Foliar structure and mesophyll succulence in three Korean *Orostachys* species and its phylogenetic implications

Kim, InSun, Jae-Hong Pak¹, Bong-Bo Seo¹ and Seung-Dal Song¹
(Department of Biology, Keimyung University, Taegu 704-701,
¹Department of Biology, Kyungpook National University, Taegu 702-701, Korea)

한국산 바위솔속 식물의 엽육조직 및 다육질성
지수와 그 유연관계 연구

김인선·박재홍¹·서봉보¹·송승달¹
(계명대학교 자연과학대학 생물학과, ¹경북대학교 자연과학대학 생물학과)

Abstract

Foliar structure and mesophyll succulence of three Korean *Orostachys* species, *O. japonicus* A. Berger, *O. malacophyllum* Fisch., and *O. sikokianus* Ohwi, were examined to survey the presence of CAM in this group. Both features demonstrated that all of them were probably CAM performing species. The foliar structure of all three species was rather simple, exhibiting only epidermis, undifferentiated mesophyll, and vascular bundles. The mesophyll consisted of large water-storing cells, each containing a large central vacuole and chloroplasts in the thin peripheral cytoplasm. A well-developed intercellular space system was found in all species. In many cases, mucilaginous materials appeared in the intercellular spaces. In addition, attention was paid to the features of the vascular tissues. Paucity of vascular tissue was a general phenomenon. The arrangement of the vascular bundles differed for each species. The mesophyll succulence (Sm) is a possible indicator of CAM. This ranged from 4.65 to 7.38 for these species. This was well within the range of Sm reported for CAM plants. The anatomical implications of ecological adaptation, and a plausible evolutionary phylogeny are also discussed.
Introduction

The classification of a plant as succulent is based exclusively on morphology and does not implicate a special taxonomic status. The most conspicuous morphological trait of succulents is the possession of photosynthetic organs with high volume to surface ratios and voluminous tissues. Hence, succulents are generally characterized by their ability to store large amounts of water. There is no doubt that the storage of water by succulents represents an important ecological adaptation as succulents are xerophytes and occupy habitats where either precipitation rarely occurs or soil water is generally not available. Periods of water deficiency are overcome by endogenous water reserves (Kluge and Ting, 1978; Osmond, 1978). Xerophytes are usually adapted to a very dry climate and are almost always exposed to strong insolation. Both factors elicit certain structural responses in the plant which enables it to survive in an adverse habitat. All vegetative organs of the plant can function as water reservoirs in xerophytic succulents. Tissues clearly having the task of storing water have been called water tissues (Kluge and Ting, 1978) or water-storing tissues (Esau, 1977; Fahn, 1990). Other xeromorphic structural features are a paucity of vascular tissues (Esau, 1977; Roth, 1984; Fahn, 1990) and lower stomatal frequency (Edwards and Walker, 1993).

The Crassulaceae has many succulent species which have Crassulacean Acid Metabolism (CAM). Many phylogenetically unrelated angiosperms, including both dicotyledons and monocotyledons, also have CAM (Szarek, 1979; Edwards and Walker, 1993). CAM is generally associated with succulent leaves and stems in which the photosynthetic cells have extremely large vacuoles. Mesophyll cells of non-CAM plants have much smaller vacuoles. An index of mesophyll succulence (Sm) has been devised (Kluge and Ting, 1978). This is based on the ratio of water content vs. chlorophyll content. Sm values for a variety of plants have been determined (Kluge and Ting, 1978; Karadge et al., 1983; Guralnick et al., 1986; Kim, unpubl. data). Sm is regarded as a quick survey for the occurrence of CAM, when used with various other lines of structural evidence.

The genus Orostachys (Crassulaceae) contains succulent species adapted for drought tolerance. About three species are present in Korea (Lee, 1979). These are found in dry or saline habitats and have morphological features which make it possible to determine their Sm values and possibly to CAM mode. These features include 1) succulence and 2) leaves with high volume to surface ratios. The present study was undertaken to examine the succulent foliar structure and the Sm indices for the three Korean species of Orostachys, and to deduce the presence of CAM. Species were compared morphologically, anatomically, and ecologically. Plausible evolutionary trends are also considered.

