Nota Técnica (Technical Note)

Comparison of microscopy techniques to visualize powdery mildew (Erysiphales) conidiophores

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Abstract

Fast techniques allowing a clear view of fungal structures, specially conidiophores, are important for accurate diagnosis and identification at the genus level of powdery mildew fungi. In this study we propose a new technique for removing cuticles of leaves with a colorless nail base (cosmetic-based cellulose acetate) and transparent adhesive tape for viewing conidiophores of powdery mildew on papaya (*Carica papaya*), papayuelo (*Vasconcellea goudoutiana*), and yellow ipe (*Tabebuia serratifolia*) leaves by light microscopy. For comparison, the traditional technique of discoloration (diaphanization) of the leaves was also tested. Phase and interference contrast microscopy and liquid montant with stains were used to improve the optical contrast. The cuticle removal technique showed to be simple and easy to use, showing more clearly the conidiophores origin and the leaf topology thus evidencing that it can be used as an auxiliary routine technique for the identification of powdery mildew fungi

Additional keywords: diagnosis; diaphanization; identification; visualization technique.

Powdery mildew (Erysiphales, Ascomycota) are important obligate parasite fungi of angiosperms, able to infect approximately 10,000 species (BRAUN & COOK, 2012). Both from a practical point-of-view as for basic research involving Erysiphales it is essential the correct diagnosis and identification at the genus level. For routine work, the morphological identification of fungi structures by light microscopy is still the quickest, cheapest, and more efficient method.

To distinguish the genera of Erysiphales, *Streptopodium* sp. and *Ovulariopsis* sp. it is necessary to clearly visualize the origin of the conidiophores (STADNIK & RIVIERA, 2001). In papaya crop in Brazil several species of powdery mildew occur which differ by the morphology of conidia and conidiophores and also the origin of the conidiophores (LIBERATO et al., 2004). Two species of powdery mildew that occur in Caricaceae in Brazil, *Streptopodium caricae* Liberato & R.W. Barreto in papaya plantation (LIBERATO et al., 2004) and *Oidiopsis haplophylii* (H. Magn.) Rulamort reported attacking papayuelo (*Vasconcellea goudoutiana* Triana & Planch) in greenhouse (VIVAS et al., 2010) both present similar morphology of conidia The conidiophores of *S. caricae* are originated from the superficial mycelium while those of *O. haplophylli* usually originate from internal mycelium and emerge through stomata (LIBERATO et al., 2004). So, fast techniques allowing a clear view of the origin of conidiophores are important for accurate diagnosis and identification at the genus level of powdery mildew on Caricacaceae.

This study aimed to describe and evaluate an adapted fast technique for visualization and identification of anamorphs genera of Erysiphales based on cuticular printing (whole mounting) which is used most commonly for histopathological studies and to compare it to the diafanization (removal of pigments by

chemical methods) used in anatomical studies and recently described for visualization of the origin of conidiophores in Erysiphales (LIBERATO et al., 2005).

For this purpose, we used leaves from plants of *C. papaya* and *V. goudoutiana* grown in greenhouse, all of them showing powdery mildew symptoms and signals. All plants came from the Northern Rio de Janeiro State University ï UENF. To evaluate the application of the technique to other plant species, we tested the new technique also in yellow ipe (*Tabebuia serratifolia*) leaves with powdery mildew signs and symptoms on the abaxial surface, previously identified as *Streptopodium tabebuiae* (LIBERATO & BARRETO, 2005). All fungal structures observed on the abaxial surface were used to assess the quality of the slides preparation.

The technique of removing the cuticle (CTR) with the fungal structures was conducted by the application of colorless base nail varnish (nail base) composed of cellulose acetate dissolved in acetone (colorless, trade mark ñQ Tockò) onto the leaves abaxial surface followed by drying on a stand for 10 min. After drying, a transparent adhesive tape (Scotch tape, trade mark ñDurexò) was pasted over the enameled leaf surface. After slight pressure between fingers, the tape was removed from the leaf containing the enameled cuticle of the leaf with conidiophores of the fungus. The tape with the cuticle was then inverted as a coverslip on a microscope slide using lactophenol as mountant (fixation) liquid. For contrast, we also tested lactic acid and 85% cotton blue (1 g L^{-1}) as mountant liquid. This technique is similar to the one proposed by Hosagordar & Kapoor (1985) although CRT may be considered another form of visualizing fungal structures with more resolution.

