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ORIGINAL ARTICLE

Nitrogen and phosphorus fertilization negatively affects strigolactone production and exudation in sorghum

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Abstract Strigolactones (SLs) are essential host recognition signals for both root parasitic plants and arbuscular mycorrhizal fungi, and SLs or their metabolites function as a novel class of plant hormones regulating shoot and root architecture. Our previous study indicated that nitrogen (N) deficiency as well as phosphorus (P) deficiency in sorghum enhanced root content and exudation of 5-deoxystrigol, one of the major SLs produced by sorghum. In the present study, we examined how N and P fertilization affects SL production and exudation in sorghum plants subjected to short- (5 days) or long-term (10 days) N or P deficiency and demonstrated their common and distinct features. The root contents and exudation of SLs in the N- or P-deficient sorghum plants grown for 6, 12 or 24 h with or without N or P fertilization were quantified by LC–MS/MS. In general, without fertilization, root contents and exudation of SLs stayed at similar levels at 6 and 12 h and then significantly increased at 24 h. The production of SLs responded more quickly to P fertilization than the secretion of SLs, while regulation of SL secretion began earlier after N fertilization. It is suggested that sorghum plants regulate SL production and exudation when they are subjected to nutrient deficiencies depending on the type of nutrient and degree of deficiency.

Keywords 5-Deoxystrigol · Nitrogen deficiency · Phosphorus deficiency · Sorgomol

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Abbreviations

AM	Arbuscular mycorrhizal
HPLC	High-performance liquid chromatography
LC–MS/MS	Liquid chromatography–tandem mass spectrometry
MRM	Multiple reaction monitoring
N	Nitrogen
P	Phosphorus
SL	Strigolactone

Introduction

Root parasitic plants of the genera *Striga* and *Orobanche* cannot complete their lifecycle without forming connections with their host plants to obtain water and nutrients, which causes severe yield losses in agricultural production all over the world (Parker 2012). The seeds of root parasitic plants are tiny and stock minimal nourishment for germination. Therefore, they need to parasitize host roots within a few days of germination and have developed an ingenious strategy to find host roots in their vicinity by sensing chemical compounds called 'germination stimulants' released from host roots. The seeds of these root parasites can germinate only when they are exposed to germination stimulants (Joel et al. 2007). Strigolactones (SLs) are representative germination stimulants and widely distributed in the plant kingdom (Xie et al. 2010). Strigol was the first SL isolated from root exudate of cotton as a germination stimulant for *S. lutea* (syn. *S. asiatica*) (Cook et al. 1966) and more than ten SLs have so far been isolated and their structures were determined (Xie et al. 2010). All of these natural SLs contain a tricyclic lactone (ABC part) that connects via an enol ether bridge to a butenolide group (D ring). These compounds have one or two methyl groups on the A ring

and one or more hydroxyl or acetyloxyl groups in the A/B ring moiety (Fig. 1).

SLs function in the rhizosphere as chemical signals to commence not only parasitism of root parasitic plants but also symbiosis of arbuscular mycorrhizal (AM) fungi. AM fungi inhabit the cortical tissues of plant roots throughout their lifecycle and supply mineral nutrients, mainly phosphorus (P) and nitrogen (N), to host plants. In turn, AM fungi obtain photosynthates from host plants. Spores of AM fungi germinate under moist conditions within a particular temperature range and their hyphae elongate to some extent (Smith and Read 2008). Then, intensive hyphal branching occurs in the vicinity of host roots, which is induced by SLs released from the host. 5-Deoxystrigol was first isolated from *Lotus japonicus* L. root exudates as a branching factor for AM fungi (Akiyama et al. 2005). This is the simplest SL and lacks hydroxyl, acetyloxyl, or other oxygen atom-containing substituents on the A and B rings; therefore, 5-deoxystrigol and its isomer, *ent*-2′-*epi*-5-deoxystrigol, are proposed to be the common precursor of all other SLs (Xie et al. 2010; Yoneyama et al. 2012). Indeed, these simplest SLs were found to be distributed widely in the plant kingdom and have been detected in root exudates from both monocots and dicots (Yoneyama et al. 2008, 2011).

In addition to their role as host recognition signals in the rhizosphere for both root parasites and AM fungi, SLs or their metabolites in plants have been described as functioning as a novel class of plant hormones. Exogenously applied GR24, a synthetic SL, suppressed shoot outgrowth in SL-deficient mutants of *Arabidopsis*, pea, and rice plants (Gomez-Roldan et al. 2008; Umehara et al. 2008). Furthermore, SLs have been shown to be involved in the regulation of root system architecture and development (Koltai 2011; Ruyter-Spira et al. 2011), secondary growth (Agusti et al. 2011), and leaf senescence (Snowden et al. 2005; Umehara 2012).

P deficiency promoted SL exudation in most plant species so far examined and N deficiency also enhanced SL exudation in some plant species including sorghum, Chinese milk vetch, lettuce, wheat, and marigold (Yoneyama et al. 2007b, 2012). Our previous study suggested that, in Chinese milk vetch, decreased shoot P level induced by N deficiency rather than N deficiency itself promoted SL exudation (Yoneyama et al. 2012). By contrast in sorghum, N deficiency did not decrease shoot P level (Yoneyama et al. 2007b), and thus N deficiency and P deficiency appeared to regulate SL production and exudation independently. Although it had been found that sorghum mainly produced not only 5-deoxystrigol but also sorgomol, the structure of sorgomol had not been determined at that time. Subsequently, the structure of sorgomol was elucidated (Xie et al. 2008) and it was found that sorgomol is distributed in both monocots and dicots (Yoneyama et al. 2008, 2011). Now, both sorgomol and 5-deoxystrigol can be quantified by liquid chromatography–tandem mass spectrometry (LC–MS/MS) (Sato et al. 2005) because natural and synthetic standards have become available. In the present study, for further detailed investigation of the effects of N and P fertilization on the production and exudation of sorgomol and 5-deoxystrigol by sorghum, the amounts of both SLs exuded and their contents in the roots of plants subjected to N or P fertilization after growth under low-N or low-P conditions for a short- (5 days) or a long-term (10 days) were quantified by using LC–MS/MS.

