利用电子探针技术观测果实组织钙原位分布

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摘要：【目的】矿质营养元素的组织分布可以反映元素的运输和利用特点，本文以钙元素为例，介绍一种利用电子探针结合图像处理的精确直观展示元素组织原位分布的分析方法。【材料】以成熟期‘怀枝’荔枝和‘砂糖橘’柑橘的果柄和果皮，及‘次郎’柿的果柄为材料，将真空下镀铂的果柄或果皮薄片在扫描电子显微镜下采集二次电子图像，同时用波普仪采集钙 X-射线信号分布图像。用 Photoshop 图像处理软件提取钙分布点阵图，将白色光点改为红色光点，将之与二次电子图像合并。【结果】合并的图像可以精确直观显示钙组织原位分布，发现果柄中韧皮部钙含量明显高于木质部，韧皮部中纤维细胞含钙低，而薄壁细胞含量高，并有大量的薄壁细胞内含有富钙颗粒（草酸钙结晶）。荔枝果皮的表皮含钙高，也有钙富集细胞，而柑橘果皮钙分布相对较均匀。【结论】电子探针分析结合图像处理是精确分析植物组织元素原位分布有效的手段。从果柄钙原位分布的结果看，钙有可能是通过韧皮部向果实运输。

关键词：钙，原位分布，电子探针微区分析，图像处理，果柄

Application of Electron Probe to the Observation of *in situ* Calcium Distribution in Fruit Tissues

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Abstract:【Objective】Distribution of mineral nutrition in plant tissues reflects the patterns of nutrient transportation and utilization. Using calcium analysis in fruit tissues as an example, this paper demonstrates a method that combines electron probe microanalysis and image processing technique, which generates a direct review of *in situ* mineral distribution in plant tissues.【Materials】Pedicels and pericarps of mature fruits of litchi cv. Huaizhi and citrus cv. Shatangju and pedicels of persimmon cv. Cilang were used as materials for this study. Tissue slices coated with platinum were

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observed under scanning electron microscope equipped with an electron probe (wave dispersive spectroscopy, WDS). Secondary electron images and Ca-specific X-ray signal mapping images (Ca mapping image) were separately collected. The map of bright dots representing abundance of Ca were extracted using image processing Photoshop and combined with the secondary electron image.【Results】The combined image provided direct review of detailed *in situ* Ca distribution in fruit tissues. In the pedicels of all the tree fruit species, Ca abundance in the phloem is significantly greater than in the xylem. Within the phloem, fiber cells have the lowest Ca content, while the parenchyma cells contain higher calcium. Many of these cells have Ca-rich bodies (calcium oxalate crystals). The epiderm of litchi pericarp has high calcium and also contain Ca-rich bodies. Pericarp of citrus has a relatively even Ca distribution.【Conclusion】Electron probe combined with image processing technique provides an effective method for observing *in situ* nutrition distribution in plant tissues. According to the distribution pattern of Ca in fruit pedicel, it is suggested that Ca transport to fruit is likely through the phloem instead of xylem.
Keywords： Calcium, *in situ* distribution, electron probe microanalyzer, image processing, pedicel

When a beam of accelerated electrons hits the surface of a specimen, the specimen produces secondary electrons, back scattered electrons and X-rays. The X-rays are produced as the excited electrons return to its stable status. Each element generated its own characteristic X-rays at particular wavelengths, and their intensities are measured to determine concentration of the element. All elements except H, He, and Li can thus be detected. Electron probe microanalysis (EPMA) makes use of electron beams focused on a small area on micron to sub-micron scale of the surface of a specimen using a series of electromagnetic lenses, and the intensity of the characteristic X-rays of target element can be detected by wave dispersive spectrometer (WDS) or energy dispersive spectrometer (EDS). This analytical technique has a high spatial resolution and sensitivity. The electron microprobe can

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function like a scanning electron microscope (SEM) and obtain highly magnified secondary electron images of a sample. Electron probe microanalysis has been widely used in mining, metallurgy, machinery, electronics and biology [1]. It has been applied in *in situ* analyses of nutrients in plants and rhizosphere and in studies of heavy metal toxicology [2].