The diaphanization technique (DT) was described in LIBERATO et al. (2005). Leaf fragments with powdery mildew signs were immersed in a solution prepared with 50 g of chloral hydrate in 20 ml of distilled water and left for 1 hour at 60 °C in a water bath and then mounted on slides with 85% lactic acid and aniline blue (1 g L^{-1}). From the discolored leaf fragments, blades were mounted with lactic acid and blue cotton. During the examination of the slides, the abundance of surface mycelium and specially the origin of the conidiophores were evaluated with focus on the observation if they came out of the stomata or not as well as the morphology of conidia and conidiophores. Additionally micrometric measurements were made under magnification of 400X with the help of willing graduated micrometer scale in the eyepiece of the microscope (Nikon®, model Eclipse E400) to confrontation with the literature for confirmation of the studied species. The slides of *V. goudoutiana* and *C. papaya* were contrasted in a light interference Olympus microscope. The slides of yellow ipe leaves were photographed in a light microscope without any contrasting microscope technique.

From micrometer measurements of conidia, conidiophores and width of the basal cells, it was possible to confirm the identification of the following specimen used: *Oidiopsis haplophylli* in leaves of *V. goudoutiana* and *Streptopodium caricae* in three samples (different plants and locals) of *C. papaya*.

With the slides made by the cuticle removal technique (CRT) we could clearly distinguish the origin of conidiophores in the studied samples, from superficial mycelium in *S. caricae* and *S. tabebuiae* or emerging through stomata in *O. haplophylli* (Figure 1). Compared with the technique of leaf clearing (DT) and staining described by LIBERATO et al. (2005b) the cuticle removal technique has the advantage of showing with good accuracy what kind of Erysiphales is present in the leaf. However, the new technique was poor in the extracting of mycelium and conidia once it does not show the actual amount of conidiophores on the leaf as it was seen by leaf clearing technique. As for the extraction and visualization of the pathogen structures, the clearing and staining technique proved a higher efficiency, with the possible observation of a greater amount of the fungal structures (conidiophores and conidia).

CRT showed that during the removal of enamel with the tape there was some breakdown in hyphae and conidia released from conidiophores is not keeping them intact. On the other hand, the application of the enamel and the pressure exerted by the tape makes the structures to remain in the same focal plane, facilitating the capture of planar images. Moreover, one sees clearly the topography and morphology of the leaf surface.

The leaf discoloration was an excellent method to determine the shape and size of conidia and conidiophores. However, to take photographs there was difficulty in getting a proper focus to see that same image on a leaf surface and conidiophores together in the same focal plane (Figure 2). Contrary to the prior technique, the sharpness of images is compromised thus making impossible to get clear images of cuticle topology.

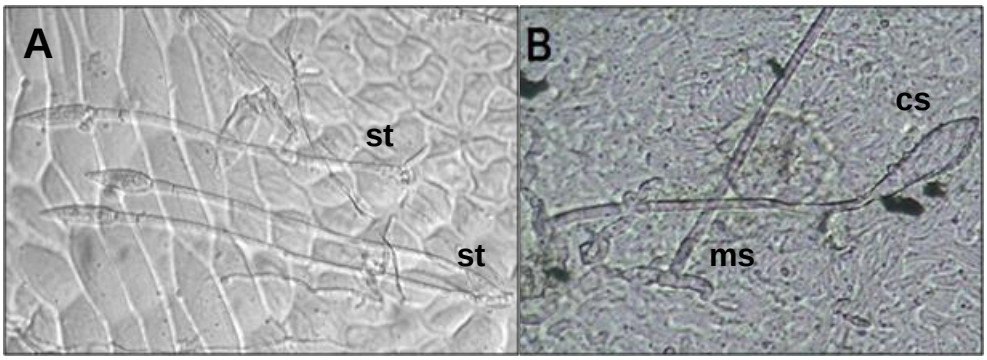

Figure 1 - Powdery mildew conidiophores in the host plant cuticle. A ï *Oidiopsis haplophylli* conidiophores emerging through stomata (st) of leaves of *V. goudotiana.* B ï *Streptopodium tabebuiae* conidiophores emerging from superficial mycelium (ms) and conidia (cs) on leaves of *T. serratifolia.*

