

New staining technique for fungal-infected plant tissues

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Abstract: The use of histological techniques, combined with the use of dyes, facilitates the visualisation of fungi present in tissues, allowing the details of plant-pathogen interactions in many different pathosystems to be revealed. The purpose of this study is to present a new double-staining method that uses cotton blue and safranin to identify fungal structures in plant tissues. With this staining method, the fungal hyphae stain blue and plant cell walls stain orange. The new method was tested on 2 pathosystems. The technique distinguishes between fungal and plant cell walls, giving it great practical utility. This is a staining technique that can be used routinely in laboratories and facilitates the creation of permanent slides.

Key words: Citrus black spot, cotton blue, postbloom fruit drop, safranin, staining

1. Introduction

Fungal diseases are responsible for structural and physiological disorders in a large number of crops, which leads to decreased productivity and economic losses for producers. Structural studies using histological techniques on the mode of penetration and colonisation of plant tissues by fungi indicate in detail the interactions of the pathogen with the plant tissue. These studies reveal important aspects for the understanding of the monocycle of diseases.

Techniques in plant anatomy that are designed to observe and characterise the behaviour of pathogens on their hosts are commonly used. These techniques are used in conjunction with a wide variety of dyes such as toluidine blue O (Ghemawat, 1977), lactophenol cotton blue (Heath, 1974), lactophenol cotton blue and aniline blue (Shipton & Brown, 1962), trypan blue and rose Bengal (Saha et al., 1988), acid fuchsin (McBride, 1936), safranin (Bandoni, 1979), and calcofluor (Trese & Loschke, 1990). Dual-staining techniques currently in use include safranin and fast green staining (Flemmer et al., 2010) and safranin staining with Astra blue (Srebotnik & Messner, 1994). However, the use of these dyes by themselves only stains fungal structures and/or plant cell walls and provides no clear distinction between the fungal and plant cell walls.

Many studies have sought to elucidate fungal development on plant surfaces, and the samples in such studies are clarified and then stained (Bruzese & Hassan, 1983; Koske & Gemma, 1989; Liberato et al.,

2005). However, the use of an infiltration method such as paraffin or plastic resins is very important for the study of the penetration and/or colonisation of tissues, because it ensures the integrity of the plant tissue, which is generally necrotic (Feder & O'Brien, 1968; Nair & Corbin, 1981; Flemmer et al., 2010).

The purpose of this study is to present a new double-staining method that uses cotton blue and safranin to identify fungal structures in plant tissues embedded in sections in HistoResin.

2. Materials and methods

2.1. Samples

For the analysis of petal tissues infected by *Colletotrichum acutatum* Simmonds, potted sweet orange plants [*Citrus sinensis* (L.) Osbeck var. Valência] were grown and maintained in a greenhouse at the Citriculture Defence Fund (FUNDECITRUS), located in Araraquara, SP, Brazil. They were induced to flower by pruning and water restriction.

Fruit of sweet orange [*Citrus sinensis* (L.) Osbeck var. Valência] with symptoms of black spot caused by *Guignardia citricarpa* Kiely and healthy fruits for the control group were collected in a commercial grove in the state of São Paulo. Fruits with hard spot symptoms were selected as described by Marques et al. (2012).

2.2. Inoculation of petals

The inoculation of *Colletotrichum acutatum* onto petals from a sweet orange tree was performed with the isolate

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142a, which is available from the FUNDECITRUS. To generate a fungal suspension, 2 mL of distilled water was spread onto petri dishes that contained the fungus. A spore suspension was generated using a flamed Drigalski loop. An aliquot of this suspension was removed and placed in a 50-mL plastic tube (Falcon), and 30 mL of distilled water was added. An aliquot of this suspension was counted using a Neubauer chamber. The concentration of the conidia suspension used was 4.3×10^5 spores/mL.

Previously selected regions of the petals were inoculated with the aid of a micropipette. Dew chambers were used to subject the petals to 24 h of leaf wetness.

2.3. Light microscopy

Petals with symptoms of postbloom fruit drop and fruits with symptoms of black spot disease were collected, sectioned, and fixed in a modified Karnovsky solution (4% paraformaldehyde, 1% glutaraldehyde in 0.1 M phosphate buffer of pH 7.2, 0.2 M phosphate buffer of pH 7.2) for 24 h (Karnovsky, 1965). During this step, a vacuum pump was applied to the samples to remove the air from the tissues.

