

Salt gland distribution in *limonium bicolor* at the individual level

B Y Leng^a, F Yuan^a, X X Dong and B S Wang*

Shandong Provincial Key Laboratory of Plant Stress, College of Life Sciences, Shandong Normal University, Ji'nan, Shandong, 250014, P.R. China

*Corresponding author: Baoshan Wang

E-mail: bswang@sdu.edu.cn

Tel: 0086 531 86180197; Fax: 0086 531 86180197

^a These authors contributed equally to the work.

Abstract. *Limonium bicolor* is a typical exo-recretohalophyte with multi-cellular salt glands. A differential interference contrast (DIC) microscope were applied to investigate the pattern of salt gland distribution in *L. bicolor* at the individual level. For a single mature leaf, more salt glands are distributed in the leaf central and apical regions than leaf base. For the leaves in different developmental stages, firstly, the density of salt glands linearly decreased at the beginning of leaf expansion and kept a relatively constant value in the later periods, which was mainly due to the rapid expansion of epidermal cells. Secondly, the total number of glands per leaf showed a reversed trend compared to the density of salt glands. These results suggested that the salt gland density was adapted to the leaf age and area as more and more salt accumulated in the saline soils.

1. Introduction

Typically, halophytes are adapted to grow in saline environments and have a substantial potential to be cultivated as vegetables, forage, and oilseed crops [1]. The salt-secretory structures (salt glands and salt bladders) are the only visible morphologic characteristics that distinguish recretohalophytes with all non-halophytes and other halophytes [2]. *Limonium bicolor* (Bunge) Kuntze is a typical exo-recretohalophyte and can excrete the excessive salt to the outside by salt glands [3]. In saline environments, salt secretion by salt glands makes it possible to maintain the ion balance required for normal metabolism [4]. In the last few decades, most studies focused on the ultrastructure and excretory mechanism of salt glands, e.g., the salt gland of *Avicennia marina* consists of two to four collecting cells, one stalk cell and usually eight secretory cells [5], and the *Limonium* salt gland develops from only one epidermal cell in five steps of cell-division to a gland complex of 20 cells [6].

The structure and function of leaves showed variations in different leaf positions and leaf age, e.g., stomata (or trichomes or salt glands) distribution and so on. The density and distribution of stomata of *Agrostis canina*, *A. palustris*, and *A. tenuis* varied with the leaf blade surface and position, and stomatal density was the highest on the youngest leaves [7]. Leaves formed early in rosette development of *Arabidopsis thaliana* lack trichomes on their abaxial surface; instead, leaves produced later have trichomes on both surfaces [8]. Moreover, there were more glands per section in third node leaves of the grey mangrove *Avicennia marina* than that in first ones which indicated that the leaf developed more glands as aged [9].

As an important salt-secreting structure in recretohalophyte, little is known about the salt gland



distribution and its relation to different leaf positions and leaf ages of *L. bicolor*. In the present study, we assessed the pattern of salt gland distribution in *L. bicolor* at the individual level using differential interference contrast microscopy combined with fluorescence excitation. Our aim was to investigate the relationship among the distribution pattern of salt glands and the leaf age at the individual level.

2. Materials and methods

2.1. Experimental materials and culture condition

Seeds of *L. bicolor* were collected from a saline inland (N37°20'; E118°36') in the Yellow River Delta, Shandong, China. Dry seeds were stored in a refrigerator at 4°C for 6 months before used. The seeds were planted in soil (a mixture of muck, vermiculite, and perlite in a 4:2:1, proportion, V/V). We chose three typical growth stages: one month (six-leaf stage), three months (twenty-leaf stage) and five months (fifty-leaf stage), respectively expressed as the sixth leaf, twentieth leaf and fiftieth leaf. The plants were grown in a growth chamber under natural light. The light intensity was 200 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, the relative humidity was 60%/80% (day/night) and the temperature was $28 \pm 3^\circ\text{C}/23 \pm 3^\circ\text{C}$ (day/night).

2.2. Distribution of salt glands in different regions of the expended fiftieth leaf

The expended fiftieth leaf was equally divided into three parts: base, central and apex (figure 1), and the salt gland density on the abaxial surface was counted under UV excitation (330-380 nm) at $\times 100$ magnification. Ten fields were randomly selected, and the procedure was repeated with ten leaves of expended fiftieth leaves.

