



Characteristics of photosynthetic function in different leaf ages of ‘Tieton’ sweet cherry in a greenhouse

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Abstract

To clarify the stages of leaf growth and development, the young leaf stage was identified at 0–20 d after emergence (DAE). The functional stage was at 20–100 DAE, with the highest functional point at 80 DAE. The leaf aging stage occurred at 100–220 DAE. During the functional stage of leaf development, optimal photosynthetic parameters and anatomical structures were achieved; the leaf area (LA) was at its largest, and the thickening rate of the palisade parenchyma was the fastest. The palisade parenchyma and LA were closely related to photosynthetic characteristics. Stomatal opening and closing, and stomatal density were greater in the functional stage than in the early stage. Both decreased during the aging stage, and the net photosynthetic rate decreased.

Keywords: aging stage; functional stage; leaf area; palisade parenchyma; stomatal density.

Introduction

Leaf age reflects the continuous life process of leaves and represents the time node of leaf development. The photosynthetic capacity of leaves changes with changes in morphology, anatomical structure, physiological structure, and content of internal substances caused by leaf age (Tholen *et al.* 2012, Wu *et al.* 2022, Jahan *et al.* 2023, Roth-Nebelsick *et al.* 2023). The newly formed chloroplast layer structure of tender leaves is underdeveloped, with low chlorophyll (Chl) content, multiple new leaves, small LA, and low photosynthetic capacity (Ai *et al.* 2024). Assimilates need to be input from other functional leaves. As the leaves grow, the number and structure of chloroplasts increase, Chl synthesis increases, and net photosynthetic rate (P_N) continues to increase. When the leaves reach maximum LA and leaf thickness (LTh), P_N reaches its maximum value and increases

as leaf mesophyll cell size increases. As leaves enter the senescence stage, their photosynthetic capacity decreases, and Chl degradation and photosystem damage reduce light energy absorption and conversion efficiency.

The differences in LA and its impact on gas exchange are related to the light environment during leaf development, as well as to changes associated with growth and aging (Reich *et al.* 1991). LA and the internal leaf anatomical structure usually affect the net gas exchange. When illumination increases during development from 900 to 42,000 lux, the leaves more than triple in thickness as mesophyll cells increase in size and frequency, with mesophyll cell surface area per unit leaf area (A_{mes}/A) increasing from 11 to 50 (Nobel *et al.* 1975). The surface area of the leaf mesophyll cells is responsible for absorbing surface CO_2 and conducting photosynthesis. Changes in cell structure and arrangement can affect leaf mesophyll conductivity in response to CO_2 , thereby limiting

Highlights

- The functional stage of sweet cherry leaves was determined at 20–100 DAE
- Palisade parenchyma was a key factor in the increase in leaf thickness
- Palisade parenchyma and leaf area were closely related to photosynthetic rate

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Abbreviations: AE – after emergence; Chl – chlorophyll; C_i – intercellular CO_2 concentration; CK – control; DAE – days after emergence; E – transpiration rate; FL – flowering; g_s – stomatal conductance; LA – leaf area; LL – leaf length; LPL – leaf petiole length; LPW – leaf petiole width; LTh – leaf thickness; LW – leaf width; P_N – net photosynthetic rate; SA – stomatal area; SD – stomatal density; SL – stomatal length; SW – stomatal width.

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photosynthetic performance (Gago *et al.* 2020, Theroux-Rancourt *et al.* 2021).

LTh is an important determinant of leaf P_N , because it is closely related to CO_2 diffusion and light energy absorption (Terashima *et al.* 2005). Most leaf cell layers differentiate toward the dorsal side during development, forming two distinct cell layers with distinct structures (Nicotra *et al.* 2011, Wright *et al.* 2017). The palisade parenchyma is usually located below the upper epidermis and consists of cylindrical cells perpendicular to the leaf surface (Borsuk *et al.* 2022). A larger surface area is beneficial for CO_2 absorption in leaf regions with abundant light and high photosynthetic rates (Ho *et al.* 2016, Borsuk and Brodersen 2019). The spongy parenchyma is located below the palisade parenchyma and consists of a loosely arranged collection of spherical or irregularly shaped cells (Chatelet *et al.* 2013, Ho *et al.* 2016). The palisade parenchyma allows light to penetrate better into the chloroplasts, whereas the spongy parenchyma enhances light capture by scattering light. The palisade parenchyma minimizes light scattering as much as possible, allowing light to penetrate deeper into the leaves (Cui *et al.* 1991).

