Applied Microscopy
Korean Society of Microscopy

pISSN 2287-5123 · eISSN 2287-4445
http://dx.doi.org/10.9729/AM.2016.46.2.76

Regular Article

# An Optimized Methodology to Observe Internal Microstructures of *Aloe vera* by Cryo-Scanning Electron Microscope

Yoon Mi Choi†, Da Hye Shin†, Chong-Hyeak Kim*

*Center for Chemical Analysis, Korea Research Institute of Chemical Technology (KRICT), Daejeon 34114, Korea*

†These authors contributed equally to this work.

*Correspondence to:
Kim CH,
Tel: +82-42-860-7721
Fax: +82-42-860-7794
E-mail: chkim@krict.re.kr

Received April 11, 2016
Revised May 27, 2016
Accepted May 29, 2016

*Aloe vera* has been used in the pharmaceutical, food and cosmetic industry for its therapeutic properties. However, there are not many current studies on the microstructure of *A. vera* compared to studies on the chemical constituents and health efficacy of *A. vera*. Therefore, we compared the morphology of an *A. vera* leaf using an optical microscope, a conventional scanning electron microscope (SEM) and a cryo-SEM. Especially, this study focused on observing the gel in the inner leaf of *A. vera*, which is challenging using standard imaging techniques. We found that cryo-SEM is most suitable method for the observation of highly hydrated biomaterials such as *A. vera* without removing moisture in samples. In addition, we found the optimal analytical conditions of cryo-SEM. The sublimation conditions of –100°C and 10 minutes possibly enable the surface of the inner leaf of *A. vera* to be observed in their "near life-like" state with retaining moisture. The experiment was repeated with *A. arborescens* and *A. saponaria* to confirm the feasibility of the conditions. The results of this study can be applied towards the basic research of aloe and further extend previous knowledge about the surface structures of the various succulent plants.

**Key Words:** *Aloe vera*, Scanning electron microscope, Cryo-scanning electron microscope, Sublimation

## INTRODUCTION

*Aloe vera* is a succulent plant species of the Liliaceae family (Surjushe et al., 2008). *A. vera* is used today in the food, cosmetics and pharmaceutical industries because of its biomedical properties and beneficial effects such as immunomodulatory, anti-inflammatory, anti-cancer, wound healing and skin hydration (Davis et al., 1994; Hamman, 2008; Maenthaisong et al., 2007; Reynolds & Dweck, 1999; Surjushe et al., 2008). The leaf of *A. vera* is lance-shaped, 40 to 60 cm long and 10 cm broad at base. The leaf of *A. vera* is mainly composed of three parts: the outer green rind, the vascular layer and the inner leaf (Fig. 1) (Hamman, 2008; Surjushe et al., 2008).

Especially the gel in the inner leaf of *A. vera* is colorless, mucilaginous and contains approximately 99% water, minerals, polysaccharides, enzymes, proteins, vitamin and inorganic compounds (Choi & Chung, 2003; Hamman, 2008; Reynolds & Dweck, 1999; Surjushe et al., 2008). It could be utilized as a biomaterial for biodegradable and bioactive compounds present in a living organism, *A. vera*. So far, many researchers have focused on the therapeutic effects of the gel consisting of various biological compounds (Davis et al., 1994; Hamman, 2008; Reynolds & Dweck, 1999; Surjushe et al., 2008). However, understanding the unique inner structure of *A. vera* can inspire application fields. More specifically, the usage of *A. vera* in the form of biomaterial-based nanostructures such as hydrogels, nanocomposites, nanoparticles and bio-inspired sponges is showing great potential for biomaterial and nanostructure based application (Balaji et al., 2015; Silva et al., 2014). Furthermore, the identification of the inherent structures of plants can provide

