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Relation	

Seedling growth, physiological characteristics, nitrogen fixation, and root and nodule phytase and phosphatase activity of a low-phytate soybean line

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Abstract

Understanding the influence of the valuable "low-phytate" trait on soybean seedling growth, physiology, and biochemistry will facilitate its future exploitation. The aim was to elucidate the physiological and biochemical characteristics of low-phytate (LP) soybean at the seedling stage. To this end, seed P and mineral content and seedling dry weight, carbon (C) and nitrogen (N) accumulation, nitrogen fixation, and root and nodule phytase and phosphatase activity levels were measured at 21 d after sowing LP and normal-phytate (NP) soybean lines. Seedling dry weight, and C and N accumulation were 31%, 38% and 54% higher, respectively, in the LP line than the NP line. The total and specific nitrogen fixation levels in the LP nodules were 46% and 78% higher, respectively, than those in the NP nodules. The phytase, phosphatase, and specific phytase levels were 1.4-folds, 1.3-folds, and 1.3-folds higher, respectively, in the LP roots than the NP roots. The phosphatase and specific phosphatase levels in LP nodules were 1.5-folds and 1.3-folds higher, respectively, than those in the NP nodules. The mineral levels were substantially higher in the LP seeds and seedings than in those of the NP line. The HCl extractabilities of P, S, Fe, Cu and Mn were higher in the LP seeds than the NP seeds. These results indicate that the LP line presented with superior seedling growth and nitrogen fixation relative to the NP line. The LP line had relatively higher root phytase and root and nodule phosphatase activity levels than the NP line and could, therefore, be better suited and more readily adapt to low P conditions.

Keywords: Low-phytate soybean, Nitrogen fixation, Nodule, Phytase, Phosphatase, Seedling growth

1. Introduction

Soybean is an important source of vegetable protein for humans. It is also used worldwide in livestock feed. Phytic acid (*myo*-inositol hexa*kis*phosphate or InsP6) is the principle storage form of phosphorus (P) in soybean seeds. It occurs mainly in phytate anion from which constitutes 70 - 80% of the seed total P in all higher plants (Raboy, 2009). However, monogastric animals such as chickens and swine have low phytase (phytic acid-specific phosphohydrolases; *myo*-inositol hexa*kis*phosphate 3- and 6-phosphohydrolase; EC 3.1.3.8 and EC 3.1.3.26) activity in their digestive systems and cannot, therefore, efficiently break down phytate. Consequently, monogastric animal feces often contain large quantities of phytate-derived P which is lost to the natural environment in arable regions and may contribute to the eutrophication of adjacent aquatic ecosystems. Moreover, phytate chelates calcium (Ca), magnesium (Mg), iron (Fe), and zinc (Zn), and reduces their bioavailability.

The aforementioned problems have been corrected by using P feed supplements or mixing phytases into feed rations (Raboy, 2009). Low-phytate (LP) strains have been identified and isolated in soybean (Wilcox *et al*., 2000), maize (Raboy *et al*., 2000), barley (Larson *et al*., 1998), rice (Liu *et al*., 2007), and wheat (Guttieri *et al*., 2004). Animal nutrition studies showed that the administration of LP soybeans to swine significantly improves P digestibility and reduces the amount of excreted P (Hill *et al*., 2009; Powers *et al*., 2006). However, LP soybean lines may have lower emergence rates than their NP counterparts (Meis *et al*., 2003). Breeding high-yield LP crops can be challenging. Nevertheless, earlier studies on barley reported successful breeding of LP lines whose agronomic performance was comparable to that of the NP (Bregitzer and Raboy, 2006). Boehm *et al*. (2017) reported no significant differences in yield or emergence between two LP soybean lines obtained by backcrossing and compared with two high-yielding NP cultivars controls. Taliman *et al*. (2019) stated the yield and seed quality of LP line and NP soybean cultivars were similar whether the plants were raised under low- or high soil P levels. In order to develop and exploit the potentially valuable "low-phytate" soybean trait, it is imperative to elucidate its physiological and biochemical impacts on seedling growth.

Symbiotic nitrogen fixation is an environmentally sustainable plant nitrogen source and an alternative to chemical nitrogen fertilizers. Soybeans develop specialized root structures called nodules that house symbiotic nitrogen-fixing rhizobium bacteria which improve nitrogen bioavailability (Oldroyd and Dixon, 2014). Nodule development may affect soybean seed yield and quality. However, earlier studies suggested that limiting phosphorus levels may reduce root growth and nodulation and decrease symbiotic nitrogen fixation (Miao *et al*., 2006). Even when the soil does contain abundant P, however, its bioavailability is low when it is fixed or bound in insoluble forms (Makoudi *et al*., 2018). Li *et al*. (1997) and Dissanayaka *et al*. (2015) reported that plants such as white lupin and rice secrete acid phosphatase from their roots under low-P conditions. In this way, they can efficiently utilize organic P in the soil. Acid phosphatase is ubiquitous in various plant organs. Roots may secrete it in order to hydrolyze soil P-monoesters, liberate inorganic phosphorus (Pi), improve P upake and assimilation, and enhance plant growth (Araújo *et al*., 2008). Phytase is a type of phosphatase that hydrolyzes phytate in seeds and releases inositol and P during germination. This reaction may be essential for plant germination and several metabolic processes (Makoudi *et al*., 2018).

To the best of our knowledge, the seedling and nodule characteristics of the LP soybeans have never been investigated. Further, the role of nodular nitrogen fixation, phytase, and phosphatase in internal LP root and nodule metabolism remain unknown. Thus, the aim of the present study was to investigate the physiological and biochemical characteristics of LP soybean seedlings including their root and root nodule nitrogen fixation and phytase and phosphatase activity. Mature seeds contain P and minerals that are subsequently assimilated by the developing embryo during early seedling growth. For this reason, the seed P and mineral levels were measured and compared in the LP and NP soybean lines.

