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Brief Report

Structure and Function of Blueberry Fruit and Flowers: Stomata, Transpiration and Photoassimilation

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Abstract: Blueberry (*Vaccinium corymbosum* L.) stands out among fruit in terms of three open physiological questions about its climacteric character, CO_2 uptake, and the absence or presence of stomata on its floral organs. The objective of the present study was to examine the structures of blueberry flowers and fruit to explain their contribution to CO_2 exchange and transpiration in order to clarify these discrepancies. Blueberries were dewaxed and the sepals/corolla removed for stomata counts, and their micromorphology was studied via LT-SEM. The fruit has stomata, contrary to beliefs in the literature, possibly because the stomata are occluded by the dense wax cover or 'bloom' and hidden on the distal part of the ovary in between and underneath the corolla. However, stomata were located on the distal part of the fruit surrounded by the sepals (calyx) and found predominantly on the abaxial sepals, while the adaxial side of the sepals and the proximal part of the ovary lacked stomata. The petals were devoid of stomata, trichomes, and chlorophyll and abscised after anthesis. In contrast, the sepals remained until maturity, contributing 5–7% to the berry surface but contributing to the majority of fruit stomata and chlorophyll. With 59–71% of the fruit's chlorophyll, sepals were a significant source of the CO_2 uptake. Similarly, with 95% of the berry stomata, sepals were a significant source of water loss, measured via porometry of fruit with and without sepals. Overall, this study identified the ovary as a minor source and sepals as the dominant source of CO_2 and H_2O exchange in blueberries.

Keywords: blueberry (*Vaccinium corymbosum* L.); cryo-preservation; LT-SEM (low-temperature SEM); CO_2 assimilation; cuticular transpiration; fruit photosynthesis; stomata; transpiration

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1. Introduction

Carbon assimilation, which governs the overall carbon uptake in plants and the productivity of horticultural crops, is predominantly a function of fully expanded leaves. The distribution of the assimilated carbon source or carbon partitioning within plants often occurs in an imbalanced manner, with unequal partitioning among various sinks, such as young leaves, meristematic tissues, flowers, fruit, and roots [1]. Except for the roots, these tissues contain chlorophyll and are designated as carbon sources [1,2], characterised by net CO_2 loss or respiration [2]. Fruit are characterised by the absence or scarcity of stomata, but fruit transpiration provides nutrient influx into the harvested and nutritious produce. A dedicated concept of fruit photosynthesis has been developed [3]. Fleshy fruit can be divided into two groups: climacteric fruit such as apples, avocados and bananas, which continue to ripen after harvest, associated with a steep rise in ethylene efflux and sensitivity and a respiratory climacteric, from which their name is derived, and a second group, such as strawberries, cherries, citrus fruit, grapes and plums, which lack these features and do not ripen after picking [4].

Blueberries (*Vaccinium corymbosum* L.) are described as "superfruits" due to their bioactive compounds [5]. The ovary develops into the fruit after dropping the petals while green sepals are retained. The fruit stand out in terms of three physiology questions about their climacteric character, CO_2 uptake, and the absence or presence of stomata on their floral organs. The first apparent contrast exists between the alleged absence of stomata in blueberries [6–9]. Another dispute is between its climacteric classification based on the

CO_2 efflux measured by Bergmann [10] and its classification as non-climacteric based on oxygen uptake [11]. Climacteric fruit are characterised by a rise in CO_2 and ethylene efflux at maturation. The last apparent discrepancy is between the berry's CO_2 effluxes reported by Flore and Hancock [12] and Birkhold et al. [13].

The flower perianth of blueberries comprises an inner ring of five petals ('corolla') and an outer ring of five sepals, designated as the calyx. After anthesis, the five petals wilt and abscise, leaving only the five green sepals attached to the berry. The berry develops a conspicuous wax bloom [6,9,14,15], which may explain these discrepancies.