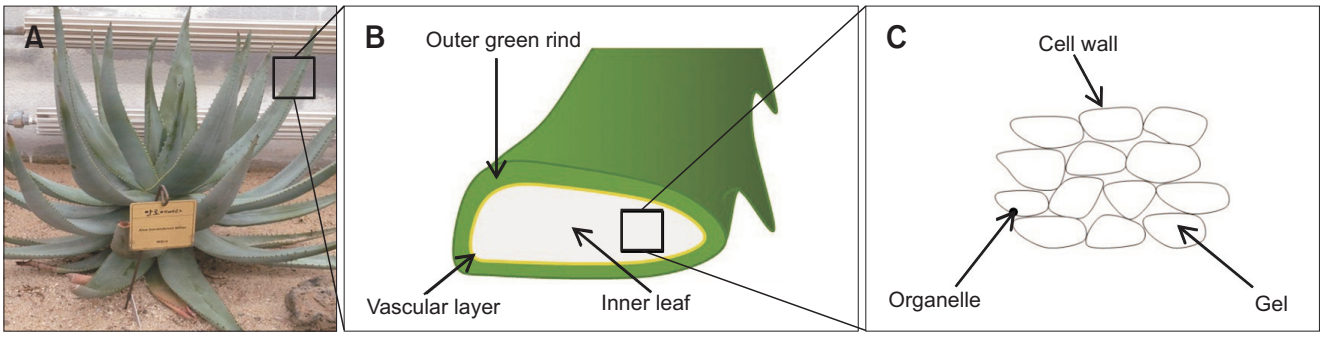


**Fig. 1.** The structural components of the *Aloe vera* leaf. (A) *A. vera*. (B) *A. vera* leaf. (C) Inner leaf of *A. vera*.

information regarding their origin, habitat and history as well as information about the effects of *A. vera*. Therefore, this study focused on observing the gel in the inner leaf of *A. vera*, which is challenging using standard imagining techniques.

The most commonly used techniques for visualizing structures are an optical microscope (OM) and an electron microscope (EM). The EM using a beam of electrons as an energy source has a greater resolution compared to the OM using visible light (Betzig et al., 1991). The widely used EM is divided into a scanning EM (SEM) and a transmission EM (TEM). Although TEM has a higher resolution than that of SEM, the specimen should be uniformly thin (tens-of-nanometer scale) for the highly absorbable electrons to penetrate a solid and form an image (Egerton, 2006; Mayer et al., 2007; Williams & Carter, 1996).

Conventional SEM images are of limited value in studying the microstructures of specimens with low conductivity or moisture (Pathan et al., 2008). For conventional SEM analysis, samples should undergo a series of preparation steps (fixation, dehydration, drying, etc.) causing damage and distortion, which make image interpretation difficult. Cryo-SEM, alternatively referred to as low temperature SEM (LT-SEM), is an optimal technique to minimize the artifacts and problems associated with conventional SEM (Choi et al., 2012; Fleck, 2015; Pathan et al., 2008; Schatten & Pawley, 2008). This technique is a rapid and effective way to observe a specimen in its natural state without significant specimen preparation (Read & Jeffree, 1991). Because of these advantages, cryo-SEM has come essential for the observation of highly hydrated biomaterials such as *A. vera* in a state of compared with OM and conventional SEM techniques. Moreover, controlling temperature and time is important in a sublimation step, one of cryo-SEM preparation procedures to reveal the underlying surface features appropriately since temperature and time for sublimation vary depending on the level of moisture contained in the sample as well as the size and shape of the sample.

The aim of this study was to find the best conditions that nearly enable the actual observation of the structure or morphology of the gel in the inner leaf of *A. vera*. The optimized conditions for cryo-SEM were also determined by the sublimation temperature. Furthermore, to demonstrate the effect of the proposed method, *A. arborescens* and *A. saponaria* used in medicine such as *A. vera* were also examined under the same conditions for cryo-SEM.

## MATERIALS AND METHODS

### *Aloe vera*

Fresh *A. vera* plants obtained from greenhouse in Daejeon, Korea were used as the raw material in all the experiments. The studied leaves, between 25 and 35 cm in length, were from approximately 7-year-old plants. The 2- to 3-year-old leaves of *A. arborescens* and *A. saponaria* used in the experiments were cultivated in the 'Aloe Farm', Geoje-si, Gyeongsangnam-do, Korea. Whole leaves were washed with distilled water and sliced into about 1 $mm^3$ samples.

### Experimental Procedures

We observed the surface morphology of an *A. vera* leaf by using an OM, a conventional SEM and a cryo-SEM. Fig. 2 shows the sample preparation procedures corresponding to the each analysis method.

### OM & SEM

The 1 $mm^3$ samples of *A. vera* were cut out with a scalpel, mounted on a glass slide and then examined in an OM (TE2000-U; Nikon, Japan).

