Original research article

Formation of densely branched lateral roots in *Sesbania cannabina* triggered by patchily distributed phosphorus in andosolic soils

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Received on July 11, 2014; Accepted on February 21, 2015

Abstract: Phosphorus (P) is patchily distributed in soil because of its slow diffusion, especially in soil with a high phosphate absorption coefficient (PAC). Root responses to localized supply of phosphate were studied in *Sesbania cannabina* grown in volcanic andosol, which has a high PAC. Seedlings were grown in soil that was supplied with 0, 10, 100, 500, or 1000 mg P kg^{-1}. After 30 days, analyses of plant P and root morphological were conducted. Further rhizobox experiments were also conducted. Seedlings were grown with layered P sources or localized P patches. Densely branched lateral roots (DBLRs) developed only in the 10 and 100 mg P kg^{-1} treatments. Although an increase in shoot dry weight (DW) was observed in the 500 and 1000 mg P kg^{-1} treatments, DBLRs were not observed. The number of DBLRs was positively correlated with shoot DW, root DW, and number of nodules, and negatively correlated with phosphorus use efficiency of shoots and roots. The rhizobox experiment showed that most DBLRs were observed in the layer with added P and in the position where P fertilizer was present. DBLRs developed so as to monopolize the P fertilizer by completely enveloping the area around it. The results suggest that DBLR formation is one of *S. cannabina*'s P acquisition strategies.

Keywords: available phosphorus, phosphorus absorption coefficient, phosphorus utilization efficiency, root length density, root system, volcanic andosol

Abbreviations: DBLR, densely branched lateral root; PUE, phosphorus utilization efficiency; PAE, phosphorus acquisition efficiency; RLD, root length density

Introduction

Phosphorus (P) is one of the major macronutrients needed for plant growth. It is a component of key molecules such as nucleic acids, phospholipids, and ATP, and plants cannot grow well without an adequate supply of P. Plant roots acquire their phosphate from solution in the external soil, where it is in equilibrium with the phosphate sorbed onto soil minerals and colloids (Smith et al. 2003). However, P is often unavailable in soils, because it rapidly forms insoluble complexes with cations, particularly Al^{3+} and Fe^{3+} in acidic soil, and Ca^{2+} in alkaline soil (Tinker and Nye 2000). Therefore, the rate of diffusion of P in soil is slow (10^{-12} to 10^{-15} m^2 s^{-1}) (Schachtman et al. 1998), and this slow diffusion results in a reduction of the phosphate concentration in solution around the roots. P availability is of particular concern in highly weathered and volcanic soils, which have a high phosphate absorption coefficient (PAC), in the humid tropics and subtropics, where crop productivity is severely compromised because of a lack of available P (Takeda et al. 2009).

Plants overcome P starvation in various ways. Root morphological changes, organic acid and phosphatase exudation, acidification of the rhizosphere, and symbiosis with arbuscular mycorrhiza are known to be main strategies employed by plants to obtain P (Tadano and Sakai 1991, Lynch 2011, Lambers et al. 2012, Gutjahr and Paszkowski 2013). Roots are generally considered as a source of P for other plant parts, but become a sink during P starvation. This appears to be a deliberate, adaptive response

Funakoshi Y, Daimon H, Matsumura A 2015 Formation of densely branched lateral roots in *Sesbania cannabina* triggered by patchily distributed phosphorus in andosolic soils. Plant Root 9: 24-33. doi:10.3117/plantroot.9.24
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by the plant to promote root proliferation and thereby enhance soil exploration and P uptake (Raghothama 1999). Changes in the architecture of the root system can profoundly affect the capacity of plants to take up P; for example, root-hair formation increases the total surface area of primary and lateral roots (López-Bucio et al. 2003). The spatial configuration of the root system determines the ability of a plant to exploit heterogeneous soil resources (Lynch and Brown 2001, Hodge 2004). The morphological plasticity of roots in nutrient-enriched patches of soil is regarded as an adaptive response in plants (Yano and Kume 2005, Li et al. 2010).

Sesbania cannabina (Fabaceae) is a multipurpose leguminous crop, used as a green manure crop, firewood, and a biofertilizer (Ladha et al. 1988, Nzioka et al. 1993, Rao and Gill 1995), while also known to be a weed (Burbidge 1965). The original distribution of *S. cannabina* remains unclear, as it has been introduced and naturalized over a wide area (Gillett 1963). It has a potentially high biomass production, high N_2-fixing activity, and vigorous root growth (Shiba and Daimon 2003). Furthermore, *S. cannabina* can adapt to climatic conditions such as waterlogging, drought, heavy metal toxicity, and high alkalinity (Kumar and Srivastava 2012). In general, the genus *Sesbania* has high P acquisition abilities (Patcharapreecha et al. 1993). However, little is known of how *S. cannabina* adapts to P-deficiency stress and obtains limited available P by root morphological change. Here, we report the development of a specific root morphology in *S. cannabina* when roots approached P under high PAC and P-deficient soil conditions.

Materials and Methods

Plant materials

S. cannabina seedlings (Yukijirushi Shubyou Co., Hokkaido, Japan) were used in all the experiments. *S. cannabina* seed coats were scarified with quartz sand to improve their germination ratio before use.

