

Epifluorescence microscopy imaging of phytoplasmas in embedded leaf tissues using DAPI and SYTO13 fluorochromes

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Summary

The use of DNA-specific dyes, i. e. DAPI, is extensively reported for phytoplasma detection in fresh plant materials. However, fluorescence-based microscopy and imaging of fresh tissues often evidences technical limitations which are more significant in infected tissues, because phenolic and other defense-related compounds accumulate in the cell wall and in the vacuole making difficult sample preparation. In this paper we describe a method based on the use of epifluorescence microscopy and the DNA probes DAPI and SYTO13® for phytoplasma visualization in resin-embedded plant tissues. The method allows detection of phytoplasmas and it is recommended for tissues that are recalcitrant to conventional imaging.

Key words: DAPI, epifluorescence microscopy, phytoplasmas, stolbur, SYTO13®, tomato.

Introduction

Phytoplasmas are wall-less prokaryotes, pleomorphic in shape (looking as filamentous, beaded or simply spheroid), belonging to the class *Mollicutes*; they are bound by a tri-layered unit membrane, contain ribosome and DNA and range up 1.2 µm in diameter (Musetti *et al.*, 1992). Phytoplasmas are associated to hundreds of diseases affecting economically important crops, such as ornamentals, vegetables, fruit trees, and grapevines (Lee *et al.*, 2000). They are located in phloem sieve elements of host plants and transmitted to other plants by phloem-sap feeding leafhoppers or psyllids in a persistent manner (Hogenhout *et al.*, 2008).

The association of these pathogens with plants exhibiting “yellows” symptoms was at first demonstrated by Doi and co-workers (1967) using transmission electron microscope (TEM). Since then, many authors have used light (for review, see Musetti, 2013) and electron microscopy to reveal phytoplasmas in the phloem tissues (Devonshire, 2013) and to

study cytological interactions occurring between these pathogens and their hosts (Kartte and Seemüller, 1991; Musetti *et al.*, 1994; 2000; 2010).

Moreover, fluorescence microscopy operating with nucleic acid specific DAPI [4,6-diamidino-2-phenylindole (for review see Andreade and Arismendi, 2013)] and SYTO13® (Christensen *et al.*, 2004) stainings has been used for the detection of phytoplasmas in infected tissues using hand-cut or freezing-microtome sectioned plant materials.

Nevertheless, fluorescence-based microscopy and imaging of fresh plant tissues often evidences technical limitations caused by plants' intrinsic properties. The transmission of light through plant cells is impeded by the heterogeneous refractive indices of the cell wall and cytoplasm, and light is often absorbed by pigments. In addition, the fluorescence of pigments and aromatic molecules further reduces the signal-to-noise ratio in images. In case of infected plants, phenolics and other defense-related compounds accumulate in the cell wall and in the vacuole making difficult sample preparation.

In the last 10 years there has been significant interest in imaging fluorescent molecules in semithin sections obtained from resin-embedded materials (for review, see Cortese *et al.*, 2009). Luby-Phelps and colleagues (2003) first described a method for retaining green fluorescence protein (GFP) signal in zebrafish embryos after fixation and resin embedding, but their method has not got widespread application.

Recently, methods to achieve information using thick optical sections of the tissue to locate cells of interest and subsequently imaged using TEM, have been described. However, there have been few attempts to retain fluorescent molecules in resin-embedded plant tissues and in particular in the sieve elements of the phloem, due to the small size of phloem cells and their deep location within plant organs (Nelson and van Bel, 1998). Pfeiffer *et al.*, (2003) were able to image sieve elements and companion cells of barley and pea leaf tissues using high-pressure freezing, followed by freeze substitution in acetone and resin embedding. Afterwards Bell *et al.*, (2013) explored a number of approaches for retaining fluorescent proteins in resin-embedded Arabidopsis tissues and described a simple method suitable for a number of conventional fluorescent

dyes, allowing localization studies.