We followed the sample preparation procedures for conventional SEM (Bozzola & Russell, 1999; Pathan et al., 2010). The 1 $mm^3$ samples of the *A. vera* were cut out with a scalpel, and then washed in 0.1 M phosphate buffer solution (PBS; Sigma-Aldrich, USA). Samples were fixed with 2.5% glutaraldehyde (Sigma-Aldrich) at 4°C for 2 hours. After washing three times with 0.1 M PBS each for 10 minutes, the 1st fixed samples were postfixed with 1% osmium tetraoxide ($OsO_4$; Sigma-Aldrich) at 4°C for 20 minutes. After washing three times with 0.1 M PBS each for 10 minutes, the 2nd fixed samples were dehydrated with an ascending sequence

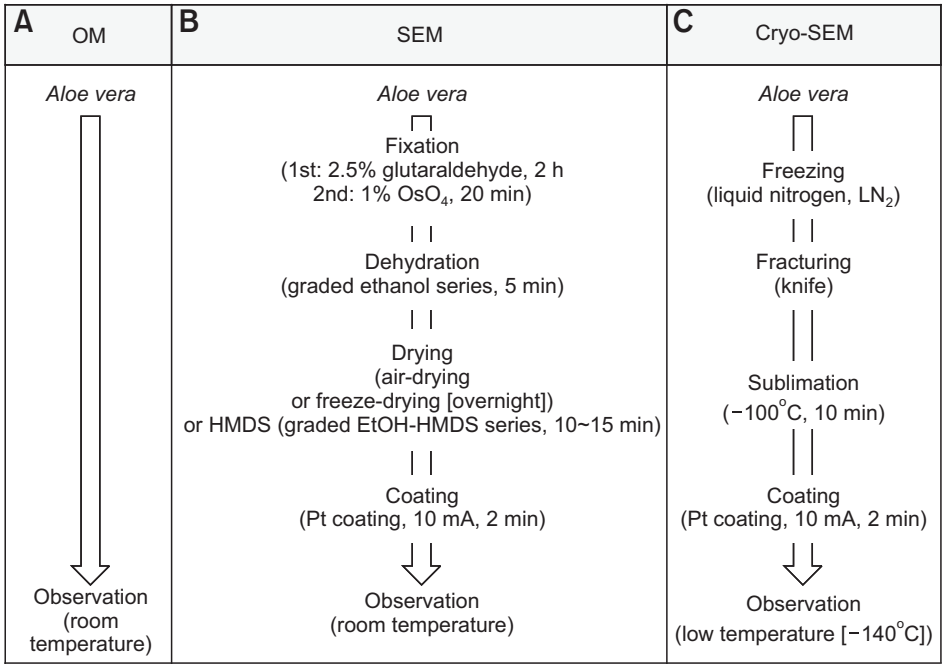


**Fig. 2.** Schematic representation of the sample preparation procedures in OM (A), conventional SEM (B), and cryo-SEM (C). OM, optical microscope; SEM, scanning electron microscope; HMDS, hexamethyldisilazane.

of ethanol concentrations (50%, 60%, 70%, 80%, 90%, 95%, and 100%) for 10 minutes at each concentration. The last dehydration step was repeated three times. The samples were dried with air-drying, hexamethyldisilazane (HMDS; Sigma-Aldrich) overnight or freeze-drying. For air-drying, the *A. vera* samples were dried with air at room temperature. For HMDS method, the samples were dried with a graded EtOH-HMDS mixture series (25%, 50%, 75%, and 100%) for 15 minutes at each concentration. For freeze-drying, the sample was freezed in liquid nitrogen for 1 hour and then dried in a freeze-dryer (ORR-FDCF-12003; Operon, Korea) at T= –120°C for overnight. The last drying step was repeated twice. Processed specimens were mounted with carbon tape on stainless steel stubs, sputter coated with Pt (Quorum Q150T ES; Quorum Technologies, United Kingdom) and examined with conventional SEM (MIRA 3 LMU FEG-SEM; Tescan, Czech Republic) operating at 5 to 10 kV.

### Cryo-SEM

Cryo-SEM was done on *A. vera* samples using an FE-SEM (MIRA 3 LMU FEG-SEM) fitted with a cryo preparation chamber (PP3000T; Quorum Technologies). The 1 $mm^3$ samples of the *A. vera* were cut out with a scalpel and mounted with medium mixing of colloidal graphite (Agar Scientific, United Kingdom) and optimal cutting temperature compound (Tissue-Tek O.C.T. compound; Sakura, Netherlands) on cryo stainless steel stubs. The samples were prepared by plunge freezing into a liquid nitrogen slush and transferring them under vacuum onto the cooled stage of the cryo-SEM preparation chamber (–140°C), which is mounted directly onto a convenient port of the SEM chamber. Samples were fractured, sublimated and sputter coated in the cryo-SEM preparation chamber. Finally the gate valve between the cryo-SEM preparation chamber and the SEM chamber was raised, and the specimen was transferred onto the cooled stage of the SEM chamber (–140°C).