Stomata are important channels for gas and water exchange between plants and their external environment. Regulation of stomatal size, opening, and density enables plants to resist adverse external factors (Buckley 2005, Cutler *et al.* 2008, Du *et al.* 2021). Leaves reduce transpiration rate (E), decrease water loss, and maintain their physiological functions by closing stomata. The thicker the leaf, the thicker the stratum corneum, the higher the stomatal density (SD), the thinner the spongy parenchyma, the more developed the palisade parenchyma, the more layers of cells, and the higher its photosynthetic efficiency. A large angle of stomatal opening and closing is beneficial to photosynthesis and, to a certain extent, to heat dissipation, which can help avoid heat damage that causes the denaturation of chloroplasts and protoplasts. The stomatal opening drives the conductivity of water vapor on the leaf surface from the leaf to the air. It regulates transpiration water loss owing to the pressure difference between the leaf and the air, and to the boundary-layer conductivity. A high stomatal conductance (g_s), which greatly promotes higher photosynthetic rates, combined with LA allocation, scales up to improve the net primary productivity of the ecosystem (Wang *et al.* 2015).

Sunlight greenhouses enable cherries to be harvested 90–100 d earlier than in open fields, and leaf growth is 90–100 d longer in sunlight greenhouses than in open fields. Maintaining leaf function and preventing premature leaf drops are critical. This study determined the continuous stages of young, functional, and senescent leaves in sweet cherries, and optimal leaf area and patterns of anatomical structural changes corresponding to leaf growth and development stages. We also analyzed the key factors influencing photosynthesis within the leaf structure, the correlation between leaf structural characteristics and photosynthetic performance, and the characteristics of leaf structural changes and their effects on photosynthetic performance indicators. This study is of significant importance for exploring the intrinsic relationship between

growth and development traits and photosynthetic performance, and for promoting the efficient cultivation of plant resources.

Materials and methods

Experimental materials and design: The experiments were conducted in the greenhouse of the Liaoning Institute of Pomology in Xiongyue town (40°11'N, 122°09'E), utilizing 8-year-old 'Tieton' sweet cherry trees with a *Prunus serrulata* rootstock. Temperatures averaged 10–12°C at night and 20–24°C during the day. The experiment began on 24 January 2024, 1 DAE, with ten branches 1 m above the ground at a similar angle, oriented to the south; five tested trees were selected. The greenhouse was opened on 12 December 2023, and the bloom season began on 25 January 2024. Samples were collected from 9:00 to 11:00 a.m. Five trees were selected with ten healthy leaves, and two leaves were taken from the 5th to 8th branches facing the sun in each tree. Leaves were sampled three times every 3 d at the young leaf stage, 12 times every 10 d at the mature stage, and five times every 20 d when the LA reached its maximum.

Leaf morphology: Using a live LA measuring instrument (YMJ-B, Zhejiang Top Aloud-Agri Technology, Zhejiang, China), LA, leaf length (LL), and width (LW) were measured. A digital Vernier caliper was used to measure the leaf petiole length (LPL) and leaf petiole width (LPW), with an average of three leaves measured.

Leaf photosynthetic characteristics: A LI-6400 (LI-COR, Lincoln, NE, USA) portable photosynthetic instrument was used to measure P_N , E , intercellular CO_2 concentration (C_i), and g_s of mature leaves on clear days. The photosynthetic system controlled the leaf chamber temperature (T_1 ; 20–24°C), humidity (RH; approximately 40%), incoming air CO_2 concentration of the chamber ($400 \pm 10 \mu\text{L L}^{-1}$), and photosynthetic effective radiation [$1,000 \mu\text{mol}(\text{photon}) \text{m}^{-2} \text{s}^{-1}$ by LED light source]. The incoming air flow rate was set to 500 mL min^{-1} .

