

# Photosynthetic Performance and Stomatal Plasticity of Six Vegetable Plants in Vegetative Phases under Controlled Hydroponic Conditions

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## Abstract

In the vegetative phase, shoot and root development are influenced by genetic and environmental factors. This research investigates changes in plant growth and stomatal plasticity among six crops grown under controlled floating raft hydroponic conditions: *Brassica chinensis* var. parachinensis (BC), *Brassica oleracea* var. sabellica (BO), *Ipomoea aquatica* L. (IR), *Brassica rapa* subsp. chinensis (BR), *Lactuca sativa* L. var. crispa (LS), and *Brassica narinosa* L. (BN). During the vegetative phase (42 days), plants adapted their stomatal development in response to growth needs, leading to increased stomatal density across all cultivars. However, this increase did not significantly affect stomatal opening width or the number of subsidiary cells, which are likely genetically controlled. A strong positive correlation was found between stomatal density, photosynthetic performance, and growth in most cultivars (BC, BR). The BN cultivar highlighted that high stomatal density does not necessarily guarantee growth functionality, emphasizing the importance of coordination with leaf area. Observations from LS and BN suggest a dynamic adaptation to balance photosynthetic demand and water use efficiency. Additionally, the consistency in stomatal aperture size and the number of subsidiary cells underscores genetic stability. Overall, the plasticity of stomatal traits demonstrates how these species optimize gas exchange and photosynthetic performance in hydroponic conditions. These findings help identify key characteristics for distinguishing plant performance, such as a photosynthetic indicator of better plant growth under hydroponic conditions, which is potentially valuable for breeding or stress resilience studies.

**Keywords:** horticulture, hydroponic, stomatal characteristics, urban plant, vegetative

## 1. Introduction

The hydroponics market is poised for notable growth, particularly in addressing the demand for food and vegetables in areas where cultivation space is limited and soil media cannot be utilized (Wang *et al.*, 2019). This positive trend has been further supported by advancements in various systems, including Nutrient Film Technique (NFT), Deep Flow Technique (DFT), vertical farming, and floating raft hydroponics. These innovative methods are increasingly embraced in urban environments to cultivate leafy greens such as lettuce, spinach, pak choi, and basil, with biomass harvests typically occurring during the mid to late vegetative stages (Palmitessa *et al.*, 2024; Vega *et al.*, 2023).

During the vegetative phase, plants primarily focus on developing their shoots, which include the stem, new

shoots, and leaves, as well as root growth (Jia *et al.*, 2023). In hydroponic systems, this stage is optimized through controlled environmental conditions—such as light, humidity, and temperature—as well as a balanced nutrient solution that is particularly rich in nitrogen, phosphorus, and potassium to promote leaf growth. Additionally, efficient water usage and oxygenation are crucial for ensuring healthy root development (Al-Gaadi *et al.*, 2024; Palmitessa *et al.*, 2024). Research indicates that hydroponics accelerates vegetative growth because plants have access to readily available nutrients and more favorable growing conditions, with fewer pathogens compared to traditional soil systems (Silva *et al.* 2018; Palsha *et al.* 2024).

Cultivating leafy greens requires a well-controlled vegetative period to maximize biomass production. This phase is the period of growth following germination and preceding flowering. During the vegetative phase, plants

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\*\* **Abbreviations:** BC, *Brassica chinensis* var. parachinensis; BN, *Brassica narinosa* L.; BO, *Brassica oleracea* var. sabellica; BR, *Brassica rapa* subsp. chinensis; IR, *Ipomoea aquatica* L.; LS, *Lactuca sativa* L. var. crispa; DFT, deep flow technique; NFT, nutrient film technique; WUE, water use efficiency.

primarily focus on expanding their leaf area to enhance photosynthesis, developing shoots and roots to facilitate nutrient uptake (Jha, 2019), and performing various metabolic processes (Kadam *et al.*, 2015). The efficiency of these processes is significantly influenced by the characteristics of the plant's leaves, particularly the stomata. Stomatal properties are vital for regulating gas exchange and conserving water through transpiration, both of which are essential for optimal growth (Chua & Lau, 2024).

Stomatal development is governed by genetic pathways involving the SPCH, MUTE, and FAMA genes (Ortega *et al.*, 2019; Verma *et al.*, 2020). Additionally, environmental factors such as light, carbon dioxide concentration, humidity, and nutrient availability significantly influence the expression of these genes. During the vegetative phase, light not only promotes stomatal development but also facilitates an increase in leaf area (Chua & Lau, 2024; Ekeke *et al.*, 2017). Furthermore, the presence of nitrogen enhances stomatal density and supports greater leaf expansion (Chen *et al.*, 2020). Water availability also plays a critical role in modulating stomatal aperture activity and density, primarily in response to variations in water content within plant tissues (Chen *et al.*, 2020; Chua & Lau, 2024).

Research has shown a positive correlation between stomatal density and biomass accumulation during the vegetative phase, which subsequently affects photosynthetic efficiency (Stinziano *et al.*, 2017; Yin *et al.*, 2020). However, it is important to note that excessively high stomatal density can negatively impact the water use efficiency (WUE) of leaves, resulting in increased transpiration rates (Bian *et al.*, 2019). Additionally, different cultivars display distinct responses and growth patterns, even when subjected to identical environmental conditions (Parvin *et al.*, 2017).

In this study, we aimed to conduct a thorough growth analysis during the vegetative phase of several plant species, specifically *Brassica chinensis* var. *parachinensis*, *Brassica oleracea* var. *sabellica*, *Ipomoea aquatica* L., *Brassica rapa* subsp. *chinensis*, *Lactuca sativa* L. var. *crispa*, and *Brassica narinosa* L. Previous research, as noted by Silva *et al.* (Silva *et al.*, 2018), has indicated that, within the *Brassica* genus, vegetative growth—quantified through leaf area expansion—follows a logistic growth pattern that positively responds to specific controlled conditions, including a temperature of around 35 °C, moderate nutrient levels (5 mS/cm EC), and high light intensity.

Conversely, a study by Zhou *et al.* (Zhou *et al.*, 2020) highlighted that in *L. sativa* var. *crispa*, relative growth rates (RGR) and various other growth components tend to fluctuate with nutrient availability, showcasing the plant's remarkable morphological plasticity during the vegetative expansion phase. Moreover, it is essential to acknowledge that several additional factors influence the growth of these six plant species, including hormonal regulation, photoperiod, sunlight intensity, environmental stimuli, mechanical signals, and the genetic control of plant architecture and anatomical traits, such as stomatal density (Araújo *et al.*, 2011; Chua & Lau, 2024).

This study seeks to enhance our understanding of the changes in plant growth during the vegetative period, as well as the stomatal plasticity of six different vegetable

crops cultivated under controlled hydroponic conditions. Over a 42-day observation period, we measured various growth parameters to elucidate the growth patterns of these crops. The focus is particularly on the stomatal plasticity exhibited by the crops during three distinct phases of vegetative growth, all within optimal hydroponic conditions. We anticipate that the insights gained from this research will be beneficial for improving plant biomass yield in hydroponic systems.