2.3. Measurement of salt gland density with different ages in the sixth leaf, twentieth and fiftieth leaves

With the leaf growth, the area of the blade extended continuously and eventually expanded in full. To measure the salt gland distribution in leaves with different ages, we traced the leaf area changes in the development of one leaf. The leaf area was measured according to the methods of O'Neal et al. and Xiao et al. [10, 11]. The area of the sixth leaf growing from one month expanded to more than 300 mm^2 ; therefore the sixth leaf can be divided into eight sections (from i to viii) according to the leaf area $\{i \in (0, 20] (0 < \text{leaf area} \leq 20 \text{ mm}^2, \text{ the same below}), ii \in (20, 50], iii \in (50, 90], iv \in (90, 150], v \in (150, 200], vi \in (200, 250], vii \in (250, 300], viii \in (300, 500]\}$. Ranging from the youngest to fully expanded leaves, the division methods were the same in the twentieth leaf growing from three months (from I to IX) $\{I \in (0, 40], II \in (40, 200], III \in (200, 300], IV \in (300, 500], V \in (500, 800], VI \in (800, 1000], VII \in (1000, 1400], VIII \in (1400, 2000], IX \in (2000, 2500]\}$ and the fiftieth leaf growing from five months (from I to X) $\{I \in (0, 150], II \in (150, 350], III \in (350, 500], IV \in (500, 700], V \in (700, 1000], VI \in (1000, 1300], VII \in (1300, 1700], VIII \in (1700, 2500], IX \in (2500, 4000], X \in (4000, 5500]\}$. To guarantee the repeatability and reproducibility, more than 300 identical seedlings were kept for further experiments. Taking the sixth leaf as an example, we marked the sixth leaf since it appeared, and measured the blade area each day. In each leaf age, the sixth leaf was drawn for clearing, ten repetitions were performed in each leaf stage, and so forth in the remaining leaf stages of the sixth leaf age. After drawing the leaves in all of the ages of the sixth leaf, we counted the salt gland density of each leaf under UV excitation (330-380 nm) at $\times 100$ magnification; we used ten fields randomly selected and ten leaves for each age. The density of salt glands of each leaf age was calculated according to the average of these 100 fields from the ten corresponding leaves. The total number of salt glands on the abaxial surfaces of *L. bicolor* leaves with different ages was calculated according to the leaf area and the density of salt glands.

2.4. Measurement of the epidermal cell area and number of the twentieth leaf

The epidermal cell area and the number of epidermal cells per unit of leaf area in the twentieth leaf were determined using DIC at $\times 400$ magnification. Ten leaves were repeated for each leaf age, and the epidermal cell area and the number of epidermal cells were counted in ten randomly selected fields.

2.5. Statistical analysis

Data were analyzed by ANOVA procedures. Differences in means between treatments were compared (Duncan's test) and considered significant at $P = 0.05$. All statistical analyses were performed with the SPSS 13.0 (SPSS Software Inc., USA).

3. Results

3.1. Salt gland density pattern in different regions of the fiftieth leaf of *L. bicolor*

The salt gland density in different regions of the same leaf is shown in figure 1. For the fiftieth leaf, the density of salt glands in the leaf base per unit leaf area was significantly lower than that of the central part and the leaf apex. There was no significant difference between the central part and the leaf apex. The salt gland density in different leaf ages of the fiftieth leaf showed a similar pattern; however, their values decreased as the leaf expanded.

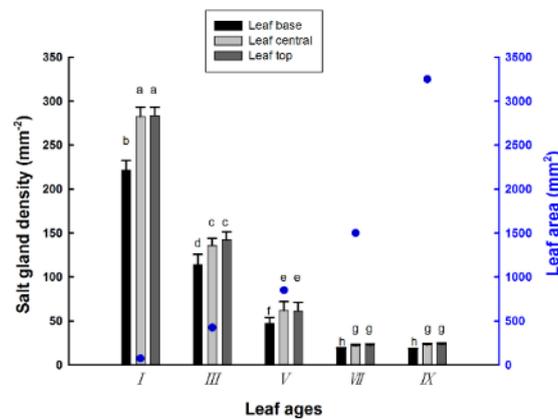


Figure 1. Salt gland density in different regions of the fiftieth leaf of *L. bicolor*. Blue dotted lines represent the trend of leaf area in different leaf areas. Values are the means \pm SE of 10 replicates, and means with the same letter are not significantly different at $P=0.05$ according to Duncan's multiple range test