Leaf anatomical structure: A $5 \times 5 \text{ mm}$ mesophyll structure adjacent to the main vein in the middle of the leaf was fixed in FAA fixative and dehydrated with alcohol and xylene several times before being embedded in paraffin. Transverse sections $10 \mu\text{m}$ thick were cut and stained with safranin green. The LTh, upper epidermis, lower epidermis, palisade parenchyma, and spongy parenchyma were measured using *LCmicro* in ten random fields under an *Olympus CX23* microscope (Olympus, Japan).

Leaf stomatal structure: The middle part of the leaf (avoiding the leaf vein) was removed using a nail polish printing film, and the epidermis was torn with tweezers to prepare a temporary mount. This mount was observed under an *Eclipse E100* (Nikon) microscope, and photographs were taken. Stomatal length (SL), stomatal width (SW), and SD were measured using *Phmias 2008 ver. 3.0* software. Indicator values were statistically

analyzed across ten visual fields for each treatment, and stomatal area (SA) was calculated using the formula $SA = 3.14 \times SL \times SW/4$ (Marchi *et al.* 2008). All measured data were systematically recorded, and subsequent statistical analyses (including the calculation of mean values and standard deviations) were conducted to ensure the reliability of the results.

Statistical analysis: *Microsoft Excel* software was used to process the data and draw figures.

Results

Morphological indicators at different leaf ages of ‘Tieton’ sweet cherry: As the age of ‘Tieton’ sweet cherry leaves increased, the LL, LW, LA, LPL, and LPW gradually increased before reaching a stable trend. The 0–20 DAE stage corresponded to the young leaf stage, during which leaf growth was relatively rapid. All studied morphological parameters showed age-related variation. The 20–100 DAE stage was the functional stage, where the leaf morphology index was the highest and the function was strongest at 80 DAE; LL, LW, LA, LPL, and LPW were 3.36, 5.33, 19.67, 5.21, and 1.94 times higher, respectively, than the lowest values in the young leaf stage. The 100–220 DAE stage was the aging stage, showing a slight change in leaf color but no change in size (Fig. 1).

Photosynthetic parameters at different leaf ages of ‘Tieton’ sweet cherry: As the reproductive stage progressed, P_N , g_s , C_i , and E gradually increased and then decreased. The 20–100 DAE stage was the functional stage. At 80 DAE, the leaf morphology index was the highest and the function was strongest, with leaf P_N , g_s , C_i , and E being $13.9 \mu\text{mol m}^{-2} \text{s}^{-1}$, $0.04 \text{ mmol}(\text{H}_2\text{O}) \text{ m}^{-2} \text{s}^{-1}$, $121.31 \mu\text{mol}(\text{CO}_2) \text{ mol}^{-1}$, and $1.03 \text{ mmol}(\text{H}_2\text{O}) \text{ m}^{-2} \text{s}^{-1}$, respectively. At this point, P_N , g_s , C_i , and E were 1.92, 2.00, 1.41, and 2.24 times higher, respectively, than those in the young leaf stage. The aging stage occurred at 100–220 DAE. At 220 DAE, leaf P_N , g_s , C_i , and E were $3.72 \mu\text{mol m}^{-2} \text{s}^{-1}$, $0.01 \text{ mmol}(\text{H}_2\text{O}) \text{ m}^{-2} \text{s}^{-1}$, $82.46 \mu\text{mol}(\text{CO}_2) \text{ mol}^{-1}$, $0.30 \text{ mmol}(\text{H}_2\text{O}) \text{ m}^{-2} \text{s}^{-1}$, respectively (Fig. 2).