The present work was conducted to examine the structures of the blueberry flower and fruit to explain their contribution to CO_2 exchange and transpiration in order to clarify some of the discrepancies described above.

2. Materials and Methods

2.1. Plant Material and Low-Temperature (LT) Cryo SEM

High-chill highbush blueberries (*Vaccinium corymbosum* L.) cv. 'Elliott' were grown at the University of Bonn, Germany, and flowered in mid-May.

Fresh berries were sampled 2–3 days before and after petal fall and mounted on microscopic stubs with a mixture of "Tissue Tek" (Agar Scientific, Stansted, Essex, UK) and colloidal and frozen graphite. These cryo-preserved samples were examined for superficial ice contamination and sublimed, if present. When the surface was clean, the sample was sputter-coated with 20 nm of gold. Coated specimens were studied and photographed at −70 °C in a Philips SEM 505 microscope (Philips, Eindhoven, The Netherlands) equipped with a Hexland cryo stage [16]. Stomata were counted according to Baker [17] in ten 0.2 × 0.2 mm squares on fruit dewaxed by two subsequent 3 s dips in chloroform.

2.2. Fruit Porometry and Chlorophyll

Photosynthesis, respiration, transpiration and stomatal conductance in attached flowers and fruit were measured in the orchard using an LCA 3 porometer (ADC Hoddesdon, Herts, UK) as described by Blanke and Cooke [18]. Flow rates into the fruit cuvette and into the LCA 3 were 450–600 mL min^{-1}, depending on the fruit size, and were controlled by two flowmeters. The cuvette air was vigorously stirred to reduce the boundary layer resistance (rb) to 0.19 mol s^{-1}. Measurements were conducted between 8 and 10 h (400–500 µmol PAR m^{-2} s^{-1}). The combination of 19–22 °C and chamber relative humidity of 50% gave water vapour deficits (VPDs) of 1.1–1.7 kPa (values are means of three measurements).

Chlorophyll was determined by direct immersion of the berries or leaves (2.5% *w*/*v*) in DMSO after optimising the extraction time from grapes to blueberries [19].

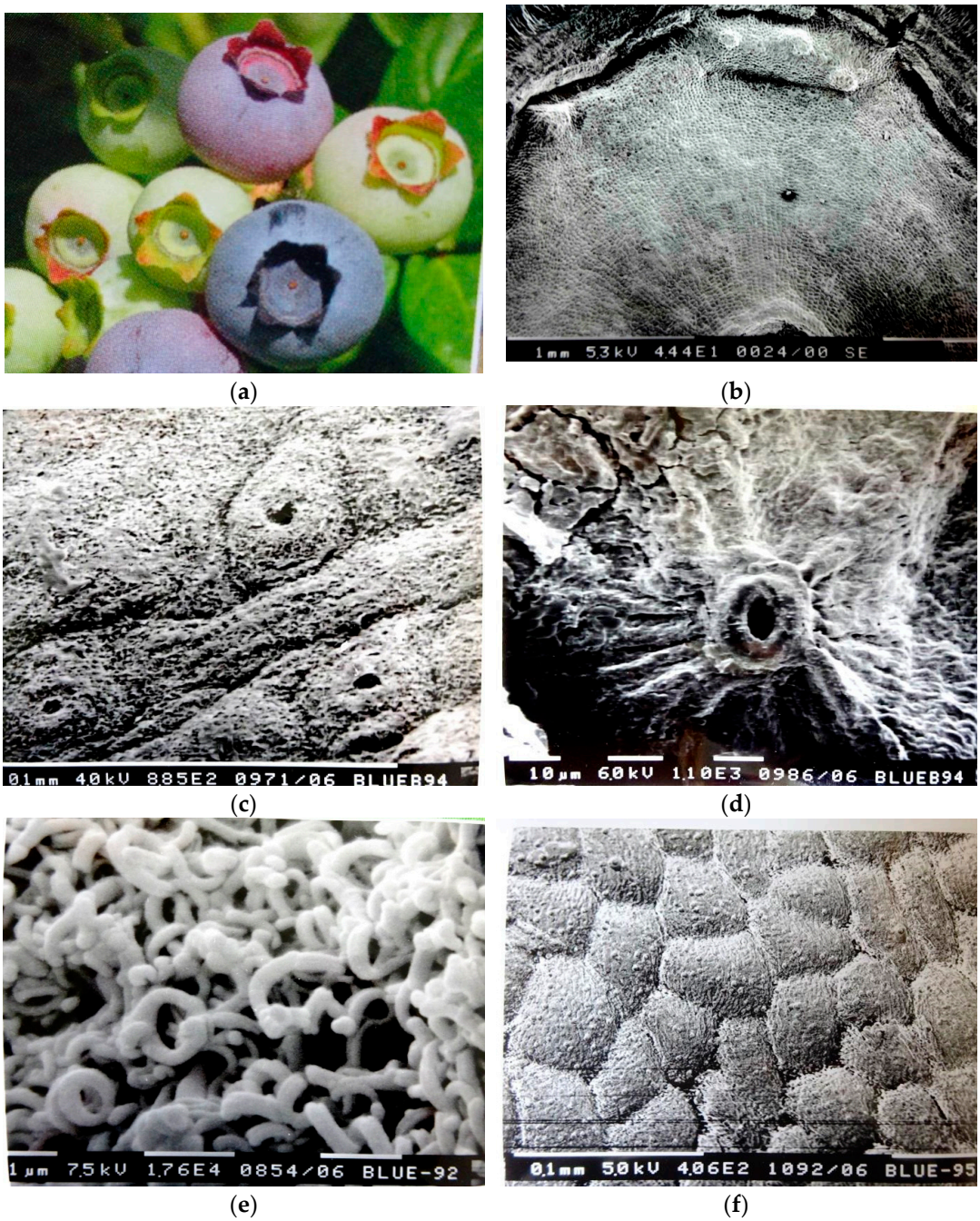
3. Results

3.1. Perianth and Sepal Development

At anthesis, the perianth comprise an inner ring of five petals (corolla) and an outer ring of five sepals (calyx). After anthesis, the five petals wilt and part, leaving the five sepals attached (Figure 1a,b). During fruit ontogeny over 2–3 months, the sepals persist and remain green, constituting a surface area of 26–45 mm^2 or 5–8% of the overall berry surface. Like the ovary, the sepals were devoid of trichomes (Figure 1c).

The adaxial, inward-facing side of the calyx or sepals was devoid of stomata (Figure 1f). On the contrary, the abaxial, outward-facing side of the calyx contained the majority of the fruit stomata (Figure 1c,d), with 170–200 stomata mm^{-2} compared with 330–380 abaxial stomata mm^{-2} on leaves.

The stomata were elliptical and functional and were 14–15 µm × 20–21 µm, with the guard cells covered by epicuticular wax ('bloom') (Figure 1e), a possible reason for why they were not discovered before.



(**a**) (**b**) (**c**) (**d**) (**e**) (**f**)

Figure 1. (**a**–**f**) From top left to bottom right. (**a**) A blueberry showing the sepals at the distal stylar end, (**b**) semicircle of the remains of the calyx or crown after the sepals were cut off (×40), space bar 1 mm, (**c**) three stomata partially obscured by dense wax crystals on the ovary (×885), space bar 100 µm, (**d**) stomata becoming apparent after dewaxing (×1100), space bar 10 µm, (**e**) wax crystals at larger magnification (×1700), space bar 1 µm, and (**f**) stomata-free inner (adaxial) side of the sepals/calyx (×400), space bar 0.1 mm.

Constituting a surface area of 26–45 mm^2, the abaxial calyx had 5200–7650 stomata and contributed 93–95% of the overall stomata count (Table 1). Since these stomata on the outside of the calyx are exposed, this Venturi effect may enhance gas exchange and transpiration and may be responsible for most of the water lost from a blueberry (Figure 2).

