development of Arabidopsis-based screening for bioactivity

Six compounds were selected as representatives of the emerging bioenergy and biofuel industry (Figure 1 and Table 1). Protocatechuate (a precursor of nylon and polyethylene terephthalate) and p-coumarate (a precursor to high-value compounds such as resveratrol and other phenolics) are plant aromatics, overproduction of which in planta can enhance the value of the lignin fraction of biomass (Eudes et al. 2016a, 2016b). The ionic liquids [Ch][Lys] and [C2C1im][OAc] are used to pretreat plant biomass prior to enzymatic deconstruction, a necessary process to liberate fermentable sugars and other valuable chemicals for microbial conversion (Cetinkol et al. 2010; Sun et al. 2014). Lastly, α-pinene and β-limonene are potential biojet fuel precursors (Tracy et al. 2009; Harvey et al. 2010) that can be produced using plant-derived sugars via microbial metabolism.

In our experimental setup, plants were germinated and grown vertically on transparent agar plates until the emergence of the first set of true leaves (6–8 d), transferred to fresh plates with or without the compound of interest, and imaged at 0 and 6 d after transfer. Finally, a semiautomated image analysis method was used to score primary root growth and total lateral root numbers, common descriptors of root development (Lobet et al. 2011). Initially, we grew a small number of plants (fewer than 20) in the presence of each compound across multiple orders of magnitude in concentration, to identify the lethal dose (Supplemental Data, Table S1). We then examined sublethal concentrations in greater detail (Supplemental Data, Table S1).
Arabidopsis root architecture is sensitive to biofuel-related compounds

Three of the selected compounds, p-coumarate, [C2C1im][OAc], and α-pinene, caused a reduction in primary root growth and lateral root initiation (Figure 2). Protocatechuate and [Ch][Lys] inhibited primary root growth at all concentrations tested but increased lateral root initiation at 1 and 0.1 mM, respectively. Protocatechuate also caused changes to primary root curvature at 1 mM, resulting in a median skew of 20° to the right from that of untreated plants (curvature at 1 mM, resulting in a median skew of 20° to the right from that of untreated plants (p < 0.0001, SE = 10.78°; Figure 2; Supplemental Data, Figure S1). α-Limonene caused inconsistent changes in root architecture both between and within plates: at least 20% of plants showed complete inhibition of primary root growth regardless of the concentration tested (Figure 2; Supplemental Data, Figure S2). The response of lateral root initiation was less consistent. For example, lateral root growth was enhanced by protocatechuate treatment but unaffected by limonene treatment (Figure 2).

Rhizobacteria alter Arabidopsis root growth

Next, we tested the effects of individual rhizobacteria on root architecture. We selected 14 strains from representative phyla (Table 2) previously described as enriched in the endophytic compartment of Arabidopsis (Lundberg et al. 2012; Levy et al. 2018) for an initial screening (Supplemental Data, Figure S3). After the emergence of true leaves, Arabidopsis roots were inoculated with cell resuspensions of microbes or mock-treated with water alone (control), then transferred to fresh agar plates. We screened each microbe individually for effects on root architecture (Supplemental Data, Table S2) and quantified changes where they were visually apparent (Figure 3).

Most candidate microbes did not significantly alter root growth (Supplemental Data, Table S2); however, 5 had strong effects. The primary roots of plants inoculated with 2 Acinetobacter species (sp. 2 and sp. 3) were shorter than controls (meancontrol = 3.780 cm; meansp.2 = 2.85 cm, standard error of the mean [SEM] = 0.205, p = 0.005; meansp.3 = 3.021 cm, SEM = 0.140, p = 0.004) and initiated fewer lateral roots (meancontrol = 4.99; meansp.2 = 2.25, SEM = 0.519, p = 0.007; meansp.3 = 2.629, SEM = 0.444, p = 0.034). A third microbe, Acinetobacter sp. 1, did not have a significant effect on root architecture, suggesting that the effects of sp. 2 and sp. 3 are specific to those strains and not a general effect of the genus. In contrast, plants inoculated with Agrobacterium rhizogenes, Flavobacterium sp., or Paenibacillus sp. grew shorter primary roots (meancontrol = 3.780 cm, meanrhizogenes = 2.693 cm, SEM = 0.116, p = 0.0001; meanFlavobacterium = 1.854 cm, SEM = 0.073, p < 0.0001; meanPaenibacillus = 0.972 cm, SEM = 0.054, p < 0.0001) but did not differ in lateral root initiation. Flavobacterium sp. and Paenibacillus sp. caused the greatest effects; reducing mean (±SE) primary root growth to 48.7 ± 0.19% and 25.5 ± 1.4% of mock-inoculated controls.

