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Enhancement of developmentally regulated daidzein secretion from soybean roots in field conditions as compared with hydroponic culture

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Analyses of metabolite secretions by field-grown plants remain scarce. We analyzed daidzein secretion by field-grown soybean. Daidzein secretion was higher during early vegetative stages than reproductive stages, a trend that was also seen for hydroponically grown soybean. Daidzein secretion was up to 10,000-fold higher under field conditions than hydroponic conditions, leading to a more accurate simulation of rhizosphere daidzein content.

Keywords: Daidzein; Rhizosphere; Simulation; Soybean

Plant specialized metabolites (PSMs) play important roles in the rhizosphere for modulation of symbiotic interactions (e.g., repelling pests and pathogens and shaping

35 microbiota), thereby promoting plant growth and improving crop production [1–3].

36 Flavonoids are a group of PSMs and consist of more than 8,000 compounds [4]. These

37 molecules function as regulators of auxin transport and reactive oxygen species and

38 protect against damage caused by ultraviolet (UV) light exposure. In legumes,

39 flavonoids are secreted from the roots to exert functions in rhizosphere plant-microbe

40 interactions, such as those necessary for defense and symbiosis [5–7].

41 Isoflavones are a subfamily of flavonoids and are found mainly in legumes [8].

42 In the rhizosphere, isoflavones such as daidzein and genistein of soybean (*Glycine max*)

43 and formononetin-7-*O*-(6″-*O*-malonylglycoside) of alfalfa (*Medicago sativa*) induce

44 *nod* genes for initiation of the nodulation process [9, 10]. In particular, daidzein was

45 recently shown to be involved in the modulation of rhizosphere bacterial communities

46 in soybean, where this compound increased the relative abundance of the

47 Comamonadaceae family of bacteria [11].

48 The secretion of metabolites from roots is a crucial process influencing
49 interactions in the rhizosphere. Daidzein is the major isoflavone secreted into
50 hydroponic media, the concentration of which is higher during the soybean vegetative
51 stage than the reproductive stage [12]. Daidzein is relatively stable in soil, with a half-
52 life of about seven days, enabling the estimation of daidzein contents in the rhizosphere
53 based on the amount secreted in hydroponic cultures [13]. Whereas sorption filters or
54 glass beads have been used to collect and analyze various metabolites in the rhizosphere
55 [14, 15], the direct measurement of secreted metabolites is technically challenging,
56 especially for field-grown plants [16]. In this study, we used the cellulose acetate
57 membrane method utilized in hydroponic culturing to analyze flavonoid secretion for
58 direct measurement of the amount of daidzein secreted by field-grown soybean plants
59 during the stages of growth.

60 All chemicals used in this study were obtained from either Wako Pure
61 Chemical Industries (Osaka, Japan) or Nacalai Tesque (Kyoto, Japan) unless otherwise

stated.

The field experiments were conducted at Kyoto University of Advanced Science, Kameoka, Kyoto, Japan (coordinates: 34°99′38″N, 135°55′14″E). Soybean seeds ("Tambaguro") were sown on May 31, 2019. The plants were irrigated as needed, and emerging weeds were manually removed weekly. No apparent symptoms of pathogen infection were observed, and pesticides were not used. Root samples and root exudates were collected on June 14 (V1 stage), July 3 (V5 stage), July 22 (V9 stage), August 14 (R2 stage), September 4 (R4 stage), and October 2 (R6 stage) of 2019 [17]. The soil around the lateral roots was partially removed with a shovel. The lateral roots were rinsed with tap water and pinched between a cellulose acetate filter (Advantec, Tokyo, Japan) using a hairpin (Fig. 1A) and then covered with soil. Additionally, cellulose acetate filters were placed in the bulk soil as a control. The cellulose acetate filters were held in the soil for 2 h, and then the filters and root tissues were collected. All samples were transferred to the laboratory in a cool container (0–10°C) within 2 h of

collection. The root samples were stored in pure water prior to fresh weight measurement. The roots and fully expanded leaves were taken from 2-week-old soybean seedlings (VE stage) for the quantification of isoflavones as described previously [13]. Rhizosphere soil was obtained from seven plants using sterile brushes and combined into one sample as described previously [18]. The samples were immediately frozen in dry ice and transferred to the laboratory for storage at −80°C. The bulk soil was sampled at least 20 cm away from the plant.