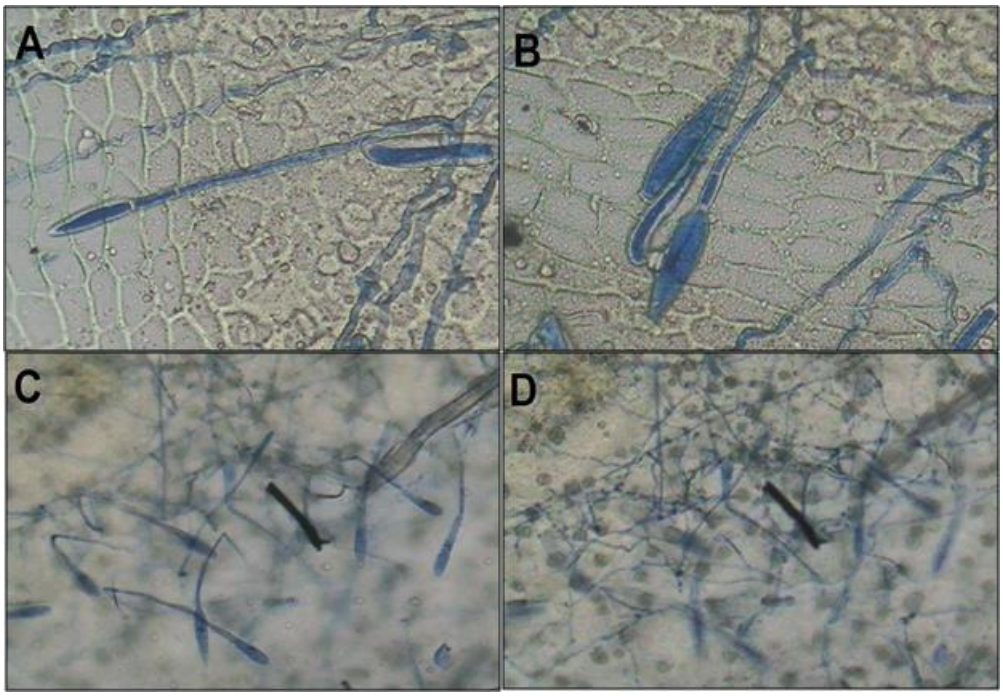

Figure 2 - *Streptopodium caricae* conidiophores in papaya leaves as seen from a light microscope. In A and B, the technique used was the cuticle removal using nail base and tape by which it can be observed the cuticle surface and conidiophores at the same plane. In C and D the slides were made by the leaf clearing technique by which large amounts of conidiophores and conidia in different focal planes are observed. In C the focus was set at the apex of conidiophores and in D on the basis of them.

As to speed and safety in diagnosis, the tested techniques were effective because, in possession of fresh material, we could immediately identify the fungus genus. Additionally, the technique of removing enamel with cuticle is more practical and faster in addition to avoiding the use of toxic reagents such as chloral hydrate. When used together with the phase contrast or interference contrast microscopy it dispenses with the use of dyes because the layers of cuticle, nail and tape are very translucent. However, even under light microscopy the visualization of the structures was not compromised (Figures 3 and 4).

For the contrast made with only the dye solution, it was observed that when the tapes with nail were glued on slides containing the dye solution instead of lactophenol, the result was not completely satisfactory, since there was no efficiency in staining the hyphae and conidiophores, which were not totally colored. An efficient way to colored all conidiophores and hyphae has not been found by CRT technique and it is recommended for the purpose of the photographic record phase contrast and interference contrast microscopy techniques.

The new technique has an additional advantage compared to that reported by LIBERATO et al. (2005), the removing sharp leaf cuticle, once we could visualize perfectly stomata, trichomes and glands and all the

cuticular topology can be seen in detail and with great clarity (Figures 1 A, 3 A to D and 4).