For a sublimation step in the cryo-SEM preparation chamber, the temperature conditions were established differently into three (no sublimation, –50°C and –100°C) and we compared the sublimation temperature effect of three conditions. We would like to refer to (–100°C, 10 minutes) when sublimation was conducted at –100°C for 10 minutes in the cryo-SEM preparation chamber.

## RESULTS AND DISCUSSION

### Comparison of OM, SEM, and Cryo-SEM Images in *Aloe vera*

The overall structure of the *A. vera* leaf could be observed on a scale of hundreds of micrometers using the OM. However, it is impossible to observe microstructures such as the cell wall and detailed morphology of the *A. vera* leaf due to limitations in magnification and resolution (Fig. 3A and B).

We compared the difference of the three drying techniques between air-drying, HMDS treatment and freeze-drying using conventional SEM. First, the gel dried at room temperature overnight was almost distorted by the drying process (Fig. 3C). HMDS solvent, which is well known as an alternative to critical point drying, is suitable for the drying of aqueous samples because of their quick and effective penetration (Braet

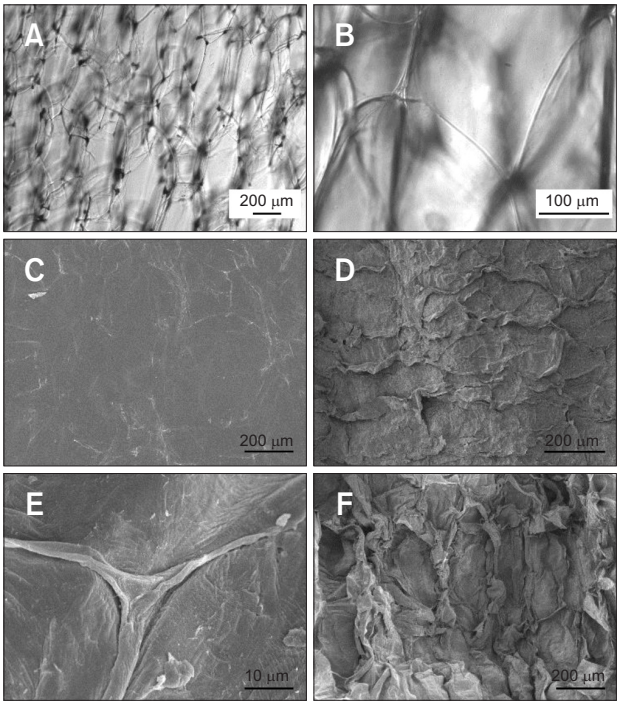


**Fig. 3.** The inner leaf of *Aloe vera* analyzed by OM (A, B) and conventional SEM (C-F). The inner leaf of *A. vera* treated with air-drying (C), HMDS (D, E), and freeze-drying (F). OM, optical microscope; SEM, scanning electron microscope; HMDS, hexamethyldisilazane.

