Manuscript Details

Manuscript number	VIRMET_2018_348
Title	IN SITU HYBRIDIZATION FOR THE LOCALIZATION OF TWO PEPINO MOSAIC VIRUS ISOLATES IN MIXED INFECTIONS
Article type	Short Communication

Abstract

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Keywords	Mixed infections; PepMV; in situ hybridization; RNA localization.

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IN SITU HYBRIDIZATION FOR THE LOCALIZATION OF TWO PEPINO MOSAIC VIRUS ISOLATES IN MIXED INFECTIONS

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ABSTRACT

In situ hybridization (ISH) is a very accurate technique for the localization of viral genomes in plant tissue and cells. However, simultaneous visualization of related plant viruses in mixed infections may be limited due to the nucleotide genome similarity and the single chromogenic detection over the same sample preparation. To address this issue, we used two *Pepino mosaic virus* (PepMV) isolates and performed ISH over consecutive serial cross-sections of paraffin-embedded leaf samples of single and mixed infected *Nicotiana benthamiana* plants. Our results, based on the overlapping of consecutive cross-sections from mixed infected plants, showed that both PepMV isolates co-localized in the same leaf tissue. In turn, both isolates were localized in the cytoplasm of the same cells. These results provide valuable information for studying mixed infections in plants by using ISH, which could help with elucidating the ecological mechanisms of virus-virus interactions within-plants.

Keywords: Mixed infections; PepMV; *Nicotiana benthamiana;* viral RNA; *in situ* hybridization; RNA localization

Mixed infections of plant viruses are common in nature (Juarez et al., 2013; Kassem et al., 2007) and can have important epidemiological and disease outcomes. Mixed viral infections can lead to competitive interactions that may create spatio-temporal distribution patterns throughout plant tissues, and hence, structured sub-populations within the plant (Tromas et al., 2014). A number of experimental approaches have been used to determine within-plant viral distribution (Bergua et al., 2016; Dietrich and Maiss, 2003; Hall et al., 2001; Takahashi et al., 2007; Takeshita et al., 2004). Many of these studies have employed proteins from among the broad

repertoire of fluorescent proteins, such as the green fluorescent protein (GFP), expressed from engineered viral genomes in order to visualize viral distribution during plant infections (Dietrich and Maiss, 2003; Oparka et al., 1997). Other studies have used *in situ* hybridization (ISH), where the viral genome can be accurately localized at the cellular level (Amari et al., 2009, 2007; Gómez-Aix et al., 2015; Gosalvez-Bernal et al., 2008, 2006), with ISH having the advantage of localizing wild type, unmodified viral genomes. Non-radioactive ISH methods have also been used for chromosome mapping (Gordon et al., 1995), gene expression localization (Zeller et al., 2001), and pathogen detection in animal (Montone and Guarner, 2013) and plant tissues (Alves-Júnior et al., 2009; Bergua et al., 2016; Rentería-Canett et al., 2011; Tanaka, 2009; Wang et al., 2010). Most of the works addressed the localization of a single mRNA type, rarely attempting to co-localize mRNAs that share a significant degree of similarity, as might be the case of RNAs from viral isolates belonging to different strains. We have addressed this issue by using two different isolates of *Pepino mosaic virus* (PepMV) in mixed infected *Nicotiana benthamiana* plants.

PepMV (genus *Potexvirus*, family *Alphaflexiviridae*) is a widespread plant virus that has caused several outbreaks worldwide in tomato crops, resulting in important economic consequences (Gómez et al., 2012; Hanssen and Thomma, 2010). The virions are filamentous and the genome is a single-stranded, positive-sense, RNA molecule of approximately 6400 nucleotides with five open reading frames (ORFs) (Aguilar et al., 2002). ORF1 encodes the putative viral replicase containing the methyl transferase, helicase and RNA-dependent RNA polymerase (RdRp) domains; ORFs 2, 3 and 4 form the triple gene block (TGB), essential for cell-to-cell movement and for RNA silencing suppression, and ORF5 encodes the coat protein (CP) (Verchot-Lubicz et al., 2007). Our previous molecular epidemiology studies showed that the PepMV populations sampled in southeastern Spain (Murcia region) between 2005 and 2008 consisted of isolates belonging to two co-circulating genotypes, Chilean (CH2) and European (EU); although the CH2 isolates predominated, the EU isolates persisted in mixed infections (Gómez et al., 2009).

