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# Rapid Sample Preparation of Plant Leaves for Scanning Electron Microscopy

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Scanning electron microscopy (SEM) is widely used to study the morphology and physiology of plant leaves. In order to be imaged in the SEM, samples have to withstand the vacuum conditions and the energy of the beam [1,2]. The most widely used protocol for plant sample preparation includes chemical fixation, dehydration with alcohol, critical point drying, and sputter coating, takes about one day, and is very labor intensive. Leaves can also be imaged at low vacuum conditions or at low temperatures after cryo-fixation which do not require the above described steps. Artifacts such as shrinkage at low vacuum and cracking of the surface of cryo-fixed cells are very common [1,3]. Additionally, cryo-fixation requires a high level of skill and expensive equipment, which limits its application. The aim of this study was to develop faster and more reliable methods of leaf sample preparation for analysis in the SEM. We tested the hypothesis that microwave irradiation during sample preparation and the production of leaf replicas with dental putties would result in shorter sample preparation time and well-preserved surface structures.

Conventional fixation of Nicotiana leaves was performed at room temperature according to Table 1 by fixing samples with 2.5% glutaraldehyde and 1 % Osmium, rinsing them with phosphate buffer (0.06M and pH 7.2), and dehydrating the specimen with ethanol. Microwave-assisted sample preparation was performed by applying 15 to 20 W microwave irradiation during sample preparation (Leica EM AMW, Leica Microsystems, Wetzlar, Germany). Samples were then critical point dried (Leica CPD 300), mounted on aluminum stubs, and sputter coated with 15 nm of iridium (Leica ACE 600). Leaf replicas were made by applying dental putty to the leaf surface for 5 minutes. The putty was then pulled off and mounted on aluminum stubs. Samples were imaged either in low vacuum mode with a Hitachi TM3030Plus table-top SEM (Hitachi High Technologies America, IL, USA) or with a FEI Versa 3D SEM (FEI, Hillsboro, OR, USA) in high vacuum mode.

The use of microwave irradiation strongly reduced sample preparation time from about 8 to 2h (Table 1). The surface of leaves was well preserved and comparable to those prepared conventionally without microwave irradiation (Figure 2 a & b). Stomatal and epidermal cells could be clearly distinguished (Figure 2 a & b). A similar situation was found on leaf replicas (Figure 2 c). Samples prepared conventionally and with the help of microwave irradiation showed signs of shrinkage in the form of wavelet structures which were especially evident around stomatal cells (Figure 2 d). Shrinkage was not observed on leaf replicas which showed a smooth surface (Figure 2 e) similar to what has been reported in cryo-fixed leaves [1,3]. All samples showed high stability when exposed to the electron beam. Cracking and hole formation as described for cryo-fixed leaves at low temperature conditions [1,3] were not observed in the SEM.

Summing up, the results of this study demonstrate that both microwave-assisted sample preparation and the production of leaf replicas with dental putty drastically decreased the sample preparation time of leaves for SEM investigations when compared to conventionally prepared samples. While conventional and microwave-assisted sample preparation induced shrinking, such effects could not be observed on the replicas. The major advantages of making dental putty replicas are that it is inexpensive, simple to apply, can be performed in the field, and that results can be obtained in a few minutes.

| | Conventional | Microwave | Dental Putty |
|---|---|---|---|
| Curing | | | 5 min |
| Primary Fixation | 90 min | 8 min | |
| Buffer Washes | 60 min | 2 min | |
| Secondary Fixation | 90 min | 12 min | |
| Buffer Washes | 40 min | 3 min | |
| Dehydration | 120 min | 7 min | |
| Critical Point Drying | 80 min | 80 min | |
| Sputter Coating | 15 min | 15 min | 15 min |
| **Total** | **495 min** | **127 min** | **20 min** |

**Figure 1.** Table 1: Time needed to prepare Nicotiana tabacum leaves conventionally, with microwave irradiation, and with dental putty.

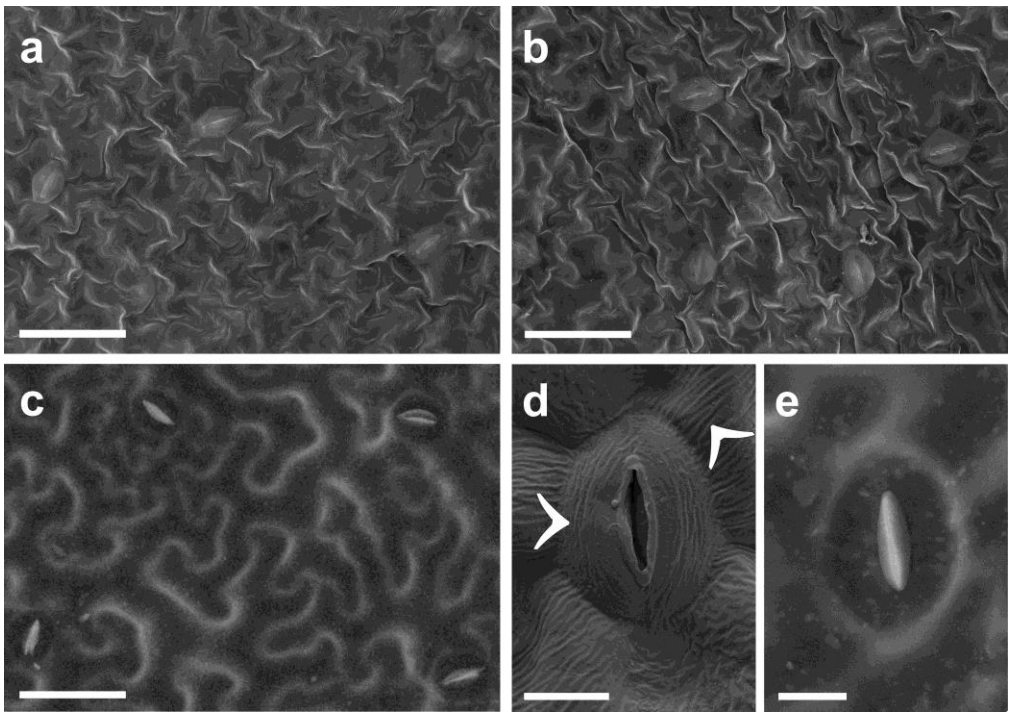


**Figure 2.** SEM micrographs showing the surface of plant leaves. (a, b, c) Overview of the surface of leaves and (d, e) close-up of a stomatal complex. Samples were prepared (a) conventionally, (b, d) with the help of microwave irradiation, and (c, e) by making replicas with dental putty. Cells prepared conventionally and with the help of microwave irradiation showed signs of shrinkage in the form of wave-let structures around stomatal cells (arrows in d). Images (a), (b), and (d) were taken with a Versa 3D SEM at (a, b) 30 kV and (d) 5 kV while images (c) and (e) were taken with a TM-3030Plus at 15kV. Bars= 50 µm in (a), (b), and (c), and 10 µm in (d), and (e).

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