2. Material and methods

2.1 Plant materials and growth condition

The LP and NP lines used in the present study were generated in August 2004 by crossing the LP

soybean line CX1834 (Wilcox *et al.*, 2000) with the Natto–kotsubu cultivar. The Natto–kotsubu is a NP and is widely grown in western Japan. The parental and F1 generations were sown in the summer of 2005 in the experimental field of the Graduate School of Biosphere Science, Hiroshima University, Higashi-Hiroshima, Japan. The LP and NP lines were selected annually. Experiments 1 and 2 were performed during the summers of 2018 and 2019, respectively. Two experiments were run on single selected LP and NP lines. The LP and NP seeds of the F11 and F12 progeny were used for Experiments 1 and 2. The seeds were produced in the field under the same fertilization and environmental conditions for both years. Five seeds per line were sown in 1-L vinyl pots filled with vermiculite and grown in a greenhouse at 25-33°C under natural light lighting. For microbial seed inoculation, the vermiculite was mixed with a small amount of fresh soil sampled from field the previous year. All experimental samples were collected at least in quadruplicate at 21 days after sowing. Nodulated root samples were used for the nitrogen fixation assay and the nodules were then separated from the roots and counted. The plants were dried for 48 h at 70°C and dry weight were measured for all organs. The samples were ground to a fine powder (IFM-800; Osaka Chemical Co. Ltd., Osaka, Japan). The nodules were stored at -80°C before until the phytase and phosphatase activity level were determinations.

2.2 Measurement of nitrogen fixation activity

The nitrogen fixation activity was measured by the acetylene reduction method of Hardy *et al*. (1968). Four lots of nodulated roots from five plants per lot were incubated at 20-25°Cin a flask containing C_2H_2 (10%, v/v) in air. Then, 0.2 mL of each sample were taken after 10 min and 60 min and analyzed for acetylene via gas chromatography (GC-14B, Shimadzu Co Ltd, Kyoto, Japan).

2.3 Measurement of total P (TP), phytate P (Phy-P), and inorganic P (Pi) concentrations

A plant material aliquot was digested in a mixture of sulfuric acid (H_2SO_4) and hydrogen peroxide (H_2O_2). The TP concentration measured by a colorimetric assay of the P in the digest according

to the method of Chen *et al*. (1956). The seed Pi and Phy-P were determined according to the methods of Raboy and Dickinson (1987).

2.4 Measurement of Ca, Mg, S, Fe, Zn, Mn, and Cu concentrations

Plant tissue was digested with a mixture of nitric acid (HNO_3) and perchloric acid ($HClO_4$) and its Ca, Mg, S, Fe, Zn, Mn, and Cu concentrations were measured by inductively coupled plasma-optical emission spectrometer (iCAP 6000; Thermo Fisher Scientific Inc., Waltham, MA, USA).

2.5 Measurement of HCl extractability of P, Ca, Mg, S, Fe, Zn, Mn, and Cu concentrations

Hydrochloric acid extractability of the minerals in the plant tissues was determined according to the method of Duhan *et al*. (2002). Finely ground plant tissue samples were shaken with 0.03M HCl at 37°C for 3h and filtered. The supernatants were oven- dried at 100°C and digested with a mixture of nitric acid (HNO_3) and perchloric acid ($HClO_4$). The P, Ca, Mg, S, Fe, Zn, Mn, and Cu levels in the samples were determined as described in Subsection 2.3 and 2.4.

2.6 Measurement of carbon and nitrogen concentrations

The carbon (C) and nitrogen (N) concentrations in the seeds and seedlings were determined by dry combustion in a CHN Analyzer (MT-5; Yanaco, Tokyo, Japan).

2.7 Measurement of root and nodule phytase and acid phosphatase activity and soluble protein content

Nodules were collected from all treatments at 21 days after sowing, ground in liquid nitrogen with 0.1M acetate-buffer (pH 5.0), and centrifuged at 13,000 × g at 4°C for 30 min. The supernatant was used to measure phytase, acid phosphatase, and soluble protein.

Phytase activity was determined according to the method of Eeckhout and De Paepe (1994). The supernatant (0.5 mL) was incubated at 37°C for 60 min with 0.5 mL of 0.2% (w/v) sodium phytate (Sigma P8810; Sigma-Aldrich Co., St. Louis, MO, USA). Then, 10% (v/v) trichloroacetic

6

acid (TCA) was added to stop the enzyme reaction. The mixture was centrifuged at $8,000 \times g$ and 4°C for 10 min. The enzyme activity of the blank was stopped with TCA immediately before starting the incubation. The phytase activity was defined as that which liberated 1nmol Pi from 0.2% (v/w) sodium phytate solution per minute per g of the nodule and root fresh mass.

For the acid phosphatase activity assay, the samples prepared and purified according to the method of Araújo *et al*. (2008). One-half milliliter supernatant was incubated at 37°C for 60 min with 1 mL substrate (0.115M disodium *p*-nitrophenyl phosphate). The enzyme reaction was stopped by adding 2 mL 0.5M NaOH and 0.5 mL 0.5M $CaCl_2$. The mixture was centrifuged at $8,000 \times g$ and 4°C for 10 min, diluted with water prior to spectrophotometer, and measured at $\lambda = 410$ nm. Phosphatase activity was defined as that which hydrolyzed 1μmol substrate per minute per gram root and nodule fresh weight.

Soluble protein was measured by the Bradford protein assay (Bradford, 1976) for specific phytase and acid phosphatase activity calculations. Aliquots (50 μL) of the extract were transferred to test tubes containing 2.45 mL of distilled water, and 2.5 mL of prepared protein assay reagent was added to them. After 5 min incubation, the color was measured in a spectrophotometer $\lambda = 595$ nm.

2.8 Statistical analysis

Statistical analyses were performed in SPSS v. 21 for Windows (IBM Inc., Armonk, NY, USA). Data were analyzed by one-way ANOVA. A *t*-test was used to identify significant differences between the NP and LP soybean lines. Differences were considered significant at $P \leq 0.05$.