## 2. Materials and methods

### 2.1. Plant materials

This research with hydroponic sets was carried out simultaneously using several types of vegetable plants, including Caisim (*Brassica chinensis* var. *parachinensis*-BC), Kale (*Brassica oleracea* var. *sabellica*-BO), water spinach (*Ipomoea aquatica* - IR), Pakcoy (*Brassica rapa* subsp. *chinensis*-BR), Lettuce (*Lactuca sativa* L. Var. *crispa*-LS), and Pagoda mustard (*Brassica narinosa* L.-BN), derived from seeds obtained from farm shops. The seeds of each cultivar were germinated in water with nutrient media consisting of AB mix. The components in AB-mix consist of macro nutrients such as NO<sub>3</sub> (9.9%), NH<sub>4</sub> (0.48%), P<sub>2</sub>O<sub>5</sub> (4.83%), K<sub>2</sub>O (16.5%), MgO (2.83%), CaO (11.48%), SO<sub>3</sub> (3.81%) and micro nutrients such as B (0.0013%), Mn (0.025%), Zn (0.015%), Cu (0.002%), Mo (0.003%), and Fe (0.037%).

### 2.2. Experimental site

This study was conducted in a greenhouse in an urban environment with temperatures between 29 °C and 32 °C between 10 am and 2 pm, and air humidity ranging from 80% in the dry season to 100% in the rainy season. The planting location was on the second floor, and a block research design was used. Each plant was planted in a different box according to its type and placed randomly, and its position was exchanged every two weeks. The number of plant replications was 3 blocks, with each block containing nine individual plants. The design for each group followed a completely randomized blocked design (CRBD).

### 2.3. Maintain hydroponic conditions

Cultivation and maintenance were conducted weekly, with plant ages ranging from seedling to harvest at 42 days old. The sterilized seeds were first soaked in a 1% chlorine solution for one minute, then rinsed three times with distilled water. They were subsequently soaked for 24 to 48 hours in trays kept in the dark. After germination, the seeds from the trays were placed into hydroponic pots, with two sprouts in each pot, with a diameter of five cm. These pots were then placed in tubs containing three liters of water, along with a growing medium that had been mixed with AB mix nutrients. The AB mix was applied by combining tap water with the AB mix solution at a ratio of 10 ml of AB mix solution to one liter of water. A total of 30 ml of AB mix was introduced into the three liters of water, which was replaced once a week. Once the plants reached the mid-vegetative stage, between 14 and 21 days after planting, the frequency of nutrient and water applications increased to twice a week until the plants entered the final vegetative period.

## 2.4. Measurement of growth characteristics

The observed growth parameters included plant height, number of leaves, leaf area of the third leaf, and leaf circumference. Plant height was measured from the base to the tip of the longest leaf. The total number of leaves was counted for each clump of plants, including both the youngest leaves that had begun to grow and the oldest leaves that had turned brown. Leaf area measurements were taken using a Leaf Area Meter (LI-3000C) and were also verified with two-dimensional digital measurements using Image Raster 3.0.

## 2.5. Observation of stomatal characteristics

Stomata were observed by taking imprints of the outer layer of the abaxial epidermis from the third leaf of each treatment group ( $n=3$ ). Each imprinted leaf was placed on an object glass and covered with several drops of water before being examined under a binocular light microscope (Boeco BM-180/SP, Germany). Observations were recorded using Optilab software (Miconos 1.0) at magnifications of 4x, 10x, and 40x. All images were subsequently analyzed using Image Raster 3, with initial calibration completed. Stomatal characteristics were measured based on several parameters, including the number and density of stomata, the width of stomatal pore openings, and the height and width of the stomata.

## 2.6. Photosynthetic and gas exchange measurement

Gas exchange and photosynthetic performance were observed during the vegetative period using the LI-6800 Portable Photosynthesis System (LI-COR Inc., Lincoln, NE, USA) to evaluate the  $\text{CO}_2$  response of A (photosynthetic rate) employing a traditional method. The measurement parameters for the chamber were established as follows:  $\text{CO}_2$  concentration at  $410 \mu\text{mol mol}^{-1}$ , photon light intensity at  $1200 \mu\text{mol m}^{-2} \text{s}^{-1}$ , flow rate at  $700 \mu\text{mol s}^{-1}$ , temperature at  $28.0 \text{ }^\circ\text{C}$ , and  $\text{H}_2\text{O}$  at  $20.93 \text{ mmol mol}^{-1}$ . Measurements were conducted between 9 am and 1 pm, using leaves that had been expanded before being placed in the leaf chamber. Measurements were taken only when environmental parameters—including flow rate, fan speed,  $\text{CO}_2$  concentration, air humidity, light intensity, and temperature—remained stable. Data corrections were applied to align with the  $\text{CO}_2$  concentration and  $\text{H}_2\text{O}$  levels

in the leaf chamber after each measurement for each treatment group. Data capture occurred once fluctuations had stabilized, typically within 30 seconds to 2 minutes after the leaves were placed in the chamber. The photosynthetic characteristics measured included the photosynthetic rate (A), transpiration rate (E), intercellular  $\text{CO}_2$  concentration ( $\text{C}_i$ ), and total stomatal conductance to  $\text{CO}_2$  ( $g_{sw}$ ).

## 2.7. Statistical analysis

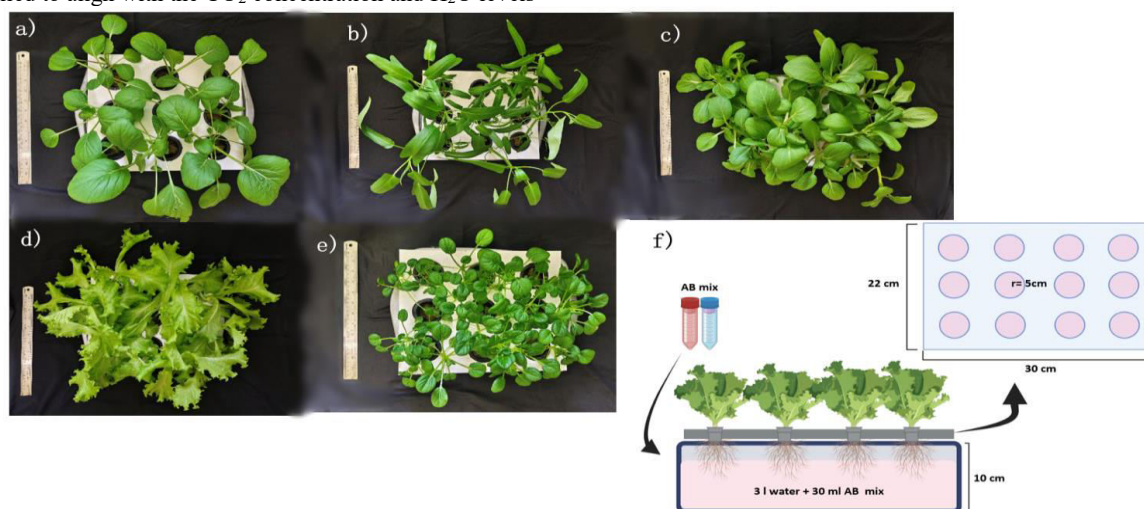
This research employed a completely randomized design, with three replications from different blocks of the same plant cultivar. Significant differences among all treatments for each parameter were tested using One-way ANOVA, followed by the Tukey range test, at a 95% confidence interval. Additionally, the correlation among all parameters was analyzed using the Pearson Correlation Test with GraphPad Prism software (version 10.3, 2025).