The samples were dehydrated using 10 washes of 10 min each of an ethanol series. After dehydration, the samples were infiltrated in mixtures of 1:1, 2:1, and 0:1 (v/v) ethanol/infiltration solution (50 mL of basic resin and 0.5 g of activator, Leica HistoResin®) for at least 2 h and then placed in a 100% infiltration solution for 24 h. Samples were transferred into plastic moulds where they were infiltrated (a mixture of 1 mL of hardener and 15 mL of infiltration solution). The polymerisation was performed for 10 min at room temperature and 10 min in an oven at 60 °C.

The samples were sectioned into 5- μ m-thick sections on a rotary microtome (model RM2245, Leica). The sections were mounted on glass slides and stained with 0.05% toluidine blue (C.I. 52040) in a phosphate-citrate buffer (pH 4–6) (Sakai, 1973) and double stained with 5% cotton blue (C.I. 42755) in lactophenol (Macedo, 1997) and 1% aqueous safranin O (C.I. 50240), as described in Section 2.4. The slides were analysed, and the images were captured with a Leica DC 300F digital camera attached to a Leica DMLD microscope.

2.4. Staining procedure

1. Staining in 5% blue cotton in lactophenol for 20 min (Macedo, 1997);
2. Three 1-min washes in distilled water;
3. Staining in 1% aqueous safranin O for 10 s;
4. Three 1-minute washes in distilled water;
5. After drying, the slide can be mounted with an Entellan® synthetic resin.

3. Results

Citrus sinensis petals infected with *Colletotrichum acutatum* (Figure) and *C. sinensis* fruits infected with the

fungus *Guignardia citricarpa* (Figure) were anatomically analysed by comparing the stain developed using the double-staining technique with cotton blue and safranin with the stain developed using only toluidine blue O dye.

The samples with lesions that were infiltrated in a glycol methacrylate resin exhibited intact fungal and plant cell walls. This feature facilitated observations of the fungal structures in the injured tissues.

Toluidine blue O staining alone could not distinguish between fungal and plant cell wall structures (Figure). Conversely, by applying blue cotton, the fungal cell walls were stained blue, whereas safranin stained all of the plant cell walls (Figure). Thus, in the double-stained petals infected with *Colletotrichum acutatum*, the appressoria were located between the epidermal cells and exhibited forms of intercellular (Figure) or intracellular penetration (Figure). In addition, the connection between the acervuli and the vascular tissues via the hyphae can be better observed using the double-staining technique (Figure).

Upon analysing the sweet orange fruit sections with symptoms of black spot disease that were stained with toluidine blue O, the formation of pycnidia could be observed (Figure). However, the distinction between the epicarp and the fungal cell walls could only be observed using the new staining technique (Figure). With the use of toluidine blue O alone, it is difficult to observe the *Guignardia citricarpa* hyphae in the mesocarp (Figure). With the new method, there is a clear distinction between the fungal hyphae and the plant cell walls (Figure).

4. Discussion

The use of HistoResin as an infiltration medium for the study of fungal-infected tissues was very efficient. The samples with lesions frequently presented a loss of cell wall integrity due to tissue necrosis. Therefore, the use of plastic resins in this type of analysis is recommended.

The routine anatomical analysis of healthy and infected tissues can be performed with the dye toluidine blue O because, due to its polychromasia, it distinguishes between the chemical composition of different plant tissues via the different shades of staining (O'Brien et al., 1964; Feder & O'Brien, 1968). However, fungal hyphae are difficult to distinguish from plant cell walls in infected tissues stained only with toluidine blue O.

Cotton blue is frequently used to stain fungal hyphae (Chesters, 1934; Macedo, 1997). This dye is typically used in diagnostic studies of fungal diseases in animals (Lecker, 1999) and mycorrhiza (Schoenlein-Crusius et al., 2006). The positive reaction of cotton blue with fungal cell walls is due to the chitin present in fungal cell walls (Lecker, 1999). Conversely, safranin imparts a metachromasia