Anatomical structure at different leaf ages of ‘Tieton’ sweet cherry: The upper epidermis, lower epidermis, palisade parenchyma, spongy parenchyma, and LTh in the transverse structure showed gradually increasing and decreasing trends. The epidermis was composed of a single layer of cells, with a thickness of 18–27 μm for the upper epidermis and 10–12 μm for the lower epidermis. The palisade parenchyma was 17–109 μm , and the spongy parenchyma was irregularly polygonal and loosely arranged below the palisade parenchyma, being 48–75 μm . The strongest functional stage of the leaves was 20–100 DAE, with an increase of 6.8, 5.3, 25.6, 10.7, 16.5, and 16.4% in the upper epidermis, lower epidermis, palisade parenchyma, spongy parenchyma, LTh, and palisade/spongy, respectively, compared to the previous stage.

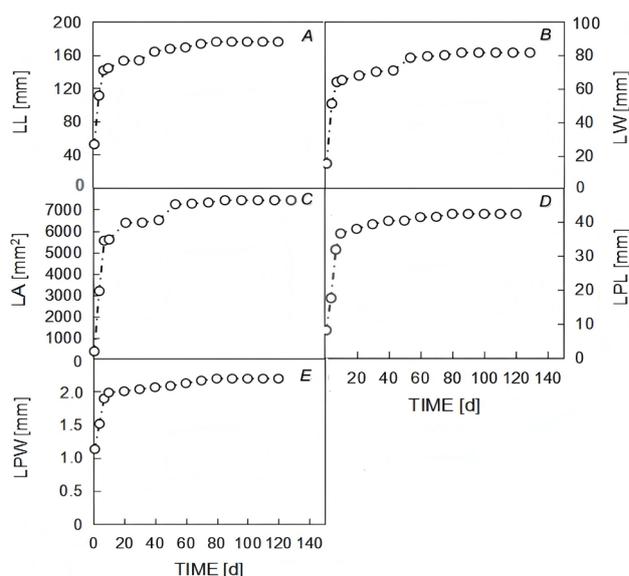


Fig. 1. Morphological changes at different leaf ages of sweet cherry. LL – leaf length; LW – leaf width; LA – leaf area; LPL – leaf petiole length; LPW – leaf petiole width.

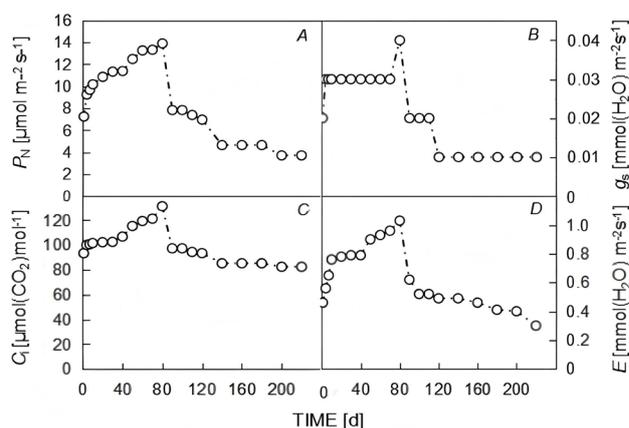


Fig. 2. Photosynthetic parameters at different leaf ages of sweet cherry. P_N – net photosynthetic rate; g_s – stomatal conductance; C_i – intercellular carbon dioxide concentration; E – transpiration rate.

At 80 DAE, leaf upper epidermis, lower epidermis, palisade parenchyma, spongy parenchyma, and LTh were the highest – 27.78 μm , 12.03 μm , 109.49 μm , 75.06 μm , and 224.36 μm , respectively. During the shedding stage at 220 DAE, the leaf upper epidermis, lower epidermis, palisade/spongy parenchyma, spongy parenchyma, LTh, and palisade parenchyma decreased by 0.5, 11.2, 2.7, 14.6, 4.8, and 15.0%, respectively, compared to the leaf functional stage. The thickness changes in the leaf upper epidermis and lower epidermis did not change throughout the entire leaf age stage. In contrast, the palisade parenchyma and spongy parenchyma showed changes, affecting leaf thickness. At 220 DAE, severe senescence of leaves was observed, the tissue structure of the leaves was destroyed, losing its integrity (Figs. 3, 4).