## 3. Results

In this study, observations made during the early, middle, and late vegetative phases provided data on changes in plant phenotypes, specifically concerning plant height, the number of leaves, and variations in leaf size.

### 3.1. Floating raft hydroponic system

The study utilized a floating raft hydroponic system, which included a five-liter container and a rack with 5 cm diameter pots. Figure 1 illustrates the model of the plants grown in this study, which were 21 days old at the time of observation. Each plant cultivar exhibited distinct shoot cover, and the number of tillers varied among them. In this hydroponic model, nutrient replacement was conducted periodically, once a week, to ensure that the plants' optimal nutritional needs were met. The growth of each cultivar in the hydroponic box was observed during three phases: early vegetative (21 days after sowing), mid vegetative (35 days after sowing), and late vegetative (42 days after sowing). Several parameters were measured to compare the changes that occurred, including plant morphological growth, leaf area, and leaf stomatal characteristics observed microscopically.



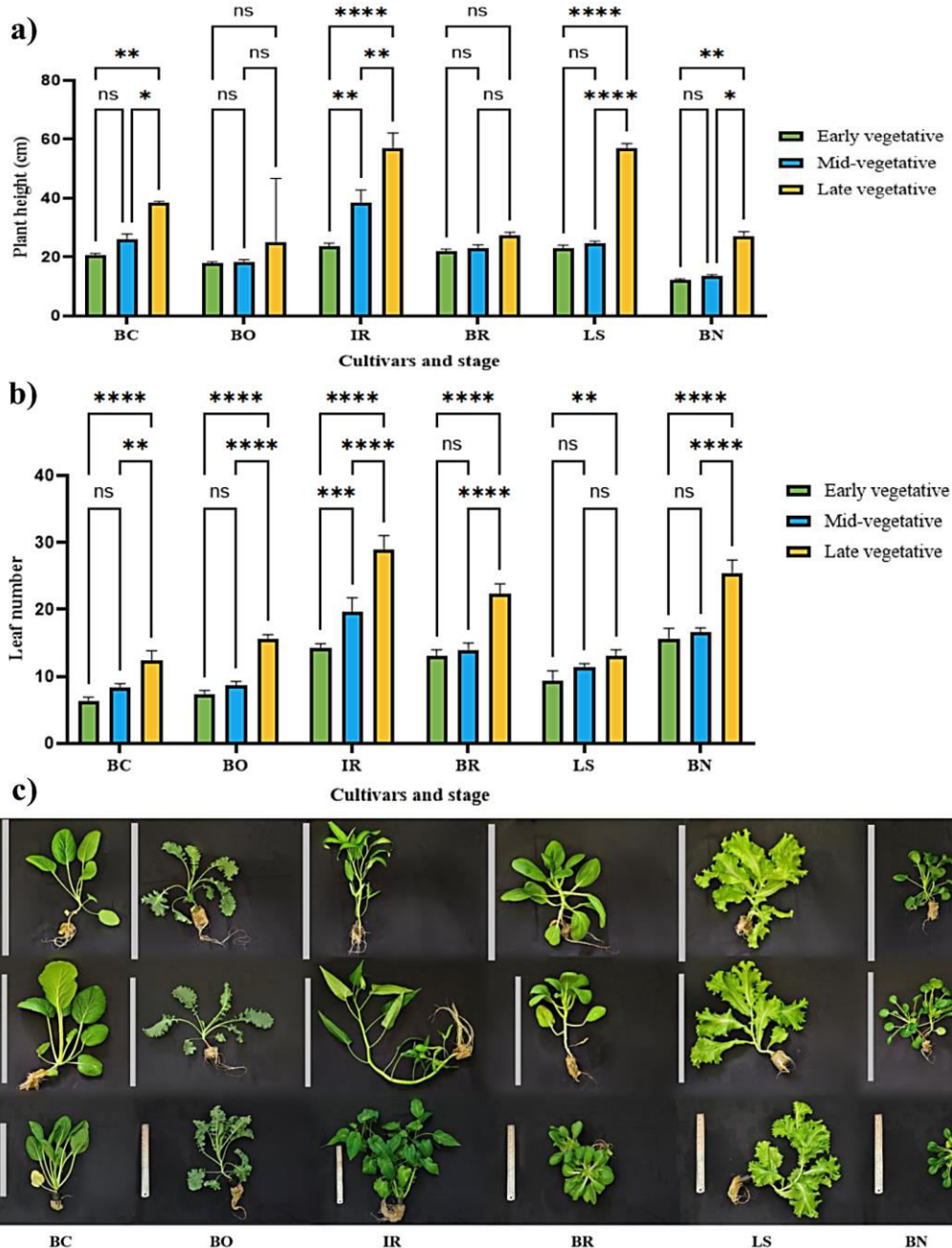
**Figure 1.** A box of a 21-day-old vegetable planting model using a floating raft hydroponic system with plants a) *Brassica chinensis* var. parachinensis, b) *Ipomoea aquatica* Poir., c) *Brassica rapa* subsp. Chinensis, d) *Lactuca sativa* L. var. crispa, e) *Brassica narinosa* L., and f) rack arrangement and growing media used—bar: 30 cm.

3.2. Plant Morphological Changes

Figure 2a illustrates the changes in plant height. The cultivars BC, IR, LS, and BN exhibited significant differences in height among the three phases. In contrast, cultivars BO and BR maintained similar heights throughout the early, mid, and late vegetative phases. Although there were significant differences in height across phases, the hydroponic vegetable cultivars did not show notable differences in height growth between the early and mid-vegetative phases. Figure 2c depicts the changes in plant height across the three stages: I, II, and III represent early vegetative, mid vegetative, and late vegetative, respectively.

Observations of growth characteristics were made based on changes in the number of leaves at each

developmental phase (see Figure 2b). All cultivars exhibited an increase in leaf count from the early vegetative stages, which is expected as plants grow. The cultivars IR and BR demonstrated a significant increase in leaf numbers across all stage comparisons. In contrast, cultivar LS showed the weakest trend, with no significant difference between the early and middle stages or between the middle and late stages. Notably, cultivar IR had the highest leaf count during the late vegetative stage, averaging around 35 leaves, followed by BR and BN. Additionally, cultivars BC, BO, BR, LS, and BN showed no significant differences between the early vegetative and middle vegetative stages, indicating a slower rate of growth during the early vegetative phase.



