https://doi.org/10.17221/225/2023-PSE

Inoculation with *Bacillus* alters nitrogen uptake and metabolism in roots of *Diospyros lotus* under wheat straw addition in soil

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Citation: Zhang P., Hao F.G., Zhu Z.T., Lang D.M., Hu H.L. (2023): Inoculation with *Bacillus* alters nitrogen uptake and metabolism in roots of *Diospyros lotus* under wheat straw addition in soil. Plant Soil Environ., 69: 463–470.

Abstract: A ^{15}N-labelling technique was carried out to investigate the effect of wheat straw co-application with *Bacillus nealsonii*, *Cohnella*, and *Paenibacillus lautus* on N uptake and assimilation in *Diospyros lotus*. Wheat straw combined with *Bacillus* increased the plant height, biomass accumulation, photosynthetic capacity, and uptake of ^{15}N by roots, with ^{15}N accumulating mainly in leaves of *D. lotus*. The NO_3^--N content in roots and leaves were decreased by wheat straw co-application with *Bacillus*, whereas NH_4^+-N, soluble protein, and total N contents were increased. Wheat straw addition promoted the activities of nitrate reductase, glutamine synthase and glutamate synthase in roots rather than in leaves. These N assimilation enzymatic activities, and glutamic-oxaloacetic transaminase and glutamic pyruvic transaminase activities were markedly increased by wheat straw combined with *Bacillus*. Moreover, the combined application of wheat straw and *Bacillus*, particularly *B. nealsonii*, improved the N use efficiency. These findings suggest that the combined application of wheat straw and *Bacillus* improved *D. lotus* growth by increasing N uptake, metabolism, and utilisation efficiency.

Keywords: ^{15}N-uptake; nitrogen assimilation; persimmon tree; nutrition; isotope

Persimmons are prominent fruits in the agricultural sector of China's northwest region, particularly in Shaanxi and Henan province (Luo and Wang 2008). However, in these areas, persimmon trees are usually planted on mountains, hills, or in areas with adverse soil conditions. *Diospyros lotus* is widely used as a rootstock of persimmon, which is initially subjected to environmental stress, particularly nutrient limitation. Nitrogen (N) is a constituent of amino acids, nucleic acids, phytohormones, and chlorophyll (Baslam et al. 2020), and is an indispensable element that is crucial for plant growth and development, and significantly affects both yield and fruit quality.

Inorganic N fertilisers are the key source of N in soil and plants. The available N in the soil is taken up by roots, and then assimilated by key enzymes, such as nitrate reductase (NR), glutamine synthase (GS), and glutamine oxoglutarate aminotransferase (GOGAT) (Rennenberg et al. 2010). NR reduces nitrate (NO_3^--N) to ammonia (NH_4^+-N), and NH_4^+-N is incorporated into 2-oxoglutarate to form glutamine *via* the GS-GOGAT cycle (Lea and Leegood 1993). Glutamate can be transaminated to other amino acids as catalysed by glutamic-oxaloacetic transaminase (GOT) and glutamic-pyruvic transaminase (GPT) (Hodges 2002). In China, excess N fertiliser has been used in

Supported by the National Natural Science Foundation of China, Grant No. 32202419, and by the Science and Technology Project of Henan Province, Project No. 202102110054/202102110051.

https://doi.org/10.17221/225/2023-PSE

agricultural production to promote yield. However, excessive N fertilisation not only results in serious environmental problems, such as nitrate leaching, NH_3 volatilisation, but also low N use efficiency (NUE). Hence, there is an urgent need to explore effective measures to improve NUE and synchronise, the reduction in chemical fertiliser application without influencing the normal growth of plants.

Crop straw and microbial biofertilisers are cost-effective and renewable sources with no detrimental effects on soil health, and their combination is considered an eco-friendly and economical method for agricultural production (Du et al. 2022, Zhu et al. 2022). *Bacillus* has been used as biofertilisers, play a crucial part in promoting growth and N uptake in plants (Masood et al. 2020, Gomez-Ramirez and Uribe-Velez 2021). Previous studies reported that straw application and *Bacillus* inoculation improved N uptake and NUE (Zhang et al. 2019b, Wang et al. 2021). However, how the co-application of crop residues with functional microbes regulate the N distribution characteristics in tissues, metabolic capacity and use efficiency of *D. lotus* remain unclear.

Therefore, the aims of this study were to: (1) analyse growth parameters and photosynthetic characteristics; (2) estimate N uptake and distribution among different tissues using ^{15}N-labeled technique, and (3) evaluate the activities of key enzymes involved in N metabolism in *D. lotus* tissues in response to the addition of wheat straw combined with *Bacillus* strains. The results provide new insights that will contribute to a comprehensive understanding and evaluation of the co-application of crop residues and functional bacteria as biological inoculants for fruit production.

