Chapter 10

DAPI Staining and Fluorescence Microscopy Techniques for Phytoplasmas

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Abstract

The 4′,6-diamidino-2-phenylindole (DAPI) stain technique is a simple method that was developed for confirming the presence of phytoplasmas in hand-cut or freezing microtome sections of infected tissues. DAPI binds AT-rich DNA preferentially, so that phytoplasmas, localized among phloem cells, can be visualized in a fluorescence microscope. The procedure is quick, easy to use, inexpensive, and can be used as a preliminary or quantitative method to detect or quantify phytoplasma-like bodies in infected plants.

Key words: 4′,6-diamidino-2-phenylindole, Phytoplasma, Fluorescence microscopy, Diagnosis, Preliminary techniques

1. Introduction

Phytoplasmas are cell wall-less, non-helical bacteria that are surrounded by a single-layer membrane, and are pleomorphic in shape with an average diameter of 200–800 nm (1). These prokaryotes live a parasitic lifestyle associated with diseases in more than a thousand plant species, and are transmitted by insect vectors, mainly leafhoppers, and psyllids (1–3). In infected plants, phytoplasmas inhabit the sieve cells of phloem tissue and induce disease symptoms related to disrupted physiological processes (such as hormonal balance, amino acid transport, and carbohydrate translocation), causing inhibition of photosynthesis and rapid senescence or morphological changes in infected tissues (4–7). Initially, these disorders were thought to be caused by viruses, but Doi and collaborators, in 1967, identified wall-less pleomorphic bodies in diseased plants which, due to their morphological similarity to mycoplasmas, were named mycoplasma-like organisms (MLOs) (2).

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Currently, PCR-based techniques are widely used to detect and identify phytoplasmas, but their major limitations are the high cost and the need for highly trained personnel to carry out the procedure, which are lacking in many laboratories in developing countries. In that sense, quick, inexpensive, and easy-to-use techniques are highly valued to detect phytoplasmas in a rapid diagnostic approach. For these reasons, techniques such as the 4′,6-diamidino-2-phenylindole (DAPI) and Dienes' stains are commonly used for quick and inexpensive phytoplasma diagnosis.

The Dienes' stain was developed as an indirect method for diagnosis of phytoplasma infection, but this method has relatively low sensitivity and is nonspecific, because the phloem of stem sections infected by phytoplasmas generally stains dark blue, while xylem can stain turquoise and the cortex pale purplish-blue (8). Thus, a stained tissue section indicates the possible presence of phytoplasmas, but there is no specific staining of phytoplasma-like bodies. On the other hand, the DAPI stain is considered more specific than the Dienes' stain. The sequenced genomes of some phytoplasmas proved to be AT-rich, with a correspondingly low GC content ranging from 21% (e.g., '*Candidatus* Phytoplasma mali') to 28% (e.g., '*Candidatus* Phytoplasma asteris') (9). These characteristics make phytoplasmas amenable to staining techniques such as the use of DAPI, a fluorescent stain that has the ability to pass through cell membranes and which binds strongly to AT-rich regions of DNA (10).

The interaction of DAPI with DNA has been the subject of numerous studies since this aromatic compound was first synthesized. Initially, several diamidine compounds were synthesized to be used as a trypanocide agent (11). However, the special spectral properties of DAPI have caused it to be used more as a DNA probe than a medication drug. It is currently used extensively in fluorescence microscopy. In phytopathology it is used especially to detect phytoplasmas and spiroplasmas in different plant species, as reported in many studies (12–18). Following DAPI staining, phloem cells of infected material show a strong fluorescence, brighter than that which is typical of the nuclei of parenchymal cells. Infected tissues generally show bright phytoplasma-like spots in the phloem sieve tubes that are not present in healthy tissues (18). The DAPI staining technique is considered to be a rapid and precise method of localization of phytoplasmas in the phloem sieve tubes of different tissues such as twigs, petioles, leaves, and roots (13, 14, 16, 18). Apart from fluorescence light microscopy for phytoplasmas, DAPI is also commonly used in the detection of DNA of mycoplasmas contaminating animal cell cultures: mycoplasma cells in the growth medium fluoresce when stained by DAPI, making them easily detectable (19). Other applications have also been explored: PCR electrophoresis (20), cytofluorometry (21), and staining chromosomes (22).