| Compound | Examples of uses in biomanufacturing |
|----------|--------------------------------------|
| Protocatechuate | Phenolic which can be directly used by aromatic-consuming microorganisms (Linger 2014). Biomass can be engineered to increase the content of protocatechuate (e.g., Eudes et al. 2015) |
| p-Coumarate | Phenolic which can be directly used by aromatic-consuming microorganisms (Linger 2014). Biomass can be engineered to increase the content of p-coumarate (e.g., Li et al. 2018) |
| Cholinium lysinate [Ch][Lys] | Biocompatible ionic liquid that can be used in the deconstruction of plant biomass (e.g., Sun et al. 2014) |
| 1-Ethyl-3-methylimidazolium acetate [C2C1im][OAc] | Ionic liquid that can be used in the deconstruction of plant biomass (e.g., Sun et al. 2014) |
| α-Pinene | Terpene that is a biogasoline candidate, a fragrance, and a component of cleaning products (e.g., Harvey et al. 2010) |
| α-Limonene | Terpene that is a biojet-fuel candidate, a fragrance, and a component of cleaning products (e.g., Tracy et al. 2009) |

*aCircled numbers refer to the process described in Figure 1.*

**TABLE 1:** Compounds tested in the present study, with proposed or known industrial applications*
respectively. Inoculation with *Flavobacterium* sp. or *Paenibacillus* sp. also shortened the zone of primary root elongation, defined here as the distance from the root apical meristem to the closest visible lateral root (Supplemental Data, Figure S4; mean\textsubscript{control} = 3.13 cm; mean\textsubscript{Flavobacterium} = 1.23 cm, SEM = 0.077, p < 0.0001; mean\textsubscript{Paenibacillus} = 0.40 cm, SEM = 0.049, p < 0.0001), facilitating lateral root initiation along a larger percentage of the primary root despite lessened primary root growth overall, thereby increasing lateral root density relative to controls (Supplemental Data, Figure S4; mean\textsubscript{control} = 0.714 lateral roots/cm primary root length; mean\textsubscript{Flavobacterium} = 1.488 roots/cm, SEM = 0.124, p < 0.0001; mean\textsubscript{Paenibacillus} = 0.3163 roots/cm, SEM = 0.186, p < 0.0001). *Agrobacterium rhizogenes* shortened the primary root elongation zone but did not significantly increase lateral root density.

**Specific rhizobacteria change the bioactivity of the selected compounds**

We next tested whether inoculation with rhizobia altered the effects of a subset of the tested compounds on plant growth. We chose 1 microbes, *A. rhizogenes* and *Acinetobacter* sp. 1, which were tolerant of 10 mM [Ch][Lys] (Supplemental Data, Figure S5) compared to 0.1 mM [Ch][Lys], which significantly
inhibited the primary root growth of Arabidopsis (Figures 2 and 4). Treatment of uninoculated plants with 0.1 mM [Ch][Lys] decreased primary root growth and increased lateral root initiation (Figures 3 and 4). Inoculation with A. rhizogenes prior to [Ch][Lys] treatment improved primary root growth (Figure 4; mean [Ch][Lys] = 0.858 cm; mean [Ch][Lys]/rhizogenes = 2.241 cm, SEM = 0.158, p < 0.0001) and lateral root initiation (mean [Ch][Lys]/rhizogenes = 7.069; mean [Ch][Lys]/rhizogenes = 4.889, SEM = 0.47, p < 0.0001) relative to uninoculated controls. In contrast, Acinetobacter sp. 1 had no significant effect on root growth regardless of [Ch][Lys] treatment. The effects of inoculation with A. rhizogenes were also evident microscopically (Figure 4G): treatment with 0.1 mM [Ch][Lys] caused irregular expansion of epidermal cells of the root apical meristem and stimulated formation of ectopic root hairs in epidermal cells immediately adjacent to the root cap (Figure 4GII). This phenotype was abolished in the A. rhizogenes–treated roots (Figure 4GIV).