In this work we describe a microscopy approach based on the use of epifluorescence microscopy and specific DNA probes (DAPI and SYTO13®) for phytoplasma visualization *in situ*, using resin-embedded plant tissues. Resin-embedded leaf sections of healthy and phytoplasma-infected tomato (*Solanum lycopersicum*) plants, [used as hosts of the 'Candidatus Phytoplasma solani' ('Ca. P. solani'), the pathogen associated to stolbur disease] were prepared, stained with DAPI or SYTO13® fluorochromes and then observed by epifluorescence microscope (EFM). In parallel, to confirm EFM analyses, transmission electron microscopy (TEM) observations have been performed using the same embedded materials.

Materials and Methods

Plant material

Three *Solanum lycopersicum* plants ('cv Micro-Tom') were infected with the stolbur phytoplasma 'Candidatus Phytoplasma solani' ('Ca. P. solani' subgroup 16 SrXII-A, Quaglino *et al.*, 2013), by grafting shoot tips from naturally infected tomato plants grown in the field onto healthy tomato plants grown

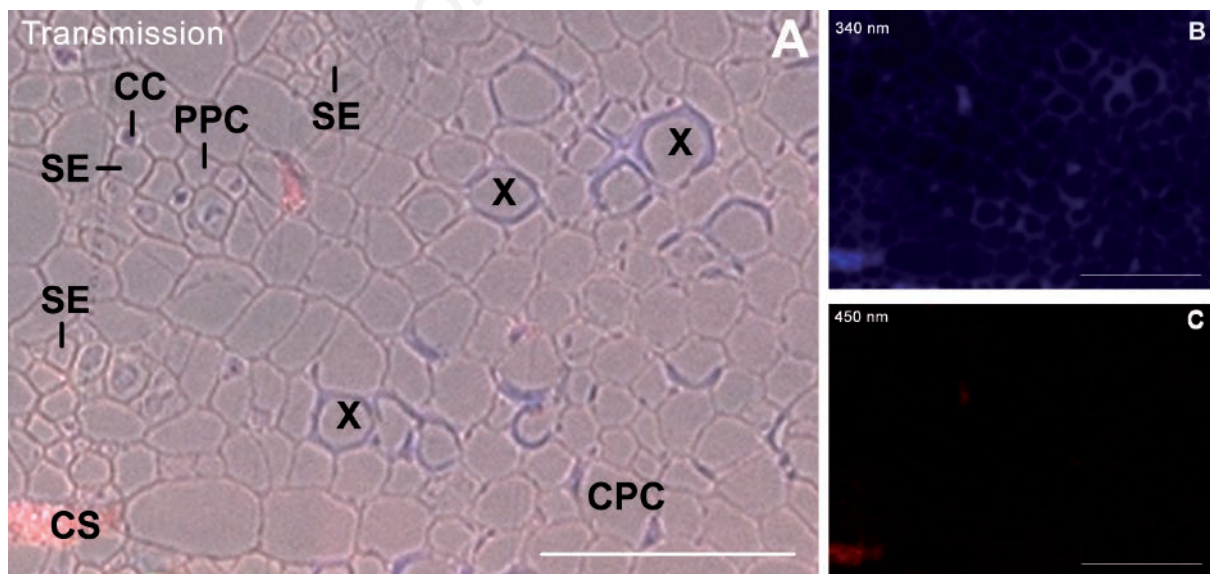


Figure 1. A through C. Autofluorescence and background images of untreated tomato tissue. Overlay (A) of bright-field image and images excited with 340 nm (B) and 450 nm (C) served as a staining control, respectively. CC, companion cell; CPC, cortex parenchyma cells; CS, crystalline structure; PPC, phloem parenchyma cell; SE, sieve element; X, xylem vessel. Scale bars: 50 µm.

from seeds in a greenhouse (27°C day, 20°C night). Three healthy uninfected tomato plants, also grown in a greenhouse, were used as controls. After two months from grafting, characteristic stolbur symptoms, such as yellowing, witches' brooms and stunting, emerged in infected tomato plants (Buxa *et al.*, 2015).