Experiment 1. Effect of applied P concentration on root development of S. cannabina

The experiment was conducted from April 22 to May 22, 2013 in a greenhouse with natural light and without temperature control located at the Experimental Farm of Osaka Prefecture University, Sakai, Osaka, Japan (34.5°N, 135.5°E). Average air temperature ranged from 10.9°C to 23.1°C during the experiment. Andosolic soil (Sandrec Co. Ltd., Japan) was used as the substrate. The properties of the soil were as follows: pH (H_2O) 5.9, electrical conductivity (EC) 0.09 dS m^{-1}, total nitrogen 0.4%, total carbon 7.3%, total P 1816 mg kg^{-1}, available P (Truog-P) 9.3 mg kg^{-1}, phosphate absorption coefficient (PAC) 2310 mg $100g^{-1}$. The soil pH (H_2O) in a soil:solution ratio of 1:2.5 was measured with a pH meter (Horiba, Japan). The conductivity of the soil samples in a soil:solution ratio of 1:5 was measured with an EC meter (Horiba, Japan). Total N and total C was analyzed by vario MAX (Elementar, Germany). Truog-P was extracted using 0.001 M H_2SO_4 including 0.3% $(NH_4)_2SO_4$ and measured with the molybdenum blue colorimetric method. For total P, soil samples were ashed in a muffle furnace at 550ºC and determined using the vanado molybdate colorimetric method. PAC was measured using the method in Nanzyo (1986). Colonization by arbuscular mycorrhizal fungi in *S. cannabina* roots was not observed when grown for a month in the same soil as used in this experiment.

Nutrients (N and K) were added to all treatment groups. Phosphorus sources (KH_2PO_4 only or KH_2PO_4 + NaH_2PO_4) were added to 1 kg soil in a solid form at levels of 0, 10, 100, 500, and 1000 mg P kg^{-1} soil. To avoid high K concentration in the soil, NaH_2PO_4 was used as the P source instead of KH_2PO_4 in 500 and 1000 mg P treatments. Thus, five P treatments were established: 1) 0 mg P, using 60 mg of $(NH_4)_2SO_4$ and 280.7 mg K_2SO_4; 2) 10 mg P, using 43.9 mg KH_2PO_4, 60 mg of $(NH_4)_2SO_4$ and 224.5 mg K_2SO_4; 3) 100 mg P, using 438.7 mg KH_2PO_4 and 60 mg of $(NH_4)_2SO_4$; 4) 500 mg P, using 1096.8 mg KH_2PO_4,967.7 mg NaH_2PO_4 and 60 mg of $(NH_4)_2SO_4$; 5) 1000 mg P, using 2193.5 mg KH_2PO_4, 1935.5 mg NaH_2PO_4 and 60 mg of $(NH_4)_2SO_4$. Truog-P concentration after mixing P sources was as follows: 1) 9.32 mg P kg^{-1} in 0 mg P treatment; 2) 13.0 mg P kg^{-1} in 10 mg P treatment; 3) 19.6 mg P kg^{-1} in 100 mg P treatment; 4) 87.0 mg P kg^{-1} in 500 mg P treatment; 5) 160.4 mg P kg^{-1} in 1000 mg P treatment. After mixing all nutrients, 1 kg soil was put into each pot (10.5 cm diameter × 22.5 cm height).

Five seeds were sown in each pot on April 29, 2013. After germination, the seedlings were thinned to one seedling per pot. For inoculation, rhizobia (*Rhizobium* sp. strain U-9709-SC) were cultured in yeast extract mannitol (YM) medium (Somasegaran and Hoben 1994). The optical density (OD, at 620 nm) of the suspensions was adjusted to 0.2, and 2 mL of rhizobia suspension was applied to each pot after sowing. The experiment used a completely randomized design, and each treatment was replicated five times, giving a final sample size of 25 seedlings. During the experiment, plants were irrigated with tap water as needed.

Plants were harvested 30 days after sowing (DAS) and separated into shoots and roots. Roots were washed gently with tap water. Root images were

scanned using the WinRHIZO software package (Regent Instruments Incorporated, Canada) and the number of densely branched lateral root (DBLR) was counted. To evaluate the size of DBLRs, the diameter of each DBLR was calculated from root images obtained using WinRHIZO. The shoots and roots were dried at 70°C for 48 h and weighed. For P analysis, the plant materials were ground, ashed in a muffle furnace at 550°C, and measured as described above. To evaluate the relationship between the number of DBLRs and plant growth, phosphorus utilization efficiency (PUE), and phosphorus acquisition efficiency (PAE), correlation analysis was conducted. PUE and PAE was calculated by shoot or root dry weight (DW) per total P of shoot or roots and total P uptake per total P applied.

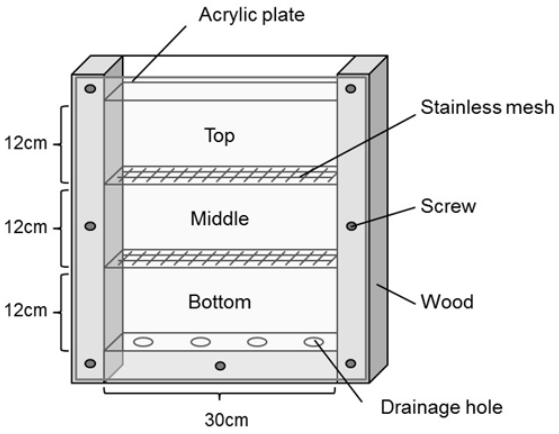

Fig. 1. Schematic diagram of the rhizobox system used in Experiment 2.

