Histological analyses of the fungal endophytes in *Rosa hybrida*

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Summary Endophytes are fungi that cause asymptomatic infections in leaves and stems of healthy plants. This study evaluated the presence of endophytic fungi on leaves of *Rosa hybrida* collected in Bogota, (Colombia) using histological techniques and light microscopy. Histological preparations showed fungal mycelium with few cells in the vascular tissue. Colonization of mesophyll cells was not observed. Visualization of fungal cells within plant tissues is a confirmatory test of the endophytic habitat of fungi isolated from leaves of *Rosa hybrida*.

Key words *Rosa hybrida*, Endophytic fungi, Histological analysis

Análisis histológico de hongos endófitos en *Rosa hybrida*

Resumen Los hongos endófitos forman infecciones asintomáticas dentro de hojas y tallos de plantas sanas. Este estudio evaluó la presencia de hongos endófitos en hojas de *Rosa hybrida* colectadas en Bogotá (Colombia) mediante el uso de técnicas histológicas y microscopia de luz. Las preparaciones histológicas muestran micelio fúngico con pocas células ubicadas dentro de los tejidos vasculares. No se observó colonización de las células del mesófilo.
La visualización de células fúngicas dentro de los tejidos de la planta es una prueba confirmatoria del hábitat endofítico de los hongos aislados de hojas de *Rosa hybrida*.

Palabras clave *Rosa hybrida*, Hongos endófitos, Análisis histológico

The term endophyte was first used by De Bary in 1886 to describe microbes that reside inside plants. The definition was subsequently limited to organisms with certain biological characteristics. Currently, the term endophyte applies only to microorganisms able to reside inside plant tissues without causing any external disease symptoms [6,18]. Over the last few years there has been an increasing interest on endophytic fungi as a source of novel bioactive compounds potentially useful in medicine, agriculture, and the industry [19,20,21,22].

Histological techniques for light or electron microscopy have been used to study structural characteristics of endophytes, the infection process, and the interaction with the host plant [2,17]. Studies including the visualization of endophytic fungi have been carried out mainly in tropical grasses and the Pinaceae [1,2,16,17,24,25] using fresh tissue or electron microscopy techniques. Only few studies have reported the use of histological techniques to demonstrate endophytic colonization of host tissues, despite recommendations encouraging its use [3,5,7,12,18]. In a previous study we reported the leaf tissue from roses collected in Bogotá, Colombia as a common habitat for endophytic fungi [14]. The objective of the present study was to demonstrate the presence of fungal mycelium in leaf tissue of *Rosa hybrida* using histological techniques.

Leaves of *Rosa hybrida* were collected from five urban zones in Bogota D.C. Twenty-two plants were chosen and six healthy mature leaves were sampled per plant. From each leaf, 20 pieces of 5 x 5 mm were taken. Leaf pieces were then fixed in formalin aceto-alcohol (FAA: 90 ml of 70% ethanol, 5 ml of formalin, 5 ml of glacial acetic acid) for 48 h and then dehydrated using an ethanol series. Pieces were embedded in Histosec (Merck) and serial sections were cut at 10 µm in a base sledge microtome (Leitz Wetzlar) and mounted on clean slides. Sections were stained with the Pianese IIIB Stain technique [7,10,11,26]. The composition of the stain used was naphthol yellow 0.1 g, malachite green 0.5 g, fuchsin acid 0.1 g, distilled water 150 ml, and 95% ethanol 50 ml. Sections were hydrated first in distilled water, then in 95% ethanol, and immersed in the stain for 45 minutes. They were rinsed in distilled water, differentiated in alcohol acid 1 minute, dehydrated using an ethanol series, and mounted with Entellan adhesive (Merck). All sections were examined under light microscope (Zeiss Axioscope).

The Pianesse IIIB stain is a differential technique that facilitates the specific detection of fungal elements within plant tissues. It has been used in many studies

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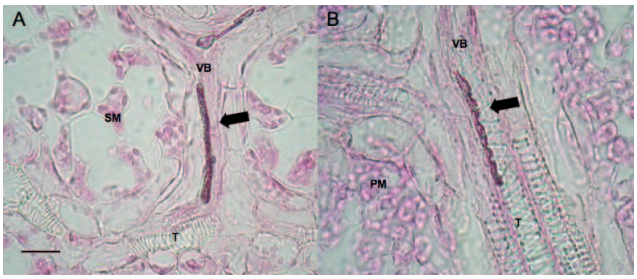

Figure 1. Light micrographs of stained endophytic mycelium inside rose foliar tissue. A, B. Mycelium (arrow) running along the host vascular bundle (VB) x1000. PM: palisade mesophyll, SM: spongy mesophyll, T: tracheids Bars = 10µm.

including pathological, mycorrhizal, and root endophytic fungi differentiation analyses [7,8,27]. Using this technique, plant tissues stain green or light pink and fungal mycelium stains deep pink [11]. Microscopic observations of rose leaves revealed fungal structures of 10-20 mm wide and 2-4 mm long approximately, formed by few cells stained with deep pink, which is in agreement with previous reports (Figure 1) [7,11,26,27]. When detected, endophyte colonization in healthy tissues was restricted to no more than a few cells, which supports previous reports by Stone et al. [18]. We are reporting the presence of endophytes in rose tissues for the first time.

Rosa hybrida leaves showed scarce endophytic colonization. In general, a low percentage of colonization by endophytic fungi (close to 17%) was observed in our previous study, using microbiological techniques [14]. It has been shown that removal of wild plants from their natural forest ecosystem causes a dramatic decrease of endophytic populations [15,23]. Home gardens in urban cities are not the optimal habitat for the plant-microbial interaction. This could account for the low percentage of colonization obtained. As shown in figure 1, fungal structures were observed only among the vascular bundles. Mycelia were not observed in the mesophyll. Based in this finding it can be concluded that endophytic fungi in rose leaves are restricted to the vascular bundles. Vascular bundles has previously been reported as a common habitat for microbial endophytes [15]. This localization could be due to a higher availability of nutrients produced by the plant. The presence of systemic endophytes in plants other than grasses has not yet been demonstrated [13]. However, we have to remember that the technique used in this study does not allow total fungal mycelia detection in the studied leaves.

This study demonstrated that conventional histology and light microscopy techniques used to detecting fungal structures in plant tissues, like Pianese stain technique, are useful tools. These tests can be also used as confirmatory tests to determine the endophytic behaviour of the fungal isolates under investigation.

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