The extraction of daidzein was performed as previously described [13, 19]. The cellulose acetate filters were rinsed with tap water, and the compounds were extracted twice using 1 ml methanol with shaking on a Labo shaker BC-730 (Bio craft, Tokyo, Japan) for 5 min each time. The combined supernatant from each sample was dried under a nitrogen stream at 50°C, dissolved in 150 µl of methanol, and filtered through a Minisart RC4 syringe filter (Sartorius, Gottingen, Germany) for LC-MS (Liquid chromatography–mass spectrometry) analysis. The exudates were analyzed by UPLC-

MS on an ACQUITY UPLC system (Waters Corporation) coupled with Xevo TQD. The LC was performed by injecting a 2 μl sample onto an ACQUITY UPLC BEH C18 column (2.1 mm × 50 mm, 1.7 μm; Waters Corporation) at 40 °C. The LC mobile phase consisted of (A) water containing 0.1% (v/v) formic acid and (B) acetonitrile. The gradient program was linear over the range of 10%–35% B, 0–1 min; linear 35%–85% B, 1–11 min; isocratic 85% B, 11–11.1 min; isocratic at 100% B, 11.1–15.5 min; and isocratic at 10% B, 15.5–20 min. The flow rate was 0.2 ml min^{-1}. Isoflavones were detected at 260 nm. The contents of daidzein were estimated from the peak areas in comparison with calibration curves constructed using known concentrations of the authentic compound.

The extraction of isoflavones was performed as described [13]. The frozen tissues were pulverized in liquid nitrogen using a mortar and pestle and then freeze-dried. The tissues were extracted in 80% methanol at 60°C for 1 h, followed by centrifugation at 12,000×g for 5 min to remove debris. The supernatant was filtered

104 through a Minisart RC4 syringe filter (Sartorius). Soil samples (1 g) were extracted in
105 500 µl of methanol at 50°C three times (10 min each) and centrifuged at 4,800 rpm for 5
106 min. The combined supernatant from each sample was dried under a nitrogen stream at
107 50°C and redissolved in 150 µl methanol. Isoflavones were analyzed by LC-MS/MS as
108 described [20].

109 The movement of daidzein secreted by a single cylindrical root was simulated
110 using a two-dimensional asymmetric system. The equations, model domains, and
111 relevant initial/boundary conditions were previously described [11]. The daidzein
112 secretion rate at the root surface was assumed to be constant (1.06 nmol m^2 s^{-1}), based
113 on the daidzein extraction for the roots sampled on June 14 (V1 stage). The simulation
114 period was set at 14 days with a 0.1-day time interval. The parameters used in this study
115 were summarized in Table S1. A cylinder of soil with a diameter of 20 cm and a depth
116 of 20 cm with a single root of diameter 2 mm and length 10 cm in the center was set as
117 a model domain for the simulation. Root length and diameter were assumed to be

118 constant for 14 days.

119 The isoflavones secreted from field-grown soybean were analyzed at three
120 vegetative growth stages V2, V5, and V8, corresponding to 2, 5, and 8 weeks after
121 sowing, respectively. Moreover, samples from three reproductive growth stages R2, R4,
122 and R6, respectively corresponding to 12, 15, and 19 weeks after sowing, were
123 analyzed. Of all the detected isoflavones collected using cellulose acetate membranes
124 that adsorb flavonoid aglycones [13,19], only daidzein was identified at each growth
125 stage (Fig. 1B). The amount of secreted daidzein changed over the growth stages and
126 peaked at V5, whereas it was constant over the reproductive stages. The trend of
127 daidzein secretion was similar to that of hydroponically grown soybean. In contrast,
128 field-grown soybean secreted up to a 10,000-fold higher amount of daidzein than
129 hydroponically grown soybean (about 36 fmol mg FW^{-1} day^{-1} at V3) [12]. While the
130 secretion from whole roots was analyzed in the hydroponic culture media, the secretion
131 from the field-grown soybean was analyzed using a 3 cm root-tip section. The

132 possibility that partial soil removal induced the isoflavone biosynthesis and increased
133 the isoflavone levels within 2 h is probably small because it is suggested to take more
134 than 3 h for the roots to accumulate isoflavones after gene induction in *Arabidopsis*
135 *thaliana* and soybean [20, 21]. The difference in the magnitude of secretion is,
136 therefore, presumably attributable to environmental conditions, i.e., sterile hydroponics
137 vs. non-sterile field environments. The contents of isoflavones in the root tissue at
138 steady-state under the field-grown conditions were similar to those under hydroponic
139 conditions [12, 13], suggesting that both the isoflavone synthesis and secretions are
140 remarkably enhanced in the rhizosphere, probably due to the presence of various
141 microorganisms.