Experiment 2. Effect of P location on DBLR formation in S. cannabina

The rhizobox experiment to examine the influence of vertical P location (Fig. 1) was conducted from September 14 to October 18, 2012 in the same greenhouse as experiment 1. Average air temperature ranged from 16.7°C to 29.8°C during the experiment. Rhizoboxes were filled with approximately 3 kg of air-dried andosolic soil with 60 mg kg^{-1} $(NH_4)_2SO_4$ and 126 mg kg^{-1} K_2SO_4 added. The front wall of each rhizobox was transparent and made of acrylic board. To ensure that roots were growing in the dark, the transparent wall of the rhizobox was covered with a black plastic sheet. The remaining walls of the rhizobox were made of wood. The soil profile was stratified into three layers: the top layer at 0-12 cm, middle layer at 13-24 cm, bottom layer at 25-36 cm. Each layer was divided by a stainless steel net (5 mm mesh size). Three treatments were established: (1) P-Top treatment, where boxes were filled with 24 cm of andosol and topped with homogeneous P-mixed andosol (100 mg P kg^{-1} of KH_2PO_4); (2) P-Middle treatment, where boxes were filled with 12 cm of andosol, topped with homogeneous P-mixed andosol, in turn topped with 12 cm of andosol; (3) P-Bottom treatment, where boxes were filled first with 12 cm of homogeneous P-mixed andosol and topped with 24 cm of andosol. There were four holes at the bottom of each pot for drainage.

Non-inoculated, pre-germinated *S. cannabina* seedlings in andosolic soil were transplanted into the rhizobox. The boxes were irrigated with tap water as needed. Each treatment was replicated three times in a completely randomized design. After 34 days after transplanting (DAT), the plants were separated into shoots and roots. Roots were recovered from each layer separately and washed with tap water. All the roots in each layer were scanned and analyzed for total root length and a DBLR survey, using the WinRHIZO software package as described above. After measurement of DW, shoot P content was determined as described above. In this experiment, in addition to the number of DBLRs, root length density (RLD, cm cm^{-3}) was also determined. After the experiment, Truog-P concentration in each layer was measured.

A further experiment was conducted at the same time to confirm the positional relationship between the DBLR and P. Andosol, supplemented with 60 mg kg^{-1} $(NH_4)_2SO_4$ and 126 mg kg^{-1} K_2SO_4, was added to the three rhizoboxes. Nine pellets (~3 mm diameter) of superphosphate of lime including 17.5% soluble P_2O_5 (56 mg P) (Kounoshima Chemical Co., Ltd., Japan) were placed in each rhizobox, on top of the soil next to the transparent acrylic board so that they were visible to the naked eye. Non-inoculated, pre-germinated *S. cannabina* seedlings were then transplanted into the rhizobox. The plants were grown for 57 d and root development was observed through the acrylic board. The changes of stem length, node number and the number of DBLRs were evaluated during the experiment. Plants were harvested at 57 DAT. Roots were washed carefully with tap water, and final numbers of DBLRs were counted.

Statistical analysis

Statistical analysis was conducted using IBM SPSS Statistics, version 19, and Excel Tokei, version 1.04. Because of the skewed distribution of the number of DBLRs, a Spearman rank correlation was used to calculate the correlation between the number of DBLRs and the other parameters. The difference 10 and 100 P mg kg^{-1} treatments in DBLR number and DBLR diameter was examined using Mann Whitney

U tests. Differences at $p < 0.05$ were considered significant. DBLR numbers were analyzed using a Kruskal Wallis test, and Steel's test was used for multiple comparison between layers with and without P applied. Overall differences between treatments in DW and P content of *S. cannabina* were tested using one-way ANOVA. If the ANOVA showed a significant difference ($p < 0.05$), individual groups were compared using Tukey multiple comparison tests. Relationships between DW or P content and supplied P concentrations (Experiment 1) and between RLD and each soil layer (Experiment 2) were explored by fitting non-linear regression curves to the data.

Results

Experiment 1. Effect of applied P concentration on root development of S. cannabina

Phosphorus addition increased DW of *S. cannabina*. There were significant differences in shoot and root DW between treatments (Fig. 2A–B). The shoot DW of *S. cannabina* was significantly lower under 0 and 10 mg P kg^{-1} than under 100, 500, and 1000 mg P kg^{-1}. In contrast, root DW did not differ significantly between the P-added treatments (10–1000 mg P kg^{-1}). Nodule number increased with P concentration, with 7, 19, 25, and 43 nodules per plant under 10, 100, 500, and 1000 mg P kg^{-1}, respectively. Nodule formation was not observed under 0 mg P kg^{-1}.

Lumps formed on the root system only when *S. cannabina* was grown in 10 and 100 mg P kg^{-1} (Fig. 3). A detailed observation showed that the lumps, randomly distributed across the root system, consisted of soil particles and DBLRs. DBLRs acted as a glue holding soil particles together. The number of DBLRs was 11 in 10 mg P kg^{-1}, and 15 in 100 mg P kg^{-1} (Table 1). Images of the whole root system obtained from WinRHIZO are shown in Fig. 3. The size of the DBLRs differed between the 10 and 100 mg P kg^{-1}