Embedding procedures for epifluorescence and electron microscopy

Fifteen randomly chosen leaf midrib segments were excised from symptomatic-infected or healthy tomato plants. Segments were cut into small portions (6-7 mm in length), fixed in 0.2% glutaraldehyde, rinsed in 0.1 M phosphate buffer (PB), pH 7.4 and dehydrated in grad-

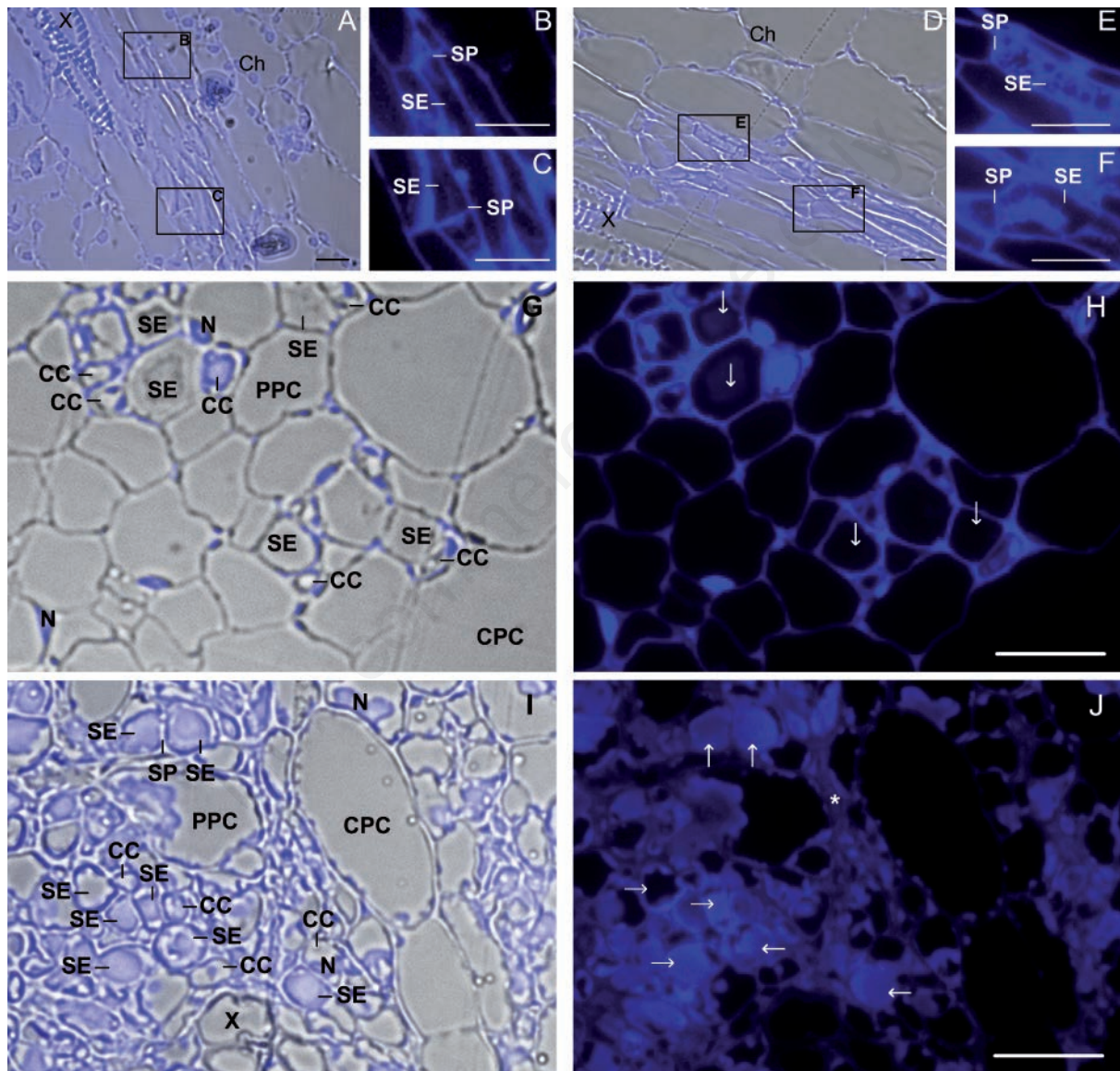


Figure 2. Light and epifluorescence microscopy images from longitudinal (A through F) and cross sections (G through J) of healthy (A through C, G and H) and stolbur-diseased (D through F, I and J) tomato leaves. Phytoplasma presence in the LRW-embedded main vein was revealed using 4',6-diamidino-2-phenylindole (DAPI) as a DNA marker. Arrows point to the sieve-element lumen and asterisks indicate altered cell-wall thickness. CC, companion cell; Ch, chloroplast; CPC, cortex parenchyma cells; N, nucleus; PPC, phloem parenchyma cell; SE, sieve element; SP, sieve plate; X, xylem vessel. Scale bars: 10 μm .

ed ethanol series (25%, 50%, 75%, 30 minutes for each step) at 4°C. After one hour of the final 100% ethanol step, the samples were infiltrated in a hard-grade London Resin White (LRW, Electron Microscopy Sciences, Fort Washington, PA, USA) -100% ethanol mixture in the proportion 1:2 for 30 minutes, followed by