Although different ionic liquids do not affect plants in the same way—as illustrated by the differences between [Ch][Lys] and [C2C1im][OAc] (Figure 2)—they share similar chemical properties as salts. Because improved plant salt tolerance is a known effect of some rhizobacteria (Mayak et al. 2004; Pinedo et al. 2015; Huang et al. 2017), we hypothesized that these microbes may have similar effects on plant phenotypes caused by [C2C1im][OAc] exposure. We tested the effect of 1 mM [C2C1im][OAc], a concentration that does not alter the growth of either microbe. Although 1 mM [C2C1im][OAc] caused an overall reduction in root growth (Figures 2 and 4), A. rhizogenes improved both primary root growth (Figure 4; mean [C2C1im][OAc] = 1.904 cm; mean [C2C1im][OAc]/rhizogenes = 2.912, SEM = 0.228, p = 0.0002) but had no effect on lateral root initiation. Despite having no effect on [Ch][Lys] treatment, Acinetobacter sp. 1 completely rescued primary root growth (mean [C2C1im][OAc] = 1.904 cm; mean [C2C1im][OAc]/rhizogenes = 4.011, SEM = 0.261, p < 0.0001). Both rhizobacteria tested were therefore capable of changing the bioactivity of the test ionic liquid compounds.

It is possible that the microbes mediate the effects of these ionic liquids by using them as a carbon source and thereby removing them from the environment around the root. Therefore, we next tested whether either [Ch][Lys] or [C2C1im][OAc] could be used as a sole carbon source by A. rhizogenes. A liquid culture allowed microbial growth at these concentrations. Supplemented with either 0.1 mM [Ch][Lys], 1 mM [C2C1im][OAc], or 1% w/v dextrose (Supplemental Data, Figure S5). Neither ionic liquid allowed microbial growth at these concentrations.

Alternatively, one or both ions of the ionic liquid could be sequestered or altered by the microbe in a process that does not directly impact cell growth. Choline, for instance, can be used as a precursor to the compatible solute glycine betaine in a process dependent on 2 genes, betA and betB, both of which are present in the A. rhizogenes genome (Levy et al. 2018). Glycine betaine is,

### TABLE 2: Summary of microbes used in the present study

| Microbe                | National Center for Biotechnology Information taxonomy no. |
|------------------------|-----------------------------------------------------------|
| Agrobacterium rhizogenes | 2521172625                                                |
| Acinetobacter sp. 1     | 2593339129                                                |
| Acinetobacter sp. 2     | 2643221500                                                |
| Acinetobacter sp. 3     | 2556921674                                                |
| Arthrobacter             | 2517572124                                                |
| Bacillus flexus         | 2522125133                                                |
| Brevundimonas sp.       | 2596583649                                                |
| Burkholderia sp.        | 2593339266                                                |
| Chryseobacterium sp.    | 2529292577                                                |
| Flavobacterium sp.      | 2563366720                                                |
| Leifsonia sp.           | 2522572063                                                |
| Paenibacillus sp.       | 256336613                                                 |
| Ralstonia sp.           | 2558309150                                                |
| Rhodococcus erythropolis| 2643221496                                                |

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in addition, known to improve the salt tolerance of both plants and microbes (Riou and Le Rudulier 1990; Hayashi et al. 1997). Therefore, we tested whether [Ch][Lys] improved the salt tolerance of *A. rhizogenes* by growing the microbe in M9 supplemented with 1% w/v dextrose and either 0.1 mM [Ch][Lys], 400 mM NaCl, or both (Supplemental Data, Figure S5). *Agrobacterium rhizogenes* growth was inhibited by NaCl; however, this was partially rescued by the addition of [Ch][Lys].
Notably, the microbes did not always ameliorate the effects of the tested compounds. For example, when Arabidopsis was inoculated with A. rhizogenes and grown in the presence of 1 mM protocatechuate, this resulted in a more severe growth inhibition in which both primary root growth (Supplemental Data, Figure S6; mean\textsubscript{protocatechuate} = 2.293 cm; mean\textsubscript{protocatechuate/rhizogenes} = 0.631, SEM = 0.201, \( p < 0.0001 \)) and lateral root initiation (mean\textsubscript{protocatechuate} = 7.989; mean\textsubscript{protocatechuate/rhizogenes} = 2.214, SEM = 0.674, \( p < 0.0001 \)) were almost completely inhibited.