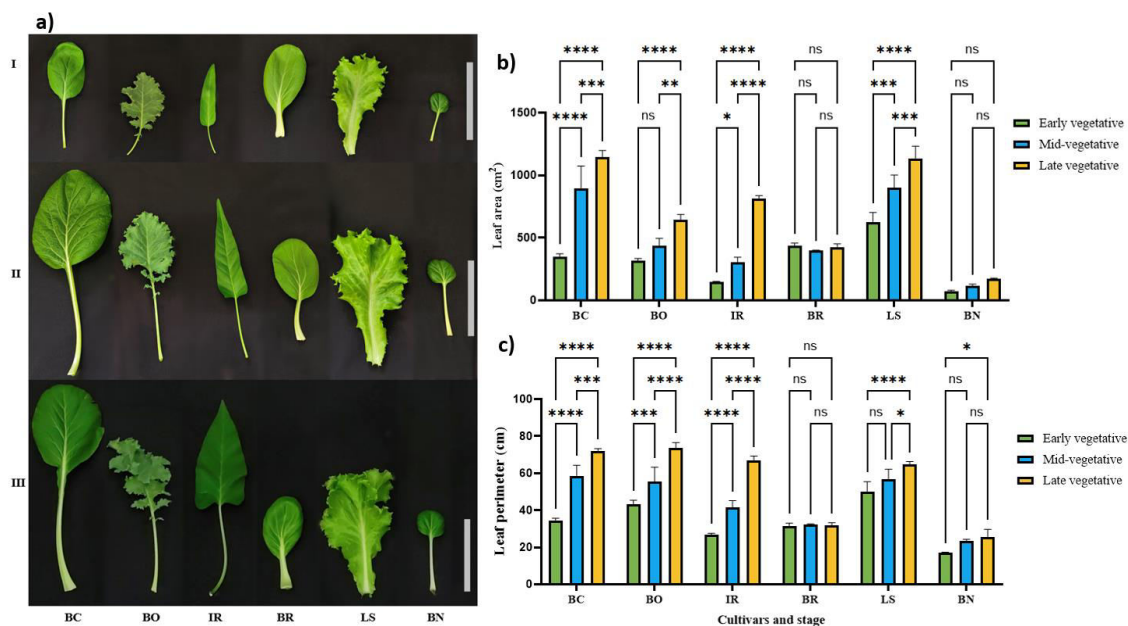
**Figure 2.** Plant growth at three vegetative phases in parameters a) plant height, b) number of leaves, c) visualization of plant growth with bar = 30 cm in plants: BC: *Brassica chinensis* var. parachinensis, BO: *Brassica oleracea* var. sabellica, IR: *Ipomoea aquatica* Poir, BR: *Brassica rapa* subsp. Chinensis, LS: *Lactuca sativa* L. var. crispa, and BN: *Brassica narinosa* L.

### 3.3. Leaf area and perimeter

During the vegetative phase of growth: early, middle, and late, plants experience significant changes in leaf area. Figure 3a illustrates the leaf morphology of six vegetable crops as they progress through this phase. The data indicate that leaf development, specifically in terms of area and perimeter, is notably influenced by both cultivar and growth phase factors. Cultivar LS exhibited the highest levels of leaf area and perimeter growth, with substantial variations observed across different phases (Figure 3b and 3c). This makes LS particularly suitable for studying changes in leaf biomass. Cultivars BC, BO, and IR also showed considerable vegetative growth, indicating their potential for improving leaf yield traits. In contrast,

cultivar BN demonstrated the smallest rate of leaf expansion. This aligns with its growth characteristics, which prioritize bud formation over increasing leaf area. Statistical analyses (Figure 3b and 3c) confirmed that leaf traits differ significantly across developmental phases for all cultivars, except for BN and, in some cases, BR.

Growth in plant height and leaf number was statistically correlated with each other. However, in some cultivars, no significant changes were observed in leaf area as the growth phase progressed. This lack of change is thought to contribute to variations in the size, distribution, and opening of leaf stomata, particularly during the vegetative phase. To confirm this, the stomatal characteristics were measured.

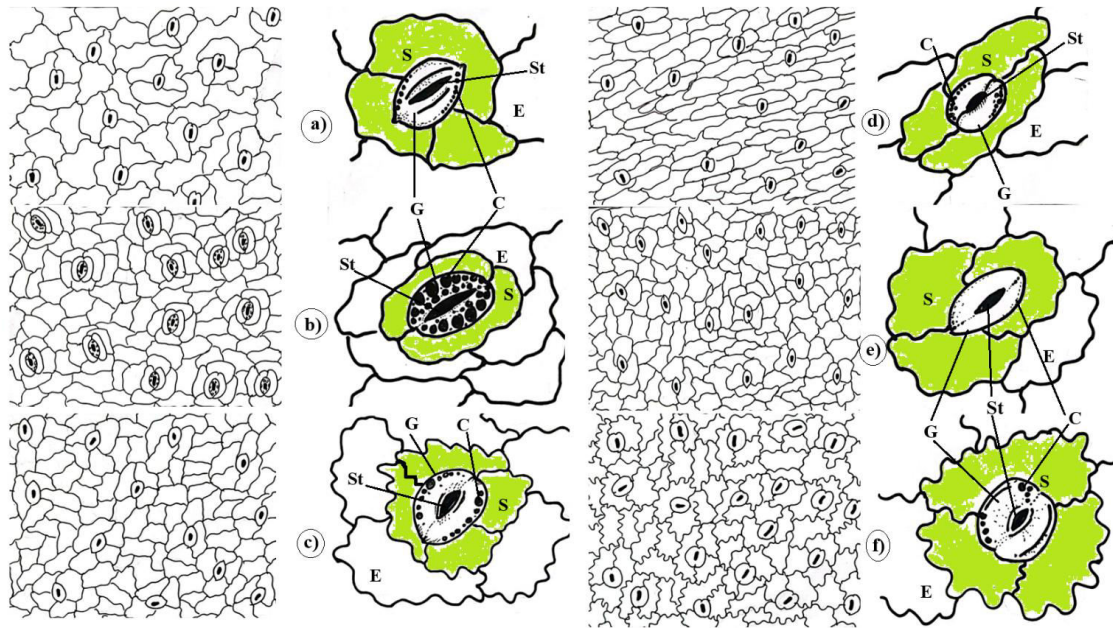


**Figure 3.** Leaf growth at three vegetative phases (I, II, and III) was observed in parameters a) visualization of leaf changes at three phases with bar = 10 cm, b) leaf area, c) leaf perimeter in BC plants: *Brassica chinensis* var. parachinensis, BO: *Brassica oleracea* var. sabellica, IR: *Ipomoea aquatica* Poir, BR: *Brassica rapa* subsp. Chinensis, LS: *Lactuca sativa* L. var. crispa, and BN: *Brassica narinosa* L. I: early vegetative, II: mid-vegetative, and III: late vegetative.

### 3.4. Stomatal size and auxiliary cell types

Figure 4 provides an overview of the shape of stomata from six different vegetable species. Each species exhibits distinct stomatal characteristics, which are characterized by the positions of guard cells (which control stomatal opening), subsidiary cells (which assist guard cell movement), chloroplasts, and epidermal cells (the cells surrounding the stomata and subsidiary cells). According to the observations presented in Figure 4, cultivars BC, BR, and BN display anisocytic stomatal characteristics, characterized by unequal cells surrounding the stomata. Cultivar IR has paracytic stomatal characteristics, with

subsidiary cells arranged in parallel, while cultivar LS shows anomocytic stomatal characteristics, featuring irregular subsidiary cell structures. Additionally, cultivar BO also exhibits unique stomatal characteristics, with either diacytic or anisocytic stomatal complexes. Overall, all species share a similar stomatal arrangement but differ in terms of density, complex type, and the size of their subsidiary cells. Understanding these stomatal characteristics is essential for identifying their function and their relationship with physiological responses such as transpiration, gas exchange, and adaptation to stress.