LRW:ethanol 2:1 for 30 minutes, and 100% LRW (two immersion periods: the first for 1 hour, followed by overnight infiltration) at room temperature. The samples were embedded in Eppendorf tubes using fresh LRW containing benzoyl peroxide 2% (w/w) according to manufacturer's protocol and polymerized for 24 h at 60°C (Musetti *et al.*, 2002).

Epifluorescence microscopy imaging

Embedded samples were cut to obtain semithin sections (1 to 2 µm) using a diamond knife on a

Reichert Leica Ultracut E ultramicrotome (Leica Microsystems, Wetzlar, Germany). Serial semi-thin sections of at least 100 samples from each healthy and stolbur-diseased plant were placed on glass slides. To detect phytoplasmas inside sieve elements of diseased plants sections were incubated with fluorescent dyes as reported below. Stained tissues were observed under an automated epifluorescence microscope (Leica Microsystems, Wetzlar, Germany) equipped with a digital camera (Leica DFC, Leica Microsystems, Wetzlar, Germany). Cell imaging was performed using Leica Application Suite Advanced Fluorescence (LAS AF®). As visual examination of primary fluorescence unstained samples were observed at the same excitation wavelengths used for the fluorochromes (340 nm and 450 nm, respectively) as visual controls (Figure 1).