Calcium is one of the essential minerals in plants, playing three categories of unreplaceable roles: (1) structural role, where calcium participates the construction of cell walls and membranes; (2) signaling role, where calcium serves as a signal involved in plant responses to environmental and developmental cues; (3) ion balancing role, where calcium sequesters and detoxifying oxalate [3,4]. Fruits are terminal organs with succulent tissues and poorly developed vascular system and are thus highly susceptible to calcium deficiency with various symptoms that cause quality loss [5]. There is dispute over the pathway of calcium transport towards fruit. It is generally believed that calcium is fed to fruit via xylem [6]. However, the are authors suggested that calcium transport to fruit depends upon phloem pathway [7]. Distribution of calcium in fruit tissues might reflect the pathway of calcium move-in. EPMA enables *in situ* observation of element distribution in micro regions of plant tissues. We used this technique to observe the changes in Ca distribution pattern in litchi fruit [8,9]. However, the secondary electron image that displays surface structure of the sample and calcium mapping image had to be separately collected, and they did not give a direct view of *in situ* calcium distribution. With the development of digital imaging and image processing techniques, it is possible to combine the images of secondary electron and element mapping, which enables precise location of elements and direct view of element distribution. In this paper, we demonstrate a method that combines electron probe analysis and image processing technique to generate a direct review of *in situ* mineral distribution in plant tissues, using calcium in fruit tissues as an example.

1 Materials and methods

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Mature fruits of litchi cv. Huaizhi and citrus cv. Shatangju taken from the experimental orchard of South China Agricultural University, and persimmon cv. Cilang from Institute of Horticulture, Guizhou Academy of Agricultural Science were used as materials. Pedicels or pericarps were cut into 0.5 mm thick slices, stuck onto a cupper sample stand with electricity-conductive carbon glue, and coated with platinum in a JFC-1600 vacuum auto-coater for 90s. The structure and calcium distribution were observed with a JXA-8100 electron probe microanalyzer. During WDS analysis, working distance of all samples was kept at 11 mm, with a probe accelerating voltage of 20 kv and an exciting current of 2×10^{-8} A. Under a magnification of over 100, secondary electron image of the samples and map image of calcium-characteristic X-ray signal (calcium mapping image) were separately collected. Intensity of calcium-characteristic X-ray signal which reflects calcium abundance was displayed by density of bright dots. We used Adobe Photoshop CS5 to extract the bright dot map, change it into red color, and combine the red dot map with the secondary image to generate a direct view of *in situ* calcium distribution in the fruit tissues.

2 Results and Analysis

Under a magnification of 100-150, the structures of pedicels of litchi, citrus and persimmon are clearly displayed by their secondary images (Plate I-1~3). Important structures in the pedicel include the pith, xylem, phloem and cortex. No periderm was observed in the three species. Xylem cells are highly lignified with scattered vessel elements, which is distinct from the phloem with dense and mostly thin-walled cells. The pith and cortex have enlarged cells, which are less dense than the xylem and phloem. The pedicel of persimmon has relatively denser xylem with smaller vessels than those of the other two species, whereas litchi has the largest vessels. Calcium mapping (Plate I-4~6) displayed an uneven distribution of the element in the pedicel. By visual comparison of the secondary electron image and calcium mapping image, it can roughly seen that calcium abundance is greater in the outer regions (phloem and

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cortex) than in the inner regions (xylem and pith) in the three fruit species. In the outer regions, there are many calcium-rich bodies.

However, because the secondary image and calcium mapping image are separate, it is hard to carry out detailed localization of calcium with naked eyes. Therefore, we used Adobe Photoshop CS5 to extract the bright dots map of calcium distribution, change it into red color (Plate I-7~9) and combine it with the secondary electron image. The *in situ* calcium distribution image (Plate I-10~12) thus generated gives a direct view of calcium distribution pattern and detailed location of the element in the pedicel. Few calcium-rich bodies are seen in the xylem but many in the phloem and cortex. These calcium-rich bodies are likely crystals of calcium oxalate. Under a magnification of 500-700, detailed calcium distribution patterns in the phloem of litchi (Plate II-1) and citrus (Plate II-2) pedicel show that the fiber cells in the phloem has the lowest calcium level, while the parenchyma cells surrounding the fiber bundle have higher calcium levels. Many of the cells contained calcium-rich bodies (calcium oxalate crystals). We used the same methods to observe calcium distribution in the pericarp of litchi (Plate II-3) and citrus (Plate II-4). Epiderm of litchi pericarp has rich calcium with high frequency of cells containing calcium-rich bodies (Plate II-3). Similar pattern was also reported in apple [10]. Calcium distribution in citrus pericarp (Plate II-4) is relatively even with sparse calcium-rich bodies.