3.2. Distribution of salt gland of the sixth, twentieth and fiftieth leaves of *L. bicolor*

The autofluorescence of salt glands indicated that the density of salt glands in unit vision (at $\times 100$ magnification) obviously decreased with the increase of the leaf area (figure 2A). Figure 2B and figure 2C quantify these trends. With the increase in leaf age, the density of salt glands dropped, and the total number of salt glands on a single leaf increased until the leaf area was 90~150 mm² (iv). Interestingly, the density of salt glands was stable, but the total number of salt glands on a single leaf significantly increased from leaf area v to viii. The stable density of salt glands was about 17 per unit area, and the stable total number of salt glands was about 1,100 on a single leaf.

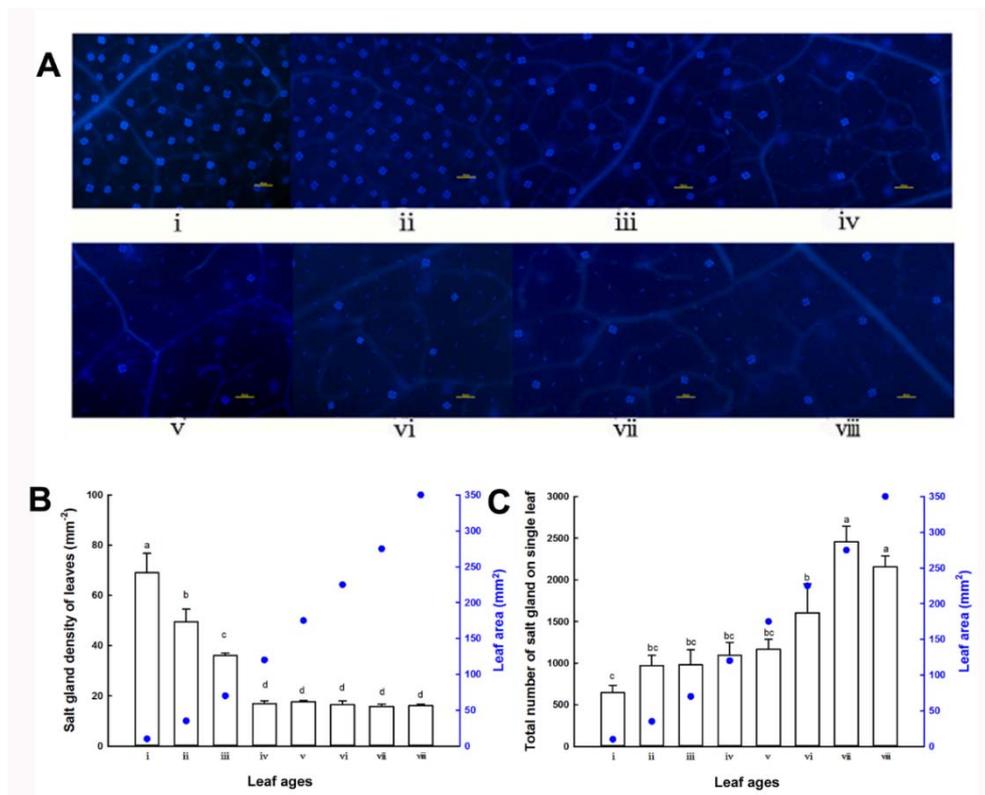


Figure 2. Distribution of salt glands in the sixth leaf of *L. bicolor* at different leaf ages. (A) Blue autofluorescence of salt glands at different leaf ages under UV excitation at 330-380 nm and at $\times 100$ magnification (bar = 10 μm); (B) Salt gland density at different leaf ages; (C) Total number of salt glands on a single leaf at different leaf ages; In B and C, values are the means \pm SE of 10 replicates, and means with the same letter are not significantly different at $P=0.05$ according to Duncan's multiple range test

The same trends can be found in the distribution of salt glands of the twentieth and the fiftieth leaves of *L. bicolor* at different leaf ages (figure 3 and figure 4). Correspondingly, the stable density of salt glands of area VII of the twentieth leaf was about 20 per unit area, and the stable total number of salt glands was about 17,600 on a single leaf. The stable density of salt glands of area VII of the fiftieth leaf was about 21 per unit area, and the stable total number of salt glands was about 56,700 on a single leaf.