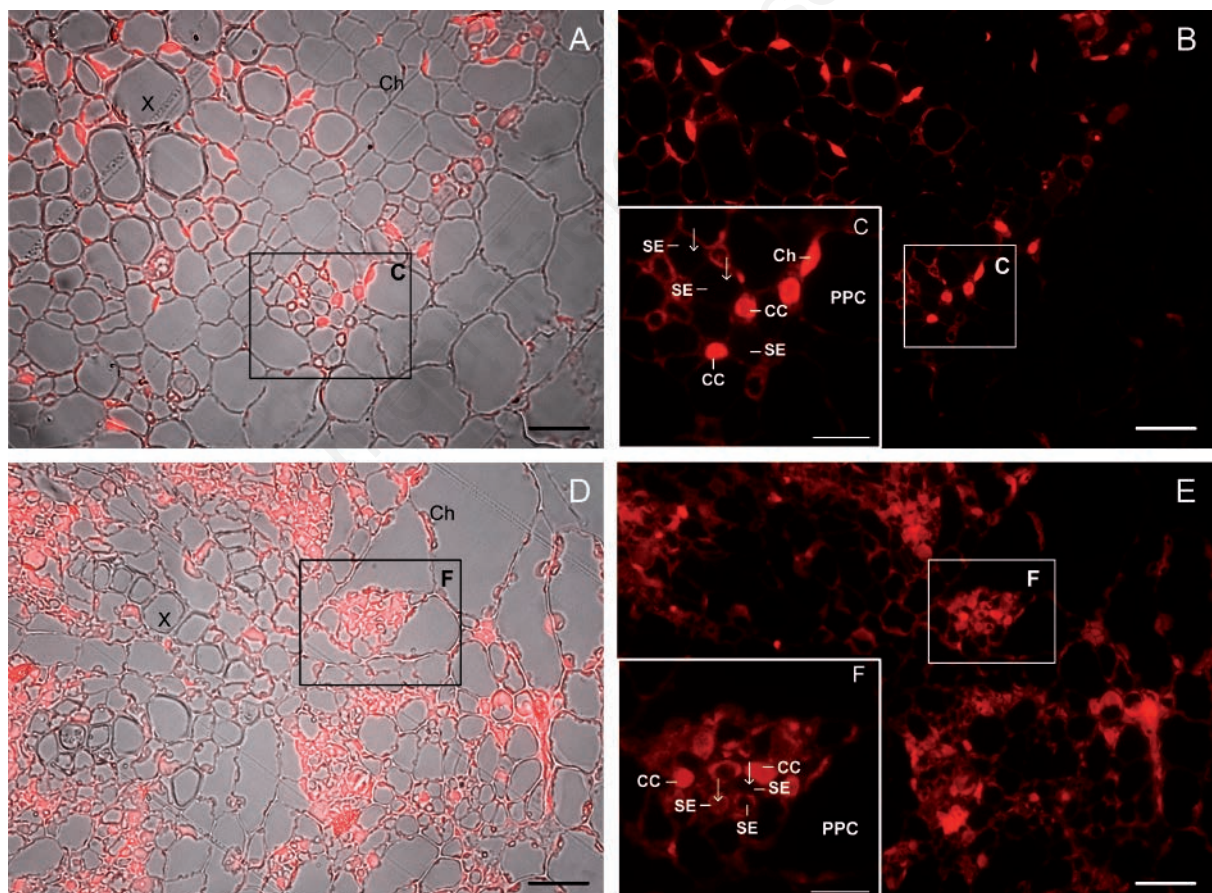


Figure 3. Light and epifluorescence microscopy images from healthy (A through C) and stolbur-diseased (D through F) tomato leaves. Phytoplasma presence in the LRW-embedded main vein was revealed using SYTO13® as a DNA/RNA specific marker. Arrows point to the sieve-element lumen. CC, companion cell; Ch, chloroplast; PPC, phloem parenchyma cell; SE, sieve element; X, xylem vessel. Scale bars: 25 µm (A,B,D,E); 10 µm (C,F).

Fluorochromes

To identify phytoplasmas inside sieve elements, as well as to detect nuclei in companion and parenchyma cells, sections were stained with 0.3 μM of DAPI [4,6-diamidino-2-phenylindole, Molecular Probes® (Invitrogen, Eugene, OR, USA)] or 0.5 $\mu\text{g}/\text{mL}$ SYTO13® dissolved in 1x phosphate saline buffer

(PBS) for 2.5 hours (Loi *et al.*, 2002; Christensen *et al.*, 2004). Briefly before observation the slides were washed twice with PB and air-dried. For observation, slides were consecutively exposed to excitation wavelengths within the spectral windows of 340 nm to 380 nm and 450 nm to 490 nm under a DM 4000 fluorescence microscope (Leica Microsystems, Wetzlar,