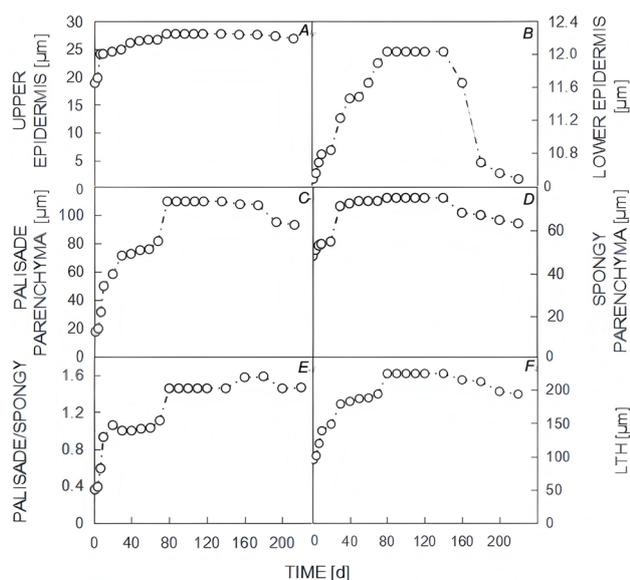


Fig. 3. Anatomical structure at different leaf ages of sweet cherry. LTh – leaf thickness.

Stomatal structure at different leaf ages of ‘Tieton’ sweet cherry: Stomata were concentrated in the lower epidermis of the leaves. As the leaves aged, the leaf stomata gradually increased, reaching a stable trend. The SL, SW, individual SA, and the SD of the leaf stomata gradually increased. As the leaves gradually entered the aging stage, SL, SW, SA, and SD decreased. At 20 DAE, a smaller SL, SW, SA, and SD were observed. At the strongest functional stage of 20–100 DAE, SL, SW, SA, and SD were all larger than at other stages – 16–19 μm , 6–8 μm , and 79–112 μm^2 , respectively. Reaching its maximum at 80 DAE, SL, SW, SA, and SD were 19.80 μm , 8.3 μm , 129.01 μm^2 , and 314.96 mm^2 , respectively. Starting at 100 DAE, the leaves gradually entered the aging stage, and the SL, SW, SA, and SD gradually decreased (Figs. 5, 6).

Discussion

LA increases with advancing developmental stages (Wright *et al.* 2004, Steppe *et al.* 2011, He and Yan 2018). With increasing leaf age, LA gradually increased, and