3. Results

3.1 Seedling growth and nitrogen fixation activity

Visual inspection revealed that the LP seedling growth was at least as good as that of the NP line (Fig. 1). The LP seedlings produced more extensive primary and lateral roots than those of the NP line. The proportion of seed total P (TP) in the form of Pi was 62% higher in the LP line than

the NP line (Fig. 2). In contrast, the ratio of Phy-P to TP in the LP line was 57% lower than that of the NP line. Experiment 1showed that the leaf dry weight (LDW), stem dry weight (SDW), and root dry weight (RDW) were not significantly different between the LP and NP lines (Fig. 3). In Experiment 2, however, the LDW, SDW, cotyledon dry weight (CDW), and RDW for the LP line were 25–58% higher than those for the NP line (Fig. 4). On the other hand, the nodule dry weights (NDW) of the LP line were 30% and 17% lower, respectively, than those of the NP line according to Experiment 1 and Experiment 2. For both experiments, the LP line the nodule number (NN) was lower than that of the NP line (data not shown). In Experiment 2, the total nitrogen fixation (TNF) and specific nitrogen fixation (SNF) activity in the LP line were 46% and 78% higher, respectively, than those of the NP line (Fig. 5).

3.2 Seed and seedling carbon (C) and nitrogen (N) content

In Experiment 1, there was no significant differences between the LP and NP lines in terms of leaf, stem, or nodule carbon (C) and nitrogen (N) content (Table 1). The N content in the NP root was lower than that in the LP root. There were no differences between the NP and LP lines in terms of their total seed and seedling C content. The net C accumulation from the seed to seedling stage increased by 185 mg and 171 mg in the NP and LP lines, respectively. The whole-plant N content slightly increased during NP and LP growth. The LP line had accumulated 4.8 mg N during seedling growth. In Experiment 2, the C content in the LP leaves, stems, cotyledons, and roots were 44%, 21%, 34%, and 65% higher, respectively, than those for NP (Table 2). The N levels in the LP leaves, stems, cotyledons and roots were 74%, 42%, 33%, and 100% higher, respectively, than those the NP. The N content in the NP cotyledons was lower than that of LP but the difference was not statistically significant. The C accumulation levels were 149 mg and 197 mg higher in the seedlings than the seeds of the NP and LP lines, respectively, and the differences were significant. The N accumulation levels were 2.4 mg and 4.2 mg higher in the seedling than seeds of the NP and LP lines, respectively. The LP line had accumulated 1.8-folds more N than the NP line during seedling growth. In Experiment 1 and 2, the C and N levels in the NP nodules

were higher than those in the LP nodules.

3.3 Phytase and acid phosphatase activities of roots and nodules

In Experiment 1, the nodular phytase, acid phosphatase, specific phytase, and specific acid phosphatase activity levels were higher for the LP than the NP line. Further, the differences between LP and NP in terms of acid phosphatase and specific acid phosphatase activity were statistically significant (Table 3). In Experiment 2, the activity levels of these enzymes in the nodule and roots were measured (Table 4). The phytase, specific phytase, and acid phosphatase activity levels in the LP roots were 5.8 nmol min^{-1} g^{-1}, 26.4 nmol min^{-1} mg^{-1}, and 0.4 μmol min^{-1} g^{-1} higher than those in the NP roots, respectively (Table 4). The acid phosphatase and specific acid phosphatase activity levels in the LP nodules were 8.8 and 6.1 μmol min^{-1} mg^{-1} higher than those in the NP nodules, respectively. The acid phosphatase activity in the LP nodules was 1.5-folds higher than that in the NP nodule. The acid phosphatase and specific phytase activity levels in the LP roots and the specific acid phosphatase activity levels in the LP nodules were both 1.3-folds higher than that those in the NP roots and nodules, respectively. There were no differences between the LP and NP lines in terms of the specific acid phosphatase activity levels in their roots or the phytase and specific phytase activity levels in their nodules. For both LP and NP lines, the phytase, acid phosphatase, and specific acid phosphatase activity levels were substantially higher in the nodules than the roots. The acid phosphatase activity level was 16 -18-folds higher in the nodules than the roots.

3.4 Total content and HCl extractability of P, Ca, Mg, S, Fe, Zn, Mn, and Cu in seeds and seedlings

In Experiment 2, we measured the levels of P and other minerals in the seeds and seedling. The TP, Ca, Mg, Zn, Cu, and Mn levels in the LP seeds were higher than those in the NP seeds (Table 5). The HCl-extractable P, S, Fe, Cu, and Mn levels in the LP seeds were higher than those in the NP seeds. The HCl-extractable P, S, and Fe levels in the LP seeds were 56%, 50%, and 30% higher than those in the NP seeds, respectively. The LP and NP seeds did not differ in terms of HCl-

extractable Ca, Mg, or Zn content.

The TP levels in the LP seedling leaves, stems, cotyledons, and roots were 37%, 40%, 29% and 90% higher, respectively, than those of NP line (Table 6). The Ca levels in the LP seedling leaves, stems, cotyledons, and roots were 36%, 45%, 38%, and 62% higher respectively, than those of the NP line. However, LP and NP did not significantly differ in terms of the cotyledon Ca contents (Table 6). The LP line had higher leaf Mg content and leaf and stem S contents than those of the NP line. The LP root Fe, Zn, Cu, and Mn levels were 99%, 56%, 62%, and 87% higher, respectively, than those of the NP line. The macronutrient and micronutrient levels in the NP nodules were higher than those in the LP nodules. The differences between LP and NP in terms of nodule Mg, S, Fe, and Zn content were statistically significant. The TP, Ca, Mg, S, Fe, Zn, Cu, and Mn levels in the LP seedling were 30–81% higher than those in the NP seedings and differences were significant.