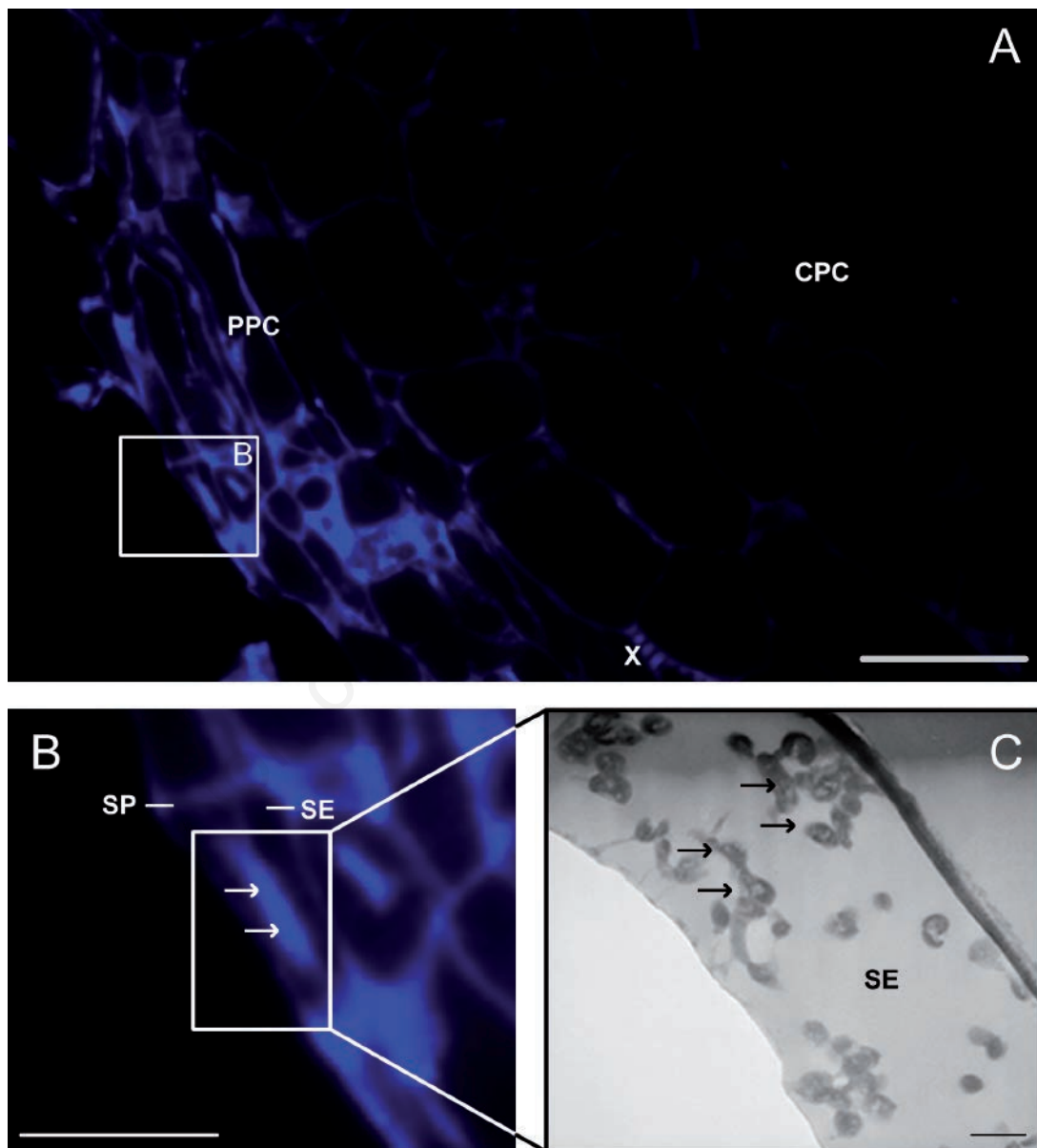


Figure 4. Representative image of combination of epifluorescence (A through B) and transmission electron microscopy (C): spatial overlap of signals in stolbur-diseased tomato tissue is well visible. Phytoplasma presence was visualized by fluorescent staining 4',6-diamidino-2-phenylindole DAPI (A through B), and heavy metals staining uranyl acetate and lead citrate (C). Arrows point to the particular signals. CPC, cortex parenchyma cells; PPC, phloem parenchyma cell; SE, sieve element; SP, sieve plate; X, xylem vessel. Scale bars: 25 μm (A); 10 μm (B); 1 μm (C).

Germany). Observations were performed using 63x oil objective and long-pass filters starting from 425 nm and 515 nm, respectively. False-color fluorescence image of DAPI (blue) and SYTO13® (red) were processed using Adobe Photoshop (CS, USA) to optimize brightness, contrast, and coloring.