Materials and Methods

Five to 10 plants of O. japonicus A. Berger, O. malacophyllus Fisch., and O. sikokianus Ohwi were
used for the study. The nomenclature of Lee (1979) was employed. Collections were made in the field, and some plants were maintained in a growth chamber or under laboratory conditions following collection. All collections are being maintained as living plants on the campus of Keimyung University.

**Foliar anatomy**: Median portions of healthy leaves were sampled. These were fixed for three hours in 1% paraformaldehyde-2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 6.8-7.2) at room temperature. Tissues were postfixed with 2% OsO₄, at 4°C, overnight in the same buffer (Kim and Fisher, 1990), dehydrated in a graded acetone series, and embedded in Spurr's (1969) resin. Two μm thick sections were made on a Reichert Ultracut ultramicrotome, stained with toluidine blue, and examined with a Zeiss photomicroscope.

**Mesophyll succulence**: To calculate the mesophyll succulence index, hereafter as mesophyll succulence, $S_m = \text{water content (g)/chlorophyll content (mg)}$. 10-15 leaves from three to five fully developed plants of each species were collected from the field. The water and chlorophyll contents were determined following the standard methods. Briefly, leaf fresh-weight was measured immediately. Dry-weight was determined by placing the leaves in an oven at 60-70°C until there was no further change in weight. Water content equaled the difference between fresh- and dry-weights. Total chlorophyll was determined by the method of Inskeep and Bloom (1985). Leaves were extracted with N, N-Dimethylformamide (Moran and Porath, 1980; Moran, 1982). Samples were shaken and kept in the dark for 72-84 hours at 4°C prior to spectrophotometric analysis. Absorbancies of the samples were read at 647 and 664.5 nm to calculate total chlorophyll (Inskeep and Bloom, 1985). Five replicates were made for each species.

**Results**

**Anatomy and morphology**: All three Korean *Orostachys* species had succulent leaves, but exhibited two types of leaf morphology. The lanceolate-leaved types included *O. japonicus* and *O. sikokianus*. *Orostachys japonicus* grew on very thin dry soils, on rocks, on roofs or on pebbles (Fig. 1). They had the longest and thickest leaves of the three (29.8 mm × 5.4 mm × 3.8 mm, length × width × thickness; see Table 1). The other ob lanceolate-leaved species, *O. sikokianus*, had the smallest leaves (7.1 mm × 1.3 mm × 1.0 mm). They grew in the most extreme habitats such as on mossy rocks on cliffs, rock wall faces, or dry slopes at high altitude (Fig. 2). The leaves of both species had short spiny tips. The broad spatulate- to ob lanceolate-leaved type, *O. malacophyllus*, grew on thin sandy soils or on rocks near the shore that were exposed to sea water spray (Fig. 3). Their leaves were 27.7 mm × 17.0 mm × 3.1 mm in size. *Orostachys japonicus* is relatively widely distributed; *O. sikokianus* has the most restricted distribution.

Transverse views of lanceolate- or ob lanceolate- and broad-leaved types showed rather simple foli-
Figs. 1–3. Plants of three Korean *Orostachys* species studied. 1. *O. japonicus* growing on pebbles. A plant in the right is in flowering. 2. *O. sikokianus* growing on mossy rocks of cliff wall faces at an elevation of ca. 1,800 m. Insert: Closeup of five *O. sikokianus* plants. 3. *O. malacophyllus* growing on rocks along the shore. Two plants in front are in flowering, and one behind is in a vegetative stage.