The aim of this study was to design digoxigenin-labelled RNA probes that targeted PepMV genomic regions with a high specificity and sensitivity for PepMV isolates of the EU or CH2 genotypes. In order to localize both types of isolates, viral RNA localization by ISH assays was performed over serial cross sections of paraffin-

embedded samples of single and mixed infected plant leaf tissue, to examine the viral RNA distribution in single and mixed infections of *N. benthamiana* plants.

We first compared PepMV-EU and -CH2 reference genomes, which have a sequence similarity ranging from 78% to 86% depending on the genomic region, in order to identify a genomic region that could be used to distinguish both isolate types. A plot similarity analysis identified regions with the lowest nucleotide similarity (Fig. 1A). A genomic region covering a portion of the RdRp ORF1 was finally selected for the synthesis of both riboprobes, which were named EU_505 and CH2_505; sequence nucleotide identity within this region among the two reference genomes was on average 60%, with a minimum of 22% and a maximum of 78% (Fig. 1A). cDNA fragments of ~500 pb from the ORF1 of both PepMV isolates (Table 1) were cloned in the pGEM-T easy vector (Promega corporation) and used for transcription of the two probes. The *in vitro* transcription reaction was incubated at 40°C for 2 h 30 min and contained 1 µl (20 units) SP6 RNA polymerase (New England Biolabs), 2 µl 10x SP6 Buffer, 4 µl DIG RNA Labeling Mix (Roche), 1 µg template DNA, 0.5 µl RNase Inhibitor and H_2O in a final volume of 20 µl. After transcription, all probes were precipitated by adding 1:10 volume of 3M sodium acetate and 2-3 volumes of 100% ethanol and finally suspended in 20 µl of sterile water. Each probe was tested for cross-detection by dot-blot molecular hybridization on total RNA extracts from mock- and single-infected *N. benthamiana* plants. Plants were inoculated and grown as described by Gómez et al., (2009). No background signal was observed from mock-inoculated plants, and both probes were specific for the corresponding virus isolate, with no cross-reaction with the heterologous viral RNA (Fig. 1B). Probe specificity was then tested in ISH assays on consecutive tissue sections from mock-, single- and mixed-infected *N. benthamiana* plants, following the procedures described by Gosalvez-Bernal et al., (2008). Apical leaves from mock, single (PepMV EU or CH2 type) and mixed (PepMV-EU+PepMV-CH2) infected plants were collected, fixed with paraformaldehyde, embedded in paraffin and sectioned (Fig. 2). For fixation, sections of leaf samples were cut with a sterile blade and immersed in recently prepared 4% paraformaldehyde (PFA, Acros organics, Belgium) in 1x phosphate buffered saline solution (10x PBS, 136 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.2) with 0.1% Triton X-100 (Merck). Immediately after the immersion, samples were vacuum infiltrated for ≈1 min to help with the penetration of the fixative into the cells and left overnight at 4ºC. The next day, the PFA was eliminated by performing two washes with 1x PBS (30 min each), then,

samples were dehydrated with ethanol (Merck), followed by increasing concentrations of a xylene substitute (Shandon™ Xylene Substitute, Thermo Fisher Scientific), at room temperature. Finally, wax paraffin (Paraplast Plus, Sherwood Medical Co., St Louis, MO) was slowly added and the xylene substitute was replaced slowly for three days. 5-7 µm serial cross sectioning of paraffin-embedded leaves was performed with a HM 325 Micron microtome (Micron International, Germany). Each individual cross-section was placed in a poly-l-lysine covered slide (VWR International BVBA, Leuven, The Netherlands), and the slides were maintained in order. The order of the slides was very important for the mixed infected samples, as the slides were alternatively hybridized with EU_505 and CH2_505 probes (Fig. 2), resulting in side-by-side comparisons. For ISH, poly-l-lysine slide mounted tissue sections were first dewaxed with Shandon™ Xylene Substitute (Várallyay and Havelda, 2011). Then, the samples were hydrated in decreasing concentrations of ethanol and the endogenous alkaline phosphatase was inactivated with 0.2 M HCl for 20 min. Afterwards, digestion with proteinase K (1 µg/ml in 100 mM Tris–HCl, 50 mM EDTA, pH 8.0) was performed at 37°C for 30 min. (Lee et al., 2013), followed by a glycine wash (2 mg/ml in 1x PBS for 2 min.) to block proteinase K, and PBS washes. Post-fixation was done with 4% PFA in 1x PBS (12 min), washed again twice in 1x PBS (5 min) and dehydrated in an increasing ethanol series. Probes were hybridized at 55°C overnight in a humid chamber inside an oven. Before hybridization, the probes were denatured at 92ºC for 3 min, and put back on ice. Probes were used at a final concentration of 500 ng/ml and they were diluted in the hybridization solution (50% formamide, 6x SSC, 1x Denhardt's solution, 10% dextran sulfate and 10 mM DTT) (Lee et al., 2013). After hybridization, a RNase/2x SSC (RNase A, Sigma-Aldrich®) treatment was done in order to eliminate the RNA that did not hybridize (20 µg/ml at 37°C for 10 min), followed by washes with 2x SSC (0.30 M NaCl plus 0.03 M sodium citrate, pH 7.0) with 0.1% SDS (2 times, 5 min each) and washes with 0.2x SSC with 0.1% SDS (2 times, 10 min each). A TBS wash (100 mM Tris-HCl, 400 mM NaCl, pH 7.5, 5 min) and an incubation with 0.5% BSA/TBS for 1 h (Bovine serum albumin fraction V, Acros organics, Fisher Scientific, USA) to block unspecific labelling, were done. Then, the samples were incubated with an anti-digoxigenin antibody conjugated to alkaline phosphatase (Anti-Digoxigenin-AP, Roche Molecular Biochemicals) for 1h (1:500 in 0.5% BSA/TBS), and additional washes in TBS (3 times, 5 min each) were performed. Samples were washed in detection buffer (100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5) and incubated for ≈ 1 h in