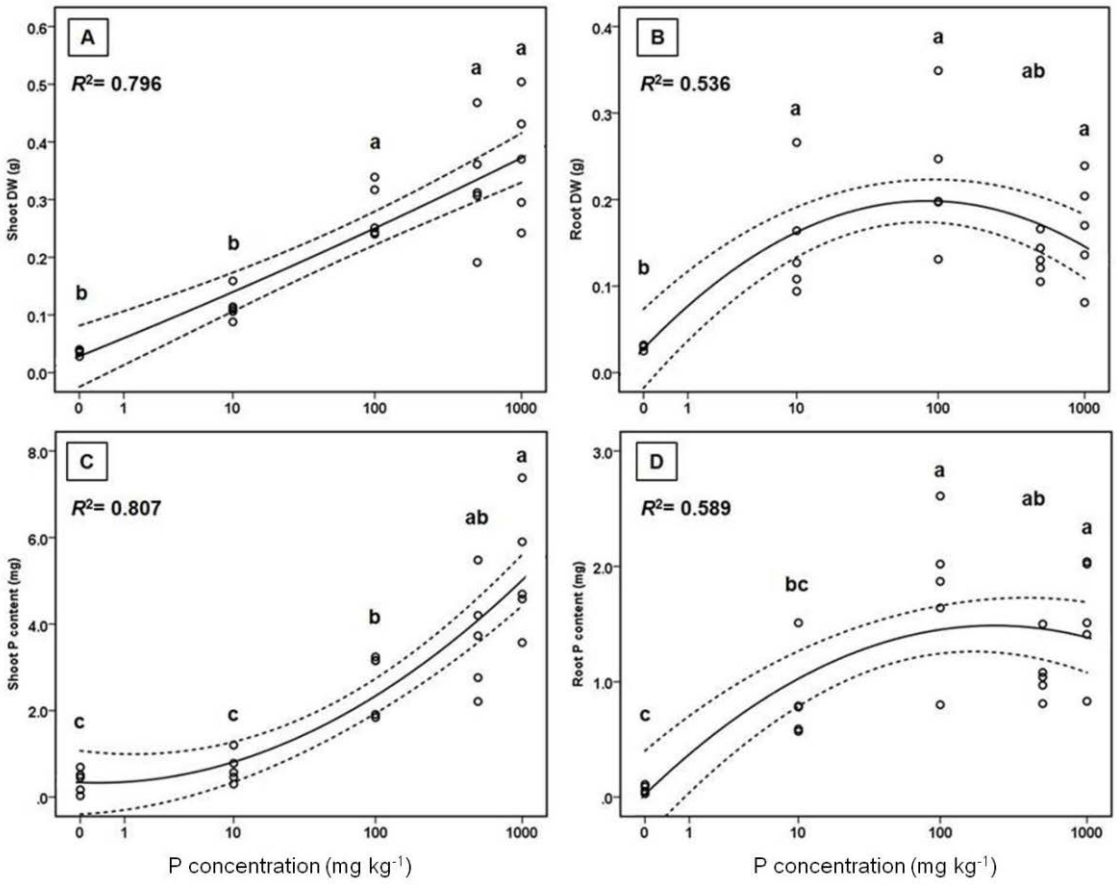

Fig. 2. Relationship between the dry weight (DW) and phosphorus (P) content of shoot or root and P concentration. Quadratic curve approximation: $y = 0.11+7.71E^{-4}*X-5.18E^{-7}*X^2$ ($R^2 = 0.796$) (A), $y = 0.12+1.42E^{-4}*X-1.05E^{-7}*X^2$ ($R^2 = 0.536$) (B), $y = 0.84+8.45E^{-3}*X-4.15E^{-6}*X^2$ ($R^2 = 0.807$) (C) and $y = 0.76+1.81E^{-3}*X-1.07E^{-6}*X^2$ ($R^2 = 0.589$) (D). Dotted line indicates the 95% confidence interval of mean. Different letters indicate statistically significant differences at $p < 0.05$.

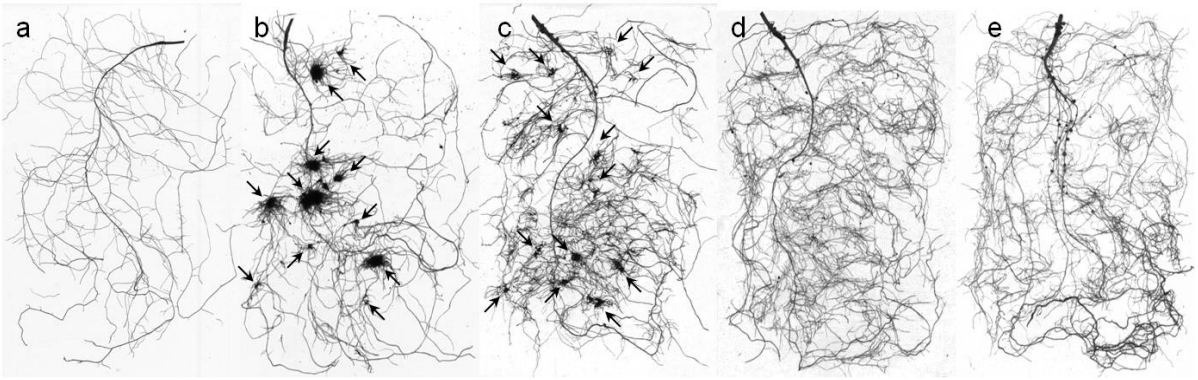

Fig. 3. Whole root system images of *Sesbania cannabina* using WinRHIZO software at 30 DAS. (a) 0 mg P kg^{-1}, (b) 10 mg P kg^{-1}, (c) 100 mg P kg^{-1}, (d) 500 mg P kg^{-1}, (e) 1000 mg P kg^{-1}. Black arrows (in b and c) indicate densely branched lateral roots (DBLRs).

treatments: *S. cannabina* supplied with 10 mg P kg^{-1} formed significantly bigger DBLRs than when supplied with 100 mg P kg^{-1} (Table 1).