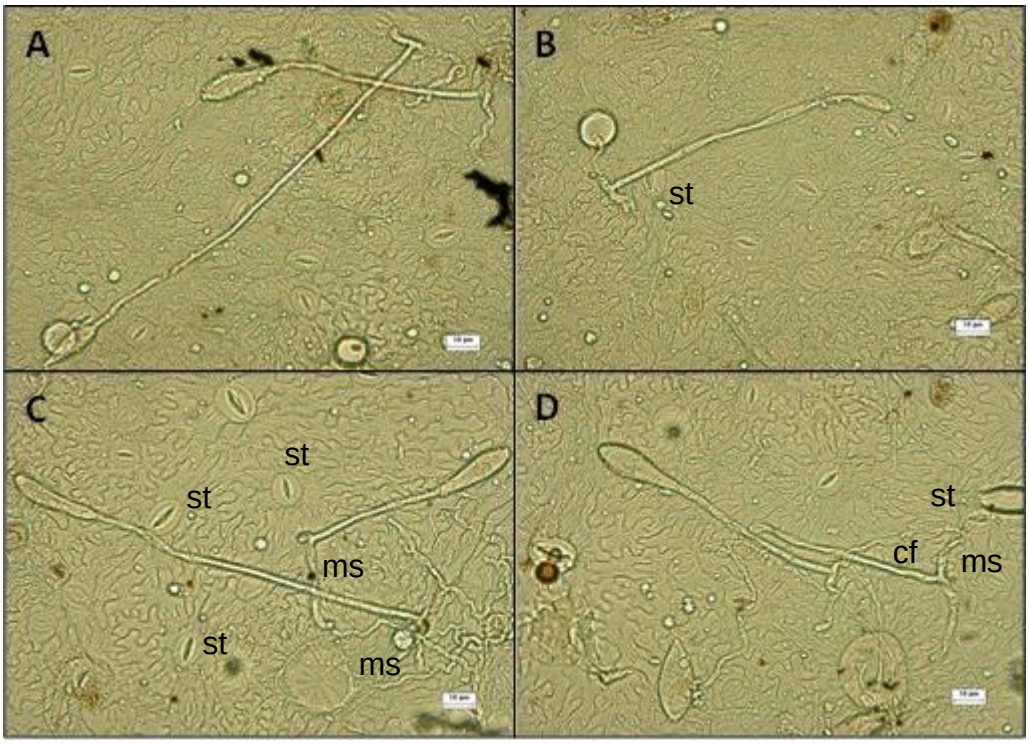

Figure 3 - Powdery mildew conidiophores on the abaxial leaf cuticle of yellow ipe leaves. A to D ï slides of *Streptopodium tabebuiae* made by the cuticle removal technique using nail base and tape and optical microscopy with interference contrast. (st) stomata, (ms) superficial mycelium, (cf) conidiophore.

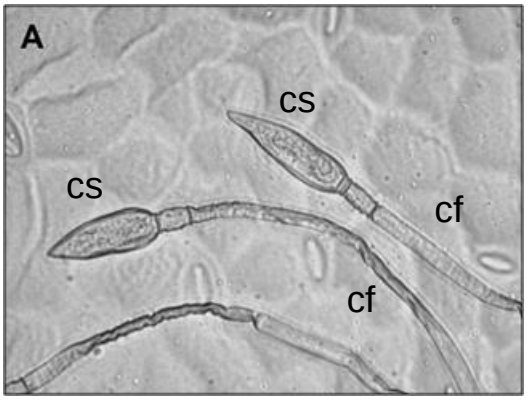

Figure 4 - Conidiophores of *Oidiopsis haplophylli* on the abaxial leaf cuticle of *Vasconcellea goudoutiana.* Lanceolate primary conidia (cs) on conidiophores (cf) (A) and conidiophores emerging through stomata (st). Slides made by the removal technique with nail base and tape. Image generated by interference contrast microscopy.

Finally, it is concluded that the cuticular removal technique using nail base and tape proved to be fast and efficient, allowing the visualization of the conidiophores origin, as well as the morphology of conidia, which are important for identifying Erysiphales and, hence, can replace to technique of leaf discoloration. The new technique developed should be tested in herborized materials and others hosts with hairy leaves, which also are widely used in classic taxonomic studies.

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