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BRIEF COMMUNICATION

# Role of nitrogen utilization in facilitating photosynthetic compensation of soybean under vertically heterogeneous light

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## Abstract

Photosynthetic compensation enables high-density planted crops to use the available light efficiently. However, the underlying mechanism remains unclear. Herein, soybeans (*Glycine max* L. Merr.) were treated with vertically heterogeneous light (HL) to simulate the light conditions in high-density planting. The net photosynthetic rate ($P_N$) increased in upper unshaded leaves (UL) while it decreased in lower shaded leaves (LL) under HL. This evident photosynthetic compensation was accompanied by a preferential distribution of N to UL. Correspondingly, the amounts of chlorophyll (Chl) and Rubisco increased in UL. At the same time, the ratio of Rubisco to Chl (Rubisco/Chl) and the photosynthetic N-use efficiency (PNUE) of UL decreased under HL. In contrast, there was little change in the PNUE of LL. This study suggests that the within-plant distribution and within-leaf allocation of N play significant roles in photosynthetic compensation. The decline in PNUE at the whole-plant level should be viewed as a cost associated with facilitating this process.

*Keywords*: heterogeneous light; nitrogen utilization; photosynthetic compensation.

High-density planting is an effective practice for increasing crop yield per unit area, which holds significant importance in addressing the global food crisis (Postma *et al*. 2021, Xu *et al*. 2021). When crops are planted at high densities, however, intraspecific competition for environmental resources, especially sunlight, intensifies significantly (Postma *et al*. 2021). The distribution of sunlight around high-density planted crops shows obvious heterogeneity in the vertical direction since severe mutual shading occurs at lower parts, while the upper parts are exposed to relatively high light (Wu *et al*. 2022, Yan *et al*. 2024). Such a kind of heterogeneous light not only causes shade-avoidance response in plants, such as accelerated elongation of hypocotyls and internodes (Lyu *et al*. 2023), but also limits assimilation driven by light energy, especially in lower leaves (Yamazaki and Shinomiya 2013, Yao *et al*. 2015). In this case, the upper leaves perform a strong photosynthetic capacity to compensate for the shade-caused decline in photosynthesis of lower leaves, termed photosynthetic compensation, which functions to use the available light efficiently, thus representing a strategy for plants to acclimate to heterogeneous light conditions like high-density planting (Huang *et al*. 2022, Sun *et al*. 2023).

Previous studies indicate that nitrogen (N) distribution within a plant has a significant response to light conditions.

## Highlights

- Soybean shows photosynthetic compensation under vertically heterogeneous light
- Photosynthetic compensation is correlated with within-plant N distribution
- Photosynthetic compensation comes at a cost of photosynthetic nitrogen-use efficiency

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*Abbreviations*: Chl – chlorophyll; $g_s$ – stomatal conductance; HL – heterogeneous light; LL – lower leaves; NS – no shade; $P_N$ – net photosynthetic rate; PNUE – photosynthetic nitrogen-use efficiency; TLs – trifoliate leaves; UL – upper leaves.

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When a plant is subjected to heterogeneous light, N content usually increases in unshaded leaves while it decreases in shaded leaves (Araya *et al.* 2008, Li *et al.* 2015, Huang *et al.* 2022). Since there is a strong positive correlation between leaf N content and photosynthetic performance, the preferential distribution of N to unshaded leaves is regarded to be of importance to support photosynthetic compensation (Huang *et al.* 2022). However, studies on different species showed that the N content of unshaded leaves increased, accompanied by a decline in photosynthesis under heterogeneous light (Ribeiro *et al.* 2017, Huang *et al.* 2022). Consequently, the increase of leaf N content does not always result in improved photosynthesis, further indicating a potential role of N investment in photosynthetic components in facilitating photosynthetic compensation. Unfortunately, only a few studies report the response of photosynthetic N-use in plants to heterogeneous light. The underlying mechanism remains to be elucidated.

As an economically important crop, soybean (*Glycine max* L. Merr.) is widely cultivated worldwide (Ramlal *et al.* 2023). However, there is still a big gap between the actual and the potential yield (Wang *et al.* 2023, Omondi *et al.* 2024). So far, increasing planting density is an effective practice to increase soybean yield, especially in regions with limited arable land (Xu *et al.* 2021, Li *et al.* 2025). In this case, it is imperative to interpret the response of soybean to a high-density planting environment, of which photosynthesis is a subject of much attention since it is the basis of yield formation. In the present study, HL surrounding high-density planted soybean was simulated in a greenhouse. Measurements suggest evident photosynthetic compensation accompanied by a response in within-plant N distribution. Representative N components were further measured to discuss photosynthetic N use, to provide a better understanding of photosynthetic performance under HL.