4. Discussion

The LP soybean seeds presented with higher inorganic P and lower phytate P levels than that the NP soybean seeds. Therefore, the LP line should provide comparatively higher bioavailable P and mineral levels for non-ruminant animals than the NP line. The use of LP soybean in animal feeds could mitigate the negative environmental impact of waste P derived from undigested phytate in feces (Raboy, 2002). Previous studies showed that the yield and agronomic performance of certain LP lines were comparable to those of NP lines (Raboy *et al*., 2015; Taliman *et al*. 2019). However, other reports also indicated that LP lines have relatively lower germination and emergence rates than NP lines (Oltmans *et al*., 2005). Here, we investigated the seedling growth and root and nodule characteristics of LP soybean. TNF, and SNF, and seedlings growth were all significantly higher for the LP than the NP line (Fig. 5). Experiment 1 revealed that seedling growth did not significantly differ between the LP and NP lines (Fig.3). In contract, Experiment 2 disclosed that the dry weights were higher for the LP than the NP seedling (Fig. 4). The low phytate trait have had little positive influence on the physiological and may have improved physiological

performance during seedling growth. Carbon is fixed by photosynthesis and accumulates during seedling growth (Murchie *et al*., 2009). Experiment 2 showed that the LP seedlings had higher dry weights (Fig. 4) and greater C accumulation (Table 2) than the NP seedlings. Therefore, the photosynthetic ability of the LP line may be superior to that of the NP line.

During seedling growth, nitrogenous compounds (enzymes, amino acids, and hormones) are translocated to the roots and released from them to the soil (Brophy and Heichel, 1989; Mayer *et al*., 2003). The relatively higher seedling N content observed in the LP line corresponded with its comparatively higher TNF and SNF levels. These findings indicated that the LP line has greater nitrogen fixation capacity than the NP line (Fig. 5). Here, LP accumulated more leaf and root C and N than NP. Thus, LP seedling may have relatively larger leaf and root surface areas which may, in turn, enhance photosynthate storage and soil mineral uptake (Table 3). Moreover, the LP seedling had relatively lower nodule number (data not shown) and comparatively higher TNF and SNF levels than the NP seedlings. Therefore, the LP root nodules more effectively fixed nitrogen than those of the NP line. Further, the genetic modifications responsible for the low phytate trait may have also enhanced nitrogen fixation.

In normal plants, root and nodule phosphatase and phytase improve P utilization during P deficiency (Araújo *et al*., 2008). Here, the nodule phytase and root and nodule acid phosphatase activity levels were higher in the LP than the NP line (Tables 3 & 4). Experiments 1 and 2 showed that the total acid phosphatase activity levels in the LP nodules were 1.7-folds and 1.5-folds higher, respectively, than those in the LP nodules. Previous studies demonstrated that transgenic phytase and phosphatase expression improved root P absorption from the soil (Ma *et al*., 2012). The presented study showed that the LP trait was associated with upregulated root phytase and acid phosphatase compared to the NP. Thus, soil P uptake in the LP line was superior to that of the NP line. Interactions between the rhizobia and the host plant may also influence nodule establishment and organ development (Limpens and Bisseling, 2003). Upregulated enzyme activity may enhance the ability of nodules to hydrolyze organic P in the soil. Therefore, rhizobial symbiosis in the LP line may have upregulated nodular phosphatase and facilitated soil P uptake and

assimilation.

Phosphorus is vital for germination and seedling development. The HCl-extractable (available inorganic) P level was higher in the LP than the NP seeds. This difference might explain the relatively superior physiological performance of the LP line (Table 5). Calcium contributes to plant plasma membrane and cell wall integrity while magnesium is an enzyme cofactor and chlorophyll ligand (Maathuis, 2009). The comparatively higher HCl-extractable Ca and Mg levels in the LP seeds may have promoted their post-germination growth and development. Sulfur occurs in the sulfur-containing amino acids cysteine and methionine. However, soybean has substantially lower sulfur-containing amino acid levels than meat (Maathuis, 2009; Zarkadas et al., 2007). The HCl-extractable S content in LP was 2.6-folds higher than that in NP. Therefore, LP seeds may be better sources of bioavailable sulfur-containing amino acids than NP seeds. Improving crop micronutrient content and availability are critical objectives in plant breeding. These properties are vital to livestock growth and human health (Wang et al., 2008). To increase their soil mineral uptake, plants enhance the development of their root systems by producing more extensive primary and lateral roots (White and Broadley, 2009). Here, the LP seedlings had greater root biomass and better developed root structures than the NP seedlings (Fig. 1). Therefore, the comparatively higher nutrient levels in the LP seeds indicated that the parental LP plants absorbed and accumulated relatively greater amounts of mineral nutrients from the soil during their development. In this manner, they could translocate comparatively larger quantities of these elements to their seeds. Previous studies confirmed that seed germination, cooking, and fermentation increase mineral nutrient bioavailability (Lewu et al., 2010; Osman, 2011; Kumari et al., 2015). Here, the LP line had higher micronutrient levels and more HCl-extractable Fe, Cu, and Mn than the NP line. For this reason, LP seeds could provide larger amounts of bioavailable essential nutrients than the NP seeds.

The macronutrients P, Ca, and Mg substantially affect plant biomass accumulation (Peng and Zhou, 2010). Experiment 2 showed that the leaf, stem, and root biomass levels of the LP line were greater than those of the NP line (Fig. 4). This finding corroborates the observation that

macronutrient contents in the LP leaves, stems, and roots were higher than those in the same NP organs (Table 6). However, the LP nodules had comparatively lower mineral content than the NP nodules. The LP line presented with lower nodule numbers, greater nitrogen fixation ability, and higher phosphatase activity than the NP line (Tables 3 & 4). These results indicate that LP nodules utilize minerals more efficiently than NP nodules for plant growth and development. A comparison of the cotyledon and seed mineral content (Table 6) revealed that the LP line allocated more P, Ca, and Mg for germination than the NP line. The fact the LP line had more inorganic extractable P than the NP line suggests that the LP line more readily assimilates its P reserves than the NP line.