MATERIAL AND METHODS

Study site and experimental design. The soil samples in this study were collected in the east of Henan Institute of Science and Technology (35°17'59"N, 113°56'37"E; altitude 70 m a.s.l.). Soil samples were collected from rows of *D. lotus* at the experimental site, with dimensions of 10 × 10 cm (40 cm in depth), following the removal of weeds and residues. The visible roots and debris were removed from soil samples, then air-dried, and mixed efficiently into homogenous substrates. The total organic carbon of the soil was 7.32 g/kg. Available N, phosphorus and potassium was 46.00 mg/kg, 11.79 mg/kg and 43.02 mg/kg, respectively. The crop straw in this study was made by wheat that was cut into 1–2 cm smash after oven at 80 °C. The wheat straw had total organic carbon and N of 466.42 g/kg and 3.80 mg/kg, respectively.

The seeds of *D. lotus* were stratified at 0–4 °C for 40 days, then planted in a nursery plate filled with substrate in greenhouse. After germination, the seedlings (about 10 cm) were transplanted into plastic pots (16 × 14 cm) containing 1 kg of the above soil samples, using one seedling per pot, and randomly placed outdoors. After pre-cultivation for 30 days, 60 seedlings of similar sizes were selected for the experiment.

Five treatment were investigated, including (1) seedlings only treated with wheat straw (WS); (2) seedlings treated with wheat straw combined with *Bacillus nealsonii* (WS + B); (3) seedlings treated with wheat straw combined with *Cohnella* (WS + C); (4) seedlings treated with wheat straw combined with *Paenibacillus lautus* (WS + P); and (5) untreated seedlings as control (CK). ^{15}N labeled urea (10% atom abundance, produced by the Shanghai Research Institute of Chemical Industry) was used as the basic fertiliser.

Wheat straw was mixed with soil at a concentration of 2 g/kg before treatment. Strains of *B. niescherii*, *Cohnella* and *P. lautus* had been previously isolated from the rhizosphere of *D. lotus*. The cells were grown in Luria-Bertani (LB) fluid medium at 25 °C in a shaker incubator operating at 150 r/min for 48 h. The culture was then centrifuged at 5 000 × *g* for 10 min, and the pellet was collected by washing with sterile deionised water and recentrifuged. Spores were resuspended in sterile deionised water. Each bacterial suspension was prepared to an optical density at 600 nm of approximately 1.0 before use. Each treated pot contained an inoculum volume of 25 mL. Each treatment consisted of three replicates, each containing 12 independent seedlings. After 90 days of treatment, three seedlings in each treatment were used to analyse photosynthesis and biomass. The remaining nine seedlings in each treatment were equally divided into three groups. Three seedlings were mixed as one replication. The roots, stem, and leaf samples were collected using a destructive method, packed with silver paper, and flash-frozen in liquid nitrogen. Samples were ground into powder using a ball-mill (Scientz-48L; Ningbo Scientz Biotechnology Co., Ltd., Ningbo, China) and stored at –80 °C. Half of the sample was used to determine nitrogen content and key enzyme activities involved in N metabolism, the other half was dried and used to measure the ^{15}N value. Soil samples were collected from the rooting zone of each seedling. These samples were air-dried, and sieved through a 0.85 mm sieve,

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and used to determine the total nitrogen content.

Photosynthetic characteristics and biomass. The fully expanded leaves from the middle of each plant were selected to determine the net photosynthetic rate (A), stomatal conductance (g_s) and intercellular CO_2 concentration (c_i) using an LI-6800 photosynthesis system (LiCor Inc., Lincoln, USA) between 10:00 and 11:00 h on sunny days.

Chlorophyll was extracted with 10 mL of 96% ethanol in darkness for 48 h and measured spectrophotometrically (TU-1810, Beijing Purkinje General Instrument Co., Ltd., Beijing, China) at 649 nm, and 660 nm as previously described (Lichtenthaler and Wellburn 1983).

After measuring photosynthesis, plants were harvested from the soil and divided into roots, stems and leaves. The tissues were oved dried at 80 °C for 48 h and each the dry biomass was weighed.

NO_3^--N, NH_4^+-N, soluble protein and total N contents, and $\delta^{15}N$ values. Approximately 0.1 g fresh samples of roots and leaves were used to determine NO_3^--N content using the salicylic acid-sulfuric acid method (Cataldo et al. 1975). The NO_3^--N content was measured spectrophotometrically at 410 nm with respect to a standard curve. The NH_4^+-N content of the roots and leaves was measured using the colourimetric assay described by Krom (1980). The soluble protein content in the roots and leaves of the control and treated seedlings was determined by the Coomassie brilliant blue method, as described by Bradford (1976), using bovine serum albumin as the standard.