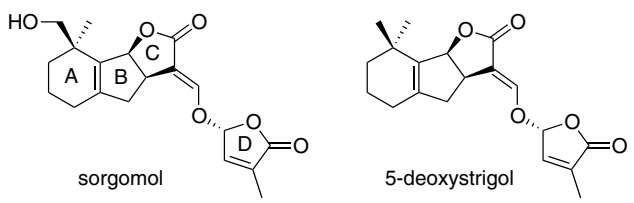

Fig. 1 Chemical structures of sorgomol and 5-deoxystrigol

Materials and methods

Chemicals

(+)-Sorgomol was purified from sorghum root exudates. (±)-5-Deoxystrigol was a generous gift from Dr. Kohki Akiyama (Osaka Prefecture University). The other chemicals of analytical grade and high-performance liquid chromatography (HPLC) solvents were obtained from Kanto Chemical Co. Ltd. (Tokyo, Japan) and Wako Pure Chemical Industries Ltd. (Osaka, Japan).

Hydroponic culture

Sorghum seeds [*Sorghum bicolor* (L.) Moench cv. Hybrid] were obtained from Snow Brand Seed Co., Ltd. (Hokkaido, Japan). The seeds were surface-sterilized in 70 % ethanol for 2 min. After thoroughly rinsing with sterile Milli-Q water, the seeds were germinated on moistened filter paper in Petri dishes for 2 days. Germinated seeds ($n = 5$) were transferred to a stainless steel sieve lined with a sheet of gauze moistened by placing it on a plastic cup (9.5 cm in diameter, 17 cm deep, approximately 550 ml in volume) containing 500 ml of tap water and incubated in tap water for 5 days in a growth chamber with a 14-h light (6 am to 8 pm) and 10-h dark photoperiod at 120 μmol photons m^{-2} s^{-1} at 28 °C/26 °C. Seedlings were then grown in 1/2 Tadano and Tanaka (TT) medium (Tadano and Tanaka 1980) containing 2.43 mM N (1.43 mM $NaNO_3$, 0.5 mM

NH_4NO_3), 0.16 mM $NaH_2PO_4{\cdot}2H_2O$, 0.5 mM K_2SO_4, 1 mM $CaCl_2{\cdot}2H_2O$, and 1 mM $MgSO_4{\cdot}7H_2O$ with 1 mM 4-morpholineethane sulfonic acid for another 5 days in the growth chamber. Then, plants were subjected to low-N conditions (0.12 mM N) or low-P conditions (8 μM P). The pH of all culture media was adjusted to 6.0 with KOH. The culture media were replaced daily at 8 am with fresh ones throughout the experimental period.

N and P fertilization

After 5 (short-term N and P deficiency) and 10 (long-term N and P deficiency) days of growth in the low-N or low-P conditions, half of the plants (three cups) were transferred to 1/2 TT media (N fertilization and P fertilization). The remaining plants (three cups) were continuously subjected to low-N and low-P conditions. At 6, 12, or 24 h after the onset of the second incubation (2, 8 pm, and 8 am on the following day, respectively), the growth media containing root exudates (plus washings) were collected (approximately 450 ml) and sorghum plants were harvested (Fig. 2).

Extraction of SLs from root exudate and root tissues

Extraction of SLs from plant root exudates and root tissues was conducted as described previously (Yoneyama et al. 2007b). The growth media containing root exudates were extracted three times with an equal volume of ethyl acetate. The ethyl acetate solutions were combined, washed with 0.2 M K_2HPO_4 (pH 8.3), dried over anhydrous $MgSO_4$, and concentrated in vacuo to afford root exudate samples. Harvested root tissues were soaked in ethyl acetate in the dark at 4 °C for 3 days. After filtration, the ethyl acetate solutions were washed with 0.2 M K_2HPO_4 (pH 8.3), dried over anhydrous $MgSO_4$, and concentrated in vacuo to afford root extracts. The samples were kept at 4 °C until use.

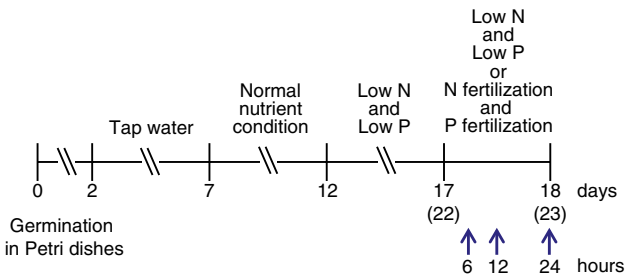

Fig. 2 Schematic diagram showing the experimental conditions. After 5 (short-term N and P deficiency) and 10 (long-term N and P deficiency) days of growth in the low-N or low-P conditions, half of the plants received N or P fertilization. The remaining plants were continuously subjected to low-N or low-P conditions. At 6, 12, and 24 h after the onset of the second incubation, the growth media containing root exudates were collected and sorghum plants were harvested