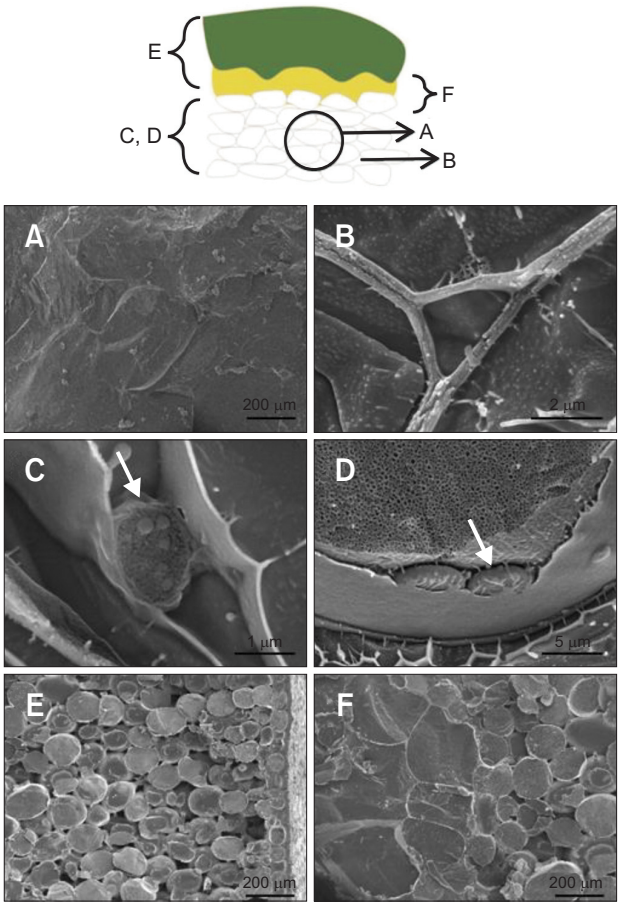


**Fig. 4.** Cryo-scanning electron microscopic images of the *Aloe vera* leaf: (A) gel, (B-D) organelles, (E) outer green rind and vascular layer, and (F) vascular layer and gel.

et al., 1997; Nation, 1983). We confirmed that the surface of the inner leaf of *A. vera* processed by a graded EtOH-HMDS mixture series maintained the boundary of cell in the gel (Fig. 3D). Although the overall structure of the cell wall surrounding the cell could be observed without difficulty, there still was some damage to the inner leaf of *A. vera*. Therefore, there are some difficulties in measuring accurately the length and observing the microstructure of the cell wall and cell (Fig. 3E).

Freeze-drying as another drying method can vaporize directly frozen water contained in a sample to gases by reducing the pressure (Wiegemann & Lehmann, 2009). After the pre-treatment process of the leaf under the same conditions as in HMDS treatment, the *A. vera* leaf dried by freeze-drying was observed. The gel surface of the inner leaf looked very similar to the sample treated with the HMDS treatment (Fig. 3F). The inner leaf of *A. vera* might get damaged by the pre-treatment process (chemical or physical method) as before.

The outer green rind is relatively hard compared to the inner leaf. Thus, the outer green rind treated with HMDS and freeze-drying was comparatively well retained when its surface was observed by SEM. However, the inner leaf of *A. vera* might get damaged by the pre-treatment process (chemical or physical method) as before.

It was confirmed using cryo-SEM analysis that the gel of the inner leaf has an apparent division among the cells without shrinkage or being rolled (Fig. 4A). Therefore, we obtained a clear image to observe the thickness of the cell wall (about 0.2 μm) (Fig. 4B). Using OM and conventional SEM analyses, we could not observe the organelles of *A. vera*. However, by using cryo-SEM analysis, various organelles of *A. vera* were observed comparatively without deformation or damage (Fig. 4C and D). Although we could not identify exactly what they are, cryo-SEM analysis is suitable for the observation of microstructures and can minimize the distortion of the gel and organelles of *A. vera*.

As a result, the outer green rind of *A. vera* can be observed clearly by HMDS treatment, freeze-drying and cryo-SEM (Fig. 4E and F). Because the outer green rind is relatively hard compared to the inner leaf, the outer green rind containing little water was not influenced by the pre-treatment process using a chemical method. However, the inner leaf of *A. vera*, which includes much water, is definitely dependent on the analysis method. The analysis using OM was not able to observe a clear morphology of the gel because of the

limitations in magnification and resolution. Because the gel of *A. vera* dried with air had changed beyond all recognition, we can confirm that the aqueous sample must be accompanied by a chemical method. However, the pre-treatment process (chemical or physical method) can distort the structure of the inner leaf. Consequently, cryo-SEM analysis is the best method, which can minimize the deformation of the sample and observe the real morphology of the inner leaf of *A. vera*.

### Optimal Conditions of Cryo-SEM for *Aloe vera*

The samples for cryo-SEM analysis were prepared with the above-mentioned procedures using a cryo-SEM preparation system. Especially, the sublimation step removes ice layers to reveal the underlying surface features, which is crucial to control the temperature and time of the frozen sample in the vacuum. The temperature and time for sublimation varies depending on the level of moisture contained in the sample as well as the size and shape of the sample.

Because water occupies up to 98% of *A. vera*, controlling the sublimation to reveal the inner structure is a crucial point in the operating procedure. Therefore, it is necessary to obtain the optimal analytical conditions of sublimation for *A. vera* to observe its microstructures. In this study, we tried to establish the best conditions of sublimation for *A. vera* by changing the time, temperature and continuity of the procedure. We imaged the surface of the inner leaf of *A. vera* varying the temperature (no sublimation, –50°C and –100°C) because the effect of temperature was much more on the structure of the inner leaf compared to the effect of time. Because sublimation is done in the cryo-SEM preparation chamber (–140°C), we compared the sublimation temperature effect of three conditions (no sublimation, –50°C and –100°C).

First, it was impossible to observe a clean surface morphology and the organelles of a cell in the inner leaf because the ice layers and ice particles were not removed without any sublimation procedure (Fig. 5A-C). Therefore, sublimation can be thought of as a necessary step for surface analysis.

Fig. 5D-F show the effect of a rapid sublimation (–50°C, 10 minutes) on the structure of the inner leaf. The rapid sublimation could have caused the fractured surface to be damaged and distorted with beehive like pores by removing lots of water in a short period. These beehive like pores inside cells support that aloe gel is mostly composed of water based components.

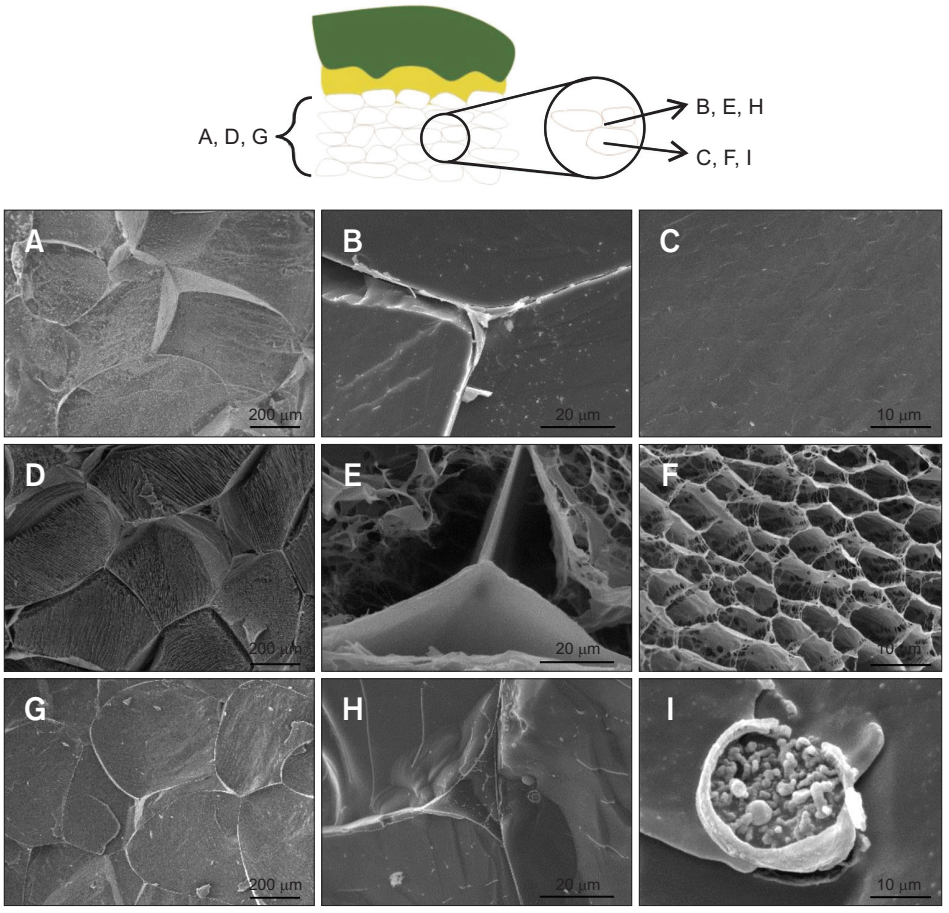


**Fig. 5.** Cryo-scanning electron microscopic images of the inner leaf of *Aloe vera* under different conditions: (A-C) without sublimation, (D-F) -50°C and 10 minutes for sublimation, and (G-I) -100°C and 10 minutes for sublimation.

Additionally, there were empty spaces between the cells, which are regarded as a sign of water loss. Even though –50°C is the temperature at which ice layers can be removed, this excessive sublimation process resulted in the distortion of the inner leaf of *A. vera*.

We can assume that the beehive like pores inside cells by excessive sublimation is similar to the formed structure when *A. vera* undergoes the dry environment and weather of deserts or high mountains, etc. Furthermore, we guess that the beehive like pores inside cells are formed when *A. vera* tries to capture as much water as possible in extreme environments.

