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Helium Ion Microscopy of Uncoated Pine Leaves

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A recently introduced helium ion microscopy (HIM) was employed to observe uncoated pine leaf specimens. Adult leaves were collected from the seedlings of *Pinus densiflora* and *P. rigida*, air-dried at room temperature, and observed by HIM without metal coating. Ovoid or round stomata and distinct Florin rings could be discerned. The epicuticular waxes were present in the epistomatal chambers and Florin rings of stomata on the leaf surface. The epicuticular waxes were mostly straight, cylindrical, and ca. 1 μm in length. The epistomatal chambers of *P. rigida* were filled with the epicuticular waxes, whereas those of *P. densiflora* were not filled with the epicuticular waxes. Based on their micromorphology, the epicuticular wax structures of the pine species were identified as tubules. These results suggest that the HIM could be used for the investigation of the plant stomata and epicuticular waxes of uncoated plant leaves. Due to the smaller ion probe and interaction volume, the HIM has advantages over conventional field emission scanning electron microscopy in terms of image resolution and charge neutralization.

Key Words: Helium ion microscopy, *Pinus densiflora*, *Pinus rigida*, Stomata, Wax

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INTRODUCTION

Current research in the areas of nanobiology and nanomaterials requires ultrahigh-resolution surface imaging techniques that allow observation of the specimens with minimal damage and preparation (Bell, 2009). Although the field emission scanning electron microscopy (FESEM) has been extensively employed, it has intrinsic limitations to resolution due to lens aberrations and specimen-electron beam interactions. Another challenge in the microscopy employing charged particle beams is the specimen preparation, because the observation is conducted in high vacuum (Bazou et al., 2011).

The helium ion microscopy (HIM) has recently emerged as a commercially available surface imaging instrument (Economou et al., 2012). It has all the advantages of FESEM's simple specimen preparation procedures and easily interpretable results with the added benefit of superior resolution and depth-of-field (Boden et al., 2012). The key feature of the HIM is a helium (He) ion source made of a tungsten tip sharpened to a trimer of atoms. The strong voltage at the tip ionizes atoms of the He gas; the ions then accelerate away from the tip (Vanden Berg-Foels et al., 2012). A beam of He ions (called the primary beam) is then directed onto a specimen surface (Bazou et al., 2011). Secondary electrons (SE) emanated from the specimen-He ion interactions are recorded during the He ion beam scanning. The subnanometer ion probe and the shallow escape depth of SE make the HIM ideally suited not only for high-resolution imaging but also for high-resolution nanofabrication (Alkemade et al., 2012). The positive charge from the positively charged primary beam neutralizes the negative charge of SE, resulting in a dramatic reduction in charging on the insulating specimens. Furthermore, quantitative, elementally specific microanalysis could be performed in the HIM using secondary electron spectroscopy, Rutherford backscattered ion spectroscopy, or secondary ion mass spectroscopy (Joy & Griffin, 2011). There is growing evidence that the HIM is promising for imaging and analyzing interfaces of uncoated biological specimens and nanostructures (Bazou et al., 2011; Alkemade et al., 2012;

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Boden et al., 2012). However, little information is available on the surface observations of plant specimens by HIM. Here, I report the HIM of uncoated pine leaves, and evaluate the potential of the novel microscopy for next-generation biological imaging.

MATERIALS AND METHODS

Two pine species of two-year-old seedlings were prepared: *Pinus densiflora* and *P. rigida* as previously described (Kim et al., 2011). Briefly, adult leaves (ca. 8 cm long) of each species were collected from the seedlings. To circumvent structural alteration of the micromorphology of the plant epicuticular waxes during conventional specimen preparation, the leaves were air-dried at room temperature for at least two months (Kim et al., 2010). The specimens were mounted on a metal stub (10 mm in diameter) using two-sided adhesive carbon tape. They were examined by HIM (Orion; Carl Zeiss, Oberkochen, Germany) with a working distance of 8.6 mm and accelerating voltage of 33.2 kV.

RESULTS AND DISCUSSION

The HIM revealed the surface features of uncoated leaves of *P. densiflora* (Fig. 1A). Ovoid or round stomata were positioned in longitudinal rows on the leaf surface. Stomata had distinct Florin rings, and were ca. 20 μm in width (Fig. 1B). It was feasible to observe the epicuticular waxes around the stomata at lower magnifications. The epicuticular waxes were also present on the Florin rings (Fig. 1C). The overall image quality and contrast formation were strikingly similar to those of conventional FESEM of metal coated specimens. Higher magnifications showed the epicuticular waxes were mostly straight, thin, and ca. 1 μm in length (Fig. 1D). The epistomatal chambers of *P. densiflora* were not filled with the epicuticular waxes.