Table 1. Stomata on the blueberry fruit and flower perianth.

Flower Part	Number of Stomata [mm^{-2}]	Number of Stomata [berry^{-1}]	Percentage of Fruit Stomata
Petals	0	0	0
Ring enclosed by calyx	42–45	375–400	5–7
Sepals			
Adaxial sepals	0	0	0
Abaxial sepals	170–200	5200–7650	93–95

For comparison, blueberry leaves have 330–380 abaxial stomata mm^{-2} (n = 30).

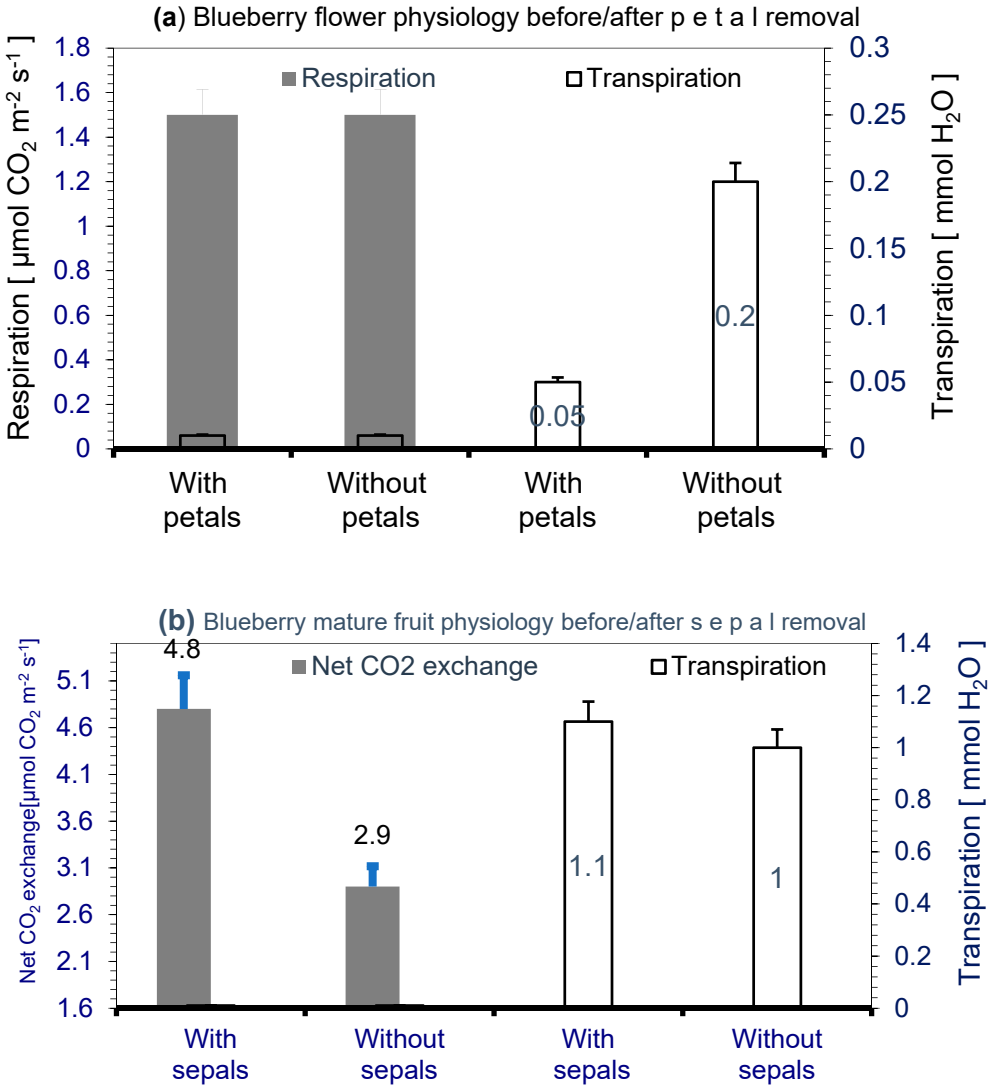


Figure 2. Transpiration and CO_2 exchange of the (**a**) blueberry inflorescence (top) before and after removal of petals and (**b**) blueberry before and after removal of sepals (400–500 µmol PAR, VPD 1.5 hPa; values ± SE).