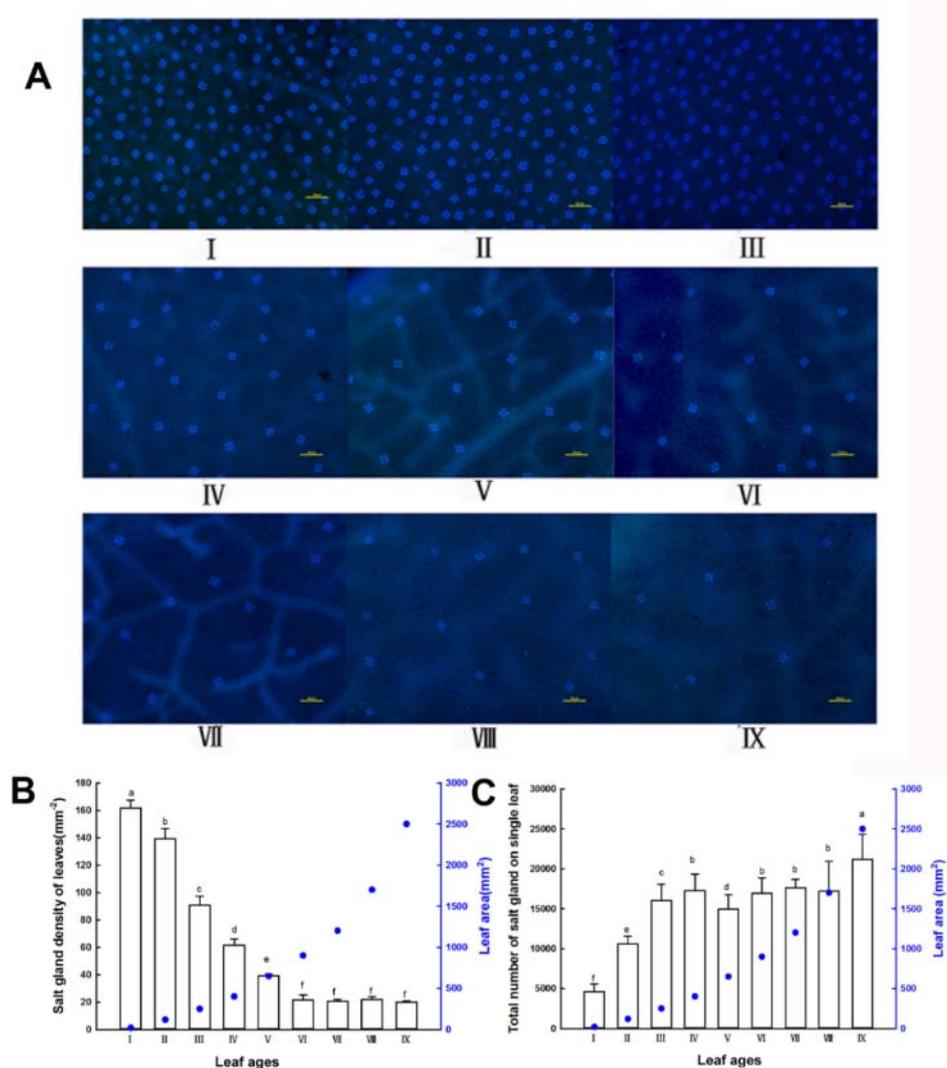


Figure 3. Distribution of salt glands in the twentieth leaf of *L. bicolor* at different leaf ages. (A) Blue autofluorescence of salt glands at different leaf ages under ultraviolet excitation at 330-380 nm and at $\times 100$ magnification (bar = 10 μm); (B) Salt gland density of leaves at different leaf ages; (C) Total number of salt glands on a single leaf at different leaf age. Blue dotted lines represent the trend of leaf area at different leaf ages. In B and C, values are the means \pm SE of 10 replicates, and means with the same letter are not significantly different at $P=0.05$ according to Duncan's multiple range test