The leaf structures. Epidermis, mesophyll, and vascular bundles are shown in Figs. 4–10. The foliar tissues consisted of relatively undifferentiated mesophyll. No palisade or spongy cells could be distinguished. However, specialized water-storing mesophyll tissues occurred between the adaxial and abaxial epidermis, occupying most of the internal leaf volume (Figs. 4–6). The mesophyll consisted of large, somewhat isodiametric, water-storing cells, each containing a large central vacuole with a thin peripheral layer of cytoplasm. Chloroplasts were pressed against the cell wall (Fig. 10). A well-developed intercellular space system was also observed in all species. Intercellular space occupied a large part of the adaxial half of *O. sikokianus* leaves. Mucilage-containing cells were present in the epidermis, and these contained large circular or ovoidal bodies or numerous small granules. On the other hand, in *O. japonicus* the mucilage occurred both in epidermal cells and in the intercellular spaces, while in *O.*
Table 1. Features of mesophyll succulence in three Korean *Orostachys* species. Each value represents a mean of 10 measurements, except Sm and total chlorophyll amount which represent five replicates. Means are given ± standard deviations.

<table>
<thead>
<tr>
<th>Species</th>
<th>Leaf size* (mm)</th>
<th>Sm</th>
<th>Water content (%)</th>
<th>Total chlorophyll amount (mg/g fr. wt.)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>O. sikokianus</em></td>
<td>7.1 × 1.3 × 1.0</td>
<td>7.38 ± 0.52</td>
<td>92.60 ± 2.96</td>
<td>0.12 ± 0.01</td>
</tr>
<tr>
<td><em>O. japonicus</em></td>
<td>29.8 × 5.4 × 3.8</td>
<td>4.65 ± 0.94</td>
<td>95.17 ± 2.23</td>
<td>0.20 ± 0.04</td>
</tr>
<tr>
<td><em>O. malacophyllus</em></td>
<td>27.7 × 17.0 × 3.1</td>
<td>6.68 ± 1.01</td>
<td>95.03 ± 1.68</td>
<td>0.14 ± 0.02</td>
</tr>
</tbody>
</table>

* Leaf length × width × thickness.

*malacophyllus* mucilage appeared mostly in the intercellular spaces (Figs. 9-10).

Features of the vascular system were also studied in transverse view, since venation can provide valuable diagnostic characters when comparing succulents. Veins were poorly developed in all species; xylem and phloem components were not easily distinguished. Tracheary elements had not developed thick cell walls. Additionally, the xylem and phloem cells were similar in size, and they were in direct contact with the neighboring water-storing cells. Neither fiber strands nor any kinds of bundle sheath cells occurred around the vascular bundles. Of particular distinction, however, was the arrangement of vascular bundles. In small, oblanceolate-leaved *O. sikokianus* (Figs. 4, 7), the veins were found mostly around the center of the leaf, with two in the mesophyll, whereas in lanceolate-leaved *O. japonicus* (Figs. 6, 9), the veins were distributed near the periphery of the somewhat inverted triangular-shaped leaf outline. Two vascular bundles were also detected in their mesophyll as in *O. sikokianus*. However, in broad-leaved *O. malacophyllus* (Figs. 5, 8) numerous veins were distributed in a horizontally paradermal plane, midway between adaxial and abaxial surfaces.