The above results show that calcium abundance is significantly greater in the phloem than in xylem in the pedicels of litchi, citrus and persimmon, indicating that phloem rather than the xylem might be the major pathway of calcium transport to these fruits.

3 Conclusions

The sample processing for electron probe microanalysis is simple and fast. Combining secondary electron image with calcium mapping image generates direct view of *in situ* calcium distribution in plant tissues and enables detailed localization of the mineral. Calcium distribution in pedicels of three fruit species revealed that the

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phloem contains significantly higher calcium than the xylem. This findings suggests that the major pathway for fruit calcium uptake is likely the phloem. This suggestion needs further experimental proofs.

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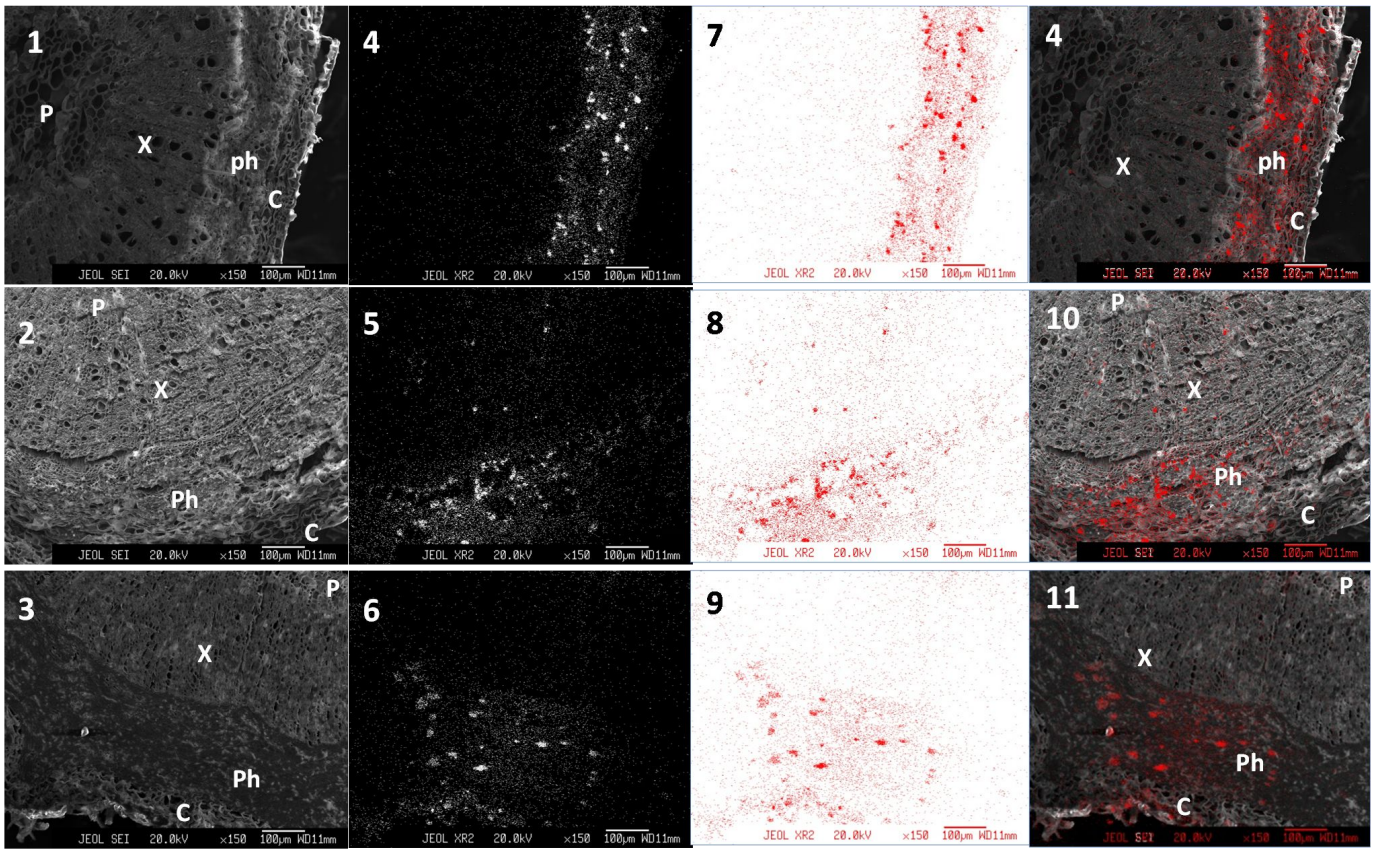