In this study, the vermiculite substate was not supplemented with nutrients or fertilizer. Vermiculite does not contain phosphorus. It is widely used as a clay mineral in plant cultivation and is moderately soluble under acidic conditions (Kalinowski and Schweda, 2007). There was no significant difference between the seed and the whole plant in terms of total P content (Tables 5 & 6). However, when plants are subjected to P deficiency, their roots increase organic acid excretion (Dong *et al*., 2004). Consequently, vermiculite mineral bioavailability and root uptake increase and could enhance plant growth. Here, mineral accumulation was higher in the LP line than the NP line. Under mineral deficiency, then, the LP line can absorb relatively more minerals from the soil than the NP line. The Fe content was much higher in the roots than in the leaves or stems. This observation aligns with an earlier report (Strasser *et al*., 1999). Iron in the apoplast might have accounted for comparatively higher measured root Fe content. Apoplastic Fe is bioavailable and can, therefore, support plant growth (Strasser *et al*., 1999) and participate in root-rhizobium symbiosis (Rotaru and Sinclair, 2009). Overall, the root Fe content was higher in the LP than the NP lines.

Cu and Fe metabolism interact with each other. Cu participates in photosynthesis and biosynthesis (Hänsch and Mendel, 2009). For both the NP and LP lines, the Cu content was higher in the leaves than the roots. Nevertheless, the LP roots presented with higher Cu and Fe content those of the NP line. In this study, the Zn and Mn levels were higher in the roots and lower in the

nodule of the LP line than the NP line. Zn and Mn are enzyme cofactors and are vital to rhizobial function (Burnell, 1988; O'hara *et al.*, 1988; Abreu *et al.*, 2017). Therefore, the relatively superior mineral assimilation efficiency of the LP nodules could account for the observed micronutrient distributions and levels in this line.

In Experiment 1, the dry weight of the LP line did not differ from that of the NP line. In contrast, it was higher for LP than NP in Experiment 2 (Fig. 3 &4). Moreover, the LP line had a higher nitrogen fixation capacity than the NP line in Experiment 2. Compared to the roots and nodules of NP, those of LP had higher acid phosphatase and phytase activity and greater leaf, stem, and root mineral accumulation (Tables 4 & 6). These findings indicate the enhanced performance of LP line under low-P conditions and suggest that seed phytate reduction either has no impact on seedling growth or improves it compared to NP.

The LP soybean lines tested here were produced by crossing cv. Natto-kotsubu (a small-seeded NP cultivar used in Natto production) with the large-seeded, LP CX1834 line. The progeny varied in seed and plant size to the same extent as their parents. Therefore, we could not attribute all observed differences in organ weight and other traits to allelic differences in genes encoding the LP trait. We concluded that the traits of these particular LP progeny were at least as good as those of the NP line used in the present study. The genetic changes in the parental LP line (CX1834; Wilcox *et al.*, 2000) used to breed the LP line evaluated here were identified as altered alleles of the two copies of the soybean *MRP* gene. *MRP* encodes a phytate-specific ABC transporter (Gillman *et al.*, 2009). Thus, the observed improvements in the LP line relative to the NP line could be explained by the alterations in phytic acid metabolism in the vegetative and/or reproductive tissues encoded by the allelic variants of the two copies of the *MRP* gene. Soil contains abundant P but most of it is fixed in insoluble forms such as phytates and other organic P (Dissanayaka *et al.*, 2015; Makoudi *et al.*, 2018). These P sources have low bioavailability which may result in P deficiency during plant growth. Phytase and phosphatase secreted from soybean roots and nodules could improve the amount and rate of P uptake from the soil. Here, the LP roots and nodules had higher phosphatase and phytase level than those of their NP counterparts.

Therefore, the LP line could assimilate comparatively more P from the soil than the NP line under P deficiency. In future studies, we will evaluate the physiological and biochemical characteristics of mature LP soybean and compare the digestibility of LP and NP soybeans in livestock.

5. Conclusion

The present study demonstrated that the LP line had less seed phytate, more seed inorganic P, and higher mineral bioavailability than the NP soybean line. The dry weight, nitrogen fixation levels, and C and N accumulation rates in the LP seedlings were comparable or superior to those of the NP seedlings. Moreover, LP seedling roots and nodules had relatively higher acid phosphatase and phytase activity than NP seedling roots. Micronutrient levels (especially P) were substantially higher in the leaves, stems, and roots of the LP line than those of the NP line. Thus, enhanced performance may be expected for LP soybeans grown on various type of soil. These findings suggest that LP seedlings have higher root and nodule mineral bioavailability and enzyme activity levels and better physiological performance than NP seedlings.

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Figuer Legend

Figure 1. Germination and growth of seedlings in normal-phytate (NP) and low-phytate (LP) soybean lines at 9 (a), 14 (b) and 21 (c) days after sowing.

Figure 2. Phosphorus fractions in the seeds of normal-phytate (NP) and low-phytate (LP) soybean lines in Experiment 1. Pi, inorganic phosphorus; Phy-P, phytate phosphorus; Other-P, calculated by subtracting Phy-P and Pi from the total phosphorus. Data are means of three replicates.

Figure 3. Leaf dry weight (LDW), stem dry weight (SDW), root dry weight (RDW), and nodule dry weight (NDW) of normal-phytate (NP) and low-phytate (LP) lines at 21 d after sowing in Experiment 1. Data are presented as means ± SD of four replicates. Means followed by different letters are significantly different at $P \leq 0.05$ according to the t-test.

Figure 4. Leaf dry weight (LDW), stem dry weight (SDW), root dry weight (RDW), cotyledon dry weight (CDW), and nodule dry weight (NDW) of normal-phytate (NP) and low-phytate (LP) lines at 21 d after sowing in Experiment 2. Data are presented as means ± SD of four replicates. Means followed by different letters are significantly different at $P \leq 0.05$ according to the t-test.