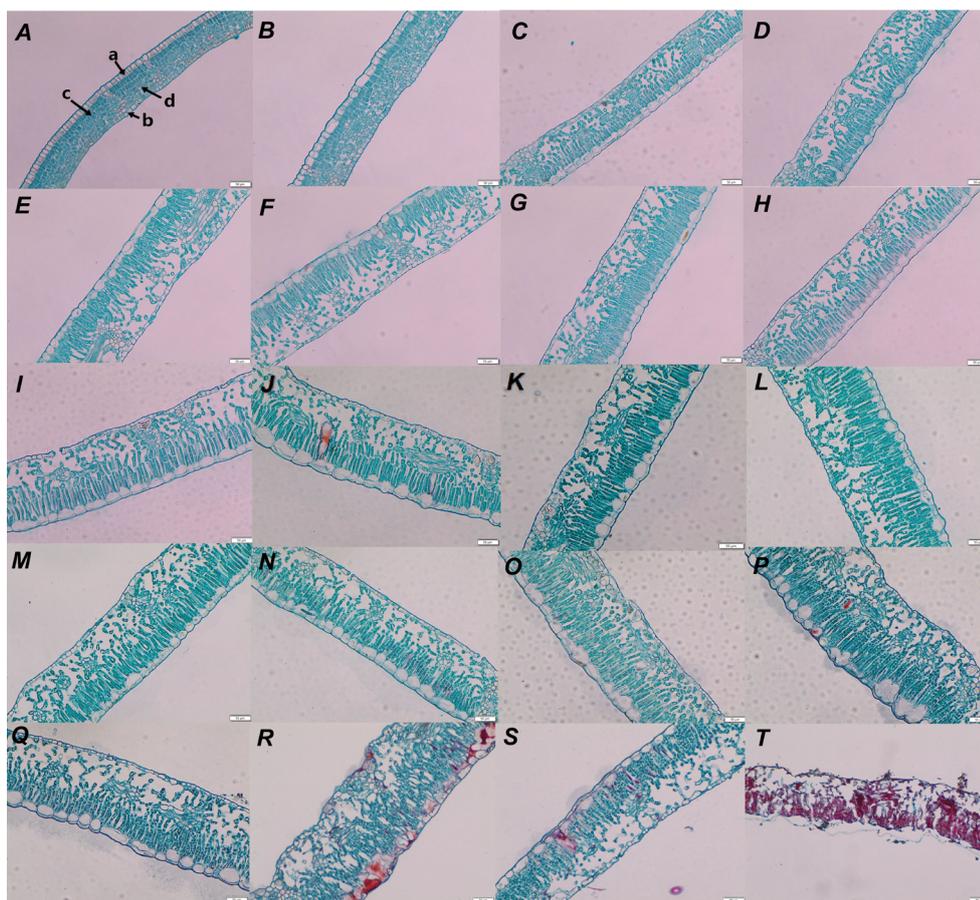


Fig. 4. Anatomical structure at different leaf ages of sweet cherry. A – 0 DAE; B – 4 DAE; C – 7 DAE; D – 10 DAE; E – 20 DAE; F – 30 DAE; G – 40 DAE; H – 50 DAE; I – 60 DAE; J – 70 DAE; K – 80 DAE; L – 90 DAE; M – 100 DAE; N – 110 DAE; O – 120 DAE; P – 140 DAE; Q – 160 DAE; R – 180 DAE; S – 200 DAE; T – 220 DAE. The images are magnified 10 \times under a microscope. a – upper epidermis; b – lower epidermis; c – palisade parenchyma; d – spongy parenchyma. DAE – days after emergence. Bar = 50 μm .

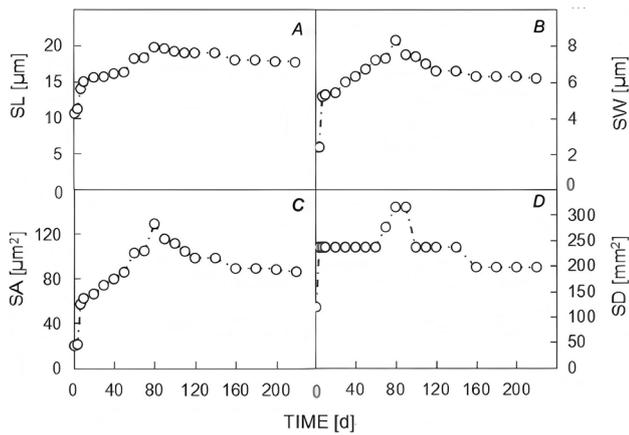


Fig. 5. Stomatal structure at different leaf ages of sweet cherry. SA – stomatal area; SD – stomatal density; SL – stomatal length; SW – stomatal width.

the leaves entered the strongest functional stage at 20–100 DAE, reaching a maximum LA and P_N at 80 DAE, which were 175.96 mm^2 and $13.9 \mu\text{mol}(\text{CO}_2) \text{ m}^{-2} \text{ s}^{-1}$, respectively. In addition, E , C_i , and g_s gradually increased with leaf age. This indicated that the newly formed chloroplast lamellar structure of tender leaves was underdeveloped, with a low Chl content, more new leaves, small LA, and low photosynthetic capacity (Ai *et al.* 2024). Therefore, it is necessary to import assimilates from other functional leaves. As the leaves grew, Chl increased, and its synthesis metabolism was vigorous, whereas P_N continued to increase. The LA and Chl contents also increased, further enhancing photosynthetic capacity. The leaves become thicker, and Chl is positively correlated with LTh (Feng *et al.* 2019). Chl was constant in young and photosynthetically active leaves. As leaves aged, P_N and photosynthetic capacity decreased, and Chl degradation and photosystem damage reduced light energy

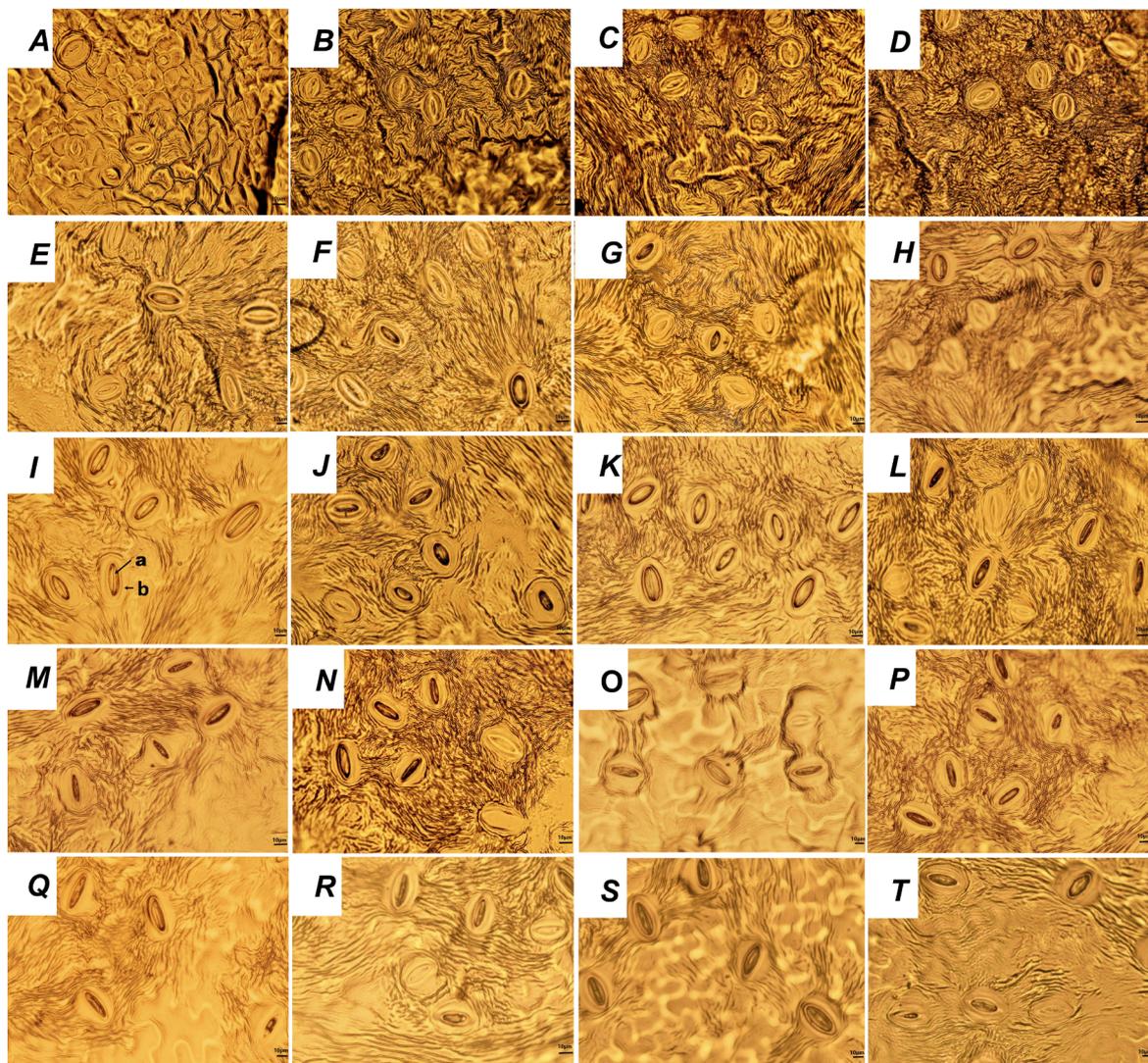


Fig. 6. Stomatal structure at different leaf ages of sweet cherry. A – 0 DAE; B – 4 DAE; C – 7 DAE; D – 10 DAE; E – 20 DAE; F – 30 DAE; G – 40 DAE; H – 50 DAE; I – 60 DAE; J – 70 DAE; K – 80 DAE; L – 90 DAE; M – 100 DAE; N – 110 DAE; O – 120 DAE; P – 140 DAE; Q – 160 DAE; R – 180 DAE; S – 200 DAE; T – 220 DAE. The images are magnified 40 \times under a microscope. a – stoma; b – guard cell; DAE – days after emergence. Bar = 10 μm .

absorption and conversion efficiency (Ghosh *et al.* 2001).