**Specific members of a microbial consortium alter the effect on plant chemical tolerance**

Finally, we tested the effects of combining all 14 rhizobiome members on Arabidopsis root growth in the presence of [Ch][Lys]. Resuspensions of the 14 rhizobacteria were pooled together (pool A), reducing the concentrations of each individual microbe to give an equivalent total inoculum as for previous experiments. Pool A decreased primary root growth (mean\subscript{control} = 3.8 cm; mean\subscript{poolA} = 1.895 cm, SEM = 0.202, \( p < 0.0001 \)) and increased the number of lateral roots (mean\subscript{control} = 4.9; mean\subscript{poolA} = 7.543, SEM = 0.735, \( p = 0.0071 \)) relative to uninoculated controls (Figure 5). Because we had previously observed that Flavobacterium sp. and Paenibacillus sp. inhibited the primary root growth of Arabidopsis by 50% or more (Figure 3, Table S2), we tested an additional pool excluding Flavobacterium sp. and Paenibacillus sp. (pool B). Pool B also inhibited primary root growth (mean\subscript{protocatechuate} = 0.464 cm; mean\subscript{protocatechuate/rhizogenes} = 1.051 cm, SEM = 0.075, \( p < 0.0001 \)) but had no effect on lateral root initiation (Figure 5). We then tested whether the pooled communities could mitigate the effects of [Ch][Lys]. When treated with 0.1 mM [Ch][Lys], plants inoculated with pool A did not differ significantly from uninoculated, [Ch][Lys]-treated controls (Figure 6). Inoculation with pool B, however, partially rescued primary root growth (mean\subscript{Ch}[Lys] = 0.858 cm; mean\subscript{poolB} = 2.072 cm, SEM = 0.227, \( p < 0.0001 \)). Therefore, although the consortium is capable of mediating [Ch][Lys] tolerance in Arabidopsis, the presence of Flavobacterium sp., Paenibacillus sp., or both represses this effect.

**A. rhizogenes increases tolerance to [Ch][Lys] in sorghum**

Arabidopsis is useful as a model system because of its fast generation time, ease of handling, and the availability of relevant genetic tools. However, we wanted to compare the effects of these treatments on a second plant species of economic relevance. We selected sorghum because it is both evolutionarily distant from Arabidopsis and an agronomically important plant for food, forage, and biomass, being cultivated over 3 300 000 ha in the United States and 40 000 000 ha globally (Deb et al. 2004; Langholtz et al. 2016). We tested [Ch][Lys] treatment and inoculation with A. rhizogenes on sorghum using a similar vertical growth system with larger agar plates. We observed no change in sorghum root growth when inoculated with A. rhizogenes alone (Figure 6). Treatment with 5 mM [Ch][Lys] was sufficient to almost completely inhibit branching lateral roots and crown root growth over 8 d. Both lateral root growth (mean\subscript{Ch}[Lys] = 0.464 cm; mean\subscript{Ch}[Lys]/rhizogenes = 1.051 cm, SEM = 0.075, \( p < 0.0001 \)) and crown root growth (mean\subscript{Ch}[Lys] = 3.267 cm; mean\subscript{Ch}[Lys]/rhizogenes = 5.028 cm, SEM = 0.4535, \( p = 0.001 \)) was partially improved when sorghum was inoculated with A. rhizogenes prior to [Ch][Lys] treatment, demonstrating that the microbe can have a positive impact on the chemical tolerance of multiple plant species.

**DISCUSSION**

In the present study we describe an efficient assay to test the effects of industrially relevant chemicals on seedling development in the presence or absence or root-associated microbes. We used this to identify previously unreported effects
of chemicals on plant growth. We found that the observed phenotypes were altered with growth in the presence of rhizobacteria. The assay, although far removed from the complexity of a natural or field ecosystem, enables future detailed exploration of plant–microbe–chemical interactions at the molecular level. Future work will add multi-omics measurements, including transcriptomics, metatranscriptomics, and untargeted chemistry and metabolomics. It will also provide a system for testing hypotheses generated from data collected in more complex environments such as higher-order microbial communities or mesocosm-scale plant–soil–microbial community experiments.

Knowledge of these effects is potentially valuable—not only to informing best practice for the use of these chemicals but also for establishing new bioactivities that may increase their value and diversify their industrial applications. The phytotoxicity of ionic liquids, for instance, has implicated them as potential herbicides and regulators of plant growth (Pernak et al. 2013). Indeed, the 0.1 mM concentration of [Ch][Lys] sufficient to completely inhibit primary root growth in Arabidopsis in the present study equates to 104 g/ha, a relatively low concentration compared to contemporary herbicides (Benbrook 2016; Coupe and Capel 2016). To be relevant in situ, however, the effect of the treatment must be demonstrated across species and in realistic environments.

Of the plant growth phenotypes induced by chemical treatment, 2 were of particular interest. The first was an increase in lateral root initiation and a right-handed root skew caused by 1 mM protocatechuate. Lateral root branching is governed by complex interactions between phytohormones and is an important aspect of adaptation to stress (Lavenus et al. 2013). Root curvature is similarly regulated by phytohormone transport, as well as other stimuli, which lead to changes in cell elongation (Muday 2001; Blancaflor and Masson 2003; Aloni et al. 2004). Therefore, although the mechanism of its effect on root growth remains ambiguous, protocatechuate may potentially alter Arabidopsis root growth through changes to hormone signaling. The second phenotype of interest was the heterogeneous response of uninoculated Arabidopsis to α-limonene—no dose–response relationship was obvious. Limonene is volatile and known to oxidize to limonene hydroperoxide under aerobic conditions, the latter being more toxic to bacteria (Chubukov et al. 2015) and a greater allergen to humans (Christennson et al. 2008). Stochastic oxidation or evaporation may explain the observed variation in the effect of α-limonene on root growth and implicate volatility as an important factor affecting bioactivity.

When the plants were inoculated with the microbes, we observed plant growth phenotypes as a result of inoculation with Acinetobacter sp. 2, Acinetobacter sp. 3, A. rhizogenes, Flavobacterium sp., and Paenibacillus sp., species that are canonically associated with changes in hormone production and transport and induction of plant defense pathways (Cartieaux et al. 2003; Contreras-Cornejo et al. 2015; Wang et al. 2015). Inoculation with either Flavobacterium sp. or Paenibacillus sp. specifically had the most significant effect by decreasing primary root length and increasing the zone of lateral root initiation, demonstrating that root-associated microbes can alter both the rate of root growth and its spatial organization. It should be noted that we only used a single inoculum density in this report and that in future it would be interesting to explore how these phenotypes are related to the colonization rate of the roots. In addition, it should be noted that these bacterial species might be expected to promote plant growth, which was not always observed in the present study. A future study which explores inoculum density, as well as the effects of a single microbe versus a consortia on
root growth, would be valuable to understand the importance of these factors in plate-based assays.

Finally, we observed that the effects of several of the test chemicals on Arabidopsis were altered by specific microbes. Both A. rhizogenes and Acinetobacter sp. 1 affected the toxicity of one or more test chemicals on Arabidopsis. In addition, A. rhizogenes altered the impact of chemical treatments in a chemical-specific fashion. Further experimentation is required to attribute this phenotype to microbial sequestration or conversion of these tested compounds. For example, both A. rhizogenes and Acinetobacter sp. 1 contain betA and betB homologs, but they did not rescue the Arabidopsis [Ch][Lys] phenotype to the same extent.

Gnotobiotic plants are not found in nature, nor do plants typically interact with a single predominant microbial species. Therefore, synergism and antagonism between diverse microorganisms are likely critical to their function in situ. Indeed, our pooled consortium of 14 test microbes caused an increase in lateral root initiation that could not be recapitulated by any of the microbes individually. Furthermore, the inclusion of Flavobacterium sp. and Paenibacillus sp.—both of which individually inhibited primary root growth in the absence of chemical stress—in the pool repressed the ability of the pool to restore primary root growth under [Ch][Lys] stress. Curiously, both pools had similar effects on primary root growth in the absence of chemical stress. Therefore, we anticipate that chemical treatment will likely affect a plant differently depending on the composition of its microbiome. Despite testing in lower-order systems, recent work is encouraging in demonstrating that such experimentation can accurately predict their interactions in larger consortia (Herrera Paredes et al. 2018; Venturelli et al. 2018). Further work is necessary to better define the impact of synthetic microbial communities and their ecological relevance as model systems. These studies are now being performed in conjunction with laboratory-scale micro- and mesocosms, including EcoFABS or ecotrons (Eisenhauer and Türke 2018; Gao et al. 2018; Mortimer et al. 2018).

As climate and economic factors drive industry away from traditional, petroleum-based fuels and commodity chemicals, the need for novel and economically viable bioproduction platforms will continue to increase. The implementation of these platforms will simultaneously diversify and increase the availability of potentially bioactive compounds produced at scale. Screens to identify unanticipated bioactivities will be important to maximize the values of these compounds as well as to identify potential risks. The present study suggests that consideration of the microbiome is essential for ecological risk assessments, at least in the case of vascular plants. Further, the methods used to test for bioactivity may be coupled with more complex devices capable of controlling additional variables during ecotoxicity screens (Eisenhauer and Türke 2018; Gao et al. 2018), thereby quickly screening conditions that are increasingly representative of those found in the field. The integration of multi-omics modalities with such platforms promises to significantly accelerate our capacity to elucidate the risks and benefits associated with new and emerging industrial chemicals.

**Supplemental Data**—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.4501

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**Disclaimer**—The views, discussions, conclusions, and future recommendations expressed in this article are those of the authors alone and do not represent the views of any other agency or organization.

**Data Accessibility**—Data, associated metadata, and calculation tools are available from the corresponding author (jcmortimer@lbl.gov or amukhopadhyay@lbl.gov).

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