Figure 5. Nitrogen fixation activity in Experiment 2. Total nitrogen fixation activity (TNF)(a), and specific nitrogen fixation activity (SNF) (b) of normal-phytate (NP) and low-phytate (LP) lines at 21 d after sowing. Data are presented as means ± SD of four replicates. Means followed by different letters are significantly different at $P \leq 0.05$ according to the t-test.
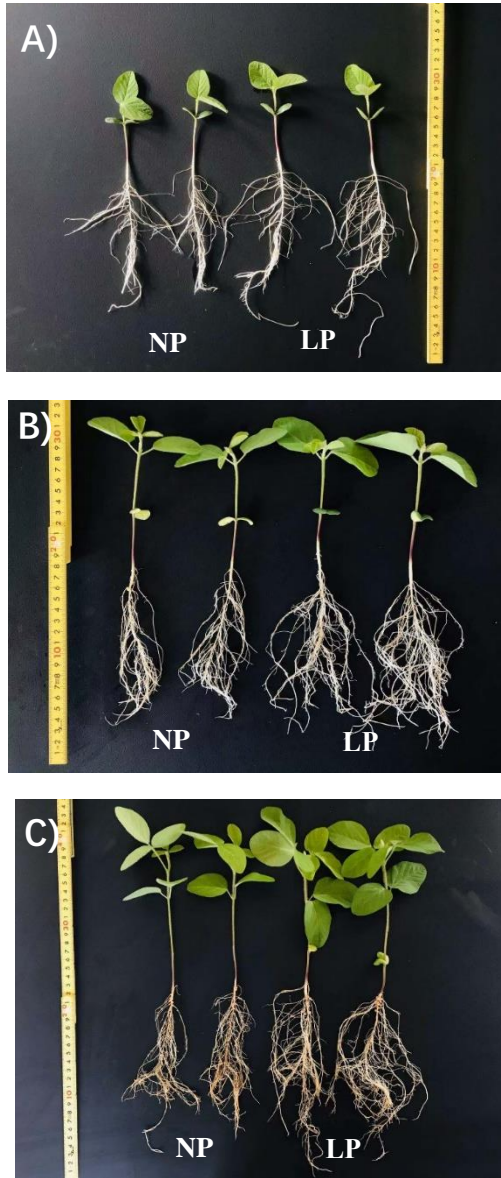
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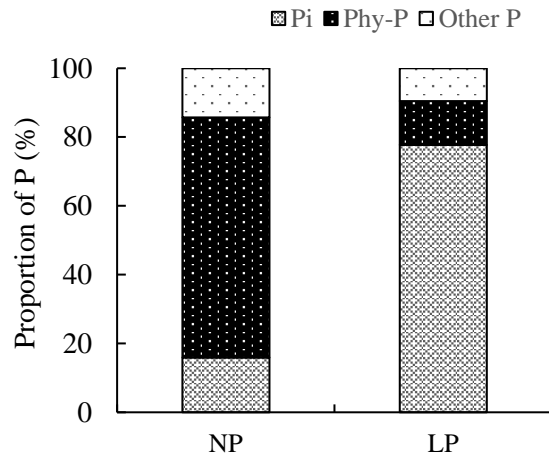
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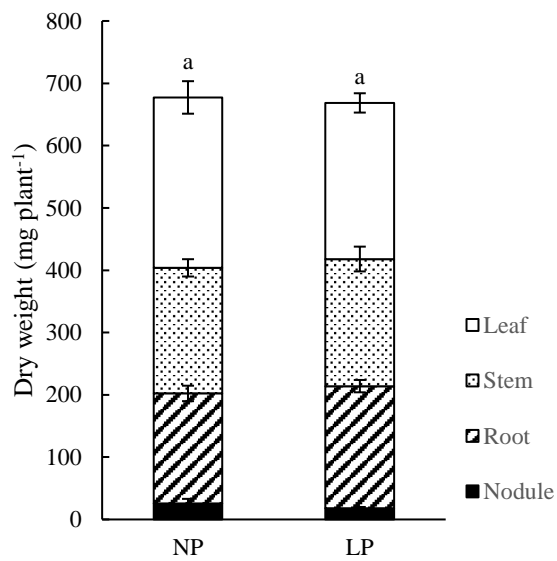Dry weight (mg plant^{-1})

700
600
500
400
300
200
100
0

b

a

☐ Leaf
⊞ Stem
▩ Cotyledon
▨ Root
■ Nodule

NP   LP

Fig. 4. Leaf dry weight (LDW), stem dry weight (SDW), root dry weight (RDW), cotyledon dry weight (CDW), and nodule dry weight (NDW) of normal-phytate (NP) and low-phytate (LP) lines at 21 d after sowing in Experiment 2. Data are presented as means ± SD of four replicates. Means followed by different letters are significantly different at $P \leq 0.05$ according to the t-test.

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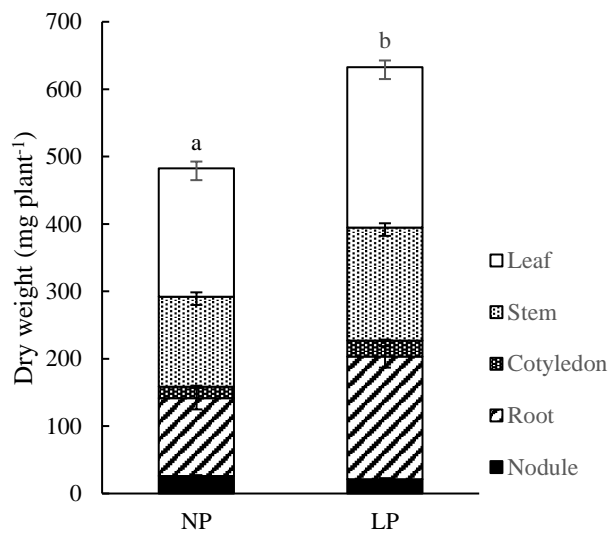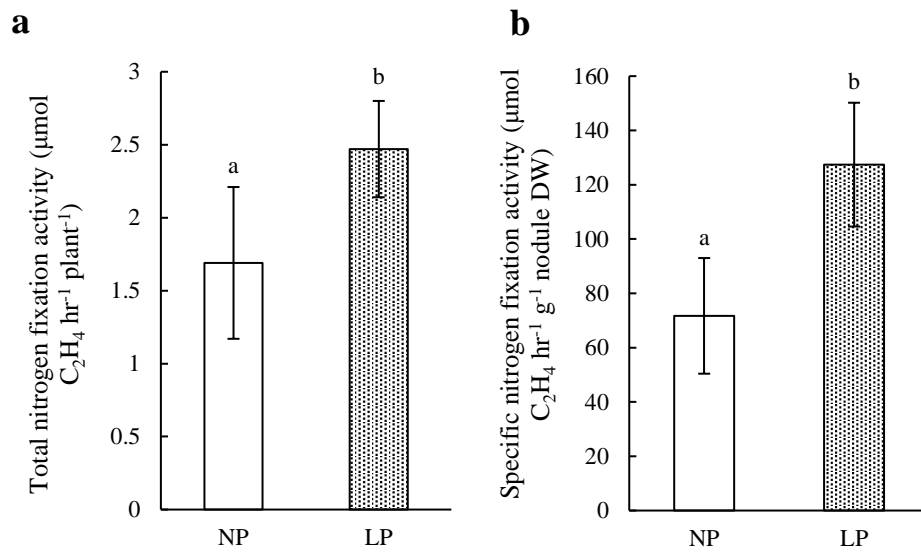
Table 1. Carbon and nitrogen levels in the seedling leaves, stems, roots, and nodules at 21 d after sowing and in seeds of the normal phytate (NP) and low phytate (LP) lines in Experiment 1.

		Carbon		Nitrogen	
		NP	LP	NP	LP
Total plant	(mg plant^{-1})	274.2^a ± 15.2	261.7^a ± 19.5	15.5^a ± 1.3	18.0^b ± 1.0
Leaf	(mg plant^{-1})	111.1^a ± 9.1	96.0^a ± 14.3	7.6^a ± 0.4	7.8^a ± 1.4
Stem	(mg plant^{-1})	83.4^a ± 5.3	82.9^a ± 7.4	3.1^a ± 0.7	3.2^a ± 0.1
Root	(mg plant^{-1})	68.3^a ± 3.6	75.0^a ± 5.8	3.5^a ± 0.2	6.0^b ± 0.8
Nodule	(mg plant^{-1})	11.5^a ± 3.5	7.9^a ± 1.0	6.0^b ± 0.8	1.0^a ± 0.2
Seed	(mg seed^{-1})	89.5^a ± 1.8	90.4^a ± 1.2	13.3^a ± 0.8	13.2^a ± 1.6

Data are presented as means ± SD of three (seeds) or four (seedlings and tissues) replicates. Different superscript letters in the same row indicate significant differences at $P \leq 0.05$ according to the t-test.

Table 2. Carbon and nitrogen levels in seedling leaves, stems, cotyledons, roots, and nodules at 21 d after sowing and in seeds of normal phytate (NP) and low phytate (LP) lines in Experiment 2.

		Carbon		Nitrogen	
		NP	LP	NP	LP
Total plant	(mg plant^{-1})	188.4^a ± 23.0	260.0^b ± 17.0	7.9^a ± 1.0	12.2^b ± 0.4
Leaf	(mg plant^{-1})	65.3^a ± 12.9	94.1^b ± 5.4	3.4^a ± 0.7	5.9^b ± 0.5
Stem	(mg plant^{-1})	57.7^a ± 4.2	69.6^b ± 3.2	1.2^a ± 0.2	1.7^b ± 0.4
Cotyledon	(mg plant^{-1})	7.3^a ± 0.5	9.8^b ± 0.7	0.3^a ± 0.0	0.4^a ± 0.1
Root	(mg plant^{-1})	46.6^a ± 5.8	76.7^b ± 10.7	1.6^a ± 0.2	3.2^b ± 0.5
Nodule	(mg plant^{-1})	11.5^b ± 0.7	9.8^a ± 0.5	1.4^b ± 0.1	1.1^a ± 0.0
Seed	(mg seed^{-1})	39.4^a ± 0.9	63.4^b ± 1.1	5.5^a ± 0.5	8.0^b ± 0.2

Data are presented as means ± SD of three (seeds) or four (seedlings and tissues) replicates. Different superscript letters in the same row indicate significant differences at $P \leq 0.05$ according to the t-test.

Table 3. Phytase, acid phosphatase, specific phytase, and specific acid phosphatase activity levels in nodules of normal phytate (NP) and low phytate (LP) lines at 21 d after sowing in Experiment 1.

Lines	Phytase activity	Phosphatase activity	Specific phytase activity	Specific phosphatase activity
	(nmol min^{-1} g^{-1}FW)	(μmol min^{-1} g^{-1}FW)	(nmol min^{-1} mg^{-1} protein)	(μmol min^{-1} mg^{-1} protein)
Nodule				
NP	48.9^a ±11.2	19.8^a ± 3.0	30.4^a ± 5.4	12.4^a ± 2.2
LP	57.7^a ± 1.5	33.7^b ± 8.3	35.5^a ± 2.1	20.6^b ± 4.7

Data are presented as means ± SD of four replicates. Different superscript letters in the same column indicate significant differences at $P \leq 0.05$ according to the t-test.

Table 4. Phytase, acid phosphatase, specific phytase, and specific acid phosphatase activity levels in nodules of normal phytate (NP) and low phytate (LP) lines at 21 d after sowing in Experiment 2.

Lines	Phytase activity $(nmol\ min^{-1}\ g^{-1}FW)$	Phosphatase activity $(\mu mol\ min^{-1}\ g^{-1}FW)$	Specific phytase activity $(nmol\ min^{-1}\ mg^{-1}\ protein)$	Specific phosphatase activity $(\mu mol\ min^{-1}\ mg^{-1}\ protein)$
Root				
NP	$14.2^a \pm 2.8$	$1.2^a \pm 0.1$	$81.4^a \pm 13.2$	$8.5^a \pm 0.6$
LP	$20.0^b \pm 1.3$	$1.6^b \pm 0.1$	$107.8^b \pm 11.3$	$8.8^a \pm 1.0$
Nodule				
NP	$62.7^a \pm 8.2$	$19.3^a \pm 0.3$	$118.1^a \pm 16.8$	$18.3^a \pm 1.6$
LP	$60.6^a \pm 3.0$	$28.1^b \pm 5.0$	$106.4^a \pm 4.8$	$24.4^b \pm 2.9$

Data are presented as means ± SD of four replicates. Different superscript letters in the same column indicate significant differences at $P \leq 0.05$ according to the *t*-test.