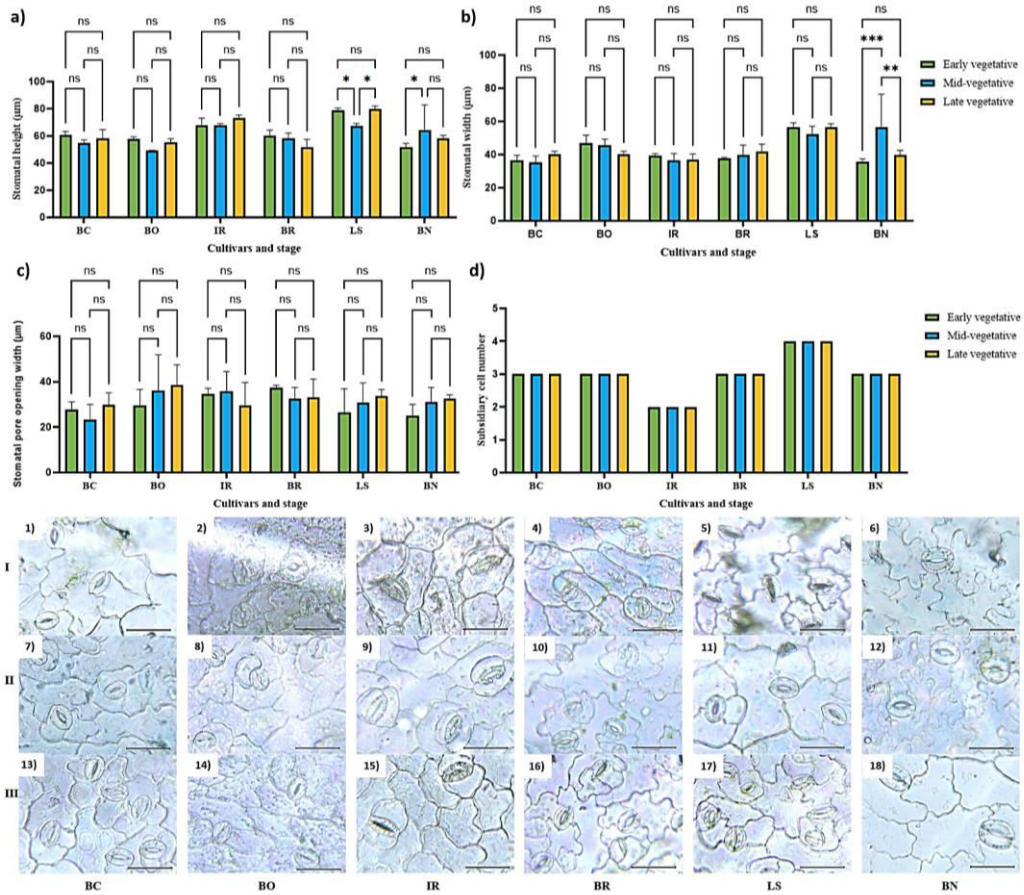
**Figure 4.** Stomatal characters based on observations on hydroponic media in plants of a) *Brassica chinensis* var. *parachinensis* (BC), b) *Ipomoea aquatica* Poir (IR), c) *Lactuca sativa* L. var. *crispa* (LS), d) *Brassica oleracea* var. *sabellica* (BO), e) *Brassica rapa* subsp. *Chinensis* (BR), and f) *Brassica narinosa* L. (BN) with the Symbols: C: chloroplast, E: epidermal cell, G: guard cell, S: subsidiary cells, St: stomatal aperture

To differentiate between stomatal characteristics during the vegetative phase of development, we measured stomatal length (Figure 5a), stomatal width (Figure 5b), stomatal aperture width (Figure 5c), and the average number of subsidiary cells (Figure 5d). The results indicated that the cultivars LS and BN exhibited a significant increase in stomatal length during the vegetative phase. Specifically, LS showed a rise from mid to late stage, while BN experienced significant growth from the early/mid stage to the late stage. In contrast, the other cultivars (BC, BO, IR, and BR) did not demonstrate any significant differences across the phases ( $p > 0.05$ ). This suggests that LS and BN are more adaptable in terms of stomatal elongation as development progresses.

It is noteworthy that no significant differences were observed across all cultivars and stages in the width of stomatal pore openings (Figure 5c). This finding suggests

that stomatal openings remain stable during the vegetative and growth phases under optimal environmental conditions. Consequently, variations in age and cultivar only influence other characteristics, not the width of the stomatal pore openings. These results indicate that different cultivars exhibit specific physiological adjustments in stomatal morphology during plant growth, particularly in the vegetative stage.

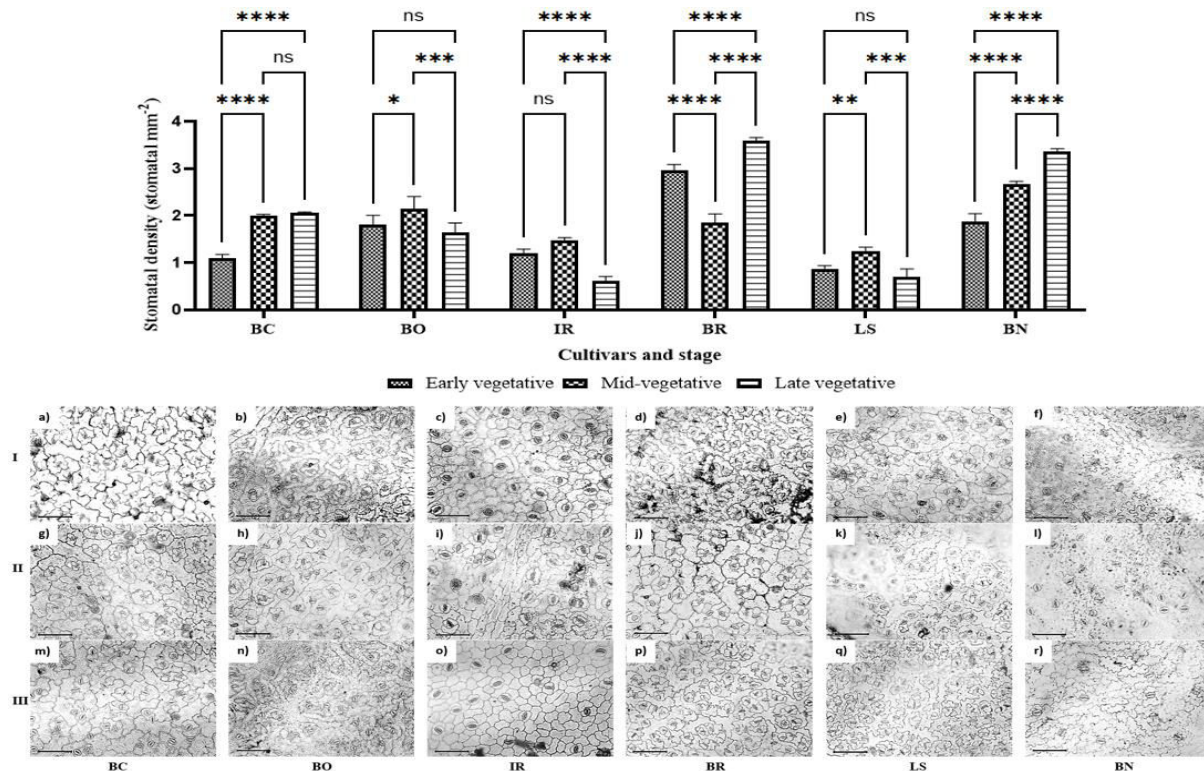
Regarding subsidiary cells (Figure 5d), uniformity was seen in the BC, BO, BR, and BN cultivars, each with three subsidiary cells surrounding the guard cell. In contrast, the LS cultivar had four subsidiary cells, while the IR cultivar had two subsidiary cells per stoma. Notably, there was no variation in the number of subsidiary cells across all vegetative stages, implying that this trait is likely genetically determined and remains consistent during vegetative development.