Seeds of soybean (cv. Nandou 12) were sown into plastic pots (15 × 15 × 15cm) filled with commercial substrates (*The Pindstrup Group*, Latvia). After germination, seedlings were grown in a greenhouse under stable artificial light provided by LEDs, with an irradiance of 600 μmol(photon) $m^{-2}$ $s^{-1}$ from 8:00 to 20:00 h. Room temperature was set to 25°C (light)/20°C (dark). When the first trifoliate leaves (TLs) were fully expanded, at which time the second TLs were approximately one-third of the area of fully expanded leaves, the primary leaves (*i.e.*, the leaves located below the first trifoliate leaf) and stem apices, including TLs younger than the second TLs, were pinched out. Subsequently, seedlings were subjected to HL with the first TLs shaded by spectrally neutral nylon net [150 μmol(photon) $m^{-2}$ $s^{-1}$], while the second TLs were kept in normal light [600 μmol(photon) $m^{-2}$ $s^{-1}$], to simulate the light condition in high-density planting. Seedlings that received no shade (NS) were designated as experimental control (Fig. 1*A*). After the second TLs were fully expanded, the middle leaflets of both upper unshaded leaves (UL, *i.e.*, the second TLs) and lower shaded leaves (LL, *i.e.*, the first TLs) were used for measurements. Throughout the experiment, all seedlings were well-watered.

All of the following results were reached from four to five replicates, each with at least three different plants. *Student*'s *t*-test was performed to statistically evaluate at the levels of both 0.01 and 0.05. Histograms were created by *Origin* (*version 8.6*) software.

Gas exchange was measured with a portable photosynthesis system (*LI-6400XT*, *Li-COR*, USA) at the light intensity corresponding to the growth conditions. During the measurement, the temperature was maintained at 25°C and the $CO_2$ concentration was kept at 400 μmol $mol^{-1}$. $P_N$ and stomatal conductance ($g_s$) were recorded when they reached steady-state values.

Leaf discs without main leaf veins were exposed to 105°C for 0.5 h and then dried to a constant mass at 80°C. The dried samples were digested in 98% $H_2SO_4$ and 30% $H_2O_2$ and diluted with 86 mM EDTA-methyl red solution. After adjusting to pH 6.0, 106 mM phenol, 0.34 mM sodium nitroprusside (SNP), and 7.05 mM NaClO were added to the mixture. Subsequently, absorbance was read on a spectrophotometer (*752N*, *INESA*, China) at 625 nm. The content of N was determined from a standard curve. Photosynthetic N-use efficiency (PNUE) was calculated as the ratio of $P_N$ to N content.

Chl was extracted by immersing finely sliced leaf filaments in 80% acetone and incubating them in complete darkness for approximately 12 h, until full discoloration was observed. During incubation, the samples were gently agitated at hourly intervals to facilitate extraction. Subsequently, absorbance of the extract was measured with a spectrophotometer (*752N*, *INESA*, China) at 646 and 663 nm. The content of Chl was calculated according to Lichtenthaler and Buschmann (2001).

Rubisco was quantified by double-antibody sandwich ELISA using a commercially available kit (*Yili Biotechnology*, China). Extracts of soluble proteins from leaf discs were added to micropores coated with a monoclonal antibody. Subsequently, horseradish peroxidase (HRP)-labeled antibody was added to form antibody-antigen-HRP-labeled antibody complexes. After thorough washing, color was developed by adding tetramethyl benzidine (TMB). Then, absorbance was read on a microplate reader (*iMark*, *Bio-Rad*, Japan) at 450 nm, and the abundance of Rubisco was determined from a standard curve.

Middle leaflets were scanned with a scanner (*CanoScan LiDE 210*, *Canon*, Japan). Subsequently, leaf area was quantified using *ImageJ* (*version 1.52a*) software. Leaf segments without main leaf veins were fixed in formalin-acetic acid-alcohol (FAA) solution and then embedded in paraffin. Cross-sections were obtained on an ultramicrotome (*RM2016*, *Leica*, Germany). After dewaxing and staining, micrographs were acquired on a light microscope (*Eclipse E100*, *Nikon*, Japan). Leaf thickness was determined using *ImageJ* (*version 1.52a*) software. The leaf mass per area was calculated from the leaf dry mass and area.