**Mesophyll succulence:** A Sm index was estimated for each of the *Orostachys* species. These species have been considered succulents basically because their leaves have high volume to surface ratios. The water content assayed in this study also supported such speculation. They all exhibited high water content in their tissues. Sm values analyzed in these species showed variation from 4.65 to 7.38 (Table 1). The highest Sm value, 7.38, was estimated in *O. sikokianus* which had the smallest leaves with the lowest water (92.6%) and lowest chlorophyll contents (0.12 mg/g fr. wt.). Another species with thick, lanceolate and long leaves, *O. japonicus*, demonstrated the lowest Sm value (4.65), but showed the highest water (95.2%) and chlorophyll contents (0.20 mg/g fr. wt.). The other broad leaved species, *O. malacophyllus*, had Sm value of 6.68 with 95.0% water in their cells. Sm values analyzed in these *Orostachys* fitted well within the range of CAM plants previously reported.
Figs. 4-10. Portions of transverse leaf sections of *Orostachys* species. Arrows point to the veins in the mesophyll. Notice the vascular bundles that are not surrounded by specialized bundle sheaths, but abut directly on mesophyll cells. The intercellular spaces are filled with mucilaginous materials. IS. Intercellular space; Mu. Mucilage cell; ^*^WT. Water-storing tissue. 4. *O. sikokianus*. An asterisk indicates mucilaginous cell. Scale bar, 200 μm. 5. *O. malacophyllus*. Scale bar, 200 μm. 6. *O. japonicus*. Only small portion of the leaf cross section is shown here. Scale bar, 200 μm. 7. Veins were found only around the central portion of leaf in *O. sikokianus*. Scale bar, 100 μm. 8. Part of vascular bundles from the peripherally arranged vascular bundle system in *O. malacophyllus*. Scale bar, 100 μm. 9. Horizontal arrangement of veins in *O. japonicus*. Scale bar, 100 μm. 10. Water-storing mesophyll cells with large central vacuoles in *O. malacophyllus*. The cytoplasm are very thin, with scattered peripheral chloroplasts (arrows) adhering to the cell membrane. Scale bar, 50 μm.
Discussion

Anatomy and morphology: Plants growing in different habitats show structural differences that are commonly interpreted as evolutionary adaptations to the specific habitat conditions. The peculiarities distinguishing plants of the various habitats are clearly expressed in leaves as they are very important in the water economy for the plant. Availability of water is a factor known to affect the plant form (Esau, 1977). According to a model for an optimal leaf size in relation to environment (Parkhurst and Loucks, 1972), leaf size is adjusted to minimize the amount of water evaporation per unit of CO₂ uptake which maintains water-use efficiency. Succulents represent one type of response to dry habitats. Succulence is evident when the leaves contain high amounts of water and appear fleshy. Succulent leaves tend to be spherical rather than disc shaped. This is the case of the lanceolate leaves in Orostachys species. The succulent leaves contain a special water-storing tissue (WST) which may develop from the epidermis itself, from subepidermal layers or from the mesophyll (Roth, 1984). WSTs occupy large portion of mesophyll and more than 40% relative volume of water-storing tissue has been reported in some succulent leaves (Glagoleva et al., 1992). The mesophyll is often homogenous, and the cells of WST are large and thin-walled as seen from the Figs. 4-10 in the present study. This is the case in many succulent leaved species which carry out CAM (Nuernbergk, 1961; Kluge and Ting, 1978; Fahn, 1990; Edwards and Walker, 1993). A specialized WST, so-called collapsible water-storage cells, detected in cactus species (Mauseth, 1995) was not observed in these Orostachys species. Large vacuoles in these water-storing tissues are known to contain dilute and/or mucilaginous cell sap (Fahn, 1990), and the mesophyll channels of intercellular spaces are reported to be filled with pectic compounds in xerophytic succulent leaves of other species (Carlquist, 1957). This was not detected in Orostachys species. Instead, well-developed intercellular spaces filled with mucilaginous substances were observed. Particularly in O. sikokianus and O. japonicus, epidermal cells containing such substances either with numerous small granules or with several to many ovoidal or circular bodies were frequently encountered. These may be the tannin sacs containing much mucilage as reported in other plant species (Heide-Jorgensen, 1980; Lees et al., 1993). The presence of a well-developed system of intercellular spaces in the mesophyll tissue can increase the internal surface area by as much as 20-25% of the relative volume (Glagoleva et al., 1992). These large intercellular spaces may provide gas reservoirs or facilitate rapid gas exchange (Kluge and Ting, 1978; Cockburn, 1983; Woolley, 1983; Fahn, 1990) despite the lower stomatal frequency reported in many CAM plants (Edwards and Walker, 1993).