142 The spatiotemporal distribution of metabolites in the rhizosphere is of particular
143 importance for deciphering their functions in inter-organismal interactions such as
144 chemotaxis response and *nod* gene induction, which are concentration-dependent [9, 22,
145 23]; however, the distribution of PSMs remains largely unknown [24]. In our previous

study, we simulated the spatiotemporal distribution of daidzein in field soil based on the advection–diffusion equation [11], and we showed that daidzein distribution was limited to within a few millimeters from the root surface [11]. To further refine the simulation of daidzein distribution in the field, we applied the secreted rate of daidzein under field conditions. The distribution of daidzein was also limited to within a few millimeters from the root surface, similar to findings from the previous simulation [11]. Limited daidzein distribution withn a few millimeters is likely due to the adsorption of daidzein by the soil. In this simulation, the average daidzein content within 1 or 3 mm soils from root surface was around 0.8 and 0.5 nmol g soil^{-1}, respectively (Fig. 2A). This concentration was within the range to induce *nod* genes in *Bradyrhizobium japonicum*, which is reported to be more than 0.1 μM [9, 25]. The isoflavone contents in the rhizosphere and plant tissues were measured in 2-week-old soybean seedlings to validate the results of this simulation. Malonylgenistin was the most predominant isoflavone in the leaves at this stage, while malonyldaidzin and daidzein were

accumulated in the roots (Fig. 2B). Rhizosphere soil was sampled from less than 3 mm layer from the root surface. In the rhizosphere soil, daidzein was the most abundant isoflavone, and the content was about 5 nmol g soil^{-1} (Fig. 2C). Collectively, rhizosphere modeling based on the amount secreted by field-grown soybean led to a more accurate simulation of daidzein distribution than our previous simulation, i.e. daidzein distribution at physiologically relevant concentrations is limited to within a few millimeters from root surface.

Despite the importance of PSMs in the rhizosphere, our current knowledge of the dynamics in the rhizosphere of field-grown plants is still preliminary. The dynamics between proteins, metabolites, and ions in the rhizosphere have been analyzed mostly using the rhizobox [23], but they should be examined in field-grown plants as well. In this study, we showed that the secretion of daidzein by field-grown soybean followed the same trends in terms of developmental regulation, but the amount was much higher than in hydroponic condition, leading to the accurate estimation of daidzein distribution

174 in the rhizosphere. The rhizosphere microbiome affects the secretion of metabolites
175 from roots [26]; therefore, we presume that the rhizosphere microbiome enhanced
176 daidzein secretion in the field, in addition to the effects of other both biotic and abiotic
177 stresses under field conditions. It is of particular importance to analyze the secretion of
178 PSMs in the rhizosphere of field-grown plants under various conditions and to integrate
179 the distribution of PSMs and the structure and functions of the microbiota in future
180 studies.
181

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186 Author Contributions: A.S. conceived and designed the research; H.T., K.Y., and A.S.
187 supervised the experiments; M.T., F.O., and M.N. conducted plant sampling and LC-
188 MS/MS analysis; S.H. conducted the simulation; M.T. and A.S. wrote the article with

contributions of all authors; A.S. agrees to serve as the author responsible for contact and ensuring communication.

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Figure Legends

Fig. 1 (A) Cellulose acetate membrane used to collect root exudates. Tips of lateral roots were washed with pure water and pinched in a cellulose acetate membrane, which was then covered with soil. The site of analysis was marked with a piece of white paper. (B) Root exudation of daidzein throughout soybean growth stages. Amount per root fresh weight of daidzein in root exudates ($n \geq 9$). Significant differences ($P < 0.05$; Tukey–Kramer test) are indicated with various letters. Root samples and root exudates were collected at three vegetative stages (V) and three reproductive stages (R).

Fig. 2 Simulation of daidzein distribution in soil and isoflavone contents in the rhizosphere. (A) Simulated daidzein distribution from 0 to 14 days in soil. The rate of daidzein secretion from roots was assumed to be constant at each depth, and the distribution at the middle of root at a depth of 5 mm was displayed in radial direction. It is noted that vertical distribution of daidzein was not obtained in this simulation. (B) Contents of isoflavones in leaves and roots (n = 3). (C) Contents of isoflavones in bulk and rhizosphere soils at VE stage (n = 3).

Suppmenentary Material

Supplementary Table 1. Parameters used in this study

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