P content exhibited a similar trend as DW. As P concentration in the soil increased, shoot P content tended to increase logarithmically (Fig. 2C), although there was no significant difference between the 0 and 10 mg P kg^{-1} treatments. In contrast, mean root P content was highest in the 100 mg P kg^{-1} treatment (Fig. 2D). Average P content under 10 mg P kg^{-1} was higher than under 0 mg P kg^{-1} treatment, but the difference was not significant. Analysis of Spearman's correlations between the number of DBLRs and DW, shoot and root PUE, number of nodules, and PAE of plants supplied with 10 and 100 mg P kg^{-1} is shown in Table 2. The number of DBLRs correlated positively with shoot DW ($\rho = 0.80$, $p < 0.01$), root DW ($\rho = 0.82$, $p < 0.01$) and, marginally significantly, with number of nodules ($\rho = 0.70$, $p < 0.05$). Shoot and root PUE was negatively correlated with the number of DBLRs ($p < 0.05$). DBLRs and PAE were positively but non-significantly correlated.

Experiment 2. Effect of P location on DBLR formation in S. cannabina

Shoot DW was significantly greater in *S. cannabina* plants supplied with P in the top and middle soil layers (P-Top and P-middle treatment) than in plants supplied with P in the bottom layer (P-Bottom treatment) (Fig. 4). However, there was no significant difference in root DW between treatments. Truog-P concentration of the top, middle and bottom layers at harvesting time was 43, 39, and 39 mg kg^{-1} in P-Top treatment, 35, 37, and 31 mg kg^{-1} in P-Middle treatment, and 25, 24, and 36 mg kg^{-1} in P-Bottom treatment, respectively. Although there was no significant difference between layers, the layer supplied with P had the highest Truog-P concentration in all treatments. Shoot P content was significantly higher in the P-Middle treatment than in the P-Bottom treatment (Fig. 5).

Table 1. Effect of phosphorus (P) concentrations on densely branched lateral root (DBLR) number and DBLR diameter.

P concentration (mg kg^{-1})	DBLR number ($plant^{-1}$)	DBLR diameter (mm)
0	0	-
10	11 a	13.7 a
100	15 a	9.8 b
500	0	-
1000	0	-

*Different letters indicate statistically significant differences at $P < 0.05$.

The changes in RLD in each layer are shown in Fig. 6. In all treatments, RLD tended to be high in the layer supplied with P. The equation of the quadratic curve describing RLD was $y = 2.02-0.93*X+0.18*X^2$ ($R^2 = 0.80$) in the P-top treatment, $y = -2.96+4.48*X-1.11*X^2$ ($R^2 = 0.87$) in the P-Middle treatment and $y = 0.52-0.58*X+0.23*X^2$ ($R^2 = 0.74$) in the P-Bottom treatment. In the P-bottom treatment, RLD was lower than in the other treatments. Whole root images, divided by layer, are shown in Fig. 7. Almost all the DBLRs were formed only in the layer supplied with P. Although very few DBLRs were

Table 2. Spearman's correlation coefficients between the number of densely branched lateral roots (DBLRs) and shoot or root dry weight (DW), shoot or root phosphorus utilization efficiency (PUE), phosphorus acquisition efficiency (PAE), and number of nodules at 10 and 100 mg kg^{-1} phosphorus (P) concentrations.

	Number of DBLRs
Shoot DW (g)	**0.80** (0.005)
Root DW (g)	**0.82** (0.004)
Shoot PUE (g mg^{-1} P)	**– 0.74** (0.014)
Root PUE (g mg^{-1} P)	**– 0.65** (0.043)
PAE (total P per applied P)	0.57 (0.086)
Number of nodules (per plant)	**0.70** (0.024)

*P values are shown within parentheses. Correlations with P values less than 0.05 are shown in bold font.

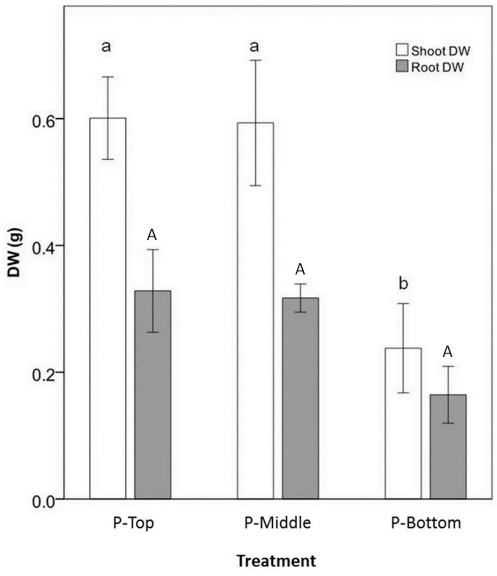

Fig. 4. Effect of different layers phosphorus (P) application on the dry weight (DW) of shoot or root. Different letters indicate statistically significant differences at $p < 0.05$.