LC–MS/MS analysis of strigolactones

Quantification of sorgomol and 5-deoxystrigol by LC–MS/MS was performed as reported previously (Yoneyama et al. 2007a, b). HPLC separation was conducted with a U980 HPLC instrument (Jasco, Tokyo, Japan) fitted with an ODS (C_{18}) column (Mightysil RP-18, 2 × 250 mm, 5 μm; Kanto Chemicals Co. Ltd.). The mobile phase was 70 % methanol in water (v/v) and was changed to 100 % methanol 14 min after injection. The column was then washed with 100 % methanol for 20 min to elute all the injected materials. The flow rate was 0.2 ml min^{-1}, and the column temperature was set to 40 °C. Mass spectrometry was performed with a Quattro LC mass spectrometer (Micromass, Manchester, UK) equipped with an electrospray source. Both the drying and the nebulizing gas was nitrogen that was generated from pressurized air in an N2G nitrogen generator (Parker-Hanifin Japan, Tokyo, Japan). The nebulizer gas flow was set to approximately 100 L h^{-1}, and the desolvation gas flow to 500 L h^{-1}. The interface temperature was set to 400 °C, and the source temperature to 150 °C. The capillary and cone voltages were adjusted to 5-deoxystrigol and to the positive ionization mode. MS/MS experiments were conducted using argon as the collision gas, and the collision energy was set to 16 eV. The collision gas pressure was 0.15 Pa. The transitions of *m/z* 353–256 and 369–272 were monitored for 5-deoxystrigol and sorgomol, respectively. Data acquisition and analysis were performed with the MassLynx software (ver. 4.1). Quantifications of sorgomol and 5-deoxystrigol were conducted using natural and synthetic standards, respectively, in a manner similar to that for orobanchol and 5-deoxystrigol (Yoneyama et al. 2007a, b).

Measurement of N and P in plant tissues

N and P measurements of plant tissues were conducted as described previously (Yoneyama et al. 2007a, b). Harvested plants were separated into shoots and roots, oven-dried at 60 °C for 72 h, and then ground by milling. The concentrations of N were determined using an N/C analyzer. For P measurements, dry materials were decomposed using 13 N nitric acid and P concentrations were then determined by the vanadomolybdate yellow method (Nanamori et al. 2004).

Plant growth determination

At the end of the experiments, plants were harvested, separated into shoots and roots, and their fresh weights were determined.

Statistical analysis

All experiments with three replications were repeated at least twice to confirm the results. The data are presented as means ± standard errors ($n = 3$) from a typical single experiment. Significance analyses were performed by Student's t test ($P < 0.05$) between cases with and without fertilization treatment at each timepoint and Tukey–Kramer's honestly significant difference (HSD) test ($P < 0.05$) among incubation periods. Root contents and exuded amount of sorgomol at each timepoint were compared statistically with those of 5-deoxystrigol by using Student's t test ($P < 0.05$).

Results

Effects of N fertilization on root content and exudation of SLs in the plants subjected to short-term N deficiency

The root contents and exudation of SLs in the sorghum plants grown for 6, 12, or 24 h either under low-N conditions (0.12 mM N) or subjected to N fertilization (2.4 mM N) after short-term (5 days) incubation under N-deficient conditions (0.12 mM N) were quantified by LC–MS/MS. Symptoms of N deficiency (e.g., yellowing of leaves) were not obvious in the sorghum plants after this short-term incubation under low-N conditions (data not shown).

In the plants continuously grown under low-N conditions, root contents of sorgomol and 5-deoxystrigol stayed at similar levels at 6 and 12 h and then significantly increased at 24 h (Fig. 3a). Sorgomol exudation also increased at 24 h. There were no significant differences in the exudation of 5-deoxystrigol among samples collected at different incubation periods (Fig. 3b). By contrast, in the plants that received N fertilization, there were no statistically significant differences in SL contents among samples collected at different incubation periods (Fig. 3a), but exudation of SLs slightly increased at 24 h (Fig. 3b).

N fertilization significantly suppressed exudation of both sorgomol and 5-deoxystrigol even at 6 h (Fig. 3b). An inhibitory effect of N fertilization on SL exudation was also observed at 12 and 24 h (Fig. 3b). By contrast, there were no statistically significant differences in root SL contents between the plants grown under low N and N fertilization conditions, and only the 5-deoxystrigol content became significantly different between the treatments at 24 h (Fig. 3a).

Under low-N conditions, sorghum roots exuded more sorgomol than 5-deoxystrigol at 24 h ($P < 0.05$); however, root contents of 5-deoxystrigol were higher than those of sorgomol at all timepoints (Fig. 3a, b). There were no statistically significant differences in root contents and exudations of these SLs in the plants that received N fertilization except for root contents at 6 h where content of 5-deoxystrigol was significantly higher than that of sorgomol ($P < 0.05$) (Fig. 3a, b).