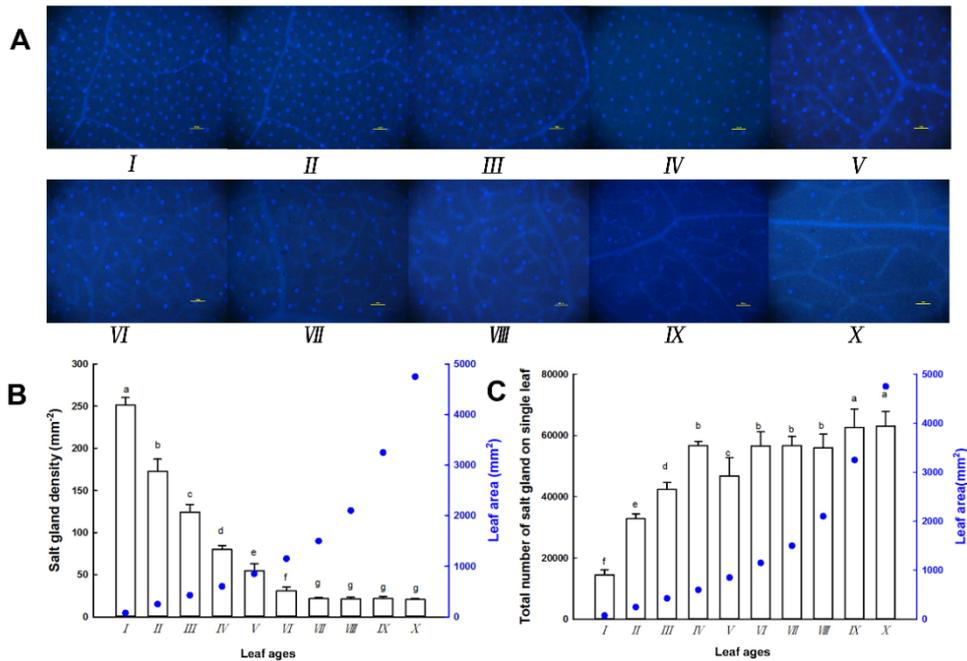


Figure 4. Distribution of salt glands in the fiftieth leaf of *L. bicolor* at different leaf ages. (A) Blue autofluorescence of salt glands at different leaf ages under ultraviolet excitation at 330-380 nm and at $\times 100$ magnification (bar = 10 μm); (B) Salt gland density of leaves at different leaf ages; (C) Total number of salt glands on a single leaf at different leaf ages. Blue dotted lines represent the trend of leaf area at different leaf ages. In B and C, values are the means \pm SE of 10 replicates, and means with the same letter are not significantly different at $P=0.05$ according to Duncan's multiple range test

3.3. Epidermal cell area and number per unit of leaf area of the twentieth leaf

Photos of epidermal cells indicated that the epidermal cell area increased apparently with the increase in leaf age, and the opposite trend was shown in the number of epidermal cells in the vision unit (at $\times 400$ magnification; figure 5A). figure 5B and figure 5C quantify these trends.

4. Discussion

In the present study, the salt gland distribution of *L. bicolor* was first reported in the current paper, which will provide a reference for further study of salt gland. These results of salt gland distribution of the sixth, twentieth and fiftieth leaves of *L. bicolor* indicates that the total number of salt glands is different at different leaf ages and leaf positions. The density of salt glands was inversely proportional to the total number of salt glands at the beginning and remained stable in later periods. Perhaps this pattern was mainly due to the rapid expansion of epidermal cells at the beginning. Furthermore, there were hardly any adjacent salt glands on the leaves of *L. bicolor*. This pattern was similar to the distribution of stomata and trichomes. In most dicot leaves, the stomata follow a "one-cell-spacing" rule in which two stomata are separated by at least one intervening nonstomatal epidermal cell [12]. In mature leaves of *Arabidopsis*, trichomes are relatively uniformly distributed, with adjacent trichomes occurring only rarely [13]. Our results indicate that the distribution of salt glands in *L. bicolor* may follow the same "one-cell-spacing" rule of the stomata. But this hypothesis needs to be further verified using different types of exo-recretohalophyte.