2. Materials

1. Petioles and stems (fresh or dried samples can be used).
2. Phosphate buffer, 0.1 M pH 6.8: KH_2PO_4. Add 6.8 g of KH_2PO_4 to 500 mL of sterile distilled water. Shake at 7 rpm for 10 min. Add 1.16 g of NaOH to 200 mL of sterile distilled water and stir at 7 rpm for 10 min. Adjust the pH of the phosphate solution to pH 6.8 by adding NaOH solution.
3. Phosphate buffer, 0.1 M pH 6.9: Add 6.8 g of KH_2PO_4 to 500 mL of sterile distilled water. Shake at 7 rpm for 10 min. Add 1.16 g of NaOH to 200 mL of sterile distilled water and stir at 7 rpm for 10 min. Adjust the pH of the phosphate solution to pH 6.9 by adding NaOH solution.
4. Glutaraldehyde (5% solution): 5 mL glutaraldehyde, 95 mL 0.1 M phosphate buffer pH 6.8.
5. DAPI stain (4′,6-diamidino-2-phenylindole dihydrochloride) (1,000× stock solution): add 1 mg of DAPI to 1 mL of sterile distilled water. Store at 4°C if not used immediately (see Note 1).
6. Sterile distilled water.
7. Stirring machine.
8. Freezing microtome.
9. Fluorescence microscope.
10. Electronic balance.
11. Conical flasks (1,000 mL).
12. Plastic bags and trays.
13. Graduated pipettes (5 and 10 mL).
14. Micropipettes (100 µL).
15. Microscope slides and coverslips.
16. Scalpel and tweezers.

3. Methods

The method described here was slightly modified from Romero (23) and can be used for determining the presence of, or quantifying, phytoplamas (see Fig. 1).

1. Dilute the stock solution to working concentration (1×): take 100 µL of stock solution (1,000×) and add 100 mL of sterile distilled water. Store the working solution at 4°C.
2. Take the young stems and petioles from the diseased plants (see Note 2).

