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Root Foraging in Soybean (*Glycine max*) under Nitrogen Deprivation

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Abstract

Nitrate is one of the key sources of nitrogen in natural and agricultural soils. The distribution and concentration of nitrate determine root system architecture in plants. Soybean (*Glycine max* L) is one of the key leguminous crops, while farmers rarely apply nitrogen in soybean crops except for a starter nitrogen dose at the time of sowing. However, the effects of severe deficiency nitrate on early seedling establishment of soybean before nodulation are not yet studied. Therefore, this study evaluated the effects of high dose of nitrate (54.3 m*M*) and its deprivationon (0 m*M*) on the root system architecture of soybean during seedling establishment. Results showed that the root traits including primary root length, fresh biomass, total length, surface area, tips, forks, and its crossings were significantly higher under no nitrate condition than nigh nitrate condition except for root volume, its dry biomass and diameter. Shoot growth attributes such as shoot length, shoot fresh biomass, shoot dry biomass, single leaf area, soil-plant analysis development value, and photosynthesis was significantly decreased while leaf dry mass per area was increased significantly under no nitrate condition. Furthermore, high nitrate supply significantly enhanced the content of nitrate in root tissue, but there was no significant difference between low and optimal nitrate supply. In summary, this study indicated that soybean root system architecture adopts a foraging strategy under nitrogen deprived environment.

Keywords: Soybean; Nitrate; Root system architecture; Foraging

Introduction

Nitrogen (N) is a primary mineral nutrient required in huge quantity for plants to support plant growth and development, but it is present in less quantity in natural and agricultural soils (Lark *et al.* 2004). There are various sources of N like nitrate (NO_3^-), ammonium (NH_4^+), organic amino acids and peptides that plants can absorb. Nitrate is the key form of N found in both natural and agricultural soils which may act as a signaling molecule that shapes the root system architecture (RSA) (Alboresi *et al.* 2005; Marín *et al.* 2011; Alvarez *et al.* 2012). The NO_3^- distribution and concentration are key players to determine the plant RSA (Gruber *et al.* 2013; Tian *et al.* 2014).

Roots are vital in plant production as roots anchor plants in soil/growth medium, provide mechanical support, ensure water and nutrient uptake, facilitate symbiosis development and serve as storage organs in plants. The root elongation, lateral root branching as well as root angles, and root longevity make the root system, while genetic, environmental, and physiological factors are the major determinants of the root system (Lynch and Brown 2012; Smith and Ive 2012). The RSA has developmental plasticity, which depends upon immediate soil environments such as soil water status, soil nutrients, soil temperature, soil pH, and soil microbes. As soil resources are distributed unevenly, therefore, the RSA is crucial for agricultural productivity and is the primary determinant of plant's capacity for the acquisition of soil resources (Lynch 1995).

The plant's ability to efficiently and quickly acquire the nutrients from natural and agricultural soils determine the comparative success rate and production of plants. As mineral nutrients interact in different ways, with each other and with soil particles, or water may carry them out of the plant's root range, which cause nutrients availability decrease and lead to nutrient scarcity. Therefore, plants activate their root foraging system to obtain nutrients from nutrient-rich patches. Root foraging consists of morphological modifications like RSA modulation or formation of root hairs, as well as physiological changes like roots release exudates to mobilize nutrients or changes the expression of nutrient transporters (Gojon *et al.* 2009;

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Hinsinger *et al.* 2009; Gruber *et al.* 2013). This root foraging enhances the interaction between root and soil and improve the ability of plant to capture immobile nutrients. Plants symbiosis with microbes also modifies RSA to some extent (Gutjahr and Paszkowski 2013).