**Figure 5.** Changes in leaf abaxial stomatal characters with parameters a) stomatal length, b) stomatal width, c) stomatal opening width, and d) number of subsidiary cells per stoma in BC plants: *Brassica chinensis* var. parachinensis, BO: *Brassica oleracea* var. sabellica, IR: *Ipomoea aquatica* Poir, BR: *Brassica rapa* subsp. Chinensis, LS: *Lactuca sativa* L. var. crispa, and BN: *Brassica narinosa* L. in the vegetative phase consisting of I: early vegetative (1-6), II: mid-vegetative (7-12), and III: late vegetative (13-18). Bar: 100 µm. Significance tests were conducted using the Tukey method with a confidence level of 95%.

In this study, we observed notable differences in stomatal density among the various cultivars during the early, middle, and late vegetative phases (see Figure 6). Stomatal density typically indicates how plants respond to their developmental and environmental conditions. According to Figure 6, the BC cultivar showed a slight increase in stomatal density from the early to the mid-vegetative phase; however, there was no significant difference between the mid and late stages. In contrast, the BO, IR, BR, LS, and BN cultivars exhibited a significant increase ( $P < 0.05$ ) in stomatal density from the early to

mid-phases and continued to rise toward the late stage. Notably, the BN cultivar had the highest final stomatal density among all the cultivars studied. Interestingly, the IR cultivar experienced a reduction in stomatal density during the late stage, which may indicate a water-conserving strategy or a shift in growth focus towards leaf expansion as compared to changes in leaf area characteristics. The photosynthetic parameters were then measured to determine the effect of stomatal configuration on physiological performance.



**Figure 6.** Leaf abaxial stomatal density in BC: *Brassica chinensis* var. parachinensis, BO: *Brassica oleracea* var. sabellica, IR: *Ipomoea aquatica* Poir, BR: *Brassica rapa* subsp. Chinensis, LS: *Lactuca sativa* L. var. *crispa*, and BN: *Brassica narinosa* L. in its vegetative phase, consisting of I: early vegetative (a-f), II: mid-vegetative (g-l), and III: late vegetative (m-r). Bar: 100 µm.

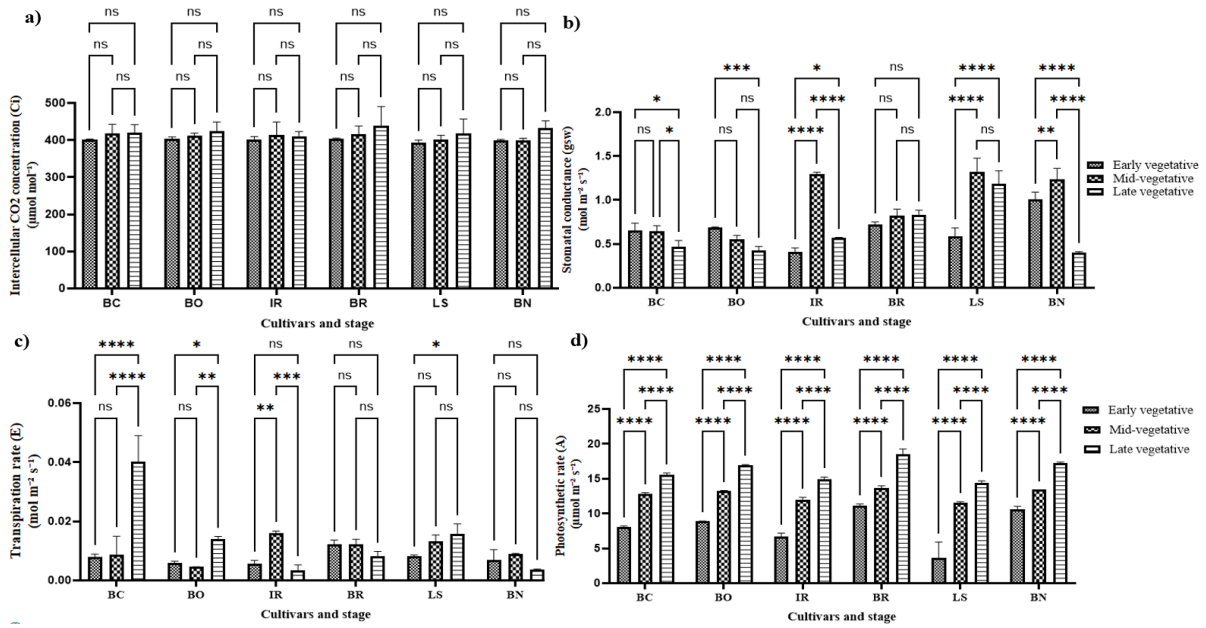
### 3.5. Photosynthetic performance of six different species under hydroponics

Figure 7 provides the physiological responses of plants at three vegetative growth stages. Intercellular CO<sub>2</sub> concentration remained relatively stable across all cultivars and stages (Figure 7a), with no significant differences marked as 'ns'. This suggests that C<sub>i</sub> is not significantly affected by growth stage or cultivar in this dataset. Figure 7b shows significant variations observed across growth stages in several cultivars. BC and BO showed a sharp decrease in g<sub>sw</sub> (stomatal conductance) from early to late stages (\* to \*\*\*\*). LS and BN exhibit higher g<sub>sw</sub> during mid stages with significant reductions in the late stage (\*\*\*\*). Cultivar IR and BR showed less variation, primarily indicated by 'ns'. This shows that stomatal regulation is active and variable depending on developmental stage and cultivar. A similar trend to stomatal conductance is shown in Figure 7c in the transpiration rate. BC and BO cultivars had the highest transpiration in the early stage, then declined sharply (\*\*\*\*). IR and BR had moderate differences showing a decline at late stages, while LS and BN maintained relatively high transpiration until late stages, where reductions occurred (\* or ns). Meanwhile, photosynthetic rate (Figure 7d) shows a significant increase in all cultivars from early to later stages (mostly with \*\*\* or \*\*\*\*). The highest photosynthetic rate was observed during the late vegetative stage for most cultivars. This suggests enhanced carbon assimilation capacity in the g<sub>sw</sub> and transpiration rate.