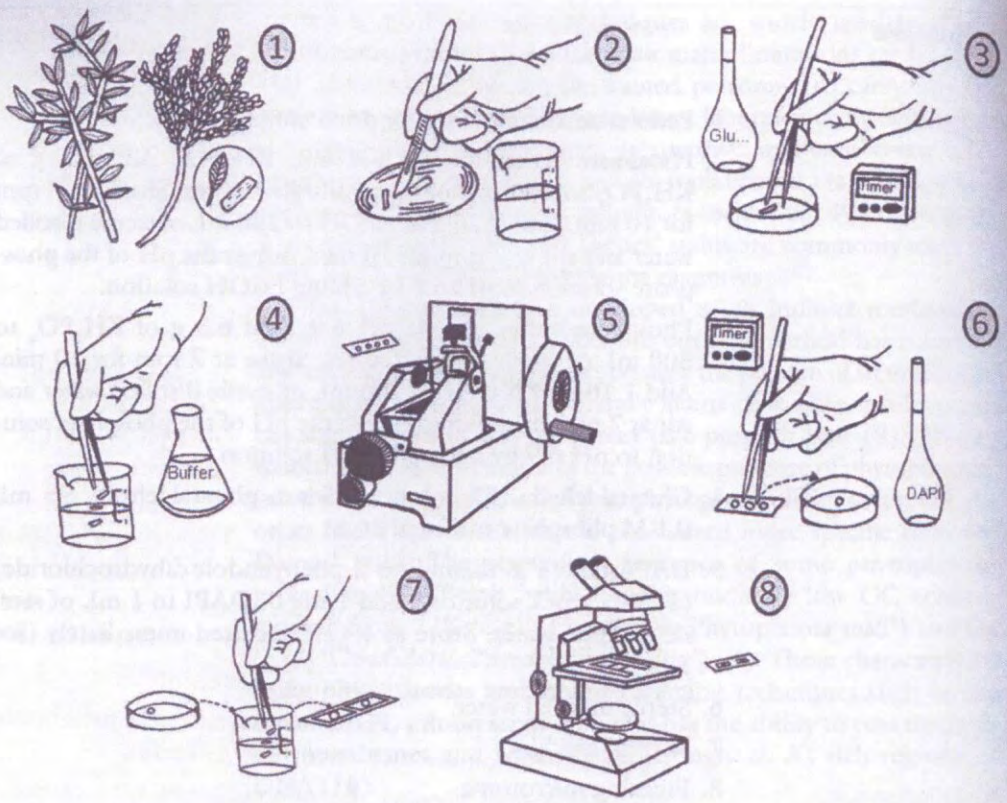

Fig. 1. Schematic illustration of the different steps for 4′,6-diamidino-2-phenylindole (DAPI) stain diagnosis in samples affected by phytoplasmas. Drawings were prepared by Francisco Beluzan, Universidad Austral de Chile.

3. Cut the samples in 1 cm lengths and put them in sterile distilled water (see Note 3).
4. Immerse the samples in 5% glutaraldehyde and fix for 20 min.
5. Remove the samples from the glutaraldehyde solution and wash with 0.1 M phosphate buffer pH 6.9 for 5 min. Transfer the samples to fresh 0.1 M phosphate buffer pH 6.9.
6. With the freezing microtome, cut longitudinal sections of 10–15 μm from the fixed samples (see Note 4).
7. Place the sample sections in the DAPI stain (1× working solution) for 25 min.

8. Remove the sections from the stain and put them in sterile distilled water to remove some of the dye (must have at least 50% of the dye when mounted). Immediately mount sections on a slide and place the coverslip (see Note 5).
9. Observe the samples with a fluorescence microscope with a range of objective lenses (10×, 40×, 100×) (see Note 6).

4. Notes

1. Caution: DAPI is labeled as a nontoxic product, but the toxicological properties of this reagent have not been fully investigated according to its Material Safety Data Sheet (http://www.sigmaaldrich.com). We recommend using gloves while handling your samples.
2. We recommend taking samples which are less affected by disease, preferably from areas that do not show fully developed symptoms, with the aim of avoiding false positives due to secondary infections by other microorganisms in the affected tissues.
3. This step reduces the dehydration of the samples.
4. We recommend having one or two layers of cells for a better fit of the samples on the slide after the DAPI stain.
5. We recommend keeping the samples in dark to extend the stain's life in the prepared sample, because light reduces the quality of the samples and tends to remove the stain from them. For further observations, it is recommended to seal the samples, e.g., using a layer of vaseline. If you do not have a special slide sealer, nail polish works very well as a sealer.
6. If more information about the fluorescence microscopy procedure is required, we recommended reading various reviews, e.g., (24). Samples that contain phytoplasmas show high DAPI fluorescence in the sieve tubes due to the specific combination of this stain with DNA, whereas no fluorescence is seen in healthy plants (Fig. 2). Generally, small points and aggregations near the cell wall of the phloem tissue cells are observed. This same procedure can be used to quantify phytoplasma-like bodies in phloem cells of infected plants (Fig. 3).