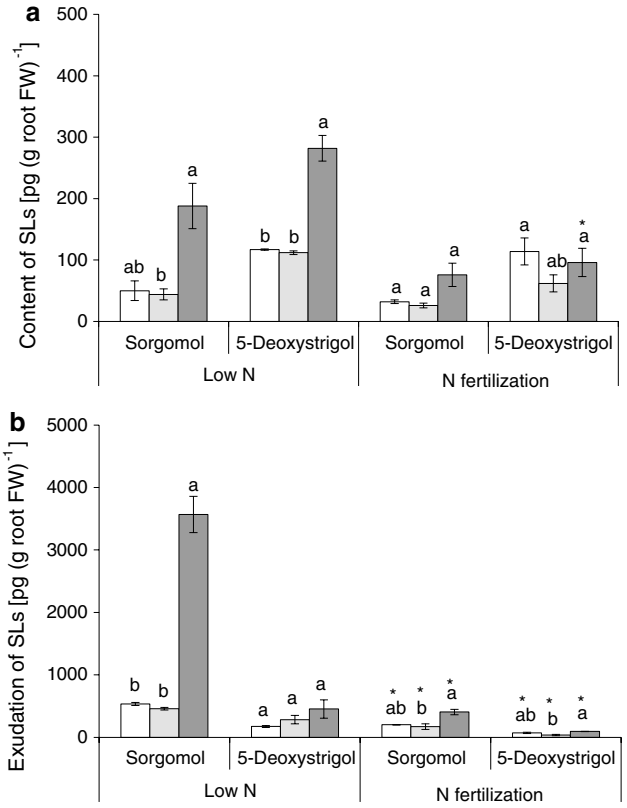

Fig. 3 Root content (**a**) and exudation of SLs [pg (g root FW)$^{-1}$] (**b**) of sorghum plants grown either under low-N (0.12 mM N) or subjected to N fertilization (2.4 mM N) after a 5-day-pre-incubation under N-deficient conditions (0.12 mM N). Root exudates and root tissues were collected at 6, 12, and 24 h after the onset of the second incubation. *White, gray*, and *dark gray* bars indicate data at 6, 12, and 24 h, respectively. The experiments were repeated twice and data were from a typical single experiment. Values are mean ± SE of three replicates. *Different letters* indicate statistically significant differences among incubation periods according to Tukey–Kramer's HSD test ($P < 0.05$). *Asterisks* indicate significant differences in SL levels between low N and N fertilization conditions at each timepoint according to Student's t test ($P < 0.05$)

Effects of N fertilization on root content and exudation of SLs in the plants subjected to long-term N deficiency

The effects of N fertilization on root content and exudation of SLs were examined in the plants subjected to long-term N deficiency for 10 days. Symptoms of N deficiency, such as yellowing of leaves, appeared in the plants grown under this long-term N deficiency and the color of the leaves did not recover by the N fertilization for 24 h (data not shown).

In the plants continuously grown under low-N conditions, root contents of sorgomol and 5-deoxystrigol stayed at similar levels at 6 and 12 h and then significantly increased at 24 h as in the case of the plants subjected to short-term N deficiency (Fig. 4a). Exudation of sorgomol increased at 24 h and that of 5-deoxystrigol gradually increased with incubation time (Fig. 4b). N fertilization clearly decreased SL contents in the roots with incubation time (Fig. 4a); however, there were no statistically significant differences in exudation of each SL among the samples collected at different incubation times (Fig. 4b).

In general, root contents and exudations of SLs were up to by 50 % greater than those observed in the plants subjected to short-term N deficiency. An inhibitory effect of N fertilization on sorgomol root content was observed at 12 and 24 h (Fig. 4a), while inhibition of sorgomol exudation was already clear at 6 h (Fig. 4b). Root content and exudation of 5-deoxystrigol were apparently suppressed by N fertilization at 24 h (Fig. 4a, b).

As in the case of short-term N deficiency, the content of 5-deoxystrigol in the roots was higher than that of sorgomol in particular at 24 h under continuous N deficiency ($P < 0.05$) and at all timepoints in the plants that received N fertilization (Fig. 4a), while the amount of sorgomol exudation was significantly greater than that of 5-deoxystrigol only under continuous N deficiency (Fig. 4b).

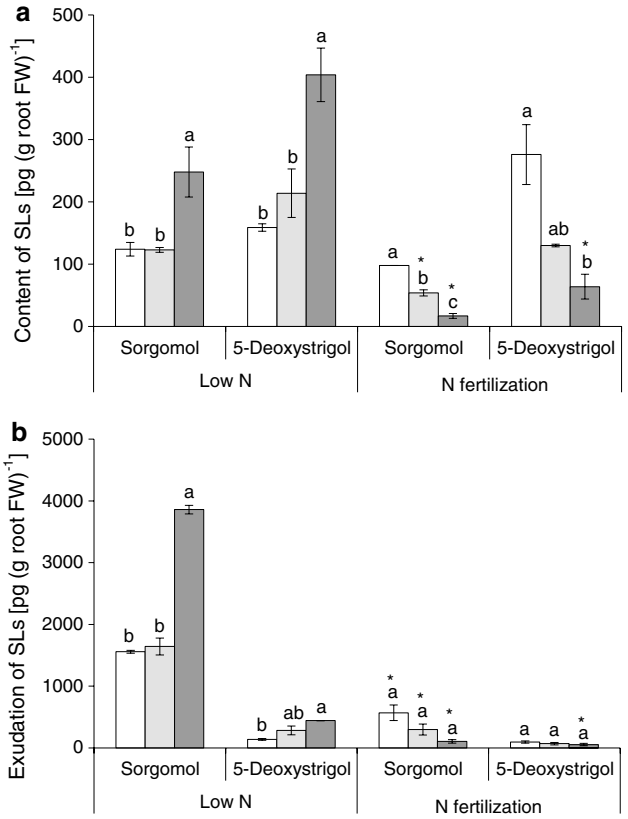

Fig. 4 Root content (**a**) and exudation of SLs [pg (g root FW)$^{-1}$] (**b**) of sorghum plants grown either under low N (0.12 mM N) or subjected to N fertilization (2.4 mM N) after a 10-day-pre-incubation under N-deficient conditions (0.12 mM N). Root exudates and root tissues were collected at 6, 12, and 24 h after the onset of the second incubation. *White, gray*, and *dark gray* bars indicate data at 6, 12, and 24 h, respectively. The experiments were repeated twice and data were from typical single experiment. Values are mean ± SE of three replicates. *Different letters* indicate statistically significant differences among incubation periods according to Tukey–Kramer's HSD test ($P < 0.05$). *Asterisks* indicate significant differences in SL levels between low N and N fertilization conditions at each timepoint according to Student's *t* test ($P < 0.05$)