The result showed that the $P_N$ increased significantly by 17% in UL. In contrast, it decreased significantly by 30% in LL under HL, as compared to NS (Fig. 1*B*). This result suggests evident photosynthetic compensation, which initially describes a phenomenon of defoliated plants and then evolved to describe the adaptive response of plants to environment cues such as heterogeneous light (He and Dong 2003, Huang *et al.* 2022, Sun *et al.* 2023). Previous studies indicated that the photosynthetic response of plants to heterogeneous light is potentially related to stomatal movement (Jiang *et al.* 2011, Li *et al.* 2015, Sun *et al.* 2020). In the present study, the $g_s$ of UL increased significantly by 78% under HL, as compared to NS (Fig. 1*C*). Therefore, the increased $P_N$ of UL is proposed to be associated with enhanced $CO_2$ uptake. In contrast, the $g_s$ of LL decreased slightly without a significant difference (Fig. 1*C*), suggesting that the decreased $P_N$ was caused mainly by nonstomatal factors.

In the present study, N content increased significantly by 37% in UL while it decreased significantly by 29% in LL under HL, as compared to NS (Fig. 1*D*). This result is consistent with previous studies on different plant species (Araya *et al.* 2008, Sugiura and Tateno 2013, Li *et al.* 2015, Huang *et al.* 2022), suggesting a preferential distribution of N to unshaded leaves under heterogeneous light conditions. Such a distribution of N is crucial to support photosynthetic compensation because of the positive correlation between leaf N content and photosynthetic performance (Niinemets *et al.* 2015, Huang *et al.* 2022). N absorbed by roots is transported to leaves through the transpiration stream within xylem, which is critically influenced by stomatal movement. Consequently, a close relationship between leaf N content and stomatal movement is proposed (Xu *et al.* 2019). Since the $g_s$ of UL increased significantly under HL (Fig. 1*C*), the preferential distribution of N to UL is potentially driven by a promoted transport of root-absorbed N *via* the transpiration stream. However, the marked decrease of N content in LL is not likely a result of the slightly decreased $g_s$ (Fig. 1*C*). Previous studies indicate significant effects of light conditions on the senescence of leaves. When a plant is subjected to heterogeneous light, the senescence of unshaded leaves is often delayed while that of shaded ones is accelerated (Ono *et al.* 2001, Weaver and Amasino 2001). Leaf senescence is one of the important reasons leading to redistribution of N within a plant, which is directly related to the degradation and