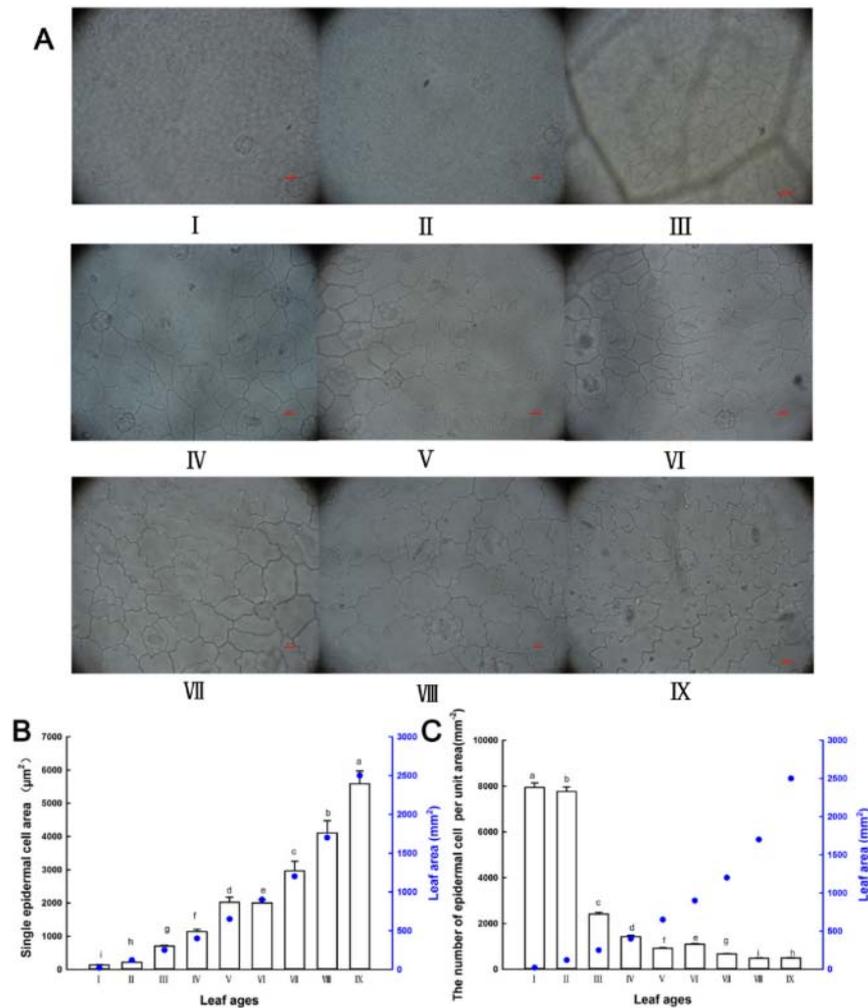


Figure 5. Epidermal cell area and number of epidermal cells per unit area in the twentieth leaf. (A) shows the epidermal cells under DIC at $\times 400$ magnification (bar = $20\ \mu\text{m}$); (B) Single epidermal cell area; (C) Number of epidermal cells per unit area. Blue dotted lines represent the trend of leaf area at different leaf ages. In B and C, the values are the means \pm SE of 10 replicates, and means with the same letter are not significantly different at $P=0.05$ according to Duncan's multiple range test.

In addition, with the increase in the leaf position (the sixth, twentieth and fiftieth leaves), the total number of salt glands evidently increased. The stable salt gland density increased slightly in different leaf positions. These results indicate that when one leaf is fully expanded, the salt gland density would not change apparently. However, the salt gland distribution may vary in different species, for example, the gland density of the old leaves of *Avicennia marina* was greater than the younger leaves [9].

In general, the amount of salt in the leaves of plants grown in saline soils depends on the transpiration rate and the leaf area. Our results indicate that the distribution pattern of salt glands of *L. bicolor* are adapted to the leaf area and leaf age. Specifically, the larger the leaf area, the more salt glands; however, the detailed mechanism needs to be further investigated and explored.

5. Conclusion

In summary, the following conclusions can be drawn:

- 1) Salt gland density in leaf central and apical regions are higher than leaf base and there are hardly any adjacent salt glands.

- 2) The rapid expansion of epidermal cells is one of the reasons for the rapid decrease of salt gland density at the beginning.
- 3) The total number of salt glands is positively related to leaf ages and leaf positions.

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