Thickening of the epidermal structure and palisade parenchyma increases the surface area available for light energy absorption and facilitates the rapid transfer of metabolites between mesophyll cells (Chartzoulakis *et al.* 2000). Leaves thicken by more than threefold as mesophyll cells increase in size and frequency (Evans 1999, Nobel *et al.* 1975). Similar results were obtained in the present study. The strongest functional stage of the leaves was 20–100 DAE, with increases of 6.8, 5.3, 25.6, 10.7, 16.5, and 16.4% in the upper epidermis, lower epidermis, palisade parenchyma, spongy parenchyma, LTh, and palisade/spongy parenchyma, respectively, compared to the previous stage. At 220 DAE, the above six indices decreased by 0.5, 11.2, 15.0, 14.6, 4.8, and 2.7%, respectively, compared to the leaf functional stage. The palisade parenchyma is rich in chloroplasts, and the vertically elongated palisade mesophyll surface area increases the cell diameter, which improves leaf photosynthetic capacity (Terashima *et al.* 2011). It is beneficial for preventing excessive water transpiration, improving photosynthesis, enhancing water retention, and improving the ability of leaves to resist adversity. The higher the ratio of leaf palisade parenchyma/spongy parenchyma and tight arrangement of cells, the higher the unit LA leaf mesophyll surface area, which is conducive to CO₂ absorption and thus maintains high photosynthetic activity (Chartzoulakis *et al.* 2000). There is a strong positive correlation between LTh and palisade parenchyma thickness (Chartzoulakis *et al.* 2000). From 100 DAE, there was little change in the upper epidermis, lower epidermis, palisade parenchyma, spongy parenchyma, palisade/spongy, and LTh. At 220 DAE, the leaves underwent severe aging, and the leaf tissue structure was broken. The regularity of palisade parenchyma in leaves improves photosynthesis. In contrast, the development of spongy parenchyma enhances the water-storage function of leaves, dilutes the concentration of harmful substances, and buffers damage to cell membranes caused by them. In addition, an increase in thickness often increases the path length from the pores to the cell wall surface, thereby increasing gas diffusion resistance (Mediavilla *et al.* 2001).

Increased stomatal size promotes gas exchange and increases CO₂ availability (Mott 2009). Increase in stomatal resistance due to closed stomata, low SD, reduced *E*, and decreased water loss results in decreased *g_s* in leaf mesophyll cells, decreased CO₂ content at carboxylation sites, and decreased *P_N* (Ai *et al.* 2025). The decrease in stomatal size mainly affects *g_s* (Maia *et al.* 2022). With increased leaf age, the stomatal opening and closing range of leaves at 20–100 DAE was larger; stomatal opening and SD were 2.5 and 1 times higher than during the early stage. A high SD could meet the needs of gas exchange and water transport, thereby facilitating better regulation of gas exchange and directly affecting transpiration and water loss, thereby increasing the photosynthetic rate (Ahmad *et al.* 2021). The increase in stomatal size, degree of opening and closing, and SD helped improve the efficiency of gas exchange, enabling leaves to fully

absorb CO₂ and release O₂. It also plays an important role in regulating transpiration. At the 100–220 DAE aging stage, the degree of stomatal opening and closing and SD slightly decreased by 25% and 17%, respectively, compared with the functional stage, leading to a rapid decrease in *P_N*, browning, and shedding of the leaves. The closure of stomata decreased *g_s*, driven by the loss of expansion pressure from osmotic pressure efflux and the contraction of guard cells, and was related to ABA signals from leaf tissues (McAdam and Brodribb 2015, Engineer *et al.* 2016). The degree of stomatal opening and closing, and SD, are indicators of leaf function.

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