The leaves of *P. rigida* showed slightly different surface features, as compared with those of *P. densiflora* (Fig. 2A). The epistomatal chambers of *P. rigida* were filled with the epicuticular waxes. The Florin rings of the stomata were elevated from the leaf surface (Fig. 2B). The presence of the epicuticular waxes could be discerned on the surrounding areas of the stomata. The micromorphology

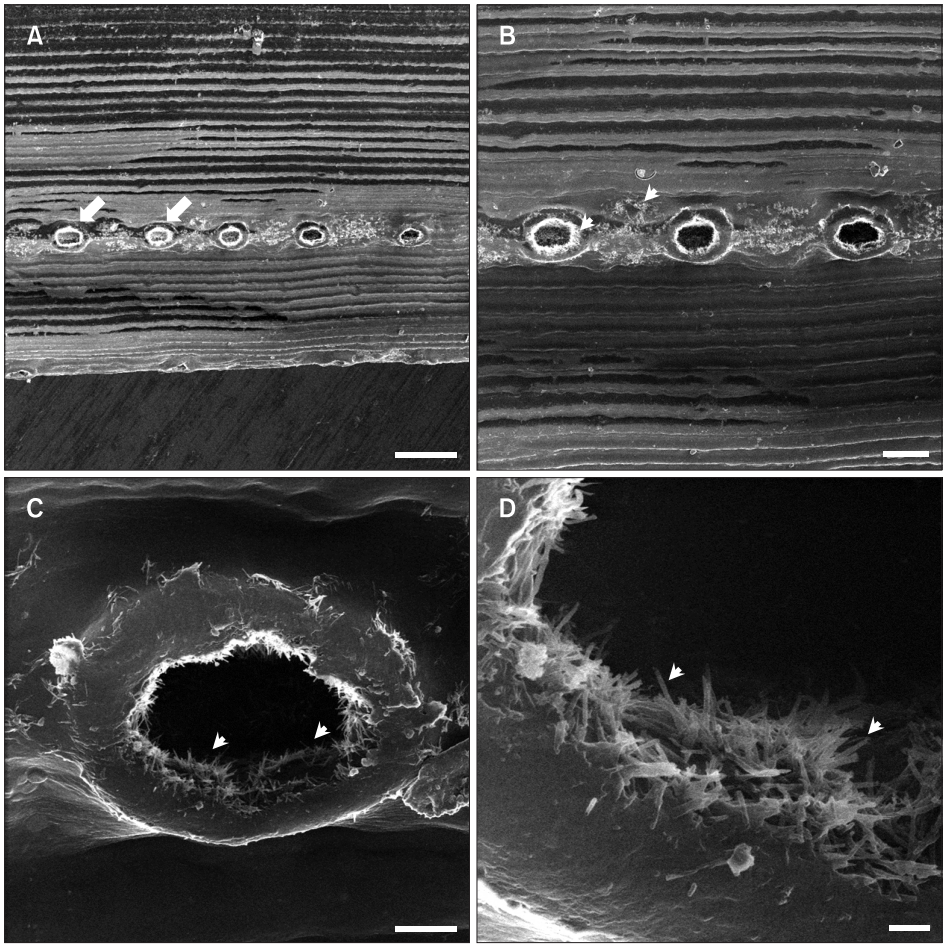

Fig. 1. Helium ion micrographs of uncoated leaves of *Pinus densiflora*. (A) Needle leaf. Stomata are positioned in longitudinal rows on the leaf surface (arrows). Bar=50 μm. (B) Stomata. Arrowheads indicate epicuticular waxes on the leaf surface. Bar=20 μm. (C) Stoma. Epicuticular waxes (arrowheads) in the epistomatal chamber could be discerned. Bar=5 μm. (D) Epicuticular waxes. Tubules (arrowheads) are rod-shaped and straight. Bar=1 μm.

of the epicuticular waxes of *P. rigida* was similar to that of *P. densiflora* (Fig. 2C). The epicuticular waxes were thin, rod-shaped, and ca. 1 μm in length. They appeared to be interconnected with each other, forming a complex network (Fig. 2D). They were mostly straight and cylindrical in shape.