Effects of P fertilization on root content and exudation of SLs in the plants subjected to short-term P deficiency

The root contents and exudation of SLs in the sorghum plants grown for 6, 12, or 24 h either under low-P conditions (8 μM P) or subjected to P fertilization (160 μM P) after short-term (5 days) incubation under P-deficient conditions (8 μM P) were quantified by LC–MS/MS.

Symptoms of P deficiency, for example, purpling of leaves, were not observed in the plants grown under these short-term P-deficient conditions (data not shown). In the plants continuously grown under low P, root content and exudation of sorgomol stayed at similar levels at 6 and 12 h and then significantly increased at 24 h (Fig. 5a, b), whereas there were no statistically significant differences in 5-deoxystrigol content among root samples collected at different incubation periods (Fig. 5a) and its exudation gradually increased with incubation time (Fig. 5b). By contrast, in the plants that received P fertilization, sorgomol content in the roots decreased at 6 h, dropped further at 12 h, and then increased at 24 h (Fig. 5a), whereas sorgomol exudation did not change during the experimental period (Fig. 5b). Similar changes of content in the roots were observed for 5-deoxystrigol (Fig. 5a). There were no statistically significant differences in 5-deoxystrigol exudation between 6 and 12 h (Fig. 5b).

Inhibitory effects of P fertilization on the root content and exudation of SLs were observed even at 6 h and continued at 12 and 24 h (Fig. 5a, b). The contents and exudation levels of SLs were slightly lower than those observed for the plants subjected to N deficiency.

The plants grown under low-P conditions contained and exuded significantly larger amounts of sorgomol than 5-deoxystrigol at 24 h ($P < 0.05$) (Fig. 5a, b). In the plants subjected to P fertilization, root contents of both sorgomol and 5-deoxystrigol decreased at 12 h and then increased at 24 h, while exudation of these SLs remained at similar level during experimental period and only a slight increase of 5-deoxystrigol was observed at 24 h (Fig. 5a, b).

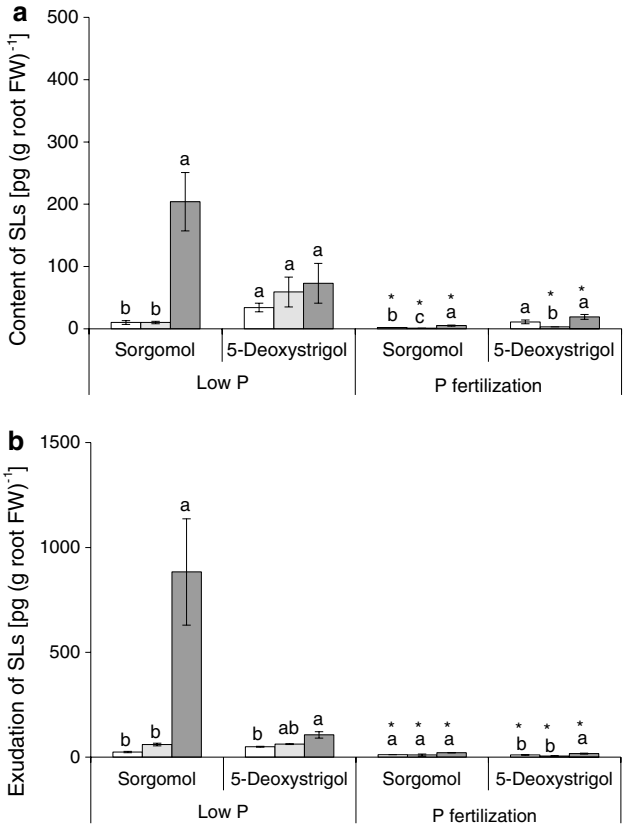

Fig. 5 Root content (**a**) and exudation of SLs [pg (g root FW)$^{-1}$] (**b**) of sorghum plants grown either under low P (8 μM P) or subjected to P fertilization (160 μM P) after a 5-day-pre-incubation under P-deficient conditions (8 μM P). Root exudates and root tissues were collected at 6, 12, and 24 h after the onset of the second incubation. *White, gray*, and *dark gray* bars indicate data at 6, 12, and 24 h, respectively. The experiments were repeated twice and data were from typical single experiment. Values are mean ± SE of three replicates. *Different letters* indicate statistically significant differences among incubation periods according to Tukey–Kramer's HSD test ($P < 0.05$). *Asterisks* indicate significant differences in SL levels between low P and P fertilization conditions at each timepoint according to Student's *t* test ($P < 0.05$)

