Wolffia brasiliensis anatomy is revealed using a simple Microscope Press

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Summary
Wolffia sp. are approximately one millimeter long when fully developed and are best viewed with a microscope. A rapid and facile technique for the study of *Wolffia* taxonomic features, without embedding or sectioning, is presented; plant material is compressed to a few planes of focus by slow drying under tension, using a slide and cover slip. The force experienced by sample tissues is moderate, as determined using patterns of optical interference. This method is particularly useful for surveys of anatomical features and numerical assessment of features which are otherwise difficult to quantify. Critical taxonomic features of *Wolffia* including stomata number and presence of dorsal papillae may be rapidly determined. In addition, features that are generally not observed with other methods are readily apparent, such as large substomatal air pockets.

Keywords: *Wolffia brasiliensis*, Newton's Rings, interference, stomata.

Introduction

Wolffia sp. are the smallest plants known with densities of between 1 and 2 million plants per square metre [3] and their longest dimension is approximately one millimeter when fully grown. Therefore, their anatomy and taxonomic features must be studied using a microscope. However, *Wolffia* are comparatively thick and roughly spherical i.e. single fields of view have several planes of focus. This makes for slow progress when studying field isolates of this genus. Also, the critical feature of *Wolffia brasiliensis*, a large but transparent dorsal papillum, is difficult to observe because it may not be focused on all at once [5 and Landolt 2008 personal communication].

Previous studies of *Wolffia* anatomy and taxonomy have depended on time consuming examination of individual plants, and comparison to model images of specimens carefully prepared for the Microtome and Electron [1] or Laser Scanning Confocal microscope [4]. Aside from prohibitive expense, these methods are unsuitable for studies that depend on processing large numbers of specimens and statistical analysis of significant datasets.

Slow and even drying under compression puts many anatomical features in the same focal plane and allows formation of prominent air bubbles in substomatal cavities and between parenchyma cells. This makes features essential for taxonomy prominent and easy to score, for example stomata density and dorsal papillae, which are used to distinguish between the five species most common in North America [2]. In addition, substomatal cavities which are essential for rising and sinking in the water column in response to physiological functions [Witty M, manuscript submitted] are revealed. Compression may be achieved using a Microscope Press, made for each sample from a slide and cover slip. This method produces images of high enough quality for use in for rapid surveys of tens of individuals per hour rather than intensive study of single individuals over tens of hours using embedding and microtome techniques.

Materials and Methods

To compare methods for viewing *Wolffia* environmental samples, live specimens were wet mounted under a cover slip (Figure 1A). These were difficult to examine because of their roughly spherical shape which makes examination of, for example, stomata numbers difficult because they may not be focused upon simultaneously and easily counted. Methods of simple pressing, by applying pressure to the cover slip, or drying did not result in enhanced clarity of these features (Figures 1B and 1C). However, a process of overnight drying under a coverslip was remarkably useful because of the slow exertion of force. This is described below and illustrated in Figures 1D and 2.