Transmission electron microscopy

For TEM analyses, serial ultrathin sections (60-70 nm) of at least 100 samples from each healthy or infected plant, were cut using the ultramicrotome and collected on 200 mesh uncoated copper grids, stained with uranyl acetate and lead citrate

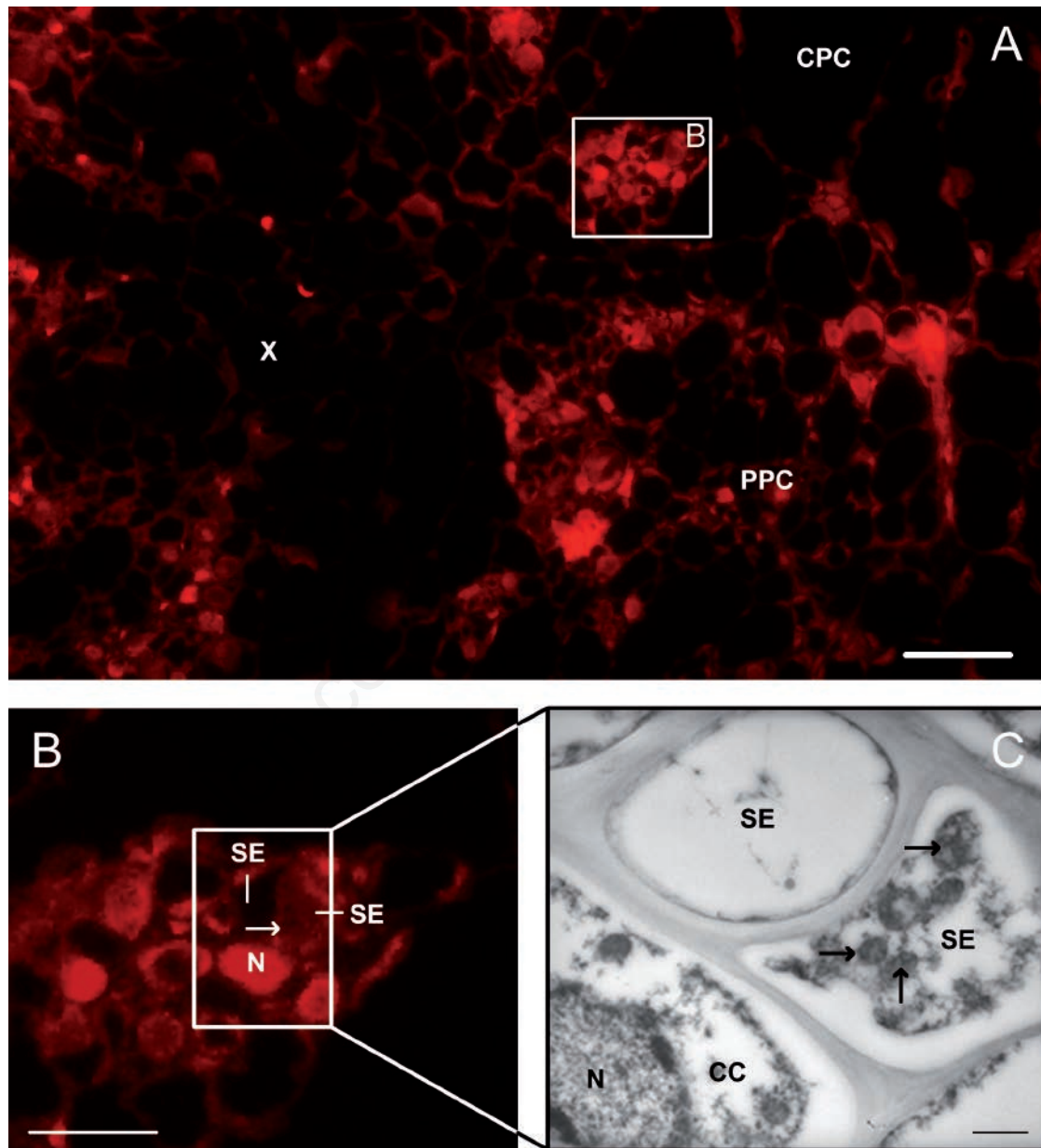


Figure 5. Representative image of combination of epifluorescence (A through B) and transmission electron microscopy (C): spatial overlap of signals in stolbur-diseased tomato tissue is well visible. Phytoplasma presence was visualized by fluorescent staining SYTO 13® (A through B), and heavy metals staining uranyl acetate and lead citrate (C). Arrows point to the particular signals. CC, companion cell; CPC, cortex parenchyma cells; N, Nucleus; PPC, phloem parenchyma cell; SE, sieve element; X, xylem vessel. Scale bars: 25 μ m (A); 10 μ m (B); 1 μ m (C).