3.2. Respiration and Transpiration of the Flower

At anthesis, the flowers transpired 0.05–0.2 mmol H_2O m^{-2} s^{-1} (Figure 2a). To determine the contribution of the petals to water and gas exchange of the inflorescence, transpiration was measured before and after the petals were removed. After their removal (20 cm^2), transpiration of the remaining 10 cm^2 surface increased four-fold from 0.05 to 0.2 mmol H_2O m^{-2} s^{-1} (Figure 2a), in line with the stomata on the distal part of the ovary as the source of water loss (Figure 1c,d) and the absence of stomata in the white petals. Respiration was measured before and after the petals were removed, and remained constant

at 1.5 µmol m^{-2} s^{-1} (Figure 2a), indicating negligible CO_2 efflux from the petals, which were devoid of stomata and chlorophyll (Table 1).

3.3. The Blueberry Ovary

The ovary was covered with a crystalline epicuticular wax in the form of cylindrical rods or wax-filled tubes of 1.0–1.2 × 0.2 µm in diameter (Figure 1c), producing the wax bloom of the blueberry. This increased with fruit ontogeny over the 50 µm wide epidermal cells. The flat, distal part (7 mm^2) sunken inside and partially covered by the protuberant perianth of the fruit comprised a smaller inner green circle of 0.4–0.6 mm in diameter surrounded by an outer ring (Figure 1a,b). Stamens and pistil originated from and had broken off the inner circle, leaving their debris and lenticels (Figure 1b) and an area devoid of stomata and trichomes. In the outer ring, stomata were difficult to count. Hence, berries were dewaxed by two dips in chloroform. Stomata on the outer ring were scarce with 42–45 stomata mm^{-2} equivalent to 380–400 stomata per berry based on an area of 9 mm^2 (Table 1). Stomatal guard cells appeared to be functional, but the ridges were covered by a dense epicuticular wax (Figure 1c) and were shielded by the perianth to further reduce gas exchange.

3.4. Transpiration and CO_2 Uptake of the Berry

After petal fall, the fruit showed a positive net CO_2 uptake. CO_2 exchange was measured before and after the sepals had been removed to determine their contribution to gas exchange. After their removal, CO_2 assimilation dropped from 4.8 to 2.9 µmol CO_2 m^{-2} s^{-1} without sepals (Figure 2b), showing the significant contribution of the sepals with their high chlorophyll content (Table 2) and the majority of the stomata (Table 1). The CO_2 assimilation of the blueberries waives the discussion about their climacteric characterisation.

Table 2. Concentration of chlorophyll in blueberry fruit and leaves and chlorophyll a:b ratio.

Content of Chlorophyll	[µg/g]	[µg/cm^2]	[µg/Berry]	Chl a:b
Ovary in May	100–120	52–72	24–29	3.9–4.2
Ovary in June	190–210	43–48	67–72	4.0–4.3
Ovary in July	370–390	27–29	38–42	4.7–5.9
Sepals in July	590–610	17–18	93–108	5.0–5.2
Blueberry **leaf**	1800–2100	41–43	26–40	5.9–6.3

3.5. Chlorophyll

The largest concentration of chlorophyll was found in the leaves, followed by the sepals and then the ovary, when expressed on a weight basis (Table 2). The fruit contained 100–390 µg/g of chlorophyll per g fresh weight compared with 2000 µg and 600 µg/g of chlorophyll in the leaves and sepals. The sepals contained 100 µg/berry and contributed 59–71% of the total fruit chlorophyll compared with 25.8 µg in small (0.25 mm), 40 µg in medium (0.5 mm) and 69 µg in large fruit (Table 2). The white petals contained no chlorophyll.