The noteworthy features in the vascular system of Orostachys leaves are the paucity and arrangement of vascular bundles within the mesophyll. Small lanceolate-leaved O. sikokianus had a few poorly-developed bundles mostly located around the central portion of the leaf, while the long lanceolated-leaved O. japonicus and the broad-leaved O. malacophyllus demonstrated numerous pe-
ripherally and horizontally arranged bundles, respectively. Two to three layers of vascular bundles are reported in other succulent species (Carlquist, 1957; Kim, 1989) which are very similar in their morphology to the latter two Orostachys species. No such bundles were found in this study. According to Fahn (1990), water conduction in leaves takes place not only by the veins, but also by the mesophyll cells. Water-storing tissue might then have replaced, to a certain degree, the function of veins in water conduction, thus leading to rudimentary vascular development. This vascularization might have some correlation with different size and shape of leaves exhibited in Orostachys occurring in different habitats. Considering a series of increasing leaf size reduction in a group of related taxa forms a semophylectic lineage (Bocher, 1979). Changes in leaf morphology in Orostachys are believed to be connected with the microclimate, which demonstrates the close connection between leaf type and environmental conditions. Small leaved-plants are common in dry habitats and a universal tendency for leaf size to decrease with an increase of altitude has been suggested (Leigh, 1975; Korner et al., 1983). The three Korean Orostachys may illustrate this tendency, since species with the smallest leaves, and with a few poorly developed vascular bundles, O. sikokianus, occurred in the most dry and adverse niches in high altitude mountains. On the other hand, two species with greater leaf size, either lanceolate or broad, with numerous vascular bundles either peripherally or horizontally arranged, occurred at low altitudes, even at sea level. Most Orostachys, however, actually occur among rocks or in shallow soils with prolonged water deficiency. This probably contributed to the different leaf morphologies established in the genus. Succulence may also develop in shore plants exposed to a sea water spray as in O. malacophyllus. A clear relationship has been revealed between soil salinity and the appearance of succulent features (Esau, 1977; Hajibagheri et al., 1983; Fahn, 1990). Since hypertrophy of parenchyma cells of some coastal plants as compared with those of inland habitats is a well-known phenomenon (Fahn, 1990), the layers of water-storing mesophyll cells found in leaves of O. malacophyllus may represent an example of this phenomenon.

**Mesophyll succulence**: If the term succulence is to be useful in considering CAM, the definition has to be made at the photosynthetic cellular or tissue level rather than the whole plant or plant organ (Kluge and Ting, 1978). Although the leaves of three Korean Orostachys were fleshy, the cellular succulence was determined by microscopy as discussed by Kluge and Ting (1978). They reported that Sm values for CAM plants range from 1.34 to 13.0, while that in non-CAM plants is 0.3-0.8. Karadge et al. (1983) also reported Sm value of 1.45 for a CAM species that resembles O. sikokianus. Sm values analyzed recently but unreported yet in eight succulent Portulaca taxa varied from 1.37 to 11.07 (Kim, unpubl. data). Likewise, Sm values of three Korean Orostachys species, 4.65-7.38, fitted well within the Sm range reported for CAM plants. Furthermore, a correlation between pigment content and CAM has been suggested, where chlorophyll level decreased in facultative succulent plants under CAM mode (Guralnick and Ting, 1988). The highest Sm value was obtained in O. sikokianus which also had the lowest amount of chlorophyll. This may be strongly related to the occurrence of
CAM in succulent plants occupying the most adverse environmental conditions.

In succulents where the water-storing tissues are identical with the photosynthetic parenchyma, the water-storing cells contain chloroplasts. All three Korean *Orostachys* examined contained chloroplasts in their water-storing cells. The number of chloroplasts per cell appears small in comparison with non-succulent photosynthetic cells, but this may be illusionary due to the large cell size. The osmotic pressure in these cells is known to be higher than those without chloroplasts (Fahn, 1990). Thus, when water is lacking, they obtain water from the water-storing tissue. Succulents without chloroplasts in their WST are considered to lack CAM, since the transport of photosynthetic C4 acids from the site of synthesis to the storage site is only effective within the distance of one or a few cells (Kluge and Ting, 1978). Only those succulents with big water-storing vacuoles accompanied by chloroplasts in the same cell are thought to possess CAM.

Most of the Crassulaceae are known to have CAM or at least may perform CAM under certain environmental conditions (Kluge and Hanscom, 1977; Kluge and Ting, 1978). The features observed in three Korean *Orostachys* species clearly demonstrated that they are good candidates for CAM. Considering CAM as largely a survival adaptation, it is believed that succulence has more functions than merely water storage (Lange *et al.*, 1976), and this appears to be the case in these *Orostachys* species.

Based on features discussed earlier such as leaf size and shape, foliar structure, vascularization, Sm values, habitats, and distribution, an assumption has been made about the evolutionary trends among three *Orostachys* studied. Upon this hypothesis, the species *O. malacophyllus* and *O. japonicus* may have evolved from either *O. sikokianus* or *O. sikokianus*-like ancestor(s) that occurred in most adverse and restricted areas in high altitude with the characters; 1) high volume to surface area and spherically shaped succulent leaves, 2) small leaves with poorly developed and few vascular bundles, mostly located around central portion of the leaf, and 3) highest Sm values. As such ancestral species have moved into lower elevation habitats with less adverse environmental conditions, leaf size has increased, especially the length, without grossly changing the leaf shape and still retaining a lanceolate shape. Anatomically, vascular bundles increased in number and developed in the periphery, rather than the center, of an inverted triangular-shaped leaf, as in *O. japonicus*. When some of *O. japonicus*-type population reached the sea level, along the shore, the leaves became widened, while the horizontally arranged bundles and other characters remained as those of *O. japonicus*, except for some adaptation to sea water exposure. The most broad distribution is encountered in *O. japonicus*, while *O. malacophyllus* showed a relatively broad distribution in shore-line habitats. The latter species, however, lacks thick cuticles or waxes over the epidermis, salt glands, or other adaptive features to sea water exposure, which might indicate that occupation of shore-line habitats may have taken placed relatively recently. Alternatively, the possibility of reverse evolutionary trends from the *O. malacophyllus*-type to *O. japonicus*-type to *O. sikokianus*-type, or evolution of *O. malacophyllus* di-
rectly from *O. sikokianus*-like ancestor(s), or even from *O. japonicus*-type to both *O. sikokianus*-type and *O. malacophyllus*-type can not be completely ruled out.

The further development of our knowledge of CAM in Korean *Orostachys* species will depend very much on finding plants performing CAM in natural habitats. Currently, structural differentiation in the cellular level of these species is also being investigated to detect whether some cellular organelles known to be observed in CAM plants are found in these species. This will help us better in understanding the succulent nature of *Orostachys* species. Moreover, molecular analysis needs to be carried out in order to reveal the precise phylogenetic relationships among these species.

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적요

한국산 바위솔속 식물 3종의 엽육구조 및 다육질성 지수(Sm)를 연구하였다. 이들의 육질성 엽육조직은 표현, 수분저장 조직 및 유관속의 비교적 단순한 구조를 보여주었다. 수분저장 조직은 매우 발달되어 있는 반면 유관속 조직은 거의 발달되어 있지 않았다. 유관속의 배열 양상은 3종 모두 다르게 나타났다. 조사된 Sm은 4.65-7.38로서 전형적인 CAM식물의 Sm에 해당되었다. 이러한 엽육구조 및 Sm 특성에 의해 한국산 바위솔속 식물 3종은 CAM을 수행하는 것으로 추정되었다. 또한 이들 엽육조직의 특성과 Sm특성에 비추어 이들 종간에 추정되는 환경적응에 대한 진화적 추이와 유연관계가 논의되었다.

**Literature Cited**