(Reynolds, 1963) and then observed under a PHILIPS CM 10 (FEI, Eindhoven, The Netherlands) transmission electron microscope operating at 100 kV.

Results and Discussion

Phytoplasmas were identified and precisely localized both in longitudinal (Figure 2A through F), and cross-sections (Figure 2G through J) obtained from LRW-embedded leaf midribs, stained with the DNA specific marker DAPI. Epifluorescence microscopic (EFM) images of longitudinal sections, showed no DAPI signal in sieve elements of healthy samples (Figure 2A through C). Only signals from nuclei in other phloem cells, chloroplasts in parenchyma cells (Figure 2A) and strong autofluorescence of the cell walls showed up (Figure 2B and C). In infected samples, phytoplasmas emerged as aggregates of fluorescent dots inside sieve elements (Figure 2D), especially at the sieve plates (Figure 2E and F). Cross-sections of healthy leaves, demonstrated a good quality of preservation (Figure 2G). No fluorescent signals were visible inside sieve elements (Figure 2H), whereas nuclei of companion cells and phloem parenchyma cells stand out clearly (Figure 2G and H). By contrast, stolbur-diseased samples showed strong fluorescent signals in sieve elements, which is indicative of phytoplasma presence (Figure 2I and J). As in controls, fluorescent signals from nuclei of companion cells and phloem parenchyma cells were detected (Figure 2I and J).

In LRW-embedded leaf midribs of healthy (Figure 3A through C) and stolbur-diseased samples (Figure 3D through F) fluorescent signal from DNA/RNA specific marker SYTO13[®] was strongly visible in nuclei in non-sieve-element cells, chloroplasts in parenchyma cells and autofluorescence of the cell walls (Figure 3B and C). As DAPI, no fluorescent signal was present in sieve element of healthy samples (Figure 3B and C). In infected samples fluorescent signal showed up SYTO13[®]-positive sieve elements (Figure 3E and F), next to background staining.

TEM observations of ultrathin sections, cut serially immediately after the semithin sections, confirmed the presence of phytoplasma cells in correspondence of the fluorescence signals observed in the sieve elements by EFM and DAPI (Figure 4) or SYTO13[®] staining (Figure 5). The nucleic acid-specific fluorescent probe DAPI was extensively used for *in situ* imaging of phytoplasmas in fixed hand-cut plant

materials (Hiruki and da Rocha, 1986; Loi *et al.*, 2002) and, more recently, *in vivo* in intact sieve elements (Musetti *et al.*, 2013). Christensen *et al.*, (2004) first used SYTO13[®] for phytoplasma detection in unfixed plant tissues, under multiphoton confocal laser scanning microscope.

Here, DAPI and SYTO13[®] were used as a novelty to detect phytoplasmas in LRW-embedded leaf samples: samples retain DAPI/SYTO13[®] fluorescence indicating phytoplasma presence in the sieve elements of stolbur-infected *S. lycopersicum*. DNA fluorescence in embedded tissue was sufficient to highlight phytoplasma accumulation in both longitudinal and cross-sections of sieve elements. Moreover the tissue morphology is of sufficient quality. The possibility to use DAPI and SYTO13[®] in this context allows various combination of dyes for multicolor labeling. The effective correspondence between the fluorescence signal imaged by EFM and phytoplasmas was confirmed by TEM visualization of ultrathin sections obtained from the same resin-embedded blocks used for fluorescence analysis.

The possibility to preserve the embedding materials for a long time may be of great utility for phytoplasma microscopical diagnosis when sectioning and observation is required at a later time. Sections could be counter-stained with a number of conventional fluorophores and antibodies, allowing co-localization studies. The described simple methods allow successive imaging of phytoplasmas with the light and electron microscope, combining the strengths of both imaging platforms. We believe this approach will have significant utility for tissues (as those collected from infected plants) that are recalcitrant to conventional imaging.

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