Soybean is a key oilseed crop with a rich source of protein. The global soybean yield is increasing continuously and significantly since last several decades (FAO 2012; Kokubun 2013). Almost all crops require a large amount of N in order to achieve higher production goals, especially the legumes due to their higher seed protein content (Sinclair and Wit 1976; Giller and Cadisch 1995). Soybean shoots accumulate on an average of 79 kg N ha^{-1} to gain additional Mg of seed with seed\ standard moisture content of 0.130 kg H_2O per kg seed (Salvagiotti *et al.* 2008; Tamagno *et al.* 2017). The soybean crop rarely recieves N fertilizer provided by farmers except for a starter dose of N that is applied at sowing time, to meet early seedling emergence/germination stage requirement until nodulation takes place. The investigation indicates whether soil N mineralization and N fixation can meet the N requirement for a seed yield of 6 to 8 Mg per hectare under well-managed field conditions (Menza *et al.* 2017). However, the effect of nitrate deficiency on seedling growth in the early seedling stage before nodulation has not been reported. Therefore, the present study examined the (a) effects of high nitrate supply or its deprivation on seedling establishment and root system architecture, and (b) variations in root foraging in soybean in response to nitrate application.

Materials and Methods

Seed selection, sterilization and sowing

The experiment was conducted using soybean (*Glycine max* L) variety Williams 82. Healthy and uniform seeds were selected and sterilized using bleach containing 5% sodium hypochlorite and hydrochloric acid in a ratio of 10:1(v/v). Briefly, the bleach was taken in a beaker and placed below the porous plate of a glass desiccator apparatus, while, seeds were placed in petri dishes in a single layer kept above the porous plate of the glass desiccator. To avoid evaporation from the glass desiccator apparatus, the lid was closed using wax and left it in a fume hood overnight. After sterilization, seeds were soaked in tap water for 3 h, and the imbibed seeds were sown in trays for germination till 7 days.

Experimental treatments

The physiological experiment was comprised of two treatments *viz*., with and without KNO_3, which started from the first day of sowing. The treatment with KNO_3 was named as high nitrate (54.3 m*M*) (N_1) while without KNO_3 was named as no nitrate (0 m*M*) (N_0). One more treatment was added for the nitrate content analysis experiment. This treatment was name as optimum nitrate (18.81 m*M*). The KCl was used to make up the concentration of K across three treatments. The nutrient medium was liquid MS with little modification that NH_4NO_3, sucrose, and agar were not used while the additional amount of KCl was used in N_0 treatment, to compensate additional potassium added as KNO_3 in high nitrate treatment. The pH of the solution was adjusted to 5.8 with NaOH solution. After 7 days, seedlings were transplanted into other trays having the same concentration of MS medium solution and replicated three times. The MS solution was changed every 5th day.

Growth environment

The experiment was conducted in a growth chamber (RXZ-500D, model number JN181018, Ningbo Jiangnan Instrument Co., Ltd., Ningbo, China), with day/night duration of 16/8 h with a relative humidity of 60% and day/night temperature of 25°C. Data were collected after 4 weeks of sowing.

Gas exchange attributes

Photosynthesis and other gas exchange attributes were recorded inside the growth chamber using Portable Photosynthesis Instrument (LI-6400XT; LI-COR Inc., Lincoln, NE, U.S.A.). The light source was red-blue LED, having 1000 μmol m^{-2} s^{-1} light intensity and carbon dioxide concentration of 399 ± 9.45 µmol mol^{-1}. The leaf temperature was kept at 25°C. The first trifoliate leaf was used to record photosynthesis data and each reading was recorded at a steady state. The SPAD value was also measured with an SPAD meter before start to each photosynthesis measurement.

Root and shoot growth attributes

Root and shoot lengths were measured in centimeters (cm). After measuring the primary root and shoot length, root and shoot fresh biomass were measured with a digital electric weighing balance (LS220A, Precisa, Shanghai, China). Tissue papers were used to absorb the water present on the root surface before recording the fresh biomass. The same roots were packed in plastic bags and kept in the refrigerator at 4°C to measure the other root parameters. The complete roots of each plant were scanned with a root scanner (Epson Expression 1680 Scanner, Seiko Epson Co., Japan), and total root length, root surface area, root diameter, root volume, root tips, forks, and crossings were determined through the Root Analyzer (Regent Instruments Inc., Quebec, Canada). Both shoot and root samples were packed in paper envelopes and kept in an oven at 80°C for 5 days until constant count, and their dry biomass was recorded with a digital electric weighing balance (LS220A, Precisa, Shanghai, China).