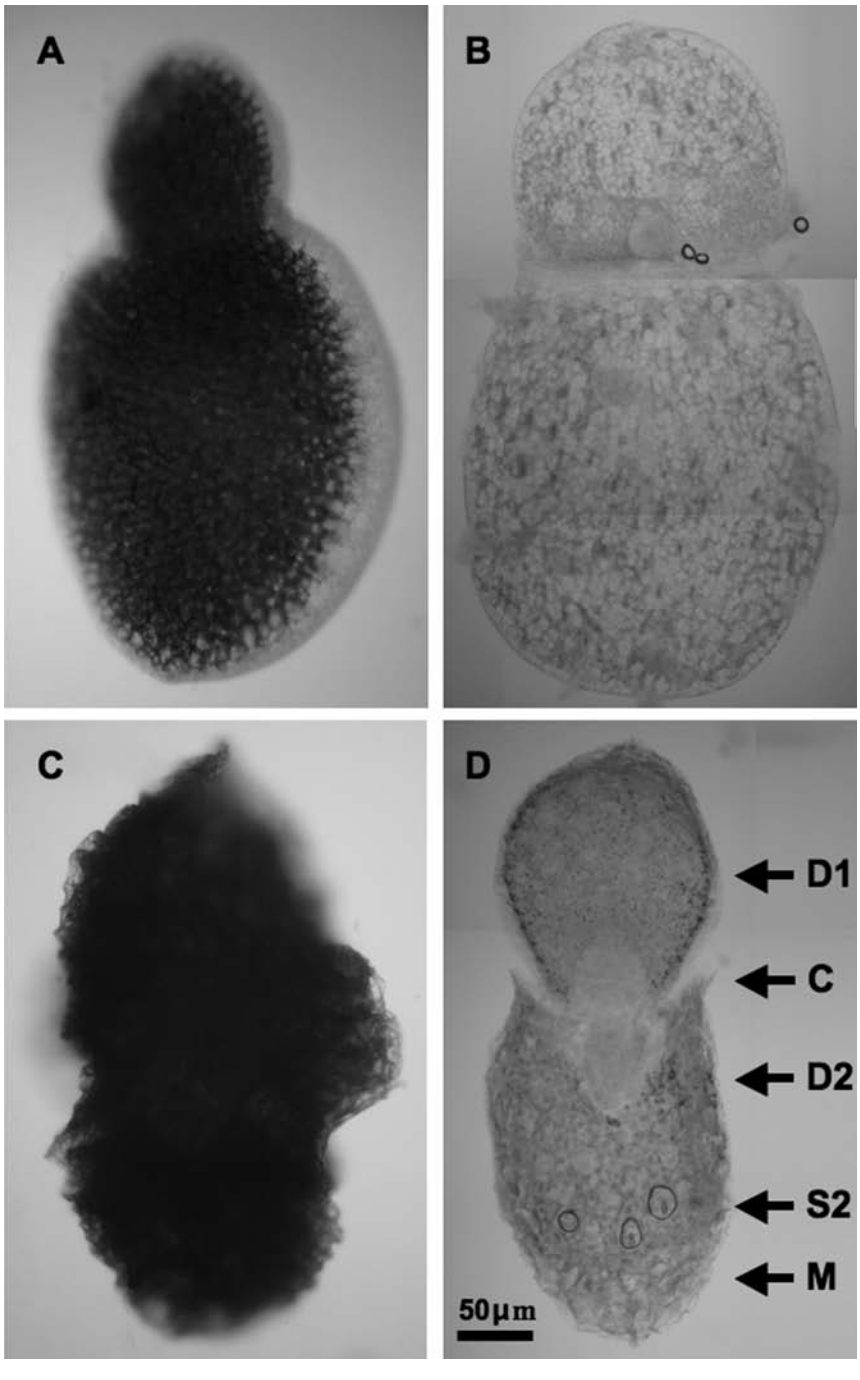

Figure 1. *Wolffia* observed under a range of pressing and drying conditions. A; live, side view, showing poor focus on most cells. B; flattened by application of thumb pressure to the coverslip. C; dried overnight. D; treated with the Microscope Press. Features seen were the mother plant (M) and two generations of daughter plant (D1 and D2), the collar around the reproductive pouch (C) and stomata with large substomatal cavities (S2). Composites of several images were used.