4. Discussion

4.1. Stomatal Frequency

Contrary to former beliefs, stomata were found on the blueberry fruit. Stomata were dominant on the abaxial side of the sepals and, to a lesser, extent, on the distal part of the ovary inside the corolla. The alleged absence of stomata in blueberries [6–9] may be because samples were taken from only the proximal, spherical part of the ovary between the peduncle and calyx, which is devoid of stomata, and the stomata are obscured by a dense cover of epicuticular wax (Figure 1c). Previous studies ignored the distal part of the ovary within the perianth and the sepals. Stomata were absent from the petals, as observed for other flowers.

The low stomatal frequency (42–45 mm^{-2}) on blueberries is comparable with the 330–380 stomata mm^{-2} (this study) or 560–640 stomata mm^{-2} [18] on the leaves. The stomatal frequency of 42–45 mm^{-2} on the distal section of the ovary is typical for fruit designated as a berry (i.e., sarcocarpium, where the pericarp remains fleshy). Examples are strawberries and grapes, with 0.5–1.5 stomata mm^{-2} [18,20]. Here, cuticular transpiration plays a larger role than stomatal transpiration in contrast to the leaves. Stomata of 14–15 × 20–21 µm in blueberry fruit are typical of those found in other fruit such as grapes (14 × 17 µm) [20].

4.2. CO_2 Exchange

Blueberry is the only fruit so far which has shown a net CO_2 uptake; this study identifies the sepals as a major contributor. Our values of 2.9 µmol CO_2 uptake m^{-2} s^{-1} are within the range of 1.2 µmol CO_2 uptake m^{-2} s^{-1} found by Flore and Hancock [11] and 8–12 µmol CO_2 uptake m^{-2} s^{-1} [12]. The smaller chlorophyll a/b ratio (3.9–5.9:1) in blueberries (Table 2) is typical of fruit compared with larger values in leaves [19,21].

4.3. Transpiration

Based on the absence of stomata in the blueberry, Freeman et al. (1979) [6] expected "pathways for water vapour through rodlets to be quite different from those in leaves". However, this study has shown that stomata exist on the distal part of the ovary within the corolla as well as on the outside of the calyx. Their exposed position on the outside of the calyx may cause a Venturi effect, which, in turn, may enhance gas exchange and transpiration. Hence, the stomatal transpiration of the sepals seems responsible for most of the water lost from the blueberry and for providing the transpirational pull for water and nutrients into the fruit depending on the environment [19].

Contrary to CO_2, the magnitude of transpiration of blueberry fruit has not been assessed before. However, the ranges found for blueberries (Figure 2) were smaller than those found for grape berries (1–8 mg H_2O $berry^{-1}$ h^{-1}) [20] and avocados [22] and smaller than in any of the leaves [20,22,23].

5. Conclusions

The present work gives pictorial evidence of stomata on blueberry fruit. Previous studies may have been hampered by the stomata being occluded by the dense wax cover (bloom) and hidden on the distal part of the ovary between and underneath the corolla. In the present experiment, the berries were dewaxed and the sepals were removed for counting the stomata. Stomata were present, contrary to former beliefs, on the distal part of the ovary and the abaxial side of the sepals. The latter contained the largest proportion of chlorophyll in the fruit (59–71%) and the majority (93–94%) of fruit stomata, but contributed little (5–7%) to the fruit surface. This study identified the ovary as a minor source and the sepals as the dominant source of CO_2 and H_2O exchange. This implies that the fruit photosynthesis concept of CO_2 accumulation within the ovary and respiratory CO_2 recycling is still standing. It also means that the blueberry is an exception in terms of net CO_2 uptake, which was found to be due to the high chlorophyll content and the large number of stomata of the sepals.

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