This study demonstrated the potential of HIM as a surface imaging tool of uncoated biological specimens. Based on the classification of plant epicuticular waxes by Barthlott et al. (1998), the epicuticular waxes of the pine species were identified as tubules. A novel finding in this study was that the classification of the epicuticular waxes could be achieved in uncoated leaf specimens by HIM. Previously, metal coated specimens have been used for the classification of epicuticular waxes by FESEM (Kim et al., 2010, 2011). Under the experimental conditions, no obvious beam damage or structural change were observed on the uncoated specimens, as shown in human colon cancer cells (Bazou et al., 2011). Such an advantage of HIM would allow for unaltered specimen structure and reduced specimen preparation. Increase in temperature during metal coating for conventional FESEM could impair surface details and alter topographic relief of biological specimens. With no need of metal coating, the HIM has overcome the limitations inherent in FESEM (Vanden Berg-Foels et al., 2012). To my knowledge, this is the first report on the use of HIM for surface imaging of plant specimens. Further studies await the diverse applications of HIM for a wide variety of biological specimens. The nanoscale imaging of uncoated specimens will provide new avenues of nanobiological research.

CONCLUSIONS

The potential of HIM was manifested as a novel microscopy of insulated objects. The stomata and epicuticular waxes of uncoated pine leaves could be observed by HIM without obvious beam damage. Due to the smaller ion probe and interaction volume, the HIM has advantages over conventional FESEM in terms of image resolution and charge neutralization.

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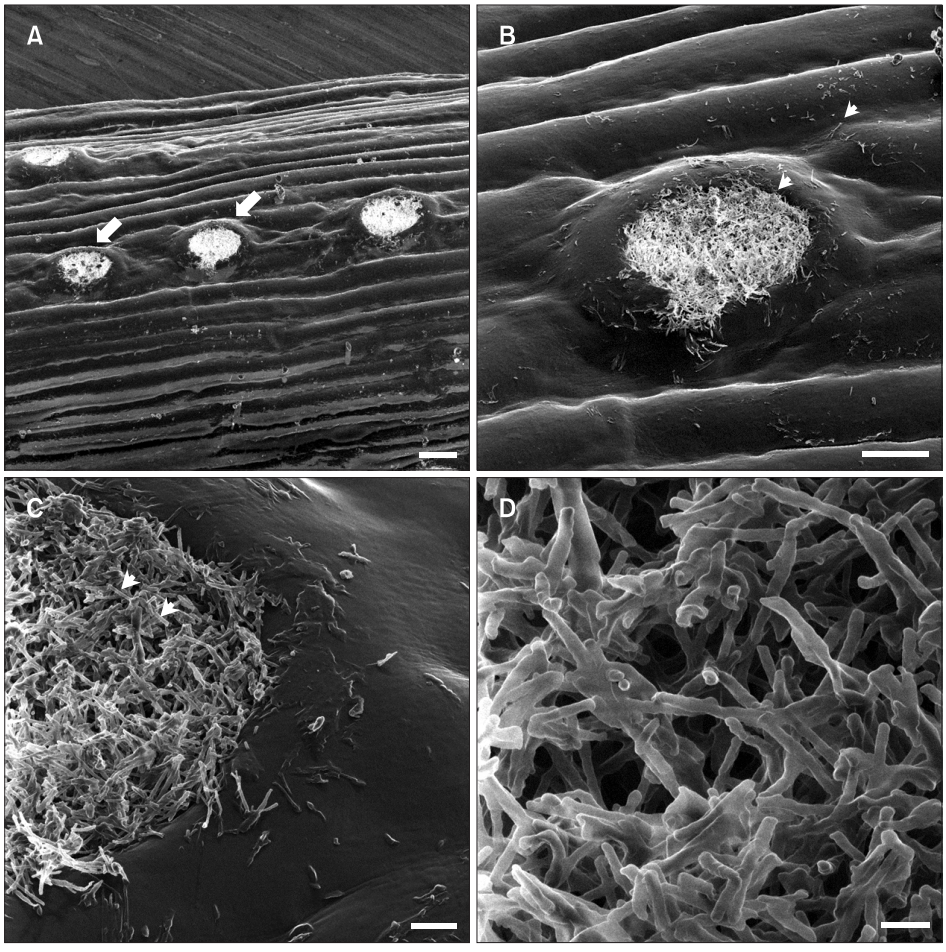

Fig. 2. Helium ion micrographs of uncoated leaves of *Pinus rigida*. (A) Needle leaf. Stomata are positioned in longitudinal rows on the leaf surface (arrows). Bar=20 μm. (B) Stoma. Arrowheads indicate epicuticular waxes on the leaf surface. Bar=10 μm. (C) Higher magnification of a stoma. Epicuticular waxes (arrowheads) in the epistomatal chamber could be discerned. Bar=2 μm. (D) Epicuticular waxes. Tubules are rod-shaped and straight. Bar=500 nm.

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