The sublimation conditions of –100°C and 10 minutes possibly enable the surface of the inner leaf of *A. vera* to be observed in their "near life-like" state with retaining moisture. Fig. 5G shows the cleanest surface by removing the ice layer properly. The sample shows well-preserved structures with observation of cell organelles such as cell walls surrounding the cell, the contact surfaces between neighboring cell walls, and the intercellular space (Fig. 5H and I). There is a lack of information about observed organelles, but these observations may be meaningful in that we give possibilities for people to show other microstructures. The sublimation conditions of –100°C and 10 minutes are the best for observing the structure of the inner leaf of *A. vera* containing water compared with other conditions.

The experiment was repeated with *A. arborescens* and *A. saponaria* to confirm the feasibility of the optimal conditions. Both *A. arborescens*, which improves blood vessels and blood circulation, and *A. saponaria*, which has an anti-cancer activity, are widely used in various fields along with *A. vera*. They have different shapes and their own functions; however, all can be used for medicinal purposes (Harlev et al., 2012; Jia et al., 2008; Picchietti et al., 2013; Silva et al., 2013). Fig. 6 shows a well-maintained inner structure and cell organelles like a cell wall for *A. arborescens* and *A. saponaria*. This study has shown that the sublimation conditions of –100°C and 10 minutes almost certainly are the optimal analytical conditions for at least aloe plant species.

## CONCLUSIONS

We did a comparative study of OM, conventional SEM, and cryo-SEM techniques and found the most suitable method and optimal analytical conditions for studying the surface morphology of *A. vera*, which has various uses in the pharmaceutical, food, cosmetic, and toiletry industry. We have shown that cryo-SEM is more suitable for studying wet and hydrated biological materials samples such as *A. vera* in the most closely native state than that of the conventional

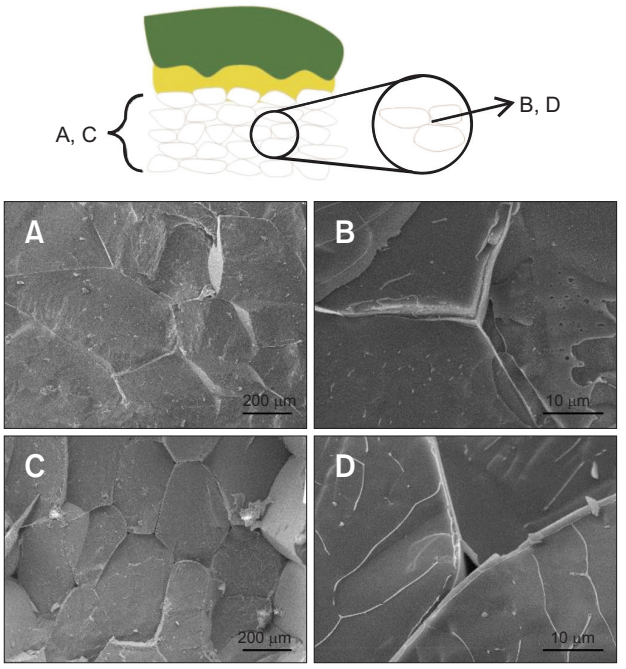


**Fig. 6.** The inner leaf of other species examined by cryo-scanning electron microscope. (A, B) *Aloe arborescens*. (C, D) *A. saponaria*.

SEM preparation method. In addition, it enables the surface morphology of wet and hydrated samples to be observed in hydrated conditions without damaging them.

We also found the optimal process conditions for cryo-SEM depending on the temperature of sublimation. Moreover, the sublimation conditions of –100°C and 10 minutes are the best result we have ever performed. Moreover, we obtained images of both *A. arborescens* and *A. saponaria* under the same conditions as that of *A. vera* and confirmed the feasibility of the proposed optimal conditions.

There are not many current studies on the morphology and microstructure of *A. vera* compared to studies on the main chemical constituents and health efficacy of *A. vera*. Therefore, established method and analytical condition would be helpful in observing internal and surface structure of *A. vera* remaining water. Understanding their microstructures for plants under optimized analytical method is likely to broaden the range of their application. In addition, it can be applied to biomaterial based nanostructure systems and basic research of aloe extending further to various succulent plants.

## CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

## REFERENCES


Balaji A, Vellayappan M V, John A A, Subramanian A P, Jaganathan S K, SelvaKumar M, bin Mohd Faudzi A A, Supriyanto E, and Yusof M (2015) Biomaterials based nano-applications of Aloe vera and its perspective: a review. *RSC Adv.* **5**, 86199-86213.

Betzig E, Trautman J, Harris T, Weiner J, and Kostelak R (1991) Breaking the diffraction barrier: optical microscopy on a nanometric scale. *Science* **251**, 1468-1470.