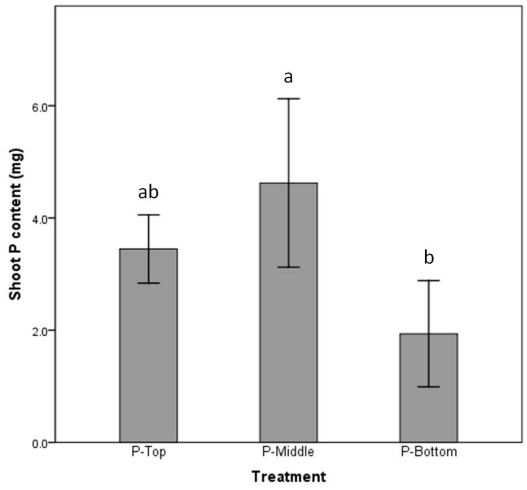

Fig. 5. Effect of different layers phosphorus (P) application on the shoot P content. Different letters indicate statistically significant differences at $p < 0.05$.

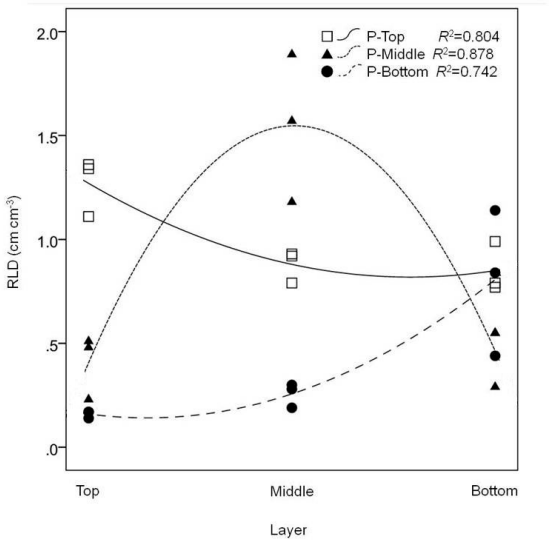

Fig. 6. Relationship between root length density (RLD) in each layer and different layers of phosphorus (P) application. Quadratic curve approximation: $y = 2.02-0.93*X+0.18*X^2$ ($R^2 = 0.804$) (P-Top), $y = -2.96+4.48*X-1.11*X^2$ ($R^2 = 0.878$) (P-Middle), $y = 0.52-0.58*X+0.23*X^2$ ($R^2 = 0.742$) (P-Bottom).

observed in the top layer, in the P-top treatment, their formation was positively affected by P application (Table 3).

An additional experiment with localized P fertilizer showed that *S. cannabina* produced DBLRs in the same location as the P fertilizer. At 33 DAT, the number of DBLRs was 0, 1, and 2 in each of the three experimental plants. The number of DBLRs was positively associated with stem length and node number, in that the plant that formed 2 DBLRs had the greatest stem length and node number (data not shown). At harvest, the number of DBLRs in each plant was 6, 3, and 2 and their stem lengths were 44, 40, and 21 cm. Fig. 8 shows the developmental process from an unspecialized DBLR to a specialized

DBLR. At 22 DAT, branching occurred near the P fertilizer pellets (Fig. 8a), and from 33 DAT to 47 DAT RLD increased around the pellets (Fig. 8b, c) until DBLRs completely covered soil clumps around the fertilizer over an area ~2 cm in diameter (Fig. 8d). Whole root system observations showed that the formation of DBLRs was observed only where P fertilizer was located (Fig. 8e, f).

Discussion

The architecture of a root system plays a major role in determining how efficiently a plant can capture water and nutrients from the soil. In this study, we investigated factors underlying significant morphological change in the roots of *S. cannabina*. Our first experiment showed that DBLR formation was stimulated under low P conditions (10 and 100 mg P kg^{-1} treatments), but not under the 0 mg P kg^{-1} treatment. Furthermore, our second experiment showed that DBLRs formed only in the P-supplied soil layer and around localized P. These results suggest that the formation of DBLRs in *S. cannabina* is triggered by the presence of P and developed in order to acquire P when its availability is relatively low. *S. cannabina* in 0 mg P kg^{-1} treatment did not form the DBLRs despite its extremely P -deficient growing conditions. A small amount of available P (9.32 mg P kg^{-1} of soil) was

Table 3. Effect of different layers of phosphorus (P) application on the number of densely branched lateral roots (DBLRs) in each layer.

	Layer	Treatment		
		P-Top	P-Middle	P-Bottom
Number of DBLRs (per layer)	Top	20 a	0 b	0 b
	Middle	1 b	15 a	0 b
	Bottom	0 b	0 b	11 a

*Different letters indicate statistically significant differences at $P < 0.05$.

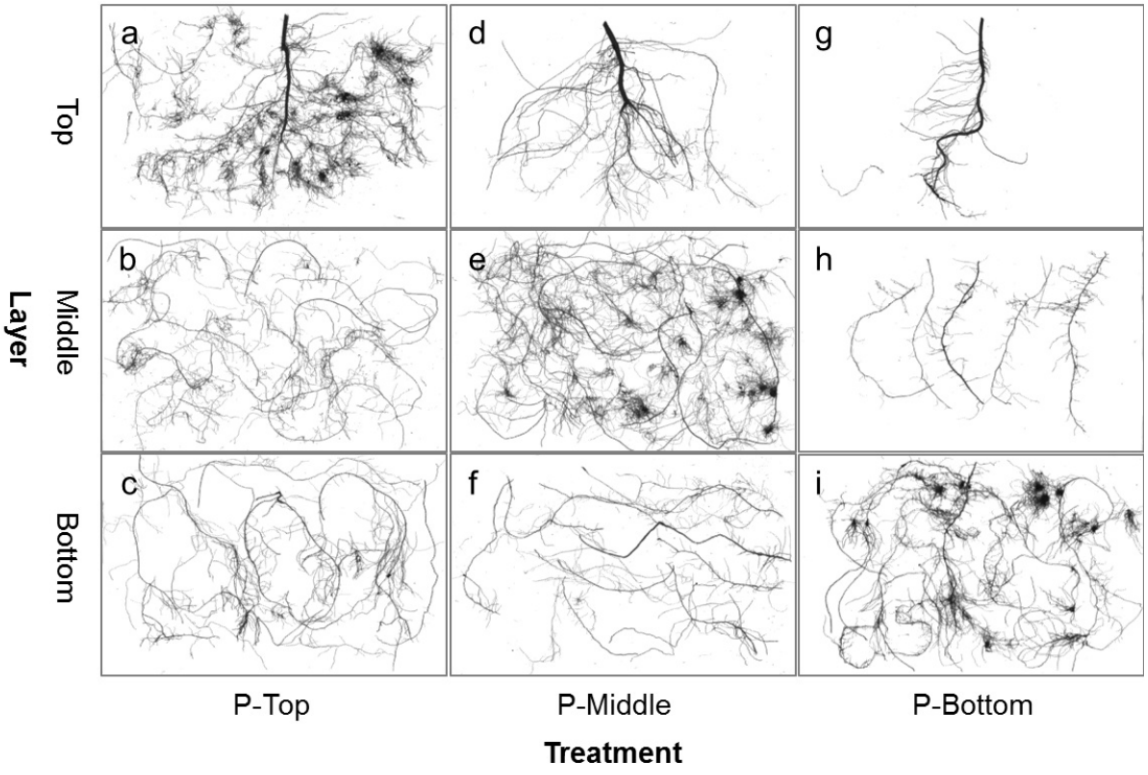

Fig. 7. Root images of *Sesbania cannabina* in each layer using WinRHIZO software under different phosphorus (P) layer applications. (a–c) P was supplied in the top layer; (d–f) P was supplied in the middle layer; (g–i) P was supplied in the bottom layer.

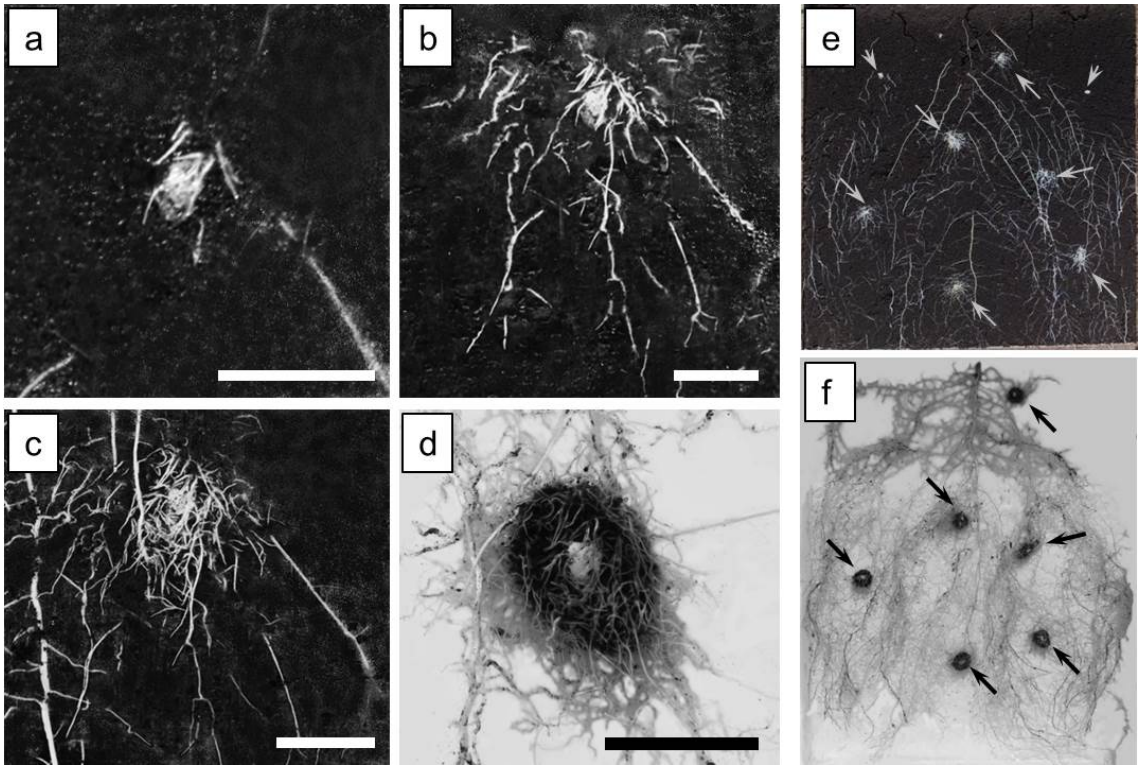

Fig. 8. Process of densely branched lateral root (DBLR) formation around P fertilizer (a–d), and whole root system containing DBLRs of *Sesbania cannabina* (e, f). (a) 22 DAT; bar is 2 cm (b) 33 DAT; bar is 2 cm (c) 47 DAT; bar is 2 cm. (d) P fertilizer was completely enveloped with DBLRs. Bar is 2 cm. (e) Root systems before the removal of soil. White arrows indicate the position of P fertilizer. (f) Root systems after the removal of soil. Black arrows indicate the position of the DBLRs.

detected in 0 mg P treatment, but most of this would have been absorbed by the soil before its collection for the experiment and P availability was extremely low compared with the supplied P treatments. Furthermore, unlike in 10 and 100 mg P treatments, P should be homogenously present throughout the soil in the 0 mg P treatment. Since DBLRs were observed around available P-enriched substrate, uniform P existence might have stimulated DBLR formation in the 0 mg P treatment.