Leaf attributes

After measuring the fresh biomass of shoots, all leaves of

each plant were separated and the leaf area was measured by LI-3100C leaf area meter (LI-COR Inc., Lincoln, NE, USA). Then leaf samples were oven-dried at 80°C for 5 days and leaf dry biomass was recorded with a digital electric weighing balance. The leaf biomass per area (LMA) was determined by dividing leaf dry mass with leaf area. The single leaf area was calculated by dividing the whole leaf area with the number of total leaves on each plant.

Nitrate assay for soybean tissues

The salicylic acid method was used to evaluate the nitrate content in soybean root, stem and leaf tissues (Zhao and Wang 2017). Briefly, 0.1 g of fresh soybean tissues was grinded into powder by liquid nitrogen using a Tissuelyser-96 (Jingxin, Shanghai, China). A 1 mL of deionized water was added into the tubes and the mixture was placed in a water bath at 100 °C for 30 min. The 0.1 mL supernatant and 0.4 mL salicylic acid-sulphuric acid was used for incubating the reaction. After adding 9.5 mL of 8% (w/v) NaOH solution into each tube, the tubes were cool down to room temperature (20–30 min), the OD410 value of each sample was measured with a visible light spectrophotometer (NanoReady FC-1100, Suizhen, Hangzhou, China) with the control (deionized water) for reference. The nitrate content were calculated using the following equation: nitrate concentration (μg/g) = (nitrate content in the standard curve × the total volume of extracted sample) / (test amount of sample solution × weight of the sample).

Statistical analysis

Statistical analysis was performed using Statistics 8.1 software and completely randomized design with three replicates to assess treatment differences.

Results

Effect of nitrate treatments on the root and shoot traits

The effect of nitrate treatment was significant on the primary root, shoot and plant length (Table 1). The primary root length was significantly increased while shoot length decreased under no nitrate condition compared to high nitrate treatment. In addition, plant length was also significantly decreased under high nitrate treatment. The primary root length showed a higher increase (42.5%) than the total plant length (14.8%) while the shoot length decreased by 17.2% under no nitrate condition compared to high nitrate treatment.

Root fresh biomass was higher while shoot and plant fresh biomass was lower under no nitrate condition compared to high nitrate treatment (Table 1). Compared with high nitrate treatment, root fresh mass was significantly increased by 20.0% while shoot and plant fresh biomasses decreased under no nitrate condition by 64.9 and 33.1%, respectively.

The effect of nitrate supply on shoot dry biomass was significant. As shown in Table 1, the shoot dry biomass was significantly decreased by 21.4% under no nitrate condition compared to high nitrate treatment. Although, there was no significant difference in root and plant dry biomass between nitrate treatments, root dry biomass increased 8.2% and plant dry biomass decreased 16.0% under no nitrate condition compared to high nitrate treatment.

Effect of nitrate treatment on gas exchange attributes

The single leaf area was significantly reduced by 25.8% in no nitrate treatment against high nitrate condition (Table 2). Leaf dry mass per area was higher in control without nitrate supply than high nitrate treatment which increased by 29.8%. The SPAD value of no nitrate treatment was significantly lower than high nitrate treatment by 25.8%.

Photosynthesis (*A*) significantly increased by 130.7% in high nitrate treatment compared with no nitrate condition. The inhibition rate of stomatal conductance (g_s) was the highest (154.9%) amongst in photosynthetic traits. Compared with high nitrate treatment, g_s decreased significantly under no nitrate treatment. No significant treatment effect was found for intercellular CO_2 concentration (C_i). However, a significant treatment effect was observed in the transpiration rate (Tr), and the Tr of high nitrate treatment recorded 117.3% higher than no nitrate treatment.

The effect of nitrate treatment on root system architecture

The effect of nitrate treatment on root related parameters was significant. The total root length was 25.7% higher under no nitrate condition than high nitrate supply condition (Table 3). Root surface area was increased by 16.9% under no nitrate treatment compared with high nitrate treatment. Contrary to total root length and root surface area, root diameter of no nitrate treatment was significantly lower than high nitrate treatment. The root volume of high nitrate treatment was increased by 7.5% compared with no nitrate treatment; but the effect was not statistically significant. Root tips, forks, and crossings also showed significant variations in response to nitrate treatment. Under no nitrate treatment, root crossings had the highest increment (32.9%), followed by root tips (30.6%) and root forks (23.6%).