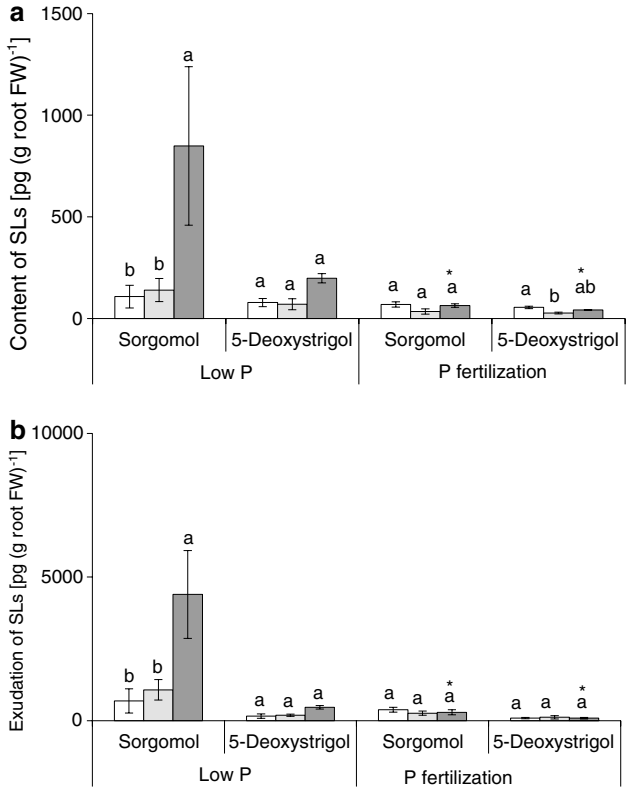

Fig. 6 Root content (**a**) and exudation of SLs [pg (g root FW)$^{-1}$] (**b**) of sorghum plants grown either under low P (8 μM P) or subjected to P fertilization (160 μM P) after a 10-day-pre-incubation under P deficient conditions (8 μM P). Root exudates and root tissues were collected at 6, 12, and 24 h after the onset of the second incubation. *White, gray*, and *dark gray* bars indicate data at 6, 12, and 24 h, respectively. The experiments were repeated twice and data were from typical single experiment. Values are mean ± SE of three replicates. *Different letters* indicate statistically significant differences among incubation periods according to Tukey–Kramer's HSD test ($P < 0.05$). *Asterisks* indicate significant differences in SL levels between low P and P fertilization conditions at each timepoint according to Student's *t* test ($P < 0.05$)

Effects of P fertilization on root content and exudation of SLs in the plants subjected to long-term P deficiency

The effects of P fertilization were then examined using the plants subjected to long-term (10 days) P deficiency. Purple pigment developed in the leaves along the veins in the plants subjected to long-term P deficiency and the plants did not recover from these typical P-deficiency symptoms by P fertilization for 24 h (data not shown). In general, root contents and exudations of SLs were up to fivefold higher than those observed in the plants subjected to short-term P deficiency (Fig. 6a, b).

In the plants grown continuously under low-P conditions, root content and exudation of sorgomol increased at 24 h (Fig. 6a, b), as in the case of the plants pre-incubated with short-term P deficiency and then grown under low-P conditions (Fig. 5a, b). The root content and exudation of 5-deoxystrigol seemed to change in a similar manner (Fig. 6a, b), while there were no significant differences in these variables of 5-deoxystrigol among samples collected at different incubation periods (Fig. 6a, b). In the plants that received P fertilization, root contents and exudations of SLs remained constant, except for 5-deoxystrigol content in the roots, which dropped slightly at 12 h (Fig. 6a, b).

Inhibitory effects of P fertilization on root content and exudation of SLs were clearly observed at 24 h (Fig. 6a, b). It took longer for significant reductions in SL contents and exudations to be exhibited in the plants pre-incubated with long-term P deficiency than for those subjected to short-term or long-term N deficiency, or short-term P deficiency.

As in the case of short-term P deficiency, both root content and exudation of sorgomol in the plants grown continuously under low-P conditions were significantly greater than those of 5-deoxystrigol at 24 h (Fig. 6a, b). In the plants that received P fertilization, the contents of these two SLs in the roots and the amounts of exudations remained at similar levels (Fig. 6a, b).

N and P contents in plant tissues

As listed in Tables 1, 2, 3, 4, pre-incubation periods affected N and P levels in plant tissues. In the plants subjected to long-term N deficiency, N levels decreased to approximately 50 % of that of the plants subjected to short-term N deficiency. P levels decreased to about 70 % in the plants subjected to long-term P deficiency when compared with those subjected to short-term P deficiency. In the present study, N and P fertilization did not significantly affect N and P levels in shoot and root tissues.

Table 1 N levels (mg g^{-1} DW) in shoot and root tissues from sorghum plants grown under low-N conditions continuously and those that received N fertilization after pre-incubation with N deficiency for 5 days

Incubation period (h)	Low N		N fertilization	
	Shoot	Root	Shoot	Root
6	28 ± 0.2	21 ± 1.1	34 ± 0.5	23 ± 0.4
12	26 ± 0.7	20 ± 0.7	27 ± 0.9	21 ± 0.6
24	26 ± 0.4	17 ± 0.5	27 ± 0.5	19 ± 0.5

Standard errors of mean are also indicated ($n = 3$)

Table 2 N levels [mg (g DW^{-1})] in shoot and root tissues from sorghum plants grown under low-N conditions continuously and those that received N fertilization after pre-incubation with N deficiency for 10 days