darkness with ready-to-use NBT/BCIP (Nitroblue tretazolium/5-bromo-4-chloro-3-indolyl-phosphate, Roche Molecular Biochemicals), prepared in detection buffer (1:20). Lastly, slides were counter stained with alcian blue (Várallyay and Havelda, 2011), carefully dried out and mounted on Merckoglass medium before observing them under bright field illumination with the Leica Leitz DMRB light microscope (Leica microsystems). All the steps were performed in Coplin jars except for the hybridization and incubations in proteinase K, RNase, anti-digoxigenin-AP and NBT/BCIP, which were done on the slides with coverslips to help with the spreading of the liquid.

The determination of hybridization specificity is essential for avoiding any background noise, and to test this, performing the ISH assays with the appropriate controls is necessary. In this study, apart from doing the pretreatment with RNase, we used different controls: performing ISH in mock-inoculated plants (Fig. 3A, B), samples from single infected PepMV EU using the PepMV CH2 probe (Fig. 3D), in addition to samples from single infected PepMV CH2 using the PepMV EU probe (Fig. 3E) as well as samples from either single infected and mixed infected plants without adding the probe to the hybridization solution (data not shown).

No ISH signal was detected in mock-inoculated consecutive leaf serial cross-sections, which showed a homogeneous blue colour of the leaf tissue due to post-staining with alcian blue (Fig. 3A and B). ISH on two consecutive leaf serial cross-sections of single PepMV-EU inoculated plants showed that only the EU_505 probe reacted with a dark-blue coloration, as a result of positive RNA target detection, while there was no reaction for the CH2_505 probe on a consecutive leaf cross-section (compare Fig. 3C and 3D). Similarly, ISH on two consecutive leaf serial cross-sections of PepMV-CH2 inoculated plants showed a dark-blue signal only for the CH2_505 probe (Fig. 3F), and no signal at all was detected with the EU_505 probe (Fig. 3E). These results demonstrated the specificity of both riboprobes for detecting their viral RNA targets. In general, we found that viral RNA distribution was discrete, creating a mosaic of infection patches of different sizes in many locations including the abaxial and adaxial epidermis, spongy mesophyll, palisade parenchyma and vascular bundle cells (Fig. 3C, F). We then performed ISH over consecutive serial sections of mixed infected samples of *N. benthamiana.* Figure 4 shows two consecutive leaf serial cross-sections hybridized either with the EU_505 (Fig. 4A) or the CH2_505 (Fig. 4B) probes, whereby both isolates were localized in the same patch of the tissue, as shown in the

leaf region with a dark-blue colour. Observing with more detail in new consecutive leaf serial cross-sections (Fig. 4C and D), viral RNAs from both isolates were localized in the cytoplasm of the same cells (arrowheads). Therefore, both PepMV isolates appeared together within the same infected tissue, showing, at least for some of the cells, a lack of spatial segregation. In all samples, the infected tissue corresponded to the abaxial and adaxial epidermis, spongy mesophyll, palisade parenchyma and bundle sheath cells. Note that a good structural preservation is essential for working at this high magnification (Fig. 4) and for clearly distinguishing the infected cells in specific, discrete plant tissue areas. In conclusion, the designed probes specifically detected their targets. This is very important for the success of our work, as sequence similarity of the two PepMV isolates is high. These and similarly designed probes and ISH experiments may assist in the characterization and distribution of plant viruses under single or mixed infections during the infection processes.