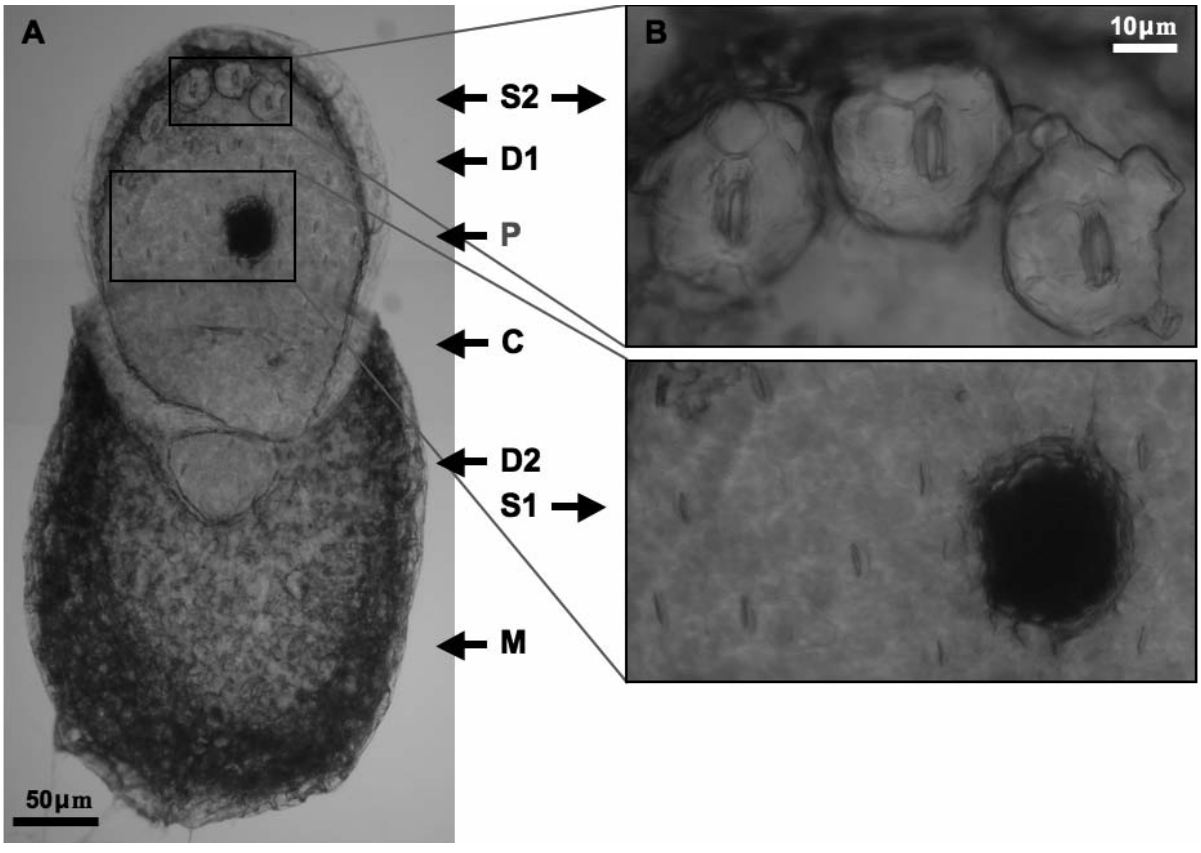

Figure 2. *Wolffia brasiliensis.* Specimen treated with the Microscope Press. A; whole plant. B; stomata with large substomatal cavities. C; conventional stomata and dorsal papillum. Features seen were the mother plant (M) and two generations of daughter plant (D1 and D2), the collar around the reproductive pouch (C), conventional stomata (S1), stomata with large substomatal cavities (S2) and dorsal papilla (P) compressed to a shadow. Composites of several images were used.

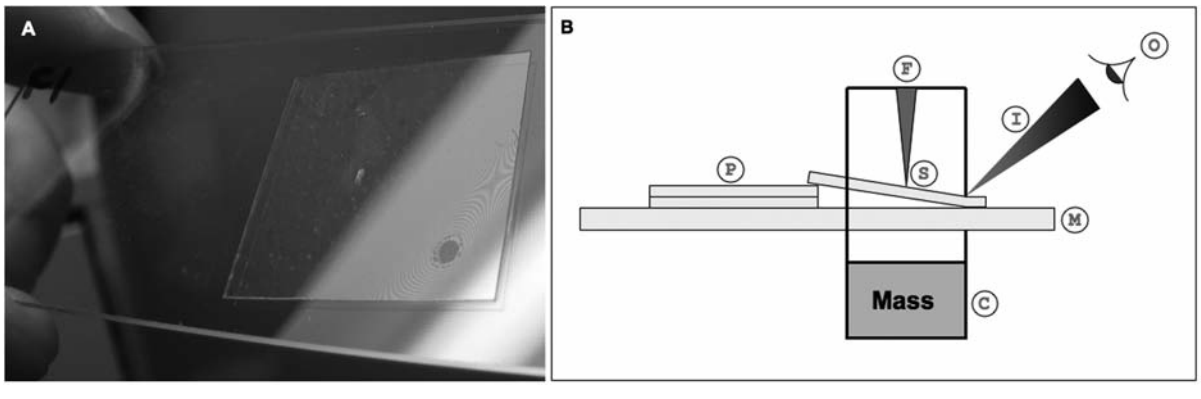

Figure 3. A; Microscope Press showing Newton's Rings. B; Apparatus for applying force to a cover slip. Microscope slide (M). Cover slip (S). Platform made of two cover slips (P). Cradle (C). Fulcrum for cradle (F) made from a razor blade. Interference pattern (I). Observer (O). The center of contact between coverslip and slide is a central zone of no interference patterns [6].