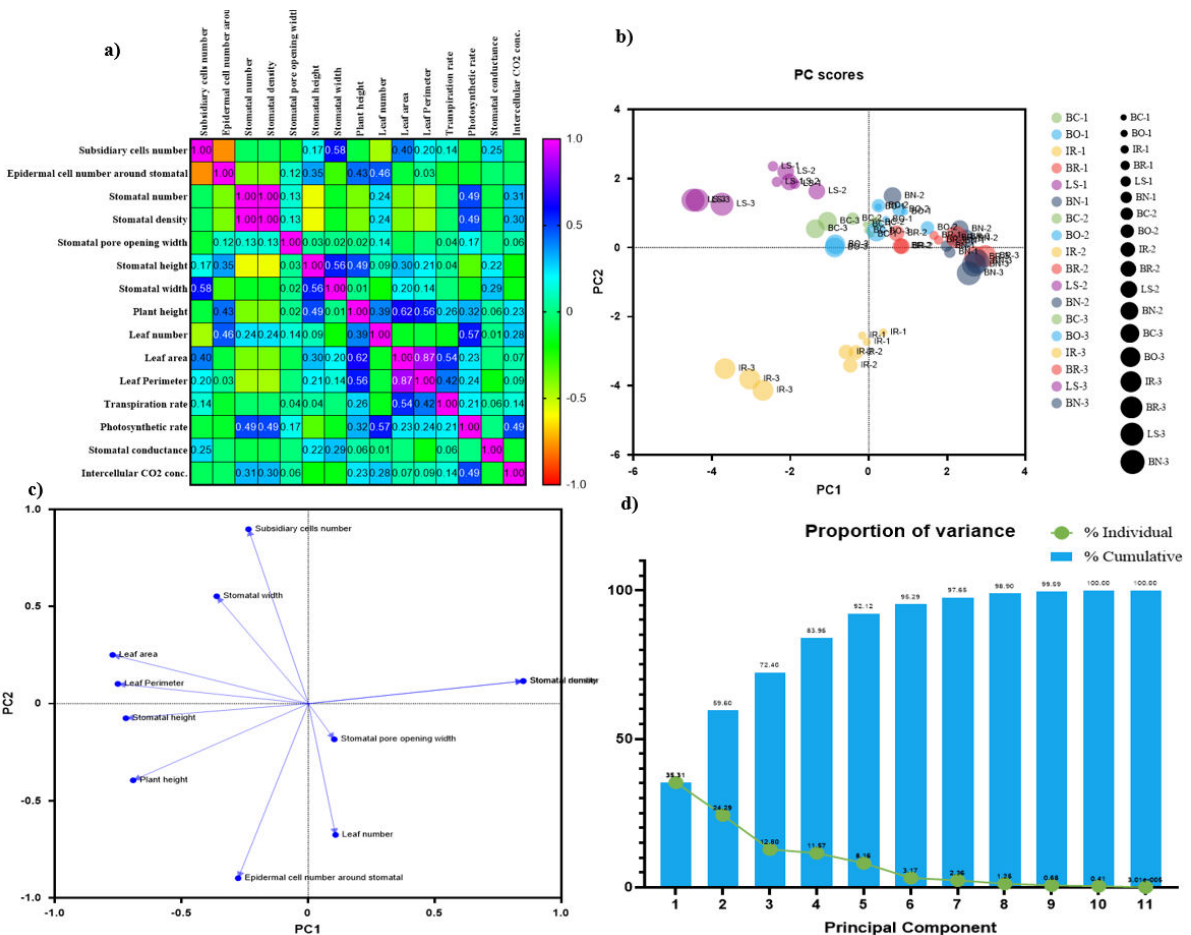
All the parameters tested in this study led to changes in the photosynthetic performance of the plant under hydroponic conditions. To know the correlation between each parameter in six species tested along three different

vegetative stages, the Pearson correlation test is performed, followed by Principal component analysis (Figure 8) to know the clustering pattern of species and stages through the parameters measured. Figure 8a shows that photosynthetic rate correlates strongly with stomatal conductance (r~0.72), transpiration rate (r~0.68), and leaf number and area (moderately). Stomatal density shows a negative correlation with stomatal size traits, indicating a trade-off between number and size. Plant height correlates positively with leaf area and perimeter, while subsidiary cell number and stomatal number are positively associated, reflecting a shared developmental pathway.

The PCA scores plot (Figure 8b-d) projects individual observations (e.g., cultivar-stage combinations) onto the first two principal components (PC1 and PC2). Samples cluster by cultivar and growth stage, suggesting genotype and stage influence trait variability. For example, the IR and BR-late stage (3) clusters are far left, suggesting distinct morpho-physiological profiles, while the LS and BN groups are toward the center, indicating similar intermediate trait patterns. Figure 8c specifically displays how traits contribute to the first two principal components. PC1 (horizontal axis) is dominated by stomatal density, leaf number, epidermal cells around stomata, and leaf perimeter, while PC2 (vertical axis) is dominated by leaf area, plant height, and stomatal height. Traits further from the origin have a stronger influence on component variance. Figure 8d shows a bar chart of the proportion of variance explained by each principal component. PC1 and PC2 together explain ~52.4% of total variation (PC1 ~35.4%, and PC2 ~17.0%). The cumulative variance reaches ~72% by PC3 and ~83% by PC4, thus 90% by PC 5-6, suggesting up to 6 PCs are needed to explain most variation.



**Figure 7.** Leaf photosynthetic characteristics shown from a) intercellular CO<sub>2</sub> concentration, b) stomatal conductance, c) transpiration rate, and d) photosynthetic rate in BC: *Brassica chinensis* var. parachinensis, BO: *Brassica oleracea* var. sabellica, IR: *Ipomoea aquatica* Poir, BR: *Brassica rapa* subsp. Chinensis, LS: *Lactuca sativa* L. var. crispa, and BN: *Brassica narinosa* L. in its vegetative phase.



**Figure 8.** a) Pearson correlation analysis and b) principal component analysis (PCA), c) PCA loading plot and d) Eigenvalues of all leaf parameters tested in this study in BC: *Brassica chinensis* var. parachinensis, BO: *Brassica oleracea* var. sabellica, IR: *Ipomoea aquatica* Poir, BR: *Brassica rapa* subsp. Chinensis, LS: *Lactuca sativa* L. var. crispa, and BN: *Brassica narinosa* L. in its vegetative phase.

#### 4. Discussion

This study evaluated the changes in morphology — including plant height, leaf number and area, and stomatal traits — length, width, stomatal aperture width, number of subsidiary cells, and stomatal density—among vegetable plant species (BC, BO, IR, BR, LS, and BN) during the early, middle, and late vegetative phases. Stomata are microscopic pores surrounded by guard cells that regulate gas exchange and plant transpiration. (Torii, 2021). Their size and density significantly influence plant water-use efficiency (WUE), photosynthetic capacity, and adaptability to environmental changes (Kitta & Katsoulas, 2020; Qiao *et al.*, 2024).

In this study, optimal environmental conditions were achieved using a floating raft hydroponic system, which ensures that each plant receives the appropriate amount of macro and micro nutrients. Under this condition, to understand the effects of age and planting method on plant growth, we analyzed plant morphology alongside stomatal anatomical characteristics to explain the growth patterns of each species, particularly during the vegetative phase.

Figure 2 and 3 clearly visualize the quantitative data showing that, during the early vegetative stage, all cultivars exhibited shorter plant heights, fewer leaves, and smaller leaf areas compared to the middle and late vegetative stages. This size developed over time, as did the number of leaves of each cultivar. The IR and BR cultivars demonstrated superior vegetative growth across all phases. Notably, in the late vegetative stage, almost all cultivars showed increased plant height, number of leaves, and leaf area. Interestingly, both the BR and BN cultivars maintained a leaf area that did not significantly differ ( $p>0.05$ ) across the vegetative phases. This observation is closely related to the characteristics of leaf stomata and their distribution on the abaxial surface of the leaves, prompting an analysis of stomatal traits, including size, aperture opening, and stomatal density.

Stomatal size, including both length and width, reflects a plant's physiological adaptations to its environment and growth stages. As a plant advances through its vegetative stages, larger stomata can enhance CO<sub>2</sub> uptake for photosynthesis, but they may also increase water loss (Ouyang *et al.*, 2017). In contrast, smaller stomata or reduced aperture width can limit water loss during later growth stages or under environmental stress (Maricle *et al.*, 2009). Surprisingly, the stomatal size of almost all cultivars in this study undergoes stability without any significant change in size along the vegetative growth phase.