Table 5. Total- and HCl-extractable minerals in normal phytate (NP) and low phytate (LP) seeds in Experiment 2.

Lines	P		Ca		Mg		S	
	Total (μg seed^{-1})	Extractable (%)	Total (μg seed^{-1})	Extractable (%)	Total (μg seed^{-1})	Extractable (%)	Total (μg seed^{-1})	Extractable (%)
NP	718.8[a] ± 63.7	22.1[a]	307.6[a] ± 1.4	67.0[a]	260.7[a] ± 7.7	81.9[a]	901.4[a] ± 22.4	32.1[a]
LP	1018.7[b] ± 121.1	78.3[b]	468.5[b] ± 50.4	54.6[a]	378.1[b] ± 24.4	81.6[a]	916.0[a] ± 16.7	82.4[b]

	Fe		Zn		Cu		Mn	
	Total (μg seed^{-1})	Extractable (%)	Total (μg seed^{-1})	Extractable (%)	Total (μg seed^{-1})	Extractable (%)	Total (μg seed^{-1})	Extractable (%)
NP	13.65[a] ± 2.88	9.5[a]	3.58[a] ± 0.16	58.4[a]	1.98[a] ± 0.11	28.9[a]	2.47[a] ± 0.12	62.9[a]
LP	17.67[a] ± 2.69	39.5[b]	5.59[b] ± 0.86	52.9[a]	2.33[b] ± 0.17	40.4[b]	3.08[b] ± 0.32	82.5[b]

Data are presented as means ± SD of three replicates. Different superscript letters in the same column indicate significant differences at $P \leq 0.05$ according to the t-test.

Table 6. Total P (TP), Ca, Mg, S, Fe, Zn, Mn, and Cu contents in whole seedling and each organ of normal phytate (NP) and low phytate (LP) lines at 21 d after sowing in Experiment 2.

Genotypes	TP (μg seedling^{-1})	Ca (μg seedling^{-1})	Mg (μg seedling^{-1})	S (μg seedling^{-1})	Fe (μg seedling^{-1})	Zn (μg seedling^{-1})	Cu (μg seedling^{-1})	Mn (μg seedling^{-1})
Leaf								
NP	276.2[a] ± 16.1	1,317.8[a] ± 114.6	642.7[a] ± 64.7	1,232.4[a] ± 70.1	22.6[a] ± 7.4	4.1[a] ± 1.1	1.1[a] ± 0.2	19.4[a] ± 1.1
LP	379.3[b] ± 29.5	1,791.4[b] ± 176.9	782.5[b] ± 70.6	1,664.5[b] ± 155.7	35.8[a] ± 15.3	4.3[a] ± 1.0	1.1[a] ± 0.1	24.2[b] ± 3.1
Stem								
NP	113.0[a] ± 4.4	716.2[a] ± 83.4	327.7[a] ± 49.9	864.1[a] ± 55.8	13.1[a] ± 3.2	1.5[a] ± 0.3	0.69[a] ± 0.09	4.1[a] ± 0.6
LP	158.3[b] ± 12.0	1,037.5[b] ± 101.2	383.3[a] ± 46.3	1,441.3[b] ± 192.3	16.8[a] ± 0.7	2.5[a] ± 1.0	0.93[a] ± 0.22	5.8[a] ± 2.1
Cotyledon								
NP	53.6[a] ± 8.3	164.3[a] ± 60.1	173.4[a] ± 51.8	85.8[a] ± 6.7	2.1[a] ± 0.3	0.19[a] ± 0.03	0.09[a] ± 0.02	1.3[a] ± 0.2
LP	68.9[b] ± 9.4	226.9[a] ± 74.6	206.1[a] ± 56.8	99.2[a] ± 9.6	8.0[b] ± 4.6	0.27[a] ± 0.06	0.13[a] ± 0.05	1.9[b] ± 0.1
Root								
NP	127.3[a] ± 29.7	357.3[a] ± 45.9	626.0[a] ± 186.4	1,286.9[a] ± 385.3	200.3[a] ± 56.0	3.6[a] ± 0.8	1.3[a] ± 0.1	1.5[a] ± 0.3
LP	241.5[b] ± 28.3	579.6[b] ± 26.2	901.5[a] ± 128.1	1,474.5[a] ± 133.0	399.1[b] ± 97.6	5.6[b] ± 1.1	2.1[b] ± 0.3	2.8[b] ± 0.3
Nodule								
NP	116.8[a] ± 11.9	53.7[a] ± 5.0	105.6[b] ± 2.3	189.8[b] ± 4.7	25.3[b] ± 5.5	0.64[b] ± 0.13	0.26[a] ± 0.04	0.58[a] ± 0.05
LP	102.8[a] ± 7.0	52.1[a] ± 9.3	79.1[a] ± 4.9	151.1[a] ± 9.0	20.6[a] ± 2.1	0.46[a] ± 0.04	0.23[a] ± 0.04	0.53[a] ± 0.03
Total plant								
NP	686.9[a] ± 50.7	2,609.3[a] ± 125.5	1,875.4[a] ± 147.9	3,659.0[a] ± 341.6	261.4[a] ± 60.0	9.9[a] ± 0.7	3.4[a] ± 0.3	25.0[a] ± 1.0
LP	950.8[b] ± 53.5	3,687.4[b] ± 317.3	2,352.5[b] ± 219.6	4,830.6[b] ± 290.4	472.2[b] ± 86.7	12.9[b] ± 2.4	4.4[b] ± 0.4	32.8[b] ± 3.7

Data are presented as means ± SD of four replicates. Different superscript letters in the same column indicate significant differences at $P \leq 0.05$ according to the *t*-test.