The andosolic soil used in this experiment includes a large amount of phosphorus as unavailable P, such as the Al phosphate and Fe phosphate as well as phytin. Because the genus *Sesbania* has a high P acquisition ability due to their exuding organic acids such as citrate and malate to mobilize P under low P conditions (Aono et al. 2001), DBLRs might be formed in response to P generated by chelating with these organic acids even under the 0 mg P treatment. In this experiment, root system architecture was evaluated in a relatively early growing stage of *S. cannabina*. To clarify the conditions of DBLR formation, further experiments that take into consideration the growing period are needed. It is unlikely that N and K induced DBLR formation, because we added N and K sources to all experimental soil. Furthermore, preliminary work showed that different doses of N (0, 12, 120 mg N kg^{-1}) and K (0, 31.5, 315 mg K kg^{-1}) fertilizer under 500 mg P kg^{-1} treatment did not influence DBLR formation (data not shown).

P deficiency stimulates expansion of the root surface area by attenuating primary root extension, which promotes the development of secondary and higher-order roots, and intensifying root hair formation (Williamson et al. 2001, Nanzyo et al. 2002, Müller and Schmidt 2004, Sanchez-Calderon et al. 2005). The DBLRs were composed of densely branched lateral roots. Moreover, some lateral roots on DBLRs could produce higher-order lateral roots, increasing the total surface area available for soil exploration. Although we did not collect data on root hairs, their development was remarkable on DBLRs. RLD increased in the layer supplied with P in the same way as DBLRs (Fig. 6, 7). Since P was not always absorbed through DBLRs, the overall root system, including DBLRs, was affected by P availability in the

soil.

The correlation analysis showed that shoot and root PUE was influenced by the number of DBLRs (Table 2). The phenotypic plasticity of *S. cannabina*, facilitated by DBLR formation, increased P concentration of *S. cannabina* and resulted in a negative correlation between shoot PUE or root PUE and the number of DBLRs. Although there was no significant association between PAE and the number of DBLRs, the marginal result supports our suggestion that increased number of DBLRs enhance P uptake of *S. cannabina*.

The production of DBLRs might require considerable carbon investment. Although no significant difference was observed in root DW and DBLR number between plants supplied with 10 and 100 mg P kg^{-1}, the shoot DW under 10 mg P kg^{-1} was significantly lower than that under 100 mg P kg^{-1}. Moreover, significantly larger DBLRs were observed in the 10 mg P kg^{-1} treatment than in the 100 mg P kg^{-1} treatment. These results indicate that *S. cannabina* may invest a great deal of carbon into the DBLRs to acquire P.

We found no significant differences in P uptake of shoots and roots between 0 and 10 mg P kg^{-1} treatments. Because of the high PAC of soil used in this experiment, there was a small difference in P concentration (Truog-P) between the treatments. Indeed, while DBLRs facilitated P uptake, they did not enhance the P status under the 10 mg P treatment. On the other hand, the P content of *S. cannabina* plants supplied with 100 mg P kg^{-1} was not statistically different to those supplied with 500 or 1000 mg P kg^{-1} (Experiment 1). An adequate P supply might inhibit DBLR enlargement in order to avoid costly, excessive loss of carbon, resulting in smaller DBLR in *S. cannabina* at 100 mg P kg^{-1} than at 10 mg P kg^{-1}. Whatever, the mechanism, the amount of P wrapped by DBLR is likely to affect the growth and P content of *S. cannabina*.

DBLR formation may depend on the interaction between the plant's P status and localized supply of P in the substrate. Root clusters occur in a range of families that are adapted to environments with little available P, such as Proteaceae (Purnell 1960), Cyperaceae (Playsted et al. 2006), and *Lupinus* in Fabaceae (Wang et al. 2013). In many cases, these roots are formed under P-deficient conditions regardless of the presence of P in the soil, and release exudates, notably carboxylates, which can free up insoluble P by chelating the metal ions that immobilize it (Wang et al. 2013). The organic acids exuded from proteoid roots in white lupin (*Lupinus albus*) can include citrate, malate, succinate, and fumarate (Gardner et al. 1981, Keerthisinghe et al. 1998). However, DBLRs observed in our experiment might be qualitatively different from cluster roots because DBLRs are triggered by the presence of P. In this experiment, DBLRs enveloped soil including P fertilizer and soil particles were observed adhered to DBLRs. Viscous substances such as mucilages might be exuded from DBLRs because the development of root structures such as lateral roots and root hair was increased. Although P is easily absorbed by high PAC soils, DBLR may prevent the P they envelop from being absorbed by root exudates. Further studies are needed to clarify the soil environment within DBLRs as well as DBLR morphology, anatomy, and physiology.

Acknowledgments

This study was supported in part by MEXT, Kakenhi, Japan (No. 26850008 and 1281300900).

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