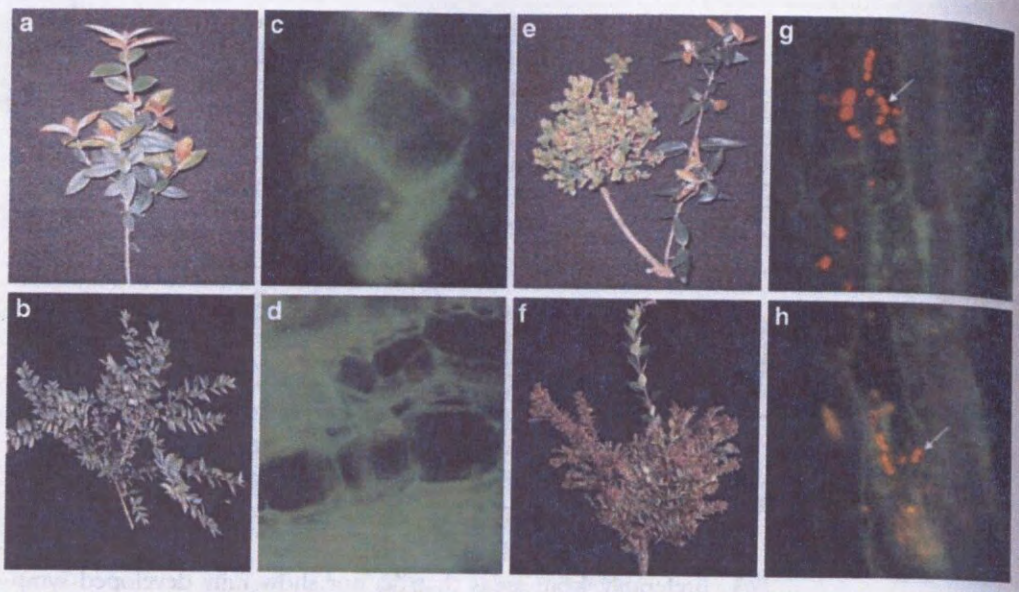

Fig. 2. Symptomless plant tissues (control) of *Ugni molinae* (**a**) and *Gaultheria phillyreifolia* (**b**), and their phloem tissues stained with DAPI ((**c**, **d**) respectively); witches' broom symptoms in *U. molinae* (**e**) and *G. phillyreifolia* (**f**), and fluorescent phytoplasma-like bodies in their phloem (indicated with *white arrows*, (**g**, **h**) respectively). The sections were examined using a fluorescence microscope (BP 450–490, FT 510, and LP 520 filters; Carl Zeiss Axiolab, New York, USA) with UV light excitation of 450–490 nm, a 100× oil immersion lens and 10× ocular lenses to achieve a magnification of 1,000× (18).

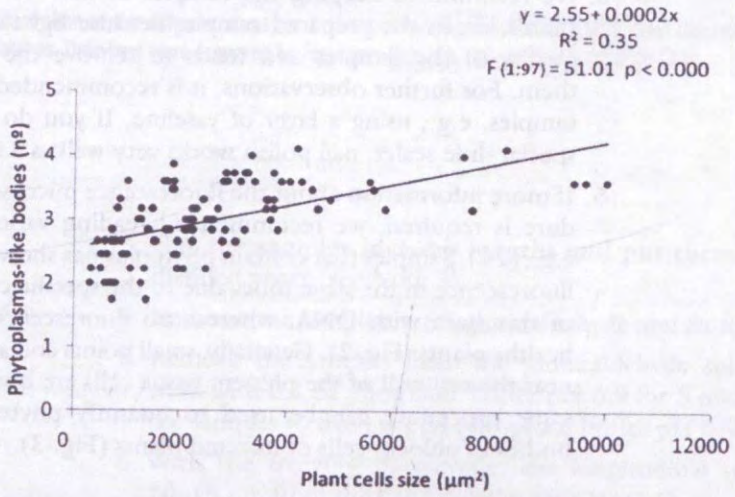

Fig. 3. Relationship between number of phytoplasma-like bodies and the size of phloem cells in petioles of *Hypochaeris radicata* L. Phloem tissues were stained with DAPI. Fluorescent phytoplasma-like bodies and phloem cells were quantified and measured with 10× grid eyepieces. The preparations were observed using a fluorescence microscope (BP 450–490, FT 510, and LP 520 filters; Carl Zeiss Axiolab, New York, USA) with a UV light excitation of 450–490 nm, a 100× oil immersion lens and total magnification of 1,000× (unpublished data).

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