Incubation period (h)	Low N		N fertilization	
	Shoot	Root	Shoot	Root
6	15 ± 0.5	13 ± 0.0	16 ± 0.3	16 ± 0.5
12	13 ± 0.1	12 ± 0.1	16 ± 0.3	15 ± 0.4
24	13 ± 0.3	13 ± 0.1	16 ± 0.3	14 ± 0.3

Standard errors of mean are also indicated ($n = 3$)

Table 3 P levels (mg g^{-1} DW) in shoot and root tissues from sorghum plants grown under low-P conditions continuously and those that received P fertilization after pre-incubation with P deficiency for 5 days

Incubation period (h)	Low P		P fertilization	
	Shoot	Root	Shoot	Root
6	2.6 ± 0.0	2.9 ± 0.5	4.1 ± 0.0	3.5 ± 0.4
12	3.0 ± 0.5	2.6 ± 0.5	3.8 ± 0.0	3.2 ± 0.0
24	2.8 ± 0.5	2.8 ± 0.5	3.8 ± 0.3	2.9 ± 0.1

Standard errors of mean are also indicated ($n = 3$)

Table 4 P levels (mg g^{-1} DW) of shoot and root tissues from sorghum plants grown under low-P conditions continuously and those that received P fertilization after pre-incubation with P deficiency for 10 days

Incubation period (h)	Low P		P fertilization	
	Shoot	Root	Shoot	Root
6	1.7 ± 0.1	2.1 ± 0.2	2.4 ± 0.1	2.9 ± 0.1
12	1.8 ± 0.0	2.3 ± 0.5	2.0 ± 0.1	2.2 ± 1.1
24	1.6 ± 0.2	2.3 ± 0.5	2.0 ± 0.1	2.5 ± 0.4

Standard errors of mean are also indicated ($n = 3$)

Effects of N and P fertilization on plant growth

Neither N nor P fertilization up to 24 h affected the plant growth in terms of shoot and root fresh weights (Tables S1–S4).

Discussion

It is now widely accepted that deficiency of mineral nutrients, especially P, affects the production and exudation of SLs, which function as plant hormones regulating shoot and root architecture, as well as being rhizosphere signaling molecules for AM fungi and root parasitic plants. In red clover plants, among mineral nutrients including N, P, K, Ca, and Mg, only P deficiency was found significantly to promote the exudation of orobanchol, one of the major SLs produced by this plant (Yoneyama et al. 2007a). By contrast, in the case of sorghum plants, N as well as P deficiency enhanced the exudation and root content of 5-deoxystrigol (Yoneyama et al. 2007b). López-Ráez et al. (2008) showed that P starvation in tomato markedly increased the root exudation of SL and the exudates from P-starved tomato plants strongly induced seed germination of root parasitic weed and hyphal branching of AM fungi. Jamil et al. (2011) reported that P deficiency significantly increased SL exudation and only a weak effect on SL exudation was observed under N deficiency in rice plants. In our recent study, only P deficiency promoted SL exudation in alfalfa and tomato, while N as well as P deficiency promoted SL exudation in Chinese milk vetch, lettuce, marigold, and wheat (Yoneyama et al. 2012). These results imply that P deficiency is a factor promoting SL production and exudation; however, it remains elusive how N deficiency affects SL production and exudation. Here,

we examined how N and P fertilization affects SL production and exudation in sorghum plants subjected to short- or long-term N or P deficiency and demonstrated their common and distinct features.

When sorghum plants were subjected to N or P deficiency and subsequently grown under the same nutrient-deficient conditions, the production and exudation of SL seemed to be rather slow from 6 to 12 h when compared with those from 12 to 24 h (Figs. 3, 4, 5, 6), since the contents and amounts of exuded SLs were similar at 6 and 12 h and then increased at 24 h. This may be related to the circadian rhythm of SL biosynthesis and metabolism. It has been well demonstrated that there are interactions between the circadian clock and hormonal signaling networks in the regulation of growth and development of plants (Robertson et al. 2009). For example, in leaves of tobacco plants grown under a 16/8 h photoperiod, peaks of indole-3-acetic acid (IAA) and physiologically active cytokinins (cytokinin bases and ribosides, CKs) were found in the subjective afternoon (sNováková et al. 2005). In *Arabidopsis*, most auxin-induced gene displayed peak clock-regulated expression in the subjective afternoon (Covington and Harmer 2007). Accordingly, it is likely that under our experimental conditions, level of endogenous auxin in shoots reached a maximum in the subjective afternoon, at around 14 h. Then a few hours later, during which auxin, positive regulator of SL biosynthesis (Hayward et al. 2009), was transported from the shoots to roots, this increase in auxin in the shoots would result in upregulation of SL biosynthesis genes in the roots and thus enhanced SL production. This would be the reason why SL levels and exudations stayed rather constant during the first 12 h and then increased during the second 12 h (Figs. 3, 4, 5, 6).

In the plants subjected to short-term N deficiency, changes in SL contents in the roots were comparable to those in the root exudates except for that ratio of sorgomol to 5-deoxystrigol was smaller in root exudates than that in root tissues (Fig. 3a, b), indicating that the availability of N directly influences the production rather than the secretion of SLs (Yoneyama et al. 2007b). Alternatively, more hydrophilic sorgomol once formed is preferably released from the roots into culture media. However, no significant differences were observed for SL contents in the roots between the plants grown under low N and N fertilization conditions. This suggests that N availability not only influences the production but also the secretion of SLs, and it is likely that regulation of SL secretion precedes that of SL production because reduction of SL exudation upon N fertilization occurred earlier than that in SL contents in the roots (Fig. 3a, b). In addition, a smaller ratio of sorgomol to 5-deoxystrigol in the root exudate from the plants that received N fertilization when compared with that of those grown in low N continuously, especially at 24 h (Fig. 3a, b), indicates that the conversion of 5-deoxystrigol to sorgomol may be promoted under low-N conditions but suppressed by N fertilization.