A very important improvement of our ISH method was the use of consecutive leaf serial cross sections of the same mixed infected samples, with a specific thickness, that allowed us to have the same cells in consecutive slides. This allowed us to undoubtedly know that after performing the ISH, by using each of the probes (EU or CH2) in each one of the consecutive slides, both isolates were present mostly in the same cells as shown by the results in Fig. 4C-D.

Among the alternative methods used to address these kinds of localizing studies that target viral RNA in mixed infected plants, recently plant researchers have used fluorescently-labeled probes to directly label transcripts (Bergua et al., 2016; Dietrich and Maiss, 2003; Takahashi et al., 2007). Nevertheless, the optical properties of plant cells and tissues bring significant challenges for fluorescence microscopy (Rost, 1992). Inherent light scattering adversely affects both the excitation and the detection efficiency; moreover, plants contain many native molecules that emit high levels of background autofluorescence as compared to other organisms (Rost, 1992, Duncan et al., 2016). Furthermore, some of the fluorochromes cannot be seen in some hosts, as it happens in tomato plants, which emit in green. On the other hand, ISH provides morphological information on the spatial distribution and heterogeneity of gene expression in complex tissues, offering an advantage over other molecular techniques in which homogenization precludes such evaluation (Hicks et al., 2004).

In conclusion, this *in-situ* hybridization technique is remarkably useful for pathologists, as it is a precise tool for the localization of nucleotide targets in plants, which in turn, could assist in the characterization and distribution of plant pathogens under single or mixed infections during infection processes.

ACKNOWLEDGEMENTS

P.G. and M.A.A. acknowledge funding from MINECO (grants AGL2014-59556-R and PCIN-2017-055, the latter within the ERANET-ARIMNet2 (ref. 302) program), and C.A. was supported by funding of the Ministerio de Economia y Competitividad (MINECO, Spain) within PhD programme grant (FPU16/02569). We also acknowledge the use of the facilities at the Servicio de Microscopia from the Servicio de Apoyo a la Investigación of the Murcia University. Mari Carmen Montesinos provided excellent technical assistance, and Mario Fon (mariogfon@gmail.com) edited the manuscript.

Conflict of interest: The authors declare that there is no conflict of interest.

Table 1. PepMV nucleotide sequences and genomic region from the PepMV-Sp13 (EU type) and PepMV-PS5 (CH2 type) isolates that were used for the synthesis of the RNA ISH probes.

Probe	PepMV sequence used for probe synthesis	Genomic region	Start and end
EU_505	5'GCAGCTGACTCAATTGGTCAAAAAAGGTACAATATCCTTAGCAGATTTTGAACA GAGAGAACCTGAAATAACTTACACTGAGTTTGAGCCTGAAACTAGGCCCCAAGTG GACTGCGTTACTAATTATAATAATGCAGTAAAAAATTTAGGTCTTTCTGCACTTGA TGAACAGCCTCAATGTTCATCTTCTAGCAGTCATATACCCTGCAATGAAATATCCTT AGCAATGACTGATGACGACAATGCTGCGGCCATTCATGAAATTGAATCTCTATTGT CTGAACCGATAATAGCTCCTCAACTCCCAGCATTGCCACACAAGACATGGGCCAG TTATGCTTCAATCACT 3'	RdRp	1302-1650
CH2_505	5'AAGGCGTAGTAACATTAGCTGATTTCAAAGAAGCGGACCAGCATGTGGAGTAC ACTCACTTTGATCCTGAGTTAAAATCCACTGTTGACCCCCACCGGAGCTATGAAAA TGCCATCAACAATCTTGGCATTGAGATTAATGAGGATGTACCTGAAAGTTCCGGC ACCAATGAAACATTGCTTAACAATGAAATATCTTTAGCAATGTCATCTGCTGAACA TGTGCAAGCTGTTCAAGAAATTGAGTCTTTACTCTCTAACCCCGAAGCGGCACCAA TATTGCCCCCTGCACATGTTAAAACATGGGCTAGCCTTGCATCTGACACTTCAAGC ACTAAAAACCGTGAAATCGAAGATATAGTGGCTAAGCTGGAAATACAAAGAAAT GAAGCTAGTTGCAGCTACCTTCAACCAAATAAGGAATTGTCAAAACCCAAGGCTG CTGