

**Rhizosphere modeling reveals spatiotemporal distribution of daidzein shaping
soybean rhizosphere bacterial community**

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23 **Running title**

24 Daidzein shapes rhizosphere bacterial communities

25

26

27 **Abstract**

28 Plant roots nurture a wide variety of microbes via exudation of metabolites, shaping the
29 rhizosphere's microbial community. Despite the importance of plant specialized
30 metabolites in the assemblage and function of microbial communities in the rhizosphere,
31 little is known of how far the effects of these metabolites extend through the soil. We
32 employed a fluid model to simulate the spatiotemporal distribution of daidzein, an
33 isoflavone secreted from soybean roots, and validated using soybeans grown in a
34 rhizobox. We then analyzed how daidzein affects bacterial communities using soils
35 artificially treated with daidzein. Simulation of daidzein distribution showed that it was
36 only present within a few millimeters of root surfaces. After 14 days in a rhizobox,
37 daidzein was only present within 2 mm of root surfaces. Soils with different
38 concentrations of daidzein showed different community composition, with reduced
39 α -diversity in daidzein-treated soils. Bacterial communities of daidzein-treated soils
40 were closer to those of the soybean rhizosphere than those of bulk soils. This study
41 highlighted the limited distribution of daidzein within a few millimeters of root surfaces,
42 and demonstrated a novel role of daidzein in assembling bacterial communities in the
43 rhizosphere by acting as more of a repellent than an attractant.

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45 Key words: advection–diffusion equation, bacterial communities, daidzein, rhizosphere

46 modelling, soybean

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49 **Introduction**

50 The rhizosphere, defined as the zone of soil surrounding the root that is affected by it
51 (Hartmann, Rothballer, & Schmid, 2008; Hiltner, 1904), is pivotal in both nutrient
52 uptake and interactions with a diverse range of soil microbes (Bakker, Pieterse, de Jonge,
53 & Berendsen, 2018). Previous studies provided evidence that plant metabolites secreted
54 from roots provoke changes in rhizosphere microbial communities and mediate plant–
55 microbe interactions, from symbiotic to commensal to pathogenic, suggesting the
56 importance of plant metabolites in the rhizosphere for promoting the growth and health
57 of plants (Berendsen, Pieterse, & Bakker, 2012; Massalha, 2017; Sasse, Martinoia, &
58 Northen, 2018). Despite the importance of root-secreted metabolites, little is known of
59 the distribution and fate of these molecules in the rhizosphere (Sugiyama, 2019). Plant
60 metabolites can be divided into primary and secondary, or plant specialized, metabolites
61 (Pichersky & Lewinsohn, 2011). A large portion of primary plant metabolites, which
62 consist of sugars and organic acids, are rapidly metabolized by soil microbes (Gkarmiri
63 et al., 2017; Gunina & Kuzyakov, 2015; Jones & Murphy, 2007). The greater abundance
64 of nutrients near roots produces an environment in which microbes flourish, and
65 microbial abundance is greater in the rhizosphere than in bulk soils (Mendes, Garbeva,
66 & Raaijmakers, 2013; Prashar, Kapoor, & Sachdeva, 2014). In contrast to primary

metabolites, the stability of plant specialized metabolites varies depending on their chemical structure and biological activity (Sugiyama & Yazaki, 2014). For example, strigolactones are unstable in soil, making them a signal of living plant roots (Ruyter-Spira, Al-Babili, van der Krol, & Bouwmeester, 2013; Seto, Kameoka, Yamaguchi, & Kyojuka, 2012), whereas flavonoids are relatively stable in soil (Sugiyama, Yamazaki, Hamamoto, Takase, & Yazaki, 2017).

Plant specialized metabolites have important ecological functions in the rhizosphere, such as acting as signals for symbiosis, repelling enemies, and modifying microbial communities (Chen et al., 2019; Huang et al., 2019; Massalha, 2017). Volatile compounds are thought to facilitate communication over larger distances (Rasman et al., 2005; Schulz-Bohm et al., 2018), whereas non-volatile compounds such as coumarins (Stringlis et al., 2018) and flavonoids appear to exert their influences near plant roots. Largely due to the difficulty of analyzing these metabolites in soil, most studies on plant specialized metabolites have been conducted in hydroponic culture or in sterile sand (Oburgera, 2018). However, root structure and metabolite fate differ in soil and under these artificial conditions (Crush J.R., 2005) (Sugiyama & Yazaki, 2014). To gain insight into the functions of plant specialized metabolites in field-grown plants, it is important to analyze rhizosphere plant–microbe interactions using soils from crop fields.

85 Rhizobox is widely used to analyze the dynamics of proteins, metabolites, and ion in the
86 rhizosphere using soils taken from fields (Kuzyakov & Razavi, 2019). The activities of
87 root-secreted enzymes such as phosphatase and β -glucosidase were visualized using
88 4-methylumbelliferone-labeled and 7-amino-4-methylcoumarin-labeled fluorogenic
89 substrates to determine the distribution of these enzymes within a few millimeters from
90 root surface (Razavi, Zarebanadkouki, Blagodatskaya, & Kuzyakov, 2016; Zhang,
91 Dippold, Kuzyakov, & Razavi, 2019). Analyses of carbon and nitrogen with
92 autoradiography or Positron Emitting Tracer Imaging System (PETIS) revealed the
93 presence of the rhizodeposits within a few millimeters from root surface as well (Holz,
94 Zarebanadkouki, Kaestner, Kuzyakov, & Carminati, 2018; Kuzyakov & Razavi, 2019).
95 In addition, advection–diffusion (dispersion) equation has been used to simulate the
96 distribution of mineral ions and water contents in soil surrounding roots (Duncan, Daly,
97 Sweeney, & Roose, 2018; Vereecken et al., 2016; Zarebanadkouki, Fink, Benard, &
98 Banfield, 2019; Zarebanadkouki, Kroener, Kaestner, & Carminati, 2014). Although the
99 simulation of the distribution of metabolites could provide valuable insights in the
100 rhizosphere interactions, it has not been employed to analyze the distribution of
101 metabolites in soil, at least partly because of the instability of root-secreted metabolites
102 and the difficulty of the measurement in rhizosphere.

Recent progress in sequencing technologies and the establishment of synthetic communities of culturable bacteria has provided insights into the molecular mechanisms underlying the assemblage and function of rhizosphere microbes (Bulgarelli et al., 2012; Duran et al., 2018; Lundberg et al., 2012); for example, *Arabidopsis* secretes coumarins that encourage assembly of a microbiome adapted to iron deficiency (Stringlis et al., 2018; M. J. E. E. Voges, Bai, Schulze-Lefert, & Sattely, 2019). Although these advancements have deepened our understanding of the rhizosphere, a large gap remains between these achievements in microbial ecology and a comprehensive understanding of the rhizosphere in field-grown plants, which are essential for the application of these insights to sustainable agriculture. One of the key limitations is rooted in the definition of the rhizosphere; it is impossible to delineate the area of the rhizosphere in field soils because the zone of root influence varies depending on which metabolites are secreted as well as the environmental or microbial conditions in the field.

Refining our understanding of the spatiotemporal distribution of key specialized metabolites in soils is indispensable for defining the area of the rhizosphere. To better define the extent of the influence of root-secreted specialized metabolites in the rhizosphere, we used daidzein, an isoflavone secreted from soybean (*Glycine max*) roots, as a model metabolite. Isoflavones are a subgroup of flavonoids predominantly found in

Fabaceae (Mazur, Duke, Wahala, Rasku, & Adlercreutz, 1998), and are produced via isoflavone synthase (IFS). The influences of isoflavones to bacterial communities were shown with *IFS*-silenced transgenic hairy roots (White, Ge, Brozel, & Subramanian, 2017) and with the addition of daidzein and genistein to soybean field soils (Guo, Kong, Wang, & Wang, 2011). Soybeans secrete daidzein into the rhizosphere to initiate symbiosis with rhizobia (Kosslak, Bookland, Barkei, Paaren, & Appelbaum, 1987). Although nodulation occurs predominantly during early vegetative stages (Calvert, Pence, Pierce, Malik, & Bauer, 1984), we previously found that daidzein was present in the rhizosphere throughout all growth stages (Sugiyama et al., 2017). These results suggest that daidzein plays additional roles in shaping the rhizosphere microbial communities.

In this report, we simulated the distribution of daidzein in the rhizosphere using the advection–diffusion equation, then validated the daidzein distribution using a rhizobox. To further characterize the effects of daidzein at the concentration measured in the rhizosphere, we set up artificial rhizospheres to analyze changes in bacterial communities. By integrating analyses of the distribution and function of daidzein, we were able to define daidzein’s zone of influence in the soybean rhizosphere as the few millimeters surrounding the roots, and to demonstrate spatiotemporal distribution of

139 daidzein and its influence on microbial community composition.

140

141 **Materials and Methods**

142 **Chemicals and soils**

143 Chemicals were obtained from Wako Pure Chemical Industries (Osaka, Japan) or
144 Nacalai Tesque (Kyoto, Japan), unless otherwise stated. Field soil was collected from a
145 soybean field at Kyoto Gakuen University, Japan. Soil chemical and physical properties
146 were analyzed at Tokachi Federation of Agricultural Cooperatives: pH of 6.8, 0.28%
147 total N, 2.5% total C, 4.3% humin, 46.1 ppm NO_3^- , 11 ppm P, 5.7 ppm K, 3.2 ppm S,
148 443 ppm Ca, 25 ppm Mg, 0.02 ppm B, 0.58 ppm Fe, 0.2 ppm Cu, 0.3 ppm Zn, and 5.51
149 ppm Mn. Soil particle density was 2.58 g cm^{-3} . Soil texture was light clay (sand: 46%,
150 silt: 23%, clay: 31%). Saturated hydraulic conductivity (K_s) was $9.6 \times 10^{-6} \text{ m s}^{-1}$, as
151 measured by the falling head method. Kaolinite was purchased from The Clay Science
152 Society of Japan, and illite was purchased from Nichika Inc. (Kyoto, Japan).

153

154 **Dynamics of isoflavones**

155 For daidzein distribution analysis, 30 g of soil was suspended in 300 mL of deionized
156 water. Different amounts of daidzein were added to the soil solution and mixed for 2 h.

157 To determine the distribution coefficient of daidzein on minerals, kaolinite or illite was
158 mixed with water and daidzein, instead of field soils. For decomposition of soil organic
159 matter, 10 g soils were treated with hydrogen peroxide (Gee & Or, 2002). Field soil
160 (10.0 g) was added to 50 mL of ion-exchanged water, then 10 mL of 30% hydrogen
161 peroxide was added. After 60 min, the mixture was heated on a hot plate to 80 °C for 4
162 h. Carbon content was measured by using CN corder (Elementar Vario EL, Elementar,
163 Germany).

164 Soil solutions were centrifuged at 5000 $\times g$ for 10 min, and supernatants were
165 filtered through filter paper (Advantech). The pH was adjusted to 3.0 using HCl. The
166 medium was passed through a Sep-pak C18 Plus short cartridge filter (Waters, Milford,
167 MA, USA), as described previously (Sugiyama et al. 2016). The eluant was dried under
168 nitrogen and reconstituted in 95% MeOH with 1% formic acid. The supernatant was
169 filtered through a Minisart 0.45- μ m filter (Sartorius, Göttingen, Germany). Isoflavones
170 were analyzed using a HPLC apparatus (LC-10A, Shimadzu, Kyoto, Japan) under the
171 following conditions: column, TSK-gel ODS-80TM (4.6 mm $\times$ 250 mm; Toso, Tokyo,
172 Japan); solvent A, 0.3% (v/v) formic acid; solvent B, 0.3% (v/v) formic acid in
173 acetonitrile; detection, 260 nm. Elution was at 0.8 mL min⁻¹ with solvent system A
174 (water containing 0.3% (v/v) formic acid) and B (acetonitrile containing 0.3% (v/v))

formic acid) with a linear gradient from 15% to 22% B over 30 min, followed by a linear gradient from 22% to 35% B over 20 min, and a linear gradient from 35% to 70% B over 5 min. The adsorption of daidzein on soils and minerals was calculated by subtracting the amount of daidzein in the aqueous phase from the added amount in the initial solution (Liang et al., 2011). The degradation of daidzein was negligible during adsorption experiments, because half-life of daidzein was calculated to be more than 7 days (Sugiyama et al., 2017).

Simulation

Movement of daidzein secreted by a single cylindrical root was simulated using a two-dimensional axisymmetric system. The model domain was assumed to be a cylinder of soil with a diameter of 20 cm and depth of 20 cm, with a single root with a diameter of 2 mm and height of 10 cm in the center. The partial differential equation describing transport of the solute (daidzein) had the following general form:

$$\frac{\partial(\theta c)}{\partial t} + \frac{\partial(\rho c_a)}{\partial t} = -\nabla \cdot q_w c + \nabla \cdot D \nabla c - kc, \quad (1)$$

where c is the solute (daidzein) concentration (kg m^{-3}), θ is the volumetric water content ($\text{m}^3 \text{m}^{-3}$), c_a is the daidzein concentration adsorbed onto the solid phase (kg kg^{-1}), ρ is the bulk density (1.34 Mg m^{-3}), q_w is the water flux induced by root uptake (m s^{-1}), D is

the solute diffusion coefficient ($\text{m}^2 \text{s}^{-1}$), k is the degradation rate constant (s^{-1}), and t is the time (s). When assuming a linear adsorption isotherm, c_a can be written as $k_d c$, where k_d is the distribution coefficient ($\text{m}^3 \text{kg}^{-1}$).

As described in Eq. (1), advective daidzein movement caused by root uptake of water was also considered in the simulation. Water movement in the soil, without considering gravity, can be described by the Richards equation as:

$$\frac{\partial \theta}{\partial t} = -\nabla \cdot q_w = \nabla \cdot [K(\nabla h)], \quad (2)$$

where h is the water pressure head (m), and K is the unsaturated hydraulic function. The van Genuchten–Mualem soil hydraulic properties model (Millington & Quirk, 1961; van Genuchten, 1980) was adopted as an unsaturated hydraulic function:

$$K(h) = K_s S_e^{0.5} \left[1 - \left(1 - S_e^{1/m} \right)^m \right]^2, \quad (3)$$

where K_s is the saturated hydraulic conductivity (m s^{-1}), m is the van Genuchten parameter, and S_e is the effective water content, described by $(\theta - \theta_r)/(\theta_s - \theta_r)$ where θ_s and θ_r are saturated water content and residual water content, respectively. Note that the van Genuchten water retention model was adopted for the relationship between h and S_e (van Genuchten, 1980).

The degradation kinetics of daidzein (k) have previously been reported (Sugiyama et al., 2017; Sugiyama et al., 2016). The solute diffusion coefficient was estimated using

211 the Millington and Quirk model (Millington & Quirk, 1961) as a function of θ . To
 212 determine the distribution coefficient (k_d), batch experiments were conducted, in which
 213 30 g of dry soils were mixed with 300 mL of daidzein solution with concentrations
 214 ranging from 17 to 270 ppb; the soil suspensions were shaken for 2 h. Based on the
 215 obtained adsorption isotherm, the distribution coefficient was set as $0.067 \text{ m}^3 \text{ kg}^{-1}$
 216 (Supplementary Fig. 1). Parameters for water retention model and unsaturated hydraulic
 217 function were estimated by a pedotransfer function (ROSETTA model) (Schaap, Leij, &
 218 van Genuchten, 2001) based on the measured soil texture data, giving $\theta_s = 0.48$, $\theta_r =$
 219 0.08 , $m = 0.28$, and $\alpha = 1.61 \text{ m}^{-1}$ as van Genuchten water retention model parameters.

220 In the simulation, root surface contact with the soil domain was considered as an
 221 influx boundary for daidzein secretion and an efflux boundary for water movement due
 222 to root uptake. The daidzein secretion rate (i.e., influx boundary at root surface)
 223 determined in a previous study was used (Sugiyama et al., 2016). Water uptake rate (i.e.,
 224 efflux boundary for water movement) was obtained from the rhizobox experiments
 225 ($5.43 \times 10^{-8} \text{ m s}^{-1}$). The upper, lower, and outer surfaces of the soil domain were
 226 considered as zero flux boundaries for solute and water movement. The initial water
 227 content of the soil domain was set as $0.43 \text{ m}^3 \text{ m}^{-3}$. Eqs. (1)–(3) were solved using the
 228 COMSOL Multiphysics 5.4 software (Keisoku Engineering System Co., Ltd., Tokyo,

229 Japan). The parameters used in this study were summarized in Supplementary Table 1.

230

231 **Growth of soybean in the rhizobox and analysis of daidzein**

232 The rhizobox was purchased from Tohoku Materials (Sendai, Japan). Soybeans were
233 grown in a plant box containing vermiculite for 4 days before being transferred to the
234 rhizobox. The water content ratio (mass of soil water / dry soil weight x 100 (%)) of the
235 soil in the rhizobox was 28%. Mixing of soil and water was performed before packing
236 the soil layer. When placing the nylon mesh, a cellulose acetate membrane capable of
237 adsorbing daidzein was placed beyond the nylon mesh on all sides (Supplementary Fig.
238 2). Soybean seedlings were removed from vermiculite, washed with distilled water,
239 dried with a Kimwipe, and sandwiched between two layers of nylon mesh. The top of
240 the rhizobox was covered with an acrylic plate. After 14 days of growth, cellulose
241 acetate membranes and soil layers within 2 mm of plant roots were removed. Extraction
242 of daidzein was performed as described previously (BolanosVasquez & Warner, 1997;
243 Sugiyama et al., 2017). In brief, the cellulose acetate filters were rinsed with distilled
244 water and then extracted with two sequential rinses with 1 ml of methanol for 5 min.
245 Extracts were dried under a nitrogen stream at 50 °C and stored at –30°C prior to HPLC
246 analysis. Soil samples were extracted in 3 × 4 mL of methanol at 50 °C (1 h each) and

centrifuged at $5000 \times g$ for 10 min. The combined supernatant was dried under a nitrogen stream at 50 °C and stored at –30°C prior to HPLC analysis. The detection limit was 0.01 nmol/g soil. Water content ratio was determined by measuring the exact weight before and after drying the sample in an oven at 105 °C for 24 hours (Gardner, 2002).

Analysis of bacterial communities after incubation with daidzein

Soybean field soil was air-dried, and 2 g of soil was mixed with sterilized water to obtain a water content ratio of 30%. To prevent the methanol from affecting the bacterial community, daidzein in methanol was first added to a plastic tube and dried under nitrogen gas. Moistened soils were transferred to a tube containing daidzein (0.04, 0.2, and 1 mM to obtain final concentrations of 10, 50, and 250 nmol, respectively), and mixed thoroughly using a vortex mixer (Supplementary Fig. 3). Daidzein was added every 3 days. The water content ratio was adjusted by addition of water after weighing the soil. After 15 days of culture at 28 °C in the dark, sample tubes were stored at –30 °C until extraction of DNA and daidzein.

DNA was extracted from 0.25 g soil with a Power Soil DNA Isolation Kit (Mo Bio, Carlsbad, CA, USA) according to the manufacturer's protocol and quantified using a

265 dsDNA HS Assay Kit for the Qubit Quantification Platform (Invitrogen, Carlsbad, CA,
266 USA). PCR amplification of 16S rRNA genes was performed in a 25-μL reaction
267 volume containing 10 ng template DNA, 0.3 μL of KOD FX neo (Toyobo, Japan), 12.5
268 μL of buffer (provided with the polymerase), 5 μL of dNTPs (2 mM), 0.5 μL of 515F
269 (5'-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-GTGCCAGCMGCCGCGGT
270 AA-3') and 806R (5'-
271 GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT-GGACTACHVGGGTWTCT
272 AAT-3') primers (10 μM) in triplicate. PCR conditions were as follows: denaturation at
273 94 °C for 2 min; 20 cycles of 98 °C for 10 s, 50 °C for 30 s, and 68 °C for 30 s. PCR
274 amplicons were purified with Ampure magnetic beads (Beckman Coulter, Danvers, MA,
275 USA) prior to the second round of PCR. To attach MiSeq adaptors, a second round of
276 PCR was performed in a 25-μL reaction volume containing 2.5 μL template DNA, 0.3
277 μL of KOD FX neo, 12.5 μL of buffer (provided with the polymerase), 5 μL of dNTPs
278 (2 mM), and 0.5 μL of primers provided with Fasmac Co. Ltd. Final PCR products were
279 purified using Ampure magnetic beads, and the concentration of the purified PCR
280 product was measured using a Qubit 2.0 Fluorometer (Life Technologies). PCR
281 products were sent to FASMAC Co. Ltd. for a single sequencing run using a MiSeq
282 reagent kit v3 on a MiSeq platform (Illumina) to obtain 2 × 300-bp paired-end

sequences. Sequence data have been deposited in the DDBJ (DNA Data Bank of Japan) Sequence Read Archive under the accession number DRA008649.

Sequence data for the amplicons were analyzed using the QIIME2 platform, version 2018.11 (Bolyen, 2018). For all paired reads, the first 20 bases of both sequences were trimmed (to remove primer sequences) and the bases after position 200 were truncated (to remove low-quality sequence data), and potential amplicon sequencing errors were corrected using DADA2 (Callahan et al., 2016) to produce an amplicon sequence variant (ASV) dataset. The resultant ASVs were aligned using MAFFT (Katoh & Standley, 2013) and then used to construct a phylogenetic tree with FastTree2 (Price, Dehal, & Arkin, 2010). The α - and β -diversity metrics were estimated from a subsampled ASV dataset, with 45,000 sequences per sample (Supplementary Table 2). Each ASV was identified using a Naïve Bayes classifier trained on 16S rRNA gene sequences from the Greengenes release 13_8 dataset (Bokulich et al., 2018) (Supplementary Table 3). Principal coordinate analysis and other statistical analyses were performed using custom R and Python scripts.

Analysis of rhizosphere bacterial communities of soybean

Field experiments were conducted in an experimental field of Kyoto University of

Advanced Science, Kameoka, Kyoto, Japan (34°99'38"N, 135°55'14"E). Soybean plants (cv. Shintambaguro) were irrigated as needed, and weeds were manually removed throughout the crop season on a weekly basis. Rhizosphere and bulk soil samples were collected at the vegetative stage (V8). Bulk soil, defined as soil that does not adhere to plant roots, was obtained at least 20 cm from the plants. Rhizosphere soil was collected using two methods: traditional dry sampling using brushes, as described previously (Sugiyama, Ueda, Zushi, Takase, & Yazaki, 2014), and fractionation using phosphate buffer with some modification, as follows (Bulgarelli et al., 2012). Roots from five plants were pooled into a 1000-mL glass beaker with 500 mL sterile PBS buffer containing 130 mM NaCl, 7 mM Na₂HPO₄, 3 mM NaH₂PO₄ (pH 7.0), and 0.02% Silwet L-77, and washed on a shaking platform for 5 min at 100 rpm. Roots were removed, the buffer was centrifuged (5000 ×g, 10 min), and the resulting pellet was defined as rhizosphere soil. DNA extraction and PCR amplification for sequencing were performed as described above.

Results

Simulation of daidzein distribution

Metabolites secreted into the rhizosphere are degraded by microbes and adsorbed onto

soil organic matter and clay minerals, depending on the soil type and microbes present (Canarini, Kaiser, Merchant, Richter, & Wanek, 2019; Soma & Soma, 1989; Sugiyama, 2019). In order to gain insight into daidzein distribution in soybean fields, we used gray lowland soils obtained from a soybean farm where soybeans had been cultivated for more than 5 years. Fig. 1 shows a simulated daidzein distribution near a single root. During early stages of vegetative growth (7, 14 days), the predicted daidzein distribution was limited to within a few millimeters of the root surface (Fig. 1A, B). These results were based on the assumption that soybean root secretes equivalent amount of daidzein from all parts of roots, and the growth of roots (i.e., enlargement of daidzein influx area) was not considered. Even during later growth stages (70 days), the predicted daidzein distribution was limited to within 1 cm of the root, as shown in the daidzein concentration profile (Fig. 1C). These results indicate that soil adsorption greatly reduces daidzein's soil mobility. Because the daidzein secretion rate is highest during earlier vegetative growth stages (Sugiyama et al., 2016), the daidzein concentration at the root surface peaked at 28 days. After that time, decomposition and reduced secretion of daidzein decreased the concentration at the root surface.

Adsorption of daidzein to organic matter in gray lowland soil

Possible adsorption sites contained in the gray lowland soil are humic substances and clay minerals. Major structural sites of humic substances include carboxyl groups, hydroxyl groups, and phenolic hydroxyl groups, which are reported to be involved in the formation of complexes with heavy metal ions and organic substances (Gan & Li, 2013). To analyze adsorption of daidzein to humic substances, organic matter in gray lowland soil was decomposed to approximately 65% with hydrogen peroxide treatment (Fig. 2). In these soils, daidzein adsorption was reduced to approximately 40% compared with controls, suggesting that humic substances are involved in adsorption of daidzein (Fig. 2).

Adsorption of daidzein to clay minerals was analyzed using two minerals typical of this lowland gray soil, kaolinite, which is classified as a 1:1 type silicate mineral, and illite, which is classified as 2:1 type silicate mineral. The adsorption data for different concentrations of daidzein were fitted by a linear line based on the Henry's law, and the distribution coefficient was calculated to be $0.0042 \text{ (m}^3 \text{ kg}^{-1}\text{)}$ (Supplementary Fig. 4).

When daidzein adsorption experiments were carried out with illite, adsorption of daidzein was not observed and distribution coefficients could not be calculated.

Validation of daidzein distribution using a rhizobox

Daidzein distribution was validated using a rhizobox. To grow soybean for 14 days without additional water supply, the water content ratio was set to 28%, which is higher than the normal field condition (24%). After 14 days, the water content ratio was reduced to 24% due to the water absorption by roots (Supplementary Table 4). A cellulose acetate membrane filter was set at 2 mm from the root surface. In HPLC analysis, daidzein could only be detected in the soil layer within 2 mm of the root surface, and could not be detected on the cellulose acetate membrane filter (Fig. 3). In addition, daidzein was not detected in soil layers > 2 mm from the root surface when cellulose acetate membrane filters were not applied (n=4, data not shown).

Effect of daidzein on bacterial community

Daidzein is known to induce the expression of *nod* genes in rhizobia to initiate symbiosis, but our previous observation that the daidzein concentration remained consistent throughout growth stages in field-grown soybean (Sugiyama et al., 2017) suggests that daidzein also performs other functions. To determine the effects of daidzein on soil bacteria, a steady daidzein concentration was maintained in a test tube containing soil collected from the soybean field. After 15 days of incubation, the daidzein concentration varied from 0.81 to 28 nmol g⁻¹ soil, depending on the amount

added to the soil (Supplementary Fig. 8). The daidzein concentration of soils supplemented with 1250 nmol was within the range of daidzein concentrations observed in the rhizosphere of field-grown soybean (10-20 nmol g⁻¹ soil) at the end of incubation.

To clarify the daidzein-mediated effects on bacterial diversity, the α -diversity of each sample was calculated using QIIME2 (Bolyen et al., 2018), as observed operational taxonomic units (OTUs) and Shannon diversity. Daidzein treatment reduced the α -diversity of bacterial communities, and observed OTUs were markedly reduced at daidzein concentrations greater than 250 nmol g⁻¹ soil (Supplementary Fig. 5), suggesting that daidzein affected the assemblage of the bacterial community. Daidzein possesses antimicrobial activity (Gorniak, Bartoszewski, & Kroliczewski, 2019) and serves as a carbon source for a particular group of bacteria that metabolize daidzein. The reduction of α -diversity is a typical change observed in the bacterial communities in the rhizosphere (Duran et al., 2018).

In the principal coordinate analysis (PCoA) of Bray–Curtis similarity (β -diversity), the microbial diversity of untreated soil was clearly distinct from that of daidzein-treated soil (Supplementary Fig. 6A and B), indicating that daidzein had a significant effect on microbiome assemblage. Especially in the weighted Unifrac distance, which compared bacterial community structures using the relative abundance

of each bacterial member, the daidzein concentration influenced bacterial community assemblage (Fig. 4A and B).

To understand the extent to which daidzein shapes the rhizosphere bacterial communities of soybean in the field, we compared the bacterial communities of daidzein-treated soils (1250 nmol) with those of bulk and soybean rhizosphere soils. Bacterial communities of daidzein-treated soils were closer to those of the soybean rhizosphere than those of bulk field soil (Fig. 5). These communities were then compared at the family level (Fig. 6, Supplementary Fig. 7, Supplementary Table 5 and 6). Seven families of microbes were enriched in the daidzein-treated soils, and two of these seven families, Comamonadaceae and Microbacteriaceae, were enriched in both daidzein-treated and soybean rhizosphere soils (Fig. 5B). The relative abundance of Comamonadaceae was positively correlated with the daidzein concentration (Supplementary Fig. 8). In contrast, 17 of the 37 families found to be depleted in daidzein-treated soil were also depleted in rhizosphere soil (Fig. 5B). These results suggest that daidzein functions more as a repellent than an attractant in assemblage of bacterial communities.

Discussion

The importance of the rhizosphere for plant growth and sustainable crop production is widely recognized (Berendsen et al., 2012; Vorholt, Vogel, Carlstrom, & Muller, 2017). Since Lorenz Hiltner coined the term rhizosphere more than 100 years ago, researchers have used various methods to define the rhizosphere, including brushes, buffers, or sensors for microbial, mineral, physical, and metabolite analyses (Kuzyakov & Razavi, 2019). Unlike heat, ions, and water, other factors that influence soil in the vicinity of plant roots, root-secreted metabolites can be metabolized by soil microbes. This characteristic makes analysis of root-secreted metabolites in the rhizosphere particularly difficult (Oburgera, 2018). Traditionally, most studies of root exudates have been conducted in hydroponic cultures, but the analysis of root-secreted metabolites in field environments has garnered increasing attention over the past decade (Oburger et al., 2013; Sugiyama et al., 2017). To gain a more precise understanding of the rhizosphere in the field, it is essential to define the zone of influence of these metabolites in the soil. In this study, we aimed to define the rhizosphere of soybean, by incorporating soil physical and microbial analysis using daidzein as a model metabolite.

Based on simulations using a fluid model, the daidzein distribution was limited to within a few millimeters of the roots, largely due to high adsorption by soil organic matter. In this simulation, the soil water content was constant; however, in actual

427 soybean fields, the water content changes depending on irrigation and precipitation. In
428 reality, daidzein has a wider distribution under wet conditions and a narrower
429 distribution during dry conditions. Our rhizobox experiments revealed that the daidzein
430 distribution is limited to within 2 mm of the root surface, concordant with the results of
431 our simulation. Incorporating a combination of wet and dry conditions into the
432 simulation would permit even more precise modeling of daidzein concentrations, and
433 would require precise measurement of field water content during soybean growth. The
434 growth of roots and the difference of daidzein secretion rate depending on the part of
435 roots are also to be incorporated into the simulation to predict the daidzein distribution
436 in soybean fields. The distribution of released carbon and nitrogen, as measured using
437 isotopes, and of root-secreted enzymes such as phosphatase and β -glucosidase is limited
438 to within a few millimeters as well (Kuzyakov & Razavi, 2019; Oburger et al., 2013).
439 The distribution and fate of plant metabolites in the rhizosphere had been analyzed in
440 rhizobox or by mass spectrometry imaging (Blossfeld, Gansert, Thiele, Kuhn, & Losch,
441 2011; Holscher et al., 2014; Veličković & Anderton, 2017). In this report, we
442 demonstrated that simulation based on a fluid model can be used to predict the
443 rhizosphere distribution of plant specialized metabolites. Plant root secretes tremendous
444 varieties of metabolites into the rhizosphere (Massalha et al. 2017). By analyzing the

degradation rate and distribution coefficient of each metabolite in field soils, we can simulate the distribution of root exudates in the rhizosphere. Revealing the spatiotemporal distribution of metabolites functioning in the rhizosphere interactions could expand our understanding of the rhizosphere into a 4-dimensional scale. One of the largest limitations of this simulation is that we need different parameters depending on the soil characteristics, for example, the distribution of daidzein could be wider in soils with low organic matters and clay minerals such as sandy soils (Supplementary Fig. 4) or in soils with lower daidzein-degradation activity. We also have to appropriately consider actual root growth and their function (e.g., root water uptake) in the field.

Recently, disruption of genes involved in synthesis of triterpene, sesterterpene, or coumarin has permitted modulation of the rhizosphere microbiome of *Arabidopsis* (Chen et al., 2019; Huang et al., 2019; Stringlis I.A., 2018; M. J. E. E. Voges, Bai, Y., Schulze-Lefert, P., Sattely, E.S., 2019). In this study, we showed that addition of daidzein at rhizosphere concentrations modulated the bacterial community in vitro and increased the abundance of Comamonadaceae. Rhizobiaceae was enriched in the rhizosphere, but depleted to one-half in daidzein treated soils (Supplementary Table 6). This is probably because daidzein is not suitable carbon source to bacteria in this family, in contrast to other bacteria enriched in daidzein treated soils. When the bacterial

communities of daidzein-treated soils were compared with those of soybean fields, daidzein treatment produced bacterial communities more similar to those in soybean rhizosphere soil than those in bulk soil. This finding supports the notion that plant specialized metabolites play key roles in formulating rhizosphere bacterial communities. Daidzein treatment of soil reduced the α -diversity and depleted more bacterial families than it enriched, as in soil from the soybean rhizosphere. These results indicate that daidzein restricts the growth of certain bacteria to shape the rhizosphere bacterial community. Enterobacteria that metabolize food-derived isoflavones have been reported to metabolize daidzein (Feng, Li, Oppong, & Qiu, 2018), but the daidzein-metabolizing enzymes in soil bacteria are still unknown. Bacteria in the Comamonadaceae and/or Microbacteriaceae may possess genes that allow them to use daidzein as a carbon source. Comamonadaceae are common in the soybean rhizosphere (Hamid et al., 2017; White et al., 2017), with >10% relative abundance (Supplementary Fig. 9). This family contains plant growth promoting bacteria such as *Delftia* sp., which enhance nodulation and pulse yield when co-inoculated with *Bradyrhizobium elkanii* (Cagide, Riviezzi, Minteguiaga, Morel, & Castro-Sowinski, 2018), and *Variovorax paradoxus*, a soybean endophyte with characteristics related to plant growth promotion (Lopes, Carpentieri-Pipolo, Oro, Pagliosa, & Degrassi, 2016). Soil type primary influences the

481 assemblage of rhizosphere microbial communities (Liu et al., 2019; Xiao et al., 2017),
482 and Comamonadaceae was abundant in the rhizosphere of successive
483 soybean-monoculture cropping (Hamid et al., 2017), which is concordant with our
484 findings using soils from soybean field under continuous cropping. It should also be
485 noted that daidzein is not the only metabolite to promote the abundance of
486 Comamonadaceae in the soybean rhizosphere, because the silencing of *IFS* gene in
487 soybean hairy roots resulted in the slight increase of Comamonadaceae in the
488 rhizosphere of IFS-silenced roots (White et al., 2017). A synthetic community-based
489 approach to the daidzein-mediated interaction among soybean, Comamonadaceae and
490 other bacteria could identify the molecular basis of these interactions.

491 Daidzein was discovered to act as a signaling molecule for initiation of rhizobial
492 symbiosis in the 1980s (Kosslak et al., 1987). Following our previous finding that
493 rhizosphere concentrations of daidzein remain high even after the key period for
494 establishment of rhizobial symbiosis, we found that daidzein has additional functions in
495 shaping the bacterial community in the rhizosphere, answering a long-unresolved
496 question. Plants modulate the rhizosphere microbial community, assembling beneficial
497 microbes to reduce damage from pathogens (Kwak M.-J., 2018; Stringlis I.A., 2018)
498 and enhance uptake of nutrients (Castrillo et al., 2017). It is tempting to speculate that

soybean secretes daidzein into the soil within the vicinity of the root surface, where daidzein helps to assemble a beneficial microbial community by acting as more of a repellant than an attractant.

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Author Contributions

Conceived and designed the experiments: SH, AS. Performed the experiments: FO, SH, NN, AS. Analyzed the data: FO, SH, YA, MN, NN, TN, KY, AS. Wrote the paper: FO,

517 SH, YA, AS, with input from all authors.

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767 **Disclosure statement**

768 No potential conflict of interest was reported by the authors.

769

770 **Figure legends**

771 Fig. 1 Simulation of daidzein distribution in soil. (A) Daidzein concentrations at 7 days
772 after germination from 0 to 6 mm from root surface. (B) Daidzein concentrations at 14
773 days after germination from 0 to 6 mm from root surface. (C) Daidzein distribution
774 from 7 to 70 days.

775

776 Fig. 2 Daidzein adsorption to soil organic matter. Soils were treated with hydrogen
777 peroxide to remove organic matter. Carbon content was measured by using a CN corder.
778 Adsorption of daidzein by gray lowland soil after hydrogen peroxide treatment to
779 remove organic matter. Values indicate means $\pm$ SD. Soil organic carbon was measured
780 in duplicate, and adsorption of daidzein was measured in triplicate. White circle:
781 control; black circle: hydrogen peroxide-treated soil.

782

783 Fig. 3 Daidzein distribution in the rhizobox. Daidzein concentrations in soil within 2

784 mm of the root surface and recovered from the cellulose acetate membrane filter ($n = 6$).

785

786 Fig. 4 Pairwise comparisons between daidzein-treated and untreated soils. Box plots

787 showing mean and variance of average pair-wise weighted UniFrac distances (A) and

788 unweighted UniFrac distance (B) between daidzein-untreated (0 nmol) and

789 daidzein-treated samples. p-values from Wilcoxon rank-sum test are shown.

790

791 Fig. 5 Comparison of bacterial communities between daidzein-treated soils and soybean

792 field soils. (A) Weighted Unifrac distance between daidzein-treated soils and bulk soils

793 and between daidzein-treated soils and rhizosphere soils. p-value from Wilcoxon

794 rank-sum test is shown. (B) Venn diagrams showing overlap of bacterial families

795 enriched or depleted in soil treated with daidzein and in the soybean rhizosphere.

796 Numbers in the circle represent number of bacterial families.

797

798 Fig. 6 Families enriched or depleted in daidzein-treated soils and soybean rhizosphere.

799 The heatmap represents the fold-changes. Colored stripes indicate whether the family

800 was enriched or depleted in daidzein-treated soils and soybean rhizosphere. Families

801 either enriched or depleted in both soybean rhizosphere and daidzein treatment are

shown. Comparisons of other Families are shown in Supplementary Fig. 7.

Supplementary Figures

Supplementary Fig. 1 Adsorption isotherms of daidzein in gray lowland soil. Initial concentrations ranging from 17 to 270 ppb were used to determine the daidzein concentration in the aqueous phase ($n = 2$). C_s ; adsorbed concentration in soil, C_w ; concentration in water in equilibrium with the soil.

Supplementary Fig. 2 Schematic illustration of the rhizobox. (A) Soybean roots were separated with nylon mesh, and a 2-mm soil layer was placed on both sides of plants. Cellulose acetate membranes were set at 2 mm from the root surface. (B) Representative images of the rhizobox.

Supplementary Fig. 3 Schematic illustration of daidzein treatment to soils.

Supplementary Fig. 4 Daidzein adsorption to kaolinite. Daidzein at initial concentrations ranging from 17 to 270 ppb was used to determine the daidzein

820 concentration in the aqueous phase.

821

822 Supplementary Fig. 5 Alpha diversity of bacterial communities of soils augmented with

823 daidzein. Observed operational taxonomic units (OTUs) (A) and Shannon diversity

824 index (B) ($n = 4$).

825

826 Supplementary Fig. 6 Principal coordinate analysis of bacterial communities of soils

827 augmented with daidzein. (A) Unweighted Unifrac and (B) weighted Unifrac analysis.

828 Different colors represent different concentrations of daidzein in soil.

829

830 Supplementary Fig. 7 Families enriched or depleted in daidzein-treated soils and

831 soybean rhizosphere. The heatmap represents the fold-changes. Colored stripes indicate

832 whether the family was enriched or depleted in daidzein-treated soils and soybean

833 rhizosphere.

834

835 Supplementary Fig. 8 Correlation between daidzein concentration after 15 days of

836 incubation and the relative abundance of Comamonadaceae.

837

838 Supplementary Fig. 9 (A) Relative abundance of Comamonadaceae in soils treated with
839 different concentrations of daidzein and in bulk and rhizosphere soils of soybean field.

840 (B) Relative abundance of Microbacteriaceae in soils treated with different
841 concentrations of daidzein and in bulk and rhizosphere soils of soybean field.

842

843 Supplementary Table 1. Parameters used in this study

844

845 Supplementary Table 2. Effective Read Number, Observed ASVs, Faith's Phylogenetic
846 Diversity, Shannon's Entropy, Pielou's Evenness of each sample.

847

848 Supplementary Table 3. Number of reads assigned to taxonomy categories.

849

850 Supplementary Table 4. Water content ratio of soils in rhizobox after soybean growth.

851

852 Supplementary Table 5. Fold changes of bacterial taxa in soybean rhizosphere and
853 daidzein-treated soils. Families either enriched or depleted in both soybean rhizosphere
854 and daidzein treatment are shown. P value of Student's t-test and Q value of false
855 discovery rate for multiple comparison are shown.

856

857 Supplementary Table 6. Fold changes of bacterial taxa in soybean rhizosphere and
858 daidzein-treated soils. All Families in Supplementary Fig. 7 are shown. P value of
859 Student's t-test and Q value of false discovery rate for multiple comparison are shown.

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861