Explanation of plates

Plate Ⅰ： 1-3 show secondary image of the pedicels litchi, citrus and persimmon respectively; 4-6 are calcium-characteristic X-ray signal mapping (calcium mapping) images collected by WDS in the pedicel of litchi, citrus and persimmon respectively; 7-9 are the dot maps of calcium extracted and changed into red color by photoshop; 10-12 are the combined images of secondary electron and dot map of calcium of litchi, citrus and persimmon, respectively. P: Pith; X: xylem; Ph: phloem; C: Cortex.

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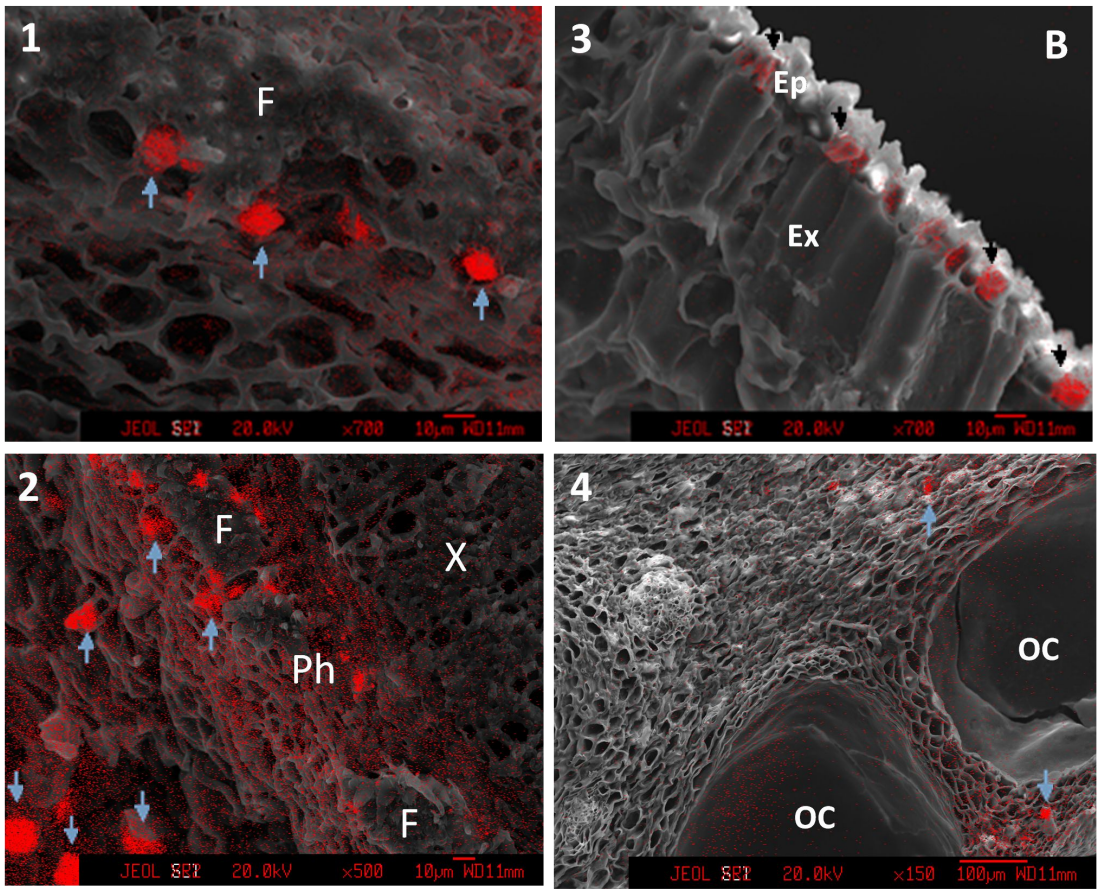

Plate II： 1 *in situ* calcium distribution image in the phloem of litchi pedicel; 2 *in situ* calcium distribution image in the phloem of citrus pedicel; 3 *in situ* calcium distribution image of litchi pericarp; 4 *in situ* calcium distribution image of citrus pericarp. Arrows indicate calcium-rich bodies; Ph: Phloem, X: Xylem; F: Fiber; Ep: epiderm; Ex: exocarp; OC: Oil chamber.

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