Total N contents and $\delta^{15}N$ values in roots, stems, leaves and soil were determined using a vario ISOTOPE CUBE elemental analyser (Elementar, Langenselbold, Germany).

Key enzymes activities involved in N metabolism. NR activity in roots and leaves was determined using the method described by Datta and Sharma (1999). The absorbance of the reaction mixture was measured using a spectrophotometer at 540 nm.

GS activity and NADH-GOGAT activity in roots, and Fd-GOGAT activity in leaves were measured using reagent kits (Suzhou Grace Biotechnology Co., Ltd, Suzhou, China), according to the manufacturer's instructions.

The activities of GPT and GOT were measured according to a previous method (Tang 1999) by measuring the reaction mixture at 500 nm. The enzyme activity was calculated using a standard curve prepared using pyruvic acid.

Statistical analysis and calculation. One-way analysis of variance was performed using PASW Statistics 18 statistical analysis software (SPSS Inc. Chicago, USA) to analyse the effect of wheat straw combined with *Bacillus* on N metabolism and growth parameters. All dates in tables and figures are expressed as mean ± stand deviation (n = 3). Differences between means were analysed using Tukey's test at a significance level of $P < 0.05$. Figures were performed by Excel 2019 (Microsoft, Redmond, USA).

The formulae for ^{15}N proportion distributions of urea were as follows:

$$\text{The amount of N derived from labelled fertiliser (Ndff, \%)} = (\delta^{15}N_{sample} - \delta^{15}N_{nature})/(\delta^{15}N_{fertiliser} - \delta^{15}N_{nature}) \times 100\% \quad (1)$$

$$\text{Total N in tissue} = \text{plant dry matter} \times \text{total N content in tissue} \quad (2)$$

$$^{15}N \text{ uptake} = \text{total N in tissue} \times \text{Ndff} \quad (3)$$

$$\text{Rate of } ^{15}N \text{ distribution (\%)} = {}^{15}N \text{ uptake in tissue}/\text{total } ^{15}N \text{ uptake (root + stem + leaf)} \times 100 \quad (4)$$

$$^{15}N \text{ use efficiency (NUE, \%)} = {}^{15}N \text{ uptake}/{}^{15}N_{fertiliser} \text{ input} \times 100 \quad (5)$$

where: $\delta^{15}N_{sample}$ – $\delta^{15}N$ value of sample; $\delta^{15}N_{fertiliser}$ – $\delta^{15}N$ value of soil; $\delta^{15}N_{nature}$ – natural $\delta^{15}N$ value that is equal to 0.366%.

RESULTS AND DISCUSSION

Wheat straw addition and it combined with *Bacillus* inoculation increased plant height and biomass accumulation in seedlings, while no marked effect on stem diameter was evident compared with that in CK (Table 1). The greatest increase in plant height was observed in the co-application of straw with *Bacillus*, especially in WS + B treatment. Straw return is beneficial for improving soil properties, and the increased soil organic carbon and available nutrient contents, contribute to preserving moisture and altering the nutrient balance of soil, which is an important factor in enhancing plant growth and biomass accumulation (Jourgholami et al. 2020). *Bacillus* strains are involved in many essential processes such as the production of phytohormones and the biosynthesis of metabolites that stimulate plant growth (Poveda and González-Andrés 2021). Photosynthesis is the basis for biomass formation in tissues (Beadle and Long 1985). The increased biomass we observed was consistent with the improved plant photosynthetic characteristics, including A,

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Table 1. The biomass and photosynthetic characteristics of *Diospyros lotus* in response to wheat straw combined with *Bacillus*

Treatment	Biomass (g)			Height (cm)	Stem diameter (mm)	A (μmol CO_2/m^2/s)	C_i (mmol/mol)	g_s (mol H_2O/m^2/s)	Chlorophyll (*a* + *b*) (mg/g)
	total	shoot	root						
CK	2.79 ± 0.18^c	1.60 ± 0.09^c	1.18 ± 0.15^b	22.63 ± 0.65^d	4.66 ± 0.40^a	4.42 ± 0.20^c	258.03 ± 8.95^a	1.73 ± 0.13^b	0.78 ± 0.02^c
WS	4.99 ± 0.16^b	3.26 ± 0.08^b	1.73 ± 0.17^a	24.57 ± 0.59^c	4.88 ± 0.03^a	5.25 ± 0.31^b	252.40 ± 2.55^a	1.92 ± 0.10^{ab}	1.59 ± 0.18^b
WS + B	5.64 ± 0.37^a	3.54 ± 0.09^a	2.11 ± 0.31^a	29.37 ± 1.21^a	4.78 ± 0.11^a	6.85 ± 0.44^a	267.13 ± 6.58^a	2.90 ± 0.15^a	2.07 ± 0.21^a
WS + C	5.43 ± 0.13^a	3.46 ± 0.09^a	1.97 ± 0.21^a	26.93 ± 1.69^b	4.92 ± 0.50^a	6.21 ± 0.73^a	254.83 ± 17.10^a	2.45 ± 0.12^{ab}	1.79 ± 0.05^{ab}
WS + P	5.44 ± 0.17^a	3.49 ± 0.05^a	1.95 ± 0.13^a	27.47 ± 0.81^b	4.84 ± 0.51^a	6.68 ± 0.17^a	245.47 ± 3.10^a	2.43 ± 0.01^{ab}	1.87 ± 0.03^{ab}

Dates showed as mean ± standard deviation (n = 3). Different lowercase letters in the same column mean significant differences among different treatments (P < 0.05; Tukey's test). A – net photosynthetic rate; g_s – stomatal conductance; C_i – intercellular CO_2 concentration. WS is treated with 2 g/kg wheat straw; WS + B, WS + C and WS + P is 2 g/kg wheat straw combined with *Bacillus nealsonii*, *Cohnella*, and *Paenibacillus lautus*, respectively; CK – control those plants without wheat straw and *Bacillus*

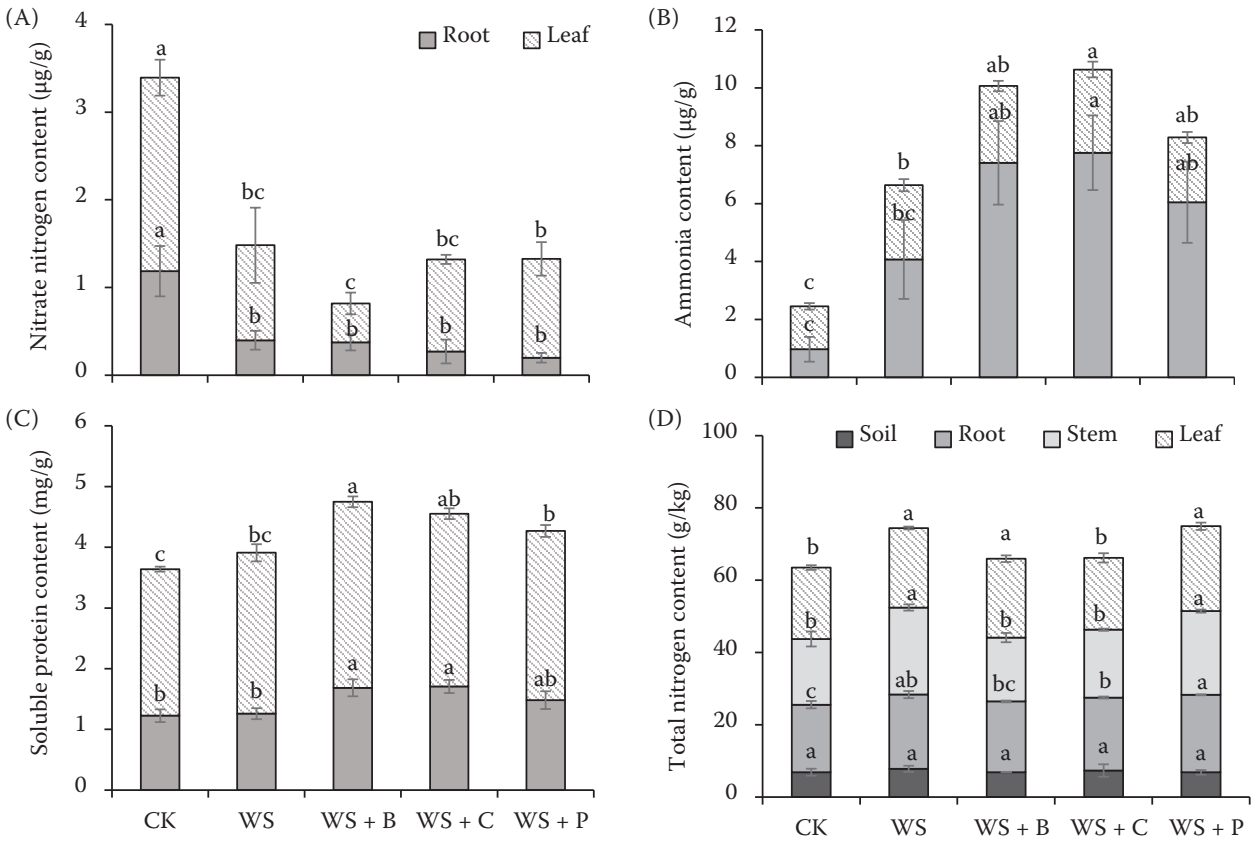

Figure 1. (A) The nitrate nitrogen; (B) ammonia; (C) soluble protein and (D) total nitrogen in tissues of *Diospyros lotus* and soil in respond to wheat straw combined with *Bacillus*. WS is treated with 2 g/kg wheat straw, WS + B, WS + C and WS + P is 2 g/kg wheat straw combined with *Bacillus nealsonii*, *Cohnella*, and *Paenibacillus lautus*, respectively; CK – control those plants without wheat straw and *Bacillus*

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g_s, and chlorophyll (*a* + *b*) content in leaves, under wheat straw addition (Table 1). Previous study reported that straw return promoted photosynthetic parameters and total aboveground biomass (Zhang et al. 2019a). Moreover, in this study, the A values and chlorophyll (*a* + *b*) contents under WS + B, WS + C, and WS + P treatments were significantly higher than those obtained with WS treatment. *Bacillus* inoculation promotes growth by improving soil fertility and photosynthesis of plants (Masood et al. 2020, Gomez-Ramirez and Uride-Velez 2021). N is a major component of chlorophyll and photosynthetic proteins (Baslam et al. 2020). Compared with CK, wheat straw and *Bacillus* inoculation reduced the NO_3^--N content, while increased the NH_4^+-N content in roots and leaves (Figure 1A, B). The content of soluble protein and total N content in roots and leaves were higher in straw combined with *Bacillus* treatments than in CK (Figure 1C, D). The increased N content in leaves promotes photosynthesis, which contributes to carbohydrate synthesis (Zhang et al. 2019c, Baslam et al. 2020), resulting in biomass accumulation in plants with wheat straw addition, especially when combined with *Bacillus* inoculation.

Plants have a high demand for N, and N fertilisation is a major source. Previous study reported that the addition of external crop straw and functional microbe promotes N uptake (Yang et al. 2022). ^{15}N-labelling has been widely used to evaluate the N distribution among plant tissues and NUE (Guo et al. 2021). In our study, the trends of Ndff values and ^{15}N content in tissues were similar, and those under the WS + B and WS + C treatments were markedly higher than those in WS treatment and the CK (Figure 2A, B). The fundings suggest the promotion of N uptake by roots. The promoted N uptake was attributed to the improved biochemical processes in the soil due to soil microbial activity and functions in response to organic matter application (Du et al. 2022). Furthermore, the functional bacteria, especially the consortia of *Bacillus*, promote the transformation of urea and increase N availability for roots (Yang

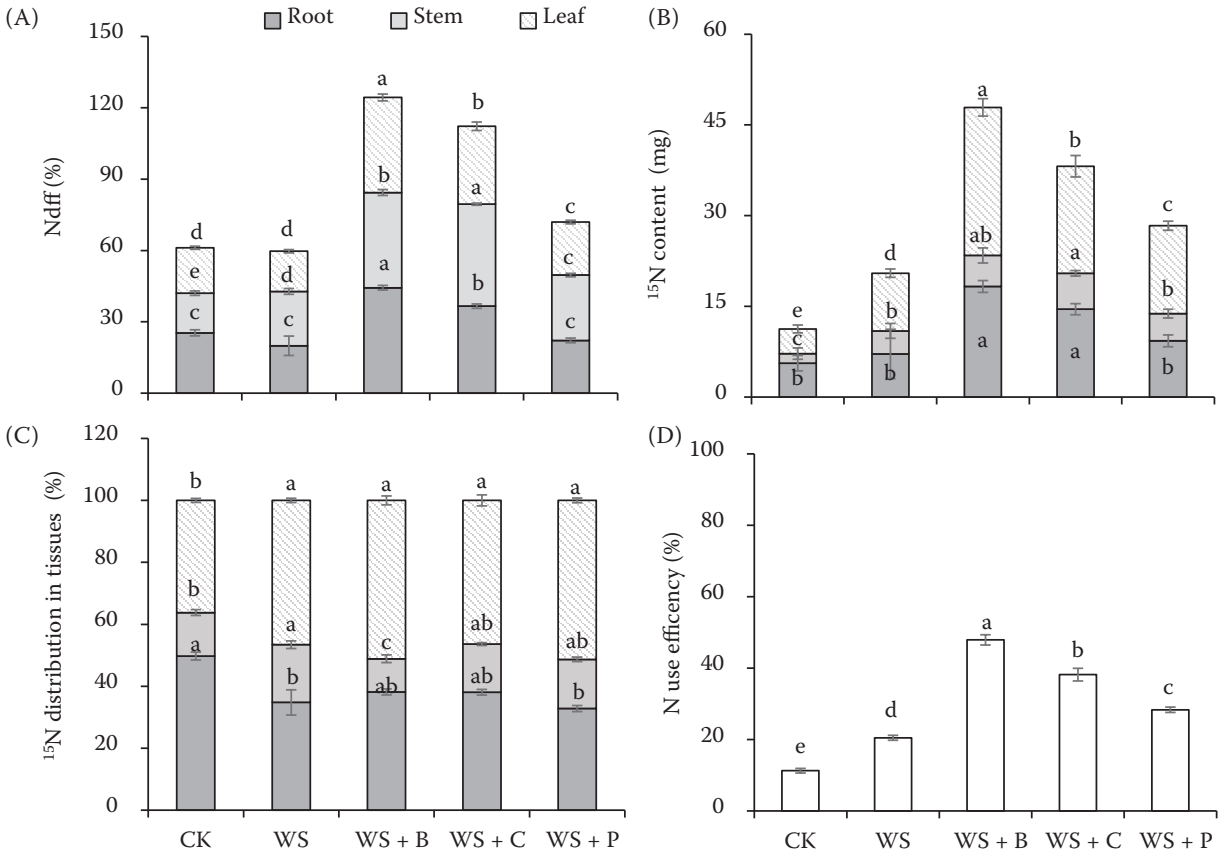

Figure 2. (A) Nitrogen (N) derived from labelled fertiliser (Ndff); (B) ^{15}N content; (C) ^{15}N distribution in tissues of *Diospyros lotus*, and (D) N use efficiency in response to wheat straw combined with *Bacillus*. WS is treated with 2 g/kg wheat straw, WS + B, WS + C and WS + P is 2 g/kg wheat straw combined with *Bacillus nealsonii*, *Cohnella*, and *Paenibacillus lautus*, respectively; CK – control those plants without wheat straw and *Bacillus*

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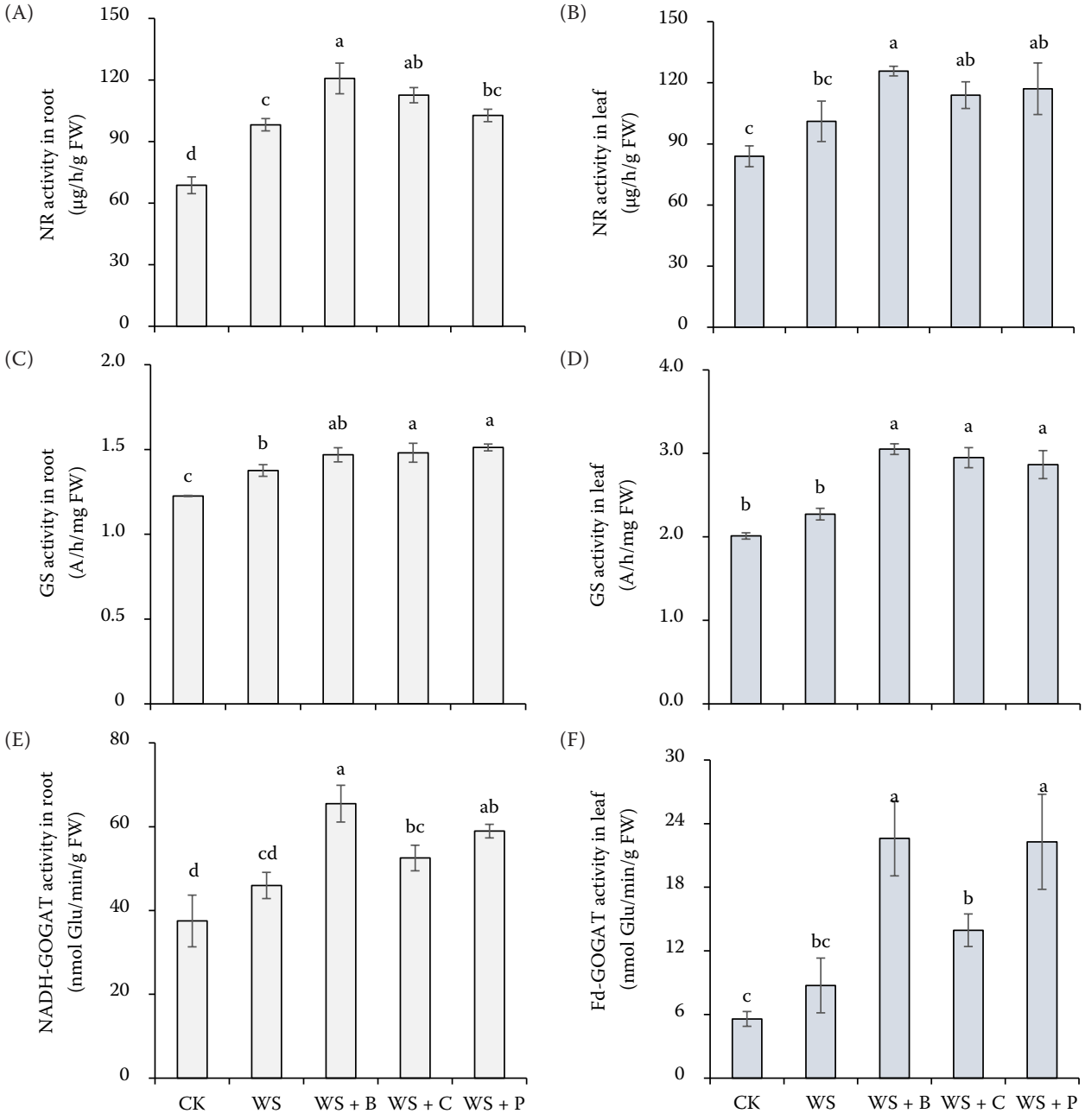

Figure 3. (A, B) The nitrate reductase (NR); (C, D) glutamate synthase (GS); (E, F) glutamate dehydrogenase activities in roots and leaves of *Diospyros lotus* in response to wheat straw combined with *Bacillus*. WS is treated with 2 g/kg wheat straw, WS + B, WS + C and WS + P is 2 g/kg wheat straw combined with *Bacillus nealsonii*, *Cohnella*, and *Paenibacillus lautus*, respectively; CK – control those plants without wheat straw and *Bacillus*; FW – fresh weight; A – absorbance; Glu – glucose

et al. 2022, Solanki et al. 2023). The majority of the absorbed N in roots is subsequently translocated to the aboveground parts of the plant. In this study, ^{15}N was mostly detected in leaves (36.20–51.31%), followed by roots (32.81–49.75%) and stems (10.71–18.63%) (Figure 2C). Wheat straw and *Bacillus* inoculation treatments significantly increased the distribution of ^{15}N in the leaves, which in WS, WS + N, WS + C, WS + P was 28.70, 41.21, 28.11 and 41.72% higher than the value in CK, respectively. These results are consistent with the increased total N content in the leaves. N predominantly allocated to the leaves contributes to assembling and operating the photosynthetic apparatus (e.g. as precursor for chlorophyll, proteins,

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or enzymes), which resulted in the accumulation of biomass and plant growth (Win et al. 2019). Wang et al. (2021) reported that straw return improves the NUE of cotton. In this study, wheat straw addition and *Bacillus* inoculation promoted the NUE, which in WS, WS + N, WS + C, WS + P was 1.82, 4.25, 3.39 and 2.52-times that of CK, respectively (Figure 2D). Compared with that in straw alone, WS + N, WS + C, WS + P increased NUE by 133.81, 86.32 and 38.29%, respectively. The NUE in the WS + B treatment was significantly higher than that in the other two strains, indicating that wheat straw combined with *B. nealsonii* efficiently improved NUE and reduced N loss. The enhancement of NUE under crop straw application and *Bacillus* inoculation has been closely attributed to improved soil quality (Zhang et al. 2019b).

Absorbed N in the roots is reduced or translocated to the leaves for assimilation by the enzymes involved in N metabolism (Aquino et al. 2021). The significantly increased NR activity caused by wheat straw and its co-application with *Bacillus* resulted in a decrease of in the endogenous NO_3^--N content in the roots and leaves (Figure 4A, B). The NO_3^--N reduced to NH_4^+-N is then rapidly converted to amino acids through the GS/GOGAT cycle (The et al. 2021). Wheat straw combined with *Bacillus* significantly increased GS and GOGAT activities in roots and leaves as compared with that in CK (Figure 3). Furthermore, the activities of GS and GOGAT in the WS + B and WS + P treatments were markedly higher than that in WS treatment suggesting the that wheat straw combined with *B. nealsonii* and *P. lautus* efficiently promotes assimilation of N in tissues and generates more glutamate as the substrate for transamination reactions. Subsequently, glutamate is transaminated to other amino acids by GOT and GPT. Therefore, increased activities of GOT and GPT in the roots and leaves were found in wheat straw combined with *Bacillus* treatments in our study (Figure 4), which accelerated the conversion

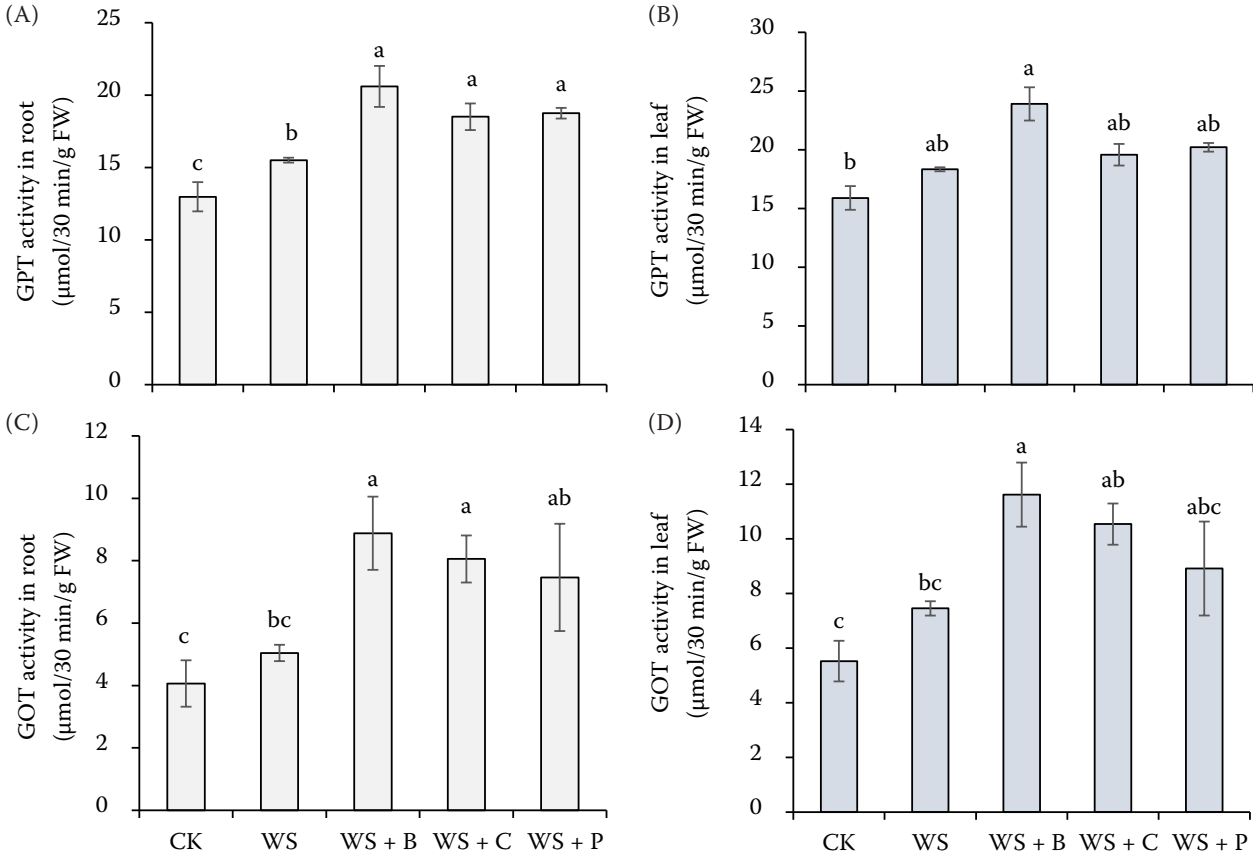

Figure 4. (A, B) The glutamic-pyruvic transaminase (GPT); (C, D) glutamic oxalacetic transaminase (GOT) activities in roots and leaves of *Diospyros lotus* in response to wheat straw combined with *Bacillus*. WS is treated with 2 g/kg wheat straw, WS + B, WS + C and WS + P is 2 g/kg wheat straw combined with *Bacillus nealsonii*, *Cohnella*, and *Paenibacillus lautus*, respectively; CK – control those plants without wheat straw and *Bacillus*; FW – fresh weight

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of N to other amino acids, especially wheat straw combined with *B. nealsonii*.

In summary, the addition of wheat straw combined with *Bacillus* inoculation increased the uptake of N by roots, N metabolism and NUE, and contributed to photosynthesis and biomass accumulation. The combined use of wheat straw and *B. nealsonii* resulted in a relatively higher NUE and N metabolism capacity, which could be considered superior candidates for biofertilisers that can effectively enhance plant growth and optimise chemical fertiliser utilisation.

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Received: June 3, 2023
Accepted: October 2, 2023
Published online: October 25, 2023