To dry *Wolffia* samples in a Microscope Press, single live plants in a drop of pond water were place on a microscope slide and a coverslip applied. Only one individual plantlet can be used per coverslip because only one fulcrum is desired. Plant orientation was observed under low power, i.e. dorsal or ventral. Water was added until liquid was in contact with the entire coverslip underside. A soft tissue was used to blot away excess liquid. This mount was allowed to dry for eight hours or overnight. Optical interference patterns, Newton's Rings, [9] show that forces between the two glass surfaces had placed the coverslip under tension and distorted it from a planar shape (Figure 3A). Pressed material was observed under bright field using a Nikon YS2-H microscope and classified by consulting The Jepson Manual [2]. Large air pockets in dried material were revealed by the increased refraction of air: water or air:cell interfaces.

Interference patterns visible by eye are a conspicuous feature of the method and show that the cover slip is under torsion and is bending slightly. However it is difficult to estimate the magnitude of force by simple examination of ring pattern [7]. To estimate the force applied, a simple apparatus was designed as shown in Figure 3B; a cover slip was supported by a platform made from two coverslips, whose height approximated that of a pressed *Wolffia* plant. This coverslip was pressed by a blade bearing a cradle which could be loaded with increasing weights (pennies) until the coverslip bent and displayed interference patterns similar to those in Figure 3A.

Results

Figure 1 A shows that *Wolffia columbiana* is difficult to examine. Simply squashing it improves views of stomata but soft tissues and cell sap are extruded, causing confusion. Drying decreases clarity but slow drying and pressing gives all the improvement seen in figure 1D and other advantages. Slow pressed material shows stomata clearly, reveals the maternal collar and at least two generations of developing daughter plants. The most important advantage of this method was the discovery of large substomatal cavities which are a previously unreported anatomical feature of *Wolffia* (Figure 2). This is important because substomatal cavities were not made prominent by embedding in paraffin and sectioning (*data not shown*); they are large features that must to be seen whole to be appreciated.

Wolffia brasiliensis have prominent a dorsal papilla, which is the critical taxonomic character for this species, but this feature is difficult to see because it is rarely in one plane of focus and is transparent. During the pressing process these papillae are transformed from transparent objects to dark shadows making them easy to identify (Figure 2A).

The Microscope Press apparatus shows prominent interference patterns (Figure 3A, Newton's Rings) and this feature was used to determine the approximate magnitude of force applied to samples. The apparatus described in figure 3B was assembled and a mass of 43.76 g was added to the cradle before interference patterns were seen, which corresponds to a force of 0.43N. This shows that for a *Wolffia* plant with radius 0.5 mm a slow increase of pressure from 0 to 273 Pa over eight hours would be experienced.

Discussion

Herbaria have used presses to preserve large specimens for anatomical and taxonomical study for hundreds of years [8]. These specimens often do not preserve three dimensional structures but are nevertheless valuable for observing overall gross anatomy and surface details. Whole plants or detached organs are usually preserved. The method reported is a similar facile method that has been used in this laboratory to screen samples of forty microscopic plants in one day with ease. Surface features were forced into one plane of focus for rapid enumeration and clearer photography.

The appearance of Newton's Rings is a useful visual indication that the method is being used properly and pressure is being applied to the sample. Initially the coverslip is bonded to the microscope slide by the hydrogen bonds of water, but later by bonds acting more directly between the two glass surfaces. In common with herbarium procedures, this Microscope Press method is a simple but revealing way of making permanent dried specimens of *Wolffia* species. It is a rapid way of producing materials of high enough quality for assessment of essential features for taxonomy such as stomata and dorsal papillae. In addition, new anatomical features may be discovered, such as the large substomatal cavities clearly revealed in this procedure for the first time.

References

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