The nitrate uptake under different nitrate concentration

To reveal the nitrate uptake in soybean, we evaluate the nitrate content in roots, stem and leaf under low (6.27 m*M*), optimum (18.81 m*M*), and high (54.3 m*M*) nitrate treatments. The nitrate content in root tissue increased with the increase of nitrate concentration in solution. The higher nitrate content was found under high nitrate treatment. No significant difference was found between low and optimal

Table 1: Effect of nitrate supply on shoot and root growth attributes of soybean variety Wm 82

Traits Name (Units)	ANOVA	Treatments (Means ± SE)		Difference (%)
Growth related		N_0 (KNO_3 = 0 m*M*)	N_1 (KNO_3 = 54.3 m*M*)	
Primary root length (cm)	***	34.15 ± 0.97a	19.63 ± 0.57b	-42.5
Shoot length (cm)	***	29.49 ± 0.68b	34.56 ± 0.65a	17.2
Plant length (cm)	***	63.64 ± 1.07a	54.19 ± 0.94b	-14.8
Root fresh biomass (g)	***	1.69 ± 0.12a	1.35 ± 0.14b	-20.0
Shoot fresh biomass (g)	***	2.82 ± 0.18b	4.65 ± 0.36a	64.9
Plant fresh biomass (g)	***	4.51 ± 0.26b	6.00 ± 0.49a	33.1
Root dry biomass (g)	ns	0.09 ± 0.006a	0.08 ± 0.009a	-8.2
Shoot dry biomass (g)	*	0.41 ± 0.03b	0.50 ± 0.05a	21.4
Plant dry biomass (g seedling^{-1})	ns	0.50 ± 0.031a	0.58 ± 0.054a	16.0

Data are presented as mean ± SE of three replications. Mean values followed by the same letters are non-significant at $P < 0.05$

ANOVA was used to test the significance of nitrate treatment. *, ** and *** show significance at $P < 0.05$, $P < 0.01$, and $P < 0.001$ levels, respectively, and ns shows non-significance at $P \geq 0.05$ level

The difference of each parameter between two treatments was calculated from the given equation, $(N_1\text{-}N_0/N_0)*100$

Table 2: Effect of nitrate supply on leaf and photosynthesis-related attributes of soybean variety Wm 82

Photosynthesis-related	ANOVA	Treatments (Means ± SE)		Difference (%)
Traits Name (Units)		N_0 (KNO_3 = 0 m*M*)	N_1 (KNO_3 = 54.3 m*M*)	
Single leaf area (cm^2)	**	20.70 ± 1.70b	26.04 ± 1.90a	25.8
Leaf dry biomass/area (gcm^{-2})	***	21.46 ± 0.018a	15.07 ± 0.013b	-29.8
Soil plant analysis development	***	18.30 ± 0.61b	23.02 ± 0.52a	25.8
Photosynthesis (μmol m^{-2} s^{-1})	***	5.11 ± 0.25b	11.78 ± 0.28a	130.7
Stomatal conductance (mol m^{-2} s^{-1})	***	0.08 ± 0.008b	0.20 ± 0.012a	154.9
Intercellular CO_2concentration (μmol mol^{-1})	ns	271 ± 6.12a	271 ± 6.66a	0.2
Leaf transpiration rate (mmol m^{-2} s^{-1})	***	1.15 ± 0.11b	2.49 ± 0.12a	117.3

Data are presented as mean ± SE of three replications. Mean values followed by the same letters are non-significant at $P < 0.05$

ANOVA was used to test the significance of nitrate treatment. *, ** and *** show significance at $P < 0.05$, $P < 0.01$, and $P < 0.001$ levels, respectively, and ns shows non-significance at $P \geq 0.05$ level

The difference of each parameter between two treatments was calculated from the given equation, $(N_1\text{-}N_0/N_0)*100$

nitrate content. In addition, there was no significant difference on the effects of the three treatments on soybean stem and leaf tissues, although optimal nitrate treatment had highest nitrate content both in leaf and stem tissues of soybean (Table 4).

Discussion

Under optimal growth conditions, plants usually have a lower root to shoot ratio as these distribute more photosynthates to above-ground plant parts, resulting in the accumulation of above-ground biomass. Nevertheless, plant growing under N deficit conditions always have a higher root to shoot ratio, which indicates that above-ground plant parts were more affected by N deficiency than the underground roots (Ruggiero and Angelino 2007; Zhang *et al.* 2009; Lima *et al.* 2010; Ju and Christie 2011). Similarly, researchers reported that moderate N fertilization favored root growth of winter wheat while higher N supply resulted in reduced root growth in subsoil (Svoboda 2006). Low N availability increased root dry biomass (Wang *et al.* 2009). Similar results were observed in present study as primary root length, root fresh biomass, and root dry biomass were increased while shoot length, shoot fresh mass, root dry mass (Table 1) and leaf expansion (Table 2) were decreased under no nitrate treatment due to reduction in photosynthesis.

The roots with longer root length and greater surface areas have resistance to nutrient diffusion, and explore higher soil volume to uptake N under low NO_3^- concentrations (Engels and Marschner 1995; Lawlor 2002). In present study, higher primary root length, total root length, root surface area, root volume, number of root tips, forks and crossings (Table 3) were observed under no nitrate treatment than high nitrate treatment. Because NO_3^- acts as an essential nutrient that limits growth and a key signaling molecule for gene expression, plant metabolism, plant growth and development, leaf expansion, root architecture, flowering time, and seed dormancy (Scheible *et al.* 2004; Zhang *et al.* 2007; Vidal and Gutiérrez 2008; Gojon *et al.* 2009; Krouk *et al.* 2010). Therefore, both primary root length and lateral root length increased (Table 1 and 3) under no nitrate environment that is consistent with previous results since NO_3^- deficient environment promotes primary root elongation and stimulates lateral root growth by regulating auxin activity (Vidal *et al.* 2010).

The NRT1.1 or CHL1 is a dual affinity transporter, while NRT 2.1 is a high-affinity transporter under low NO_3^- availability. It has been reported in *Arabidopsis* that both are involved in nitrate acquisition from the soil solution. In addition, mutation studies have shown that these transporters are either indirectly or directly involved in NO_3^-

Table 3: Effect of nitrate supply on root architecture of soybean variety Wm 82

Traits Name (Units)	ANOVA	Treatments (Means ± SE)		Difference (%)
Root related		N_0 (KNO_3 = 0 m*M*)	N_1 (KNO_3 = 54.3 m*M*)	
Total root length (cm)	***	1156 ± 79a	859 ± 66b	-25.7
Root surface area (cm^2)	**	145 ± 10.58a	120 ± 10.35b	-16.9
Root diameter (mm)	*	0.40 ± 0.007b	0.48 ± 0.037a	19.1
Root volume (cm^3)	ns	1.45 ± 0.12a	1.35 ± 0.13a	-7.0
Root tips (no)	***	1146 ± 94a	796 ± 59b	-30.6
Root forks (no)	**	3105 ± 288a	2371 ± 290b	-23.6
Root crossings (no)	***	686 ± 76a	460 ± 50b	-32.9

Data are presented as mean ± SE of three replications. Mean values followed by the same letters are non-significant at $P < 0.05$

ANOVA was used to test the significance of nitrate treatment. *, ** and *** show significance at $P < 0.05$, $P < 0.01$, and $P < 0.001$ levels, respectively, and ns shows non-significance at $P \geq 0.05$ level

The difference of each parameter between two treatments was calculated from the given equation, $(N_1\text{-}N_0/N_0)*100$

Table 4: Effect of nitrate supply on nitrate uptake of soybean variety Wm 82

Nitrate Concentration	Root (μg/g)	Stem (μg/g)	Leaf (μg/g)
Low nitrate (6.27 m*M*)	52.35 ± 5.10b	91.20 ± 15.87a	70.07 ± 3.78a
Suitable nitrate (18.81 m*M*)	68.80 ± 14.94b	102.02 ± 12.50a	78.89 ± 11.76a
High nitrate (54.3 m*M*)	107.62 ± 20.43a	85.40 ± 16.85a	64.47 ± 15.93a

Data are presented as mean ± SE of three replications. Values followed by different lowercase letters within different treatments are significantly different according to LSD test ($P < 0.05$)

signaling (Muños *et al.* 2004; Little *et al.* 2005; Remans *et al.* 2006; Ho *et al.* 2009; Wang *et al.* 2009, 2020). It was speculated that these transporters might also be present as a signaling molecule to promote root growth and reduce shoot growth in soybean under no or low nitrate condition (Table 1 and 2). However, the low affinity transporters family are active under high availability of nitrate (Krapp *et al.* 2011; Kotur *et al.* 2012; Gu *et al.* 2013, 2014; Léran *et al.* 2014; Liu *et al.* 2014). For example, high nitrate treatment promotes shoot growth and reduces root growth in the present study (Table 1 and 2).

Plant roots could sense nutrient concentration in the soil environment, increase nutrient uptake or its assimilation systems, as well as proliferate in nutrient-rich areas. This phenomenon is known as local signaling. On the other hand, when plant internal nutrient availability becomes inadequate, this phenomenon boosted the whole plant system, which is called systemic signaling (Schachtman and Shin 2007). This dual system regulation controls nutrients, such as NO_3^-, which is one of the most growth-limiting nutrients. The current model of dual regulation indicates that root growth or development and NO_3^- transport are regulated by (i) NO_3^- itself locally and (ii) by reduced N metabolites through systemic feedback repression (Zhang *et al.* 1999; Gojon *et al.* 2009). The experimental results are in accordance where plants under the absence of local N (N_0) increased root foraging by increasing root related parameters while under higher dose/presence of local N (N_1) reduced root growth and development (Tables 1 and 3). In addition, the reduction of photosynthesis metabolism due to less photosynthetic enzymes/components and activities revealed the feedback suppression of the system under no nitrate condition.

When plant roots face N deficiency, the root system architecture behaves in two ways based on the degree of N deficiency (Giehl *et al.* 2012). The survival strategy in a severe N deficiency environment constitutes elongation of primary and lateral roots as well as inhibition of new lateral roots (Giehl *et al.* 2012; Giehl and Wirén 2014). This kind of adaptation depends upon a regulatory module along with the NRT1.1 dependent auxin removal from primordia of lateral roots (Araya *et al.* 2014, 2016). The relatively mild N deficiency rather than severe N limitation stimulates the lateral root emergence as well as primary and lateral root elongation particularly (Gruber *et al.* 2013; Giehl and Wirén 2014; Ma *et al.* 2014). This stimulatory response is an interesting strategy, in which roots enhance soil foraging volume is known as the foraging strategy. The upregulation of the auxin biosynthesis gene *TAR2* was observed under low N conditions. Under mild N deficiency environment, *tar2* mutant showed inhibition in lateral root emergence , thus auxin is considered to be an active role player (Ma *et al.* 2014). However, as primary and lateral root length of *tar2* mutant was not affected, so TAR2-dependent auxin biosynthesis alone fails to explain the root elongation stimulation mechanism under mild N deficiency. In present study, soyeban root foraging strategy was found under severe nitrate deficiency as primary root length, total root length, and all other root related parameters showed increment except root diameter (Table 1 and 3). Further studies are needed to investigate the genetic behavior of root and shoot growth under excessive and deficient nitrate environments in soybean.

Conclusion

The NO_3^- deprivation or high dose/presence is a signal to monitor plant growth in soybean in the early growth stage before nodulation. The deprivation of NO_3^- promoted root growth in search of NO_3^- and showed a decrease in above-ground plant parts through local and systemic signaling. Similarly, the high dose/presence of NO_3^- promoted shoot

growth and showed a decrease in root growth through local and systemic signaling. High nitrate supply significantly enhanced the nitrate contents in root tissue, but there was no significant difference between low and optimal nitrate supply. In summary, soybean roots act as plant foraging organs under NO_3^- absence environment.

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Author Contributions

MT and LS designed experiment and wrote manuscript. YD, ZP, LC, MS helped in performing experiments and analyzing data. MA and GZ helped in revising manuscript.

Conflict of Interest

We declare that the authors have no competing interests as defined by Nature Research, or other interests that might be perceived to influence the results and/or discussion reported in this paper.

Data Availability

All data will be available upon reasonable request to the corresponding author.

Ethics Approval

Not applicable.

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