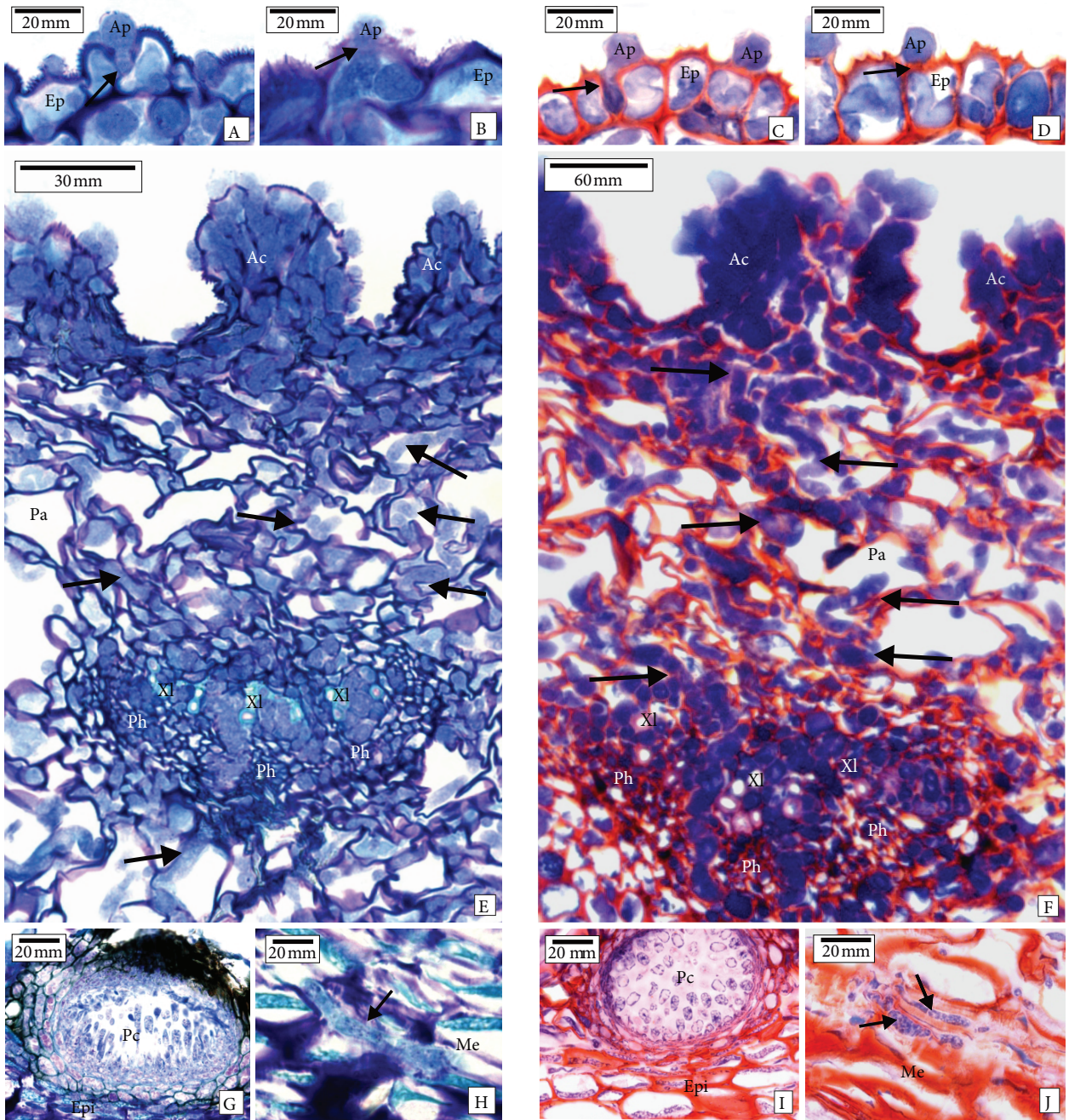


Figure. Histopathological analysis of infected tissues. Comparison between toluidine blue O staining alone (left column) and cotton blue and safranin O double-staining (right column). A–F: Photomicrographs of petals from Valencia sweet oranges infected with *Colletotrichum acutatum*. G–J: Valencia sweet orange fruits infected with *Guignardia citricarpa*. When the new double-staining technique is used (right column), there is a clear distinction between the fungal cell walls (blue) and the plant cell walls (orange). A and C: Intercellular penetration (arrows); B and D: intracellular penetration (arrows); E and F: typical view of the region where acervuli are interconnected with vascular bundles via hyphae located in the mesophyll (arrows); G and I: pycnidial lesion; H and J: details of the mesocarp containing fungal hyphae (arrows). Ac: acervulus, Ap: appressorium, Ep: epidermis, Epi: epicarp, Ph: phloem, Me: mesocarp, Pa: parenchyma, Pc: pycnidium, Xl: xylem.

orange tint on plant cells (Lillie, 1990) and is widely used in anatomical studies under different aspects (Vasic & Dubak, 2012; Serdar, 2013).

The double-staining method proved to be effective for histopathological studies involving phytopathogenic fungi. The method was effective with both *Colletotrichum*

acutatum-infected petals and *Guignardia citricarpa*-infected fruits. We recommend this processing and staining method for use on samples from other pathosystems. Moreover, the ability to produce permanent slides enables future analyses of lesioned samples.

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