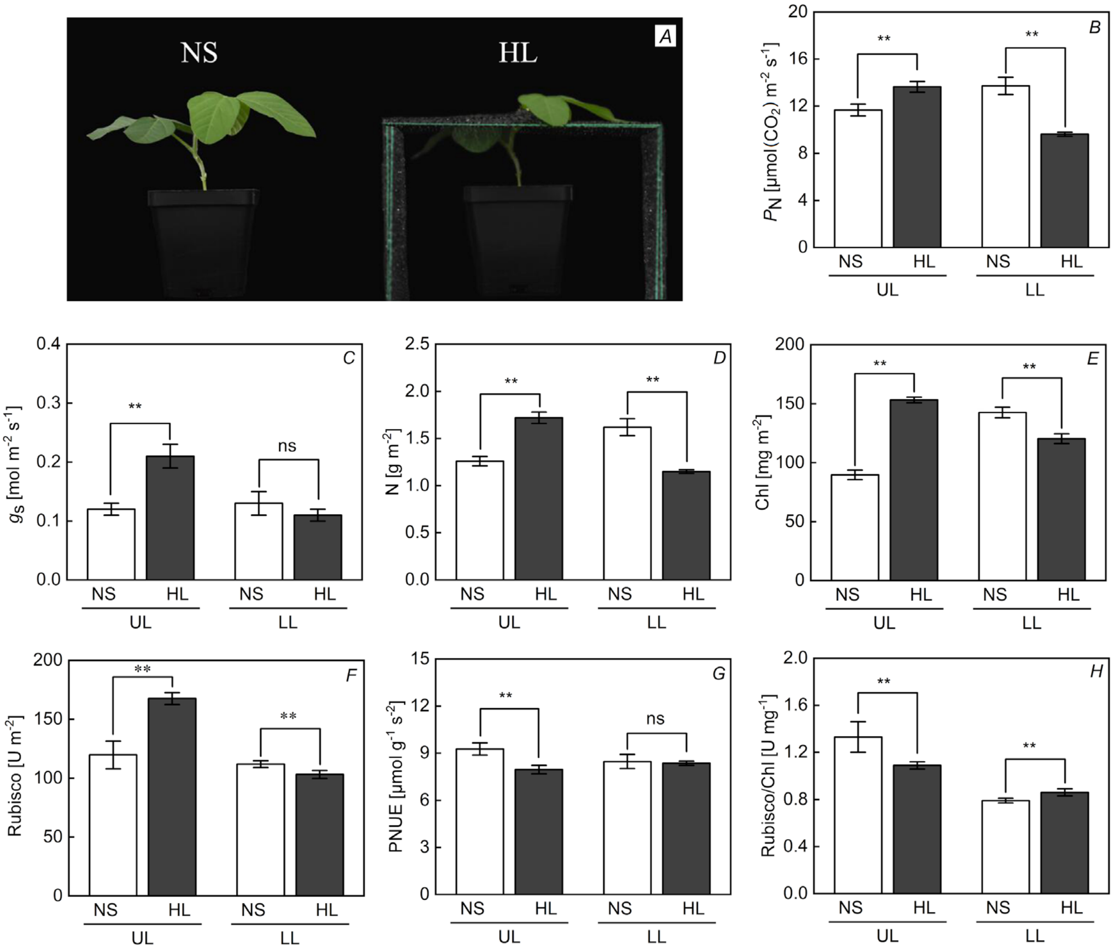

Fig. 1. Photosynthetic performance, nitrogen (N) and photosynthetic N components of upper leaves (UL) and lower leaves (LL). (*A*) The treatment of soybean; (*B*) net photosynthetic rate ($P_N$); (*C*) stomatal conductance ($g_s$); (*D*) total N content; (*E*) total chlorophyll (Chl) content; (*F*) Rubisco abundance; (*G*) photosynthetic nitrogen-use efficiency (PNUE); (*H*) ratio of Rubisco to Chl (Rubisco/Chl). Results are shown as mean values ± standard error (*n* = 5). Significance of difference (**$P<0.01$; ns – not significant) is indicated. HL – heterogeneous light; NS – no shade.

export of chloroplast components in senescing leaves (Ono *et al*. 2001, Havé *et al*. 2017). Results showed that the amounts of Chl and Rubisco decreased significantly by 16% and 8%, respectively, in LL under HL, as compared to NS (Fig. 1*E*,*F*). This result suggests a great possibility that the decrease in N content in LL was caused by accelerated senescence. Under HL, a large amount of N components in LL were degraded and then redistributed to UL, in which a further accumulation of N in addition to transpiration-driven transport was achieved.

In addition to N distribution within a plant, N allocation within a leaf also responds to heterogeneous light (Yao *et al*. 2015, Sun *et al*. 2023). In the present study, the amounts of Chl and Rubisco increased significantly by 71% and 40%, respectively, in UL under HL, as compared to NS. In contrast, they decreased significantly in LL, as mentioned above (Fig. 1*E*,*F*). Therefore, the investment of leaf N in photosynthetic components is proposed to be enhanced in UL, while it declined in LL under HL. This is consistent with a previous study on sunflower (*Helianthus annuus* L.), which also performed evident photosynthetic compensation under HL (Yamazaki and Shinomiya 2013). However, another study in kidney bean (*Phaseolus vulgaris* L.) showed that the amounts of Chl and Rubisco in upper unshaded leaves decreased with a downward trend of photosynthetic performance under HL (Murakami *et al*. 2014). Consequently, a role of within-leaf N allocation, besides within-plant N distribution, in facilitating photosynthetic compensation is proposed.