The stomatal characteristics observed among the six plant species are theoretically distinct, but are primarily governed by the genetic expression inherent to each cultivar (Akbar & Begum, 2020; Chen *et al.*, 2020). For example, in the previous research, *B. rapa* subsp. *chinensis* demonstrates a stomatal density that varies across different accessions (Akbar & Begum, 2020). Furthermore, it is noteworthy that *B. oleracea* possesses a stomatal pattern of the cruciferous type, which consists of two guard cells and two unequal subsidiary cells. The density of these stomata can differ based on genotype and environmental adaptability. In addition, cultivars of the *Ipomoea* species exhibit an amphistomatic arrangement, featuring stomata

on both the upper and lower surfaces of the leaves, alongside paracytic complexes. In contrast, BN typically showcases cruciferous-type stomata (Akbar & Begum, 2020; Ye *et al.*, 2024).

According to Figure 5b, BN showed a significant decrease in stomatal width from the early to the late stage and also from the mid to the late stage. The other cultivars did not exhibit any significant changes ( $p > 0.05$ ). This may indicate that BN reduces its stomatal aperture size during development, possibly as a water-saving adaptation. Generally, stomatal size—particularly height and width—reflects a plant's physiological adaptations to developmental and environmental conditions.

In this study, the LS and BN cultivars displayed a significant increase in stomatal length during the late vegetative stage. This suggests a developmental adjustment that could potentially enhance photosynthetic capacity as leaf area increases. However, the BN cultivar also demonstrated a notable reduction in stomatal width during this period, which can be interpreted as a compensatory mechanism to restrict excessive transpiration, despite the increase in stomatal height. These findings align with previous studies (Chua & Lau, 2024; Han *et al.*, 2021), indicating that stomatal plasticity—both in size and aperture—is a critical characteristic for development during the vegetative phase and for acclimatization to stress (Salsinha *et al.*, 2021).

Stomatal aperture opening is an important indicator of stomatal conductance. Interestingly, this study found no significant variation in stomatal aperture width across all stages and cultivars, suggesting that pore aperture may be tightly regulated and less responsive to developmental stages alone (Demicheli *et al.*, 2023; Han *et al.*, 2021). This stability reflects the plant's efforts to balance gas exchange and water conservation during vegetative growth. When examining the characteristics of subsidiary cells, it is clear that these cells play a crucial role in the rapid movement of guard cells, enabling more efficient opening and closing of stomata in response to changes in the external environment (Araújo *et al.*, 2011; Daszkowska-Golec & Szarejko, 2013). Data in this study indicate that the number of subsidiary cells remains constant across different cultivars throughout the vegetative development phase. This observation supports the notion that the formation of subsidiary cells is likely genetically determined, as indicated by previous studies (Chen *et al.*, 2020) on stomatal development.

In cultivars such as IR and BR, genetic factors play a significant role in determining stomatal characteristics, leading to variations in vegetative growth. Cultivars with optimized stomatal traits show greater adaptability and better biomass performance. During the vegetative phase, especially from the mid to late stages, plants adjust their stomatal development to meet transpiration demands as well as growth requirements, such as height increase and leaf expansion (Chatterjee *et al.*, 2016; Fayed *et al.*, 2020). Generally, a period of vigorous growth is accompanied by an increase in stomatal density (Chatterjee *et al.*, 2016; Verma *et al.*, 2020). However, this increase does not affect the width of stomatal openings or the number of subsidiary cells surrounding the likely genetically regulated stomata.

Stomatal conductance and transpiration rates are positively correlated as the stomatal pores widen, leading to increased water loss through transpiration. Although

there is a decline in both  $g_{sw}$  and transpiration rates in later stages, photosynthetic rates exhibit an increase (Figure 7). This suggests an enhancement in mesophyll efficiency, a better utilization of intercellular  $CO_2$ , and potentially improved chloroplast activity or enzyme regulation during the late vegetative growth phase (He *et al.*, 2015; Parvin *et al.*, 2017). In this study, the stable intercellular  $CO_2$  levels across different stages imply that variations in assimilation and gas exchange are primarily attributed to stomatal and biochemical adjustments rather than fluctuations in  $C_i$ .

The  $C_i$  remains stable regardless of the growth stage or cultivar, indicating that internal  $CO_2$  availability is not a limiting factor during vegetative growth. This is in line with findings (Pan *et al.*, 2023; Yang & Li, 2016) where  $C_i$  remained relatively constant under non-stress conditions. Opposite to the  $C_i$  patterns, the decline in  $g_{sw}$  at later stages may reflect stomatal closure or reduce stomatal aperture to conserve water due to the changes in endogenous hormonal signaling (Lucas *et al.*, 2013). Another study found that the strong correlation of  $g_{sw}$  was found with transpiration rate at later stages, suggesting an adaptive response to limit water loss, possibly due to changes in stomatal behaviour or leaf surface characteristics, supporting the idea of a functional linkage between transpiration and stomatal regulation (Merewitz *et al.*, 2011; Verma *et al.*, 2020).

Despite the decline in  $g_{sw}$  and transpiration, the photosynthetic performance observed in this study across six cultivars is enhanced. This indicates that while intercellular  $CO_2$  concentrations remain stable, stomatal conductance and transpiration rates decline from early to late vegetative stages in most cultivars. In contrast, photosynthetic rates significantly increase. This data suggests an improvement in photosynthetic efficiency during the later stages, along with cultivar-dependent variations in stomatal regulation. This performance shows an increased mesophyll conductance or biochemical efficiency. This implies greater internal  $CO_2$  use efficiency, which is consistent with the findings (Han *et al.*, 2021; He *et al.*, 2015) that suggest increased photosynthetic capacity at advanced growth stages.

The data on Pearson correlation and PCA reveal strong interrelationships between physiological traits (photosynthesis,  $g_{sw}$ , and transpiration) and anatomical traits (stomatal density, size, and leaf traits). PCA separates cultivar-stage combinations based on these traits, showing how growth stages and genotypes influence functional and structural parameters. The first two components are mainly driven by stomatal density and leaf dimensions (PC1) and plant height and leaf area (PC2).

## 5. Conclusion

This study shows the main contribution in providing stomatal plasticity data among six species tested under hydroponic conditions, giving a new insight into plant responses, especially in leaf photosynthetic matters, to the total growth under hydroponic conditions. This study shows consistency in stomatal aperture size, and the number of subsidiary cells highlights the developmental stability of these traits. Overall, the integrated plasticity of stomatal traits shows how vegetable plants optimize gas exchange and photosynthetic performance throughout the vegetative growth phase in hydroponic conditions. These

findings help identify key characteristics for distinguishing plant performance, such as a photosynthetic indicator of better plant growth under hydroponic conditions, which is potentially valuable for breeding or stress resilience studies. This study only highlights plant growth in the vegetative stage, so further analysis on the generative stage should be done.

## 6. Author contributions

YCFS, idea and conceptualization, observation and analysis, manuscript writing; DYWL, data analysis, visualization, manuscript writing; DSR, research design, observation and measurement, statistical analysis; SP, literature review, microscopic measurement; SS, manuscript writing and evaluation; KT, observation and manuscript writing; NK, data analysis, manuscript writing and literature review.

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