In the plants subjected to long-term N deficiency, the time-dependent suppression of SL content in the roots by N fertilization was clearer than that in the plants subjected to short-term N deficiency. The reduction of SL contents in the roots may have been not only due to reduced production but also due to enhanced metabolism.

In the sorghum plants subjected to short-term P deficiency and then continuously grown under low-P conditions, sorgomol content in the roots increased significantly from 12 to 24 h, while 5-deoxystrigol level increased constantly with incubation period, indicating that the conversion of 5-deoxystrigol to sorgomol is promoted under more intense P deficiency (Fig. 5a). P fertilization strongly reduced both sorgomol and 5-deoxystrigol contents in the roots, while exudation of these SLs remained constant (Fig. 5a, b). These results imply that the production of SLs responded more quickly to P fertilization than the secretion of SLs, and therefore the response of SL production and secretion in sorghum plants to P fertilization appears to be somewhat different from that to N fertilization, for which regulation of SL secretion begins earlier. The observed recovery of SL contents in the roots at 24 h in the plants that received P fertilization indicates that the production of SLs occurs constitutively and is enhanced under conditions of reduced P supply.

In red clover plants pre-incubated at 8 μM P and then transferred to 160 μM P, orobanchol exudation halved during the first 24 h and continued to decrease thereafter (Yoneyama et al. 2007a). Umehara et al. (2010) also showed that the expression of SL biosynthesis genes, as well as SL levels in the roots, was drastically decreased within 1 day after P was supplied to P-starved rice plants. In the present study, we found that, in general, N and P fertilization suppressed SL production and exudation within 6 h in the N- and P-deficient plants, respectively. AM fungi as obligate biotrophs can be parasitic on plants growing in well-fertilized soils with high soluble Pi content (Bucher 2007). Therefore, plants should be highly sensitive to nutrient availabilities in the rhizosphere and regulate SL production and exudation so as not to be parasitized by AM fungi when they obtain sufficient nutrients by themselves.

The N content in plant tissues continuously grown under low-N conditions was lower in the plants subjected to long-term N deficiency than in those subjected to short-term N deficiency (Tables 1 and 2). This difference in N content resulted in the increased sorgomol content in the roots and its exudation at 6 and 12 h in the plants subjected to long-term N deficiency when compared with those in the plants subjected to short-term N deficiency (Figs. 3 and 4). However, the levels of the root content and exudation of

sorgomol observed at 24 h were similar irrespective of the duration of N deficiency, suggesting that these are the maximum levels reachable under N deficiency. Therefore, sorgomol production and exudation seem to be more rapidly enhanced under stronger N deficiency, but once the production and exudation reach certain levels, they will not increase further. A similar trend was also observed for 5-deoxystrigol under N deficiency.

The amounts of SL exudation and content in the roots were higher in the plants subjected to long-term P deficiency than in those subjected to short-term P deficiency (Figs. 5, 6). This was probably due to the fact that the short-term P deficiency treatment did not create strong P deficiency in plant tissues, especially in shoot tissues. N and P deficiencies appear to affect the relative ratios of sorgomol to 5-deoxystrigol in the roots and in the root exudates. In general, the N-deficient plants were found to exude larger amounts of sorgomol than that of 5-deoxystrigol and contained smaller amounts of sorgomol than that of 5-deoxystrigol (Figs. 3, 4). On the other hand, the P-deficient plants contained and exuded larger amounts of sorgomol than that of 5-deoxystrigol (Figs. 5, 6). These results also suggest that sorghum plants regulate SL production and exudation when they are subjected to nutrient deficiencies in somewhat different ways depending on the type of nutrient and degree of deficiency.

Plants exude a mixture of SLs (Yoneyama et al. 2008, 2011); however, it remains elusive why they do this. Thus, it is important to quantify and compare the amounts of each SL to resolve this issue. Comparison of peak areas is not a reliable estimation of SL contents since ion suppression occurs in electrospray-ionization, which is commonly used in LC–MS/MS analysis of SLs. Our previous study demonstrated that Chinese milk vetch exuded larger amounts of sorgomol than that of 5-deoxystrigol, alfalfa exuded larger amounts of orobanchol than that of orobanchyl acetate, lettuce exuded larger amounts of orobanchyl acetate than that of orobanchol, and marigold exuded equal amounts of orobanchol and orobanchyl acetate when plants were grown hydroponically under P-deficiency conditions (Yoneyama et al. 2012). The compositions and amounts of SLs exuded from plants appear to change depending on the growth stage and can be influenced by various environmental factors. In the present study, both N- and P-deficient sorghum plants exuded larger amounts of sorgomol than that of 5-deoxystrigol, suggesting that sorgomol may be more important than 5-deoxystrigol as a host recognition signal for AM fungi (and also for root parasitic plants), at least in this cultivar. Alternatively, probably because we used hydroponic cultivation systems, we could not detect even a trace amount of sorgolactone. Since sorgolactone seems to be synthesized from 5-deoxystrigol via sorgomol, sorgolactone may be an important signal for AM fungi. Further study is needed to uncover the importance of individual SLs in AM symbiosis and parasite seed germination in the soil.

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Conflict of interest The authors declare that they have no conflict of interest.

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