Bozzola J J and Russell L D (1999) *Electron Microscopy: Principles and Techniques for Biologists* (Jones and Bartlett Learning, Sudbury).

Braet F, De Zanger R, and Wisse E (1997) Drying cells for SEM, AFM and TEM by hexamethyldisilazane: a study on hepatic endothelial cells. *J. Microsc.* **186**, 84-87.

Choi S and Chung M H (2003) A review on the relationship between Aloe vera components and their biologic effects. *Semin. Integr. Med.* **1**, 53-62.

Choi Y J, Lee K H, Je A R, Chae H, Jang J, Lee E, and Kweon H S (2012) Cryo-SEM methodology of Arabidopsis thaliana stem using high-pressure freezing. *Korean J. Microsc.* **42**, 111-114.

Davis R H, Donato J, Hartman G M, and Haas R C (1994) Anti-inflammatory and wound healing activity of a growth substance in Aloe vera. *J. Am. Podiatr. Med. Assoc.* **84**, 77-81.

Egerton R (2006) *Physical Principles of Electron Microscopy: An Introduction to TEM, SEM, and AEM* (Springer, New York).

Fleck R A (2015) Low-temperature electron microscopy: techniques and protocols. In: *Cryopreservation and Freeze-Drying Protocols,* eds. Willem F W and Harriëtte O, pp. 243-274, (Springer, New York).

Hamman J H (2008) Composition and applications of Aloe vera leaf gel. *Molecules* **13**, 1599-1616.

Harlev E, Nevo E, Lansky E P, Ofir R, and Bishayee A (2012) Anticancer potential of aloes: antioxidant, antiproliferative, and immunostimulatory attributes. *Planta Med.* **78**, 843-852.

Jia Y, Zhao G, and Jia J (2008) Preliminary evaluation: the effects of Aloe ferox Miller and Aloe arborescens Miller on wound healing. *J. Ethnopharmacol.* **120**, 181-189.

Maenthaisong R, Chaiyakunapruk N, Niruntraporn S, and Kongkaew C (2007) The efficacy of aloe vera used for burn wound healing: a systematic review. *Burns* **33**, 713-718.

Mayer J, Giannuzzi L A, Kamino T, and Michael J (2007) TEM sample preparation and FIB-induced damage. *MRS Bull.* **32**, 400-407.

Nation J L (1983) A new method using hexamethyldisilazane for preparation of soft insect tissues for scanning electron microscopy. *Biotech. Histochem.* **58**, 347-351.

Pathan A, Bond J, and Gaskin R (2008) Sample preparation for scanning electron microscopy of plant surfaces—horses for courses. *Micron* **39**, 1049-1061.

Pathan A, Bond J, and Gaskin R (2010) Sample preparation for SEM of plant surfaces. *Mater. Today* **12**, 32-43.

Picchietti S, Bernini C, Belardinelli M, Ovidi E, Taddei A, Guerra L, Abelli L, and Fausto A (2013) Immune modulatory effects of Aloe arborescens extract on the piscine SAF-1 cell line. *Fish Shellfish Immunol.* **34**, 1335-1344.

Read N D and Jeffree C E (1991) Low-temperature scanning electron microscopy in biology. *J. Microsc.* **161**, 59-72.

Reynolds T and Dweck A (1999) Aloe vera leaf gel: a review update. *J. Ethnopharmacol.* **68**, 3-37.

Schatten H and Pawley J B (2008) *Biological Low-Voltage Scanning Electron Microscopy* (Springer, New York).

Silva M A, Trevisan G, Klafke J Z, Rossato M F, Walker C I B, Oliveira S M, Silva C R, Boligon A A, Flores F C, and de Bona Silva C (2013) Antinociceptive and anti-inflammatory effects of Aloe saponaria Haw on thermal injury in rats. *J. Ethnopharmacol.* **146**, 393-401.

Silva S, Oliveira M, Mano J, and Reis R (2014) Bio-inspired Aloe vera sponges for biomedical applications. *Carbohydr. Polym.* **112**, 264-270.

Surjushe A, Vasani R, and Saple D (2008) Aloe vera: a short review. *Indian J. Dermatol.* **53**, 163.

Wiegemann M and Lehmann F (2009) High-throughput SEM preparation of proteinaceaous extracellular matrix. *J. Mater. Sci.* **44**, 3813-3818.

Williams D B and Carter C B (1996) *The Transmission Electron Microscope* (Springer, New York).