The present study also evaluated the effect of HL treatment on PNUE. The result showed that the PNUE decreased significantly by 14% in UL under HL as compared to NS. At the same time, it decreased slightly without a significant difference in LL (Fig. 1*G*). This result suggests a decline in PNUE at the whole-plant level under HL, which is consistent with a previous study on high-density planted sorghum (*Sorghum bicolor* L.) (Li *et al*. 2015). Moreover, Rubisco activity per Chl was previously reported to have a significant response to heterogeneous light (Araya *et al*. 2008). In the present study, the results showed that the ratio of Rubisco to Chl (Rubisco/Chl) decreased significantly by 18% in UL under HL, as compared to NS (Fig. 1*H*). Therefore, more leaf N is suggested to be allocated for light-harvesting rather than $CO_2$ fixation in UL, since Chl and Rubisco represent essential components for light-harvesting and $CO_2$ fixation, respectively. Taking into account that the light energy availability was not limited on UL, the preferential allocation of leaf N for light-harvesting is obviously not advantageous to photosynthetic N use in the case of increased N content. Simultaneously, the Rubisco/Chl increased significantly by 8% in LL under HL, as compared to NS (Fig. 1*H*). This result suggests a preferential allocation of leaf N for $CO_2$ fixation under a light-limited condition, in which enhanced light-harvesting is instead more advantageous to photosynthetic N use in the case of decreased N content. Consequently, the decline in PNUE at the whole-plant level is proposed to be a result of misallocation of leaf N among photosynthetic components. It should be regarded as a cost in facilitating photosynthetic compensation under HL.

It is worth noting that heterogeneous light has a significant impact on leaf morphology, which is an important factor affecting photosynthetic performance (Matsuda and Murakami 2016). Previous studies on various plant species suggested that the upper developing leaves usually exhibited shade-type characteristics, such as increased area and decreased thickness, when lower leaves were shaded (Yano and Terashima 2001, Jiang *et al*. 2011, Murakami *et al*. 2014). Results showed that the leaf area of UL increased significantly by 27% under HL, as compared to NS (Fig. 2*A*), while the leaf thickness and leaf mass per area responded little (Fig. 2*B*,*C*). In this case, a larger area for light interception is proposed to UL. This is also an effective way to improve the photosynthetic performance of UL and further support photosynthetic compensation under HL. In this case, a lot of leaf N in UL should be invested in promoting leaf development, instead of photosynthesis, for intercepting more light. This is another possible reason for the decline in PNUE.

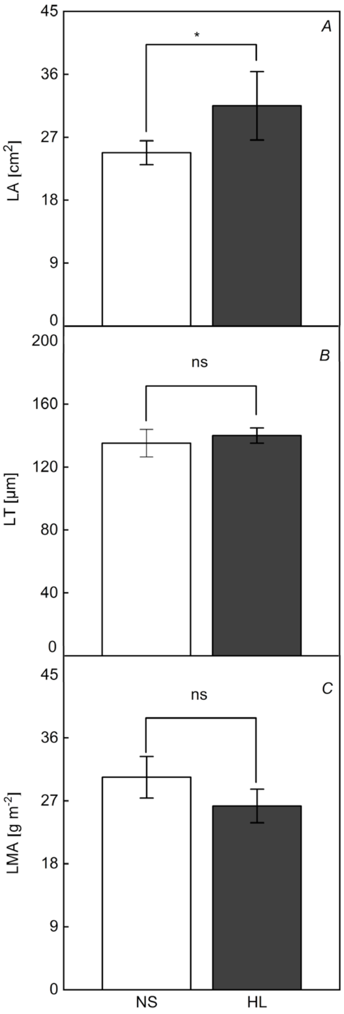

Fig. 2. (*A*) Leaf area (LA), (*B*) leaf thickness (LT), and (*C*) leaf mass per area (LMA) of upper leaves (UL). Results are shown as mean values ± standard error (*n* = 4). Significance of difference (**P*<0.05; ns – not significant) is indicated. HL – heterogeneous light; NS – no shade.

In conclusion, the present study suggests that the photosynthetic compensation of soybean under HL is closely correlated to within-plant distribution and within-leaf allocation of N. N is preferentially distributed to the large upper unshaded leaves, in which the allocation of leaf N for photosynthesis was enhanced to support photosynthetic compensation. In contrast, both the total N and photosynthetic N components were reduced in LL. Moreover, a decline in the PNUE caused by misallocation of leaf N among photosynthetic components, particularly in the UL, should be regarded as a cost in facilitating photosynthetic compensation. This conclusion provides a necessary foundation for further studies on the photosynthetic characteristics of field-grown soybeans under dense planting conditions, where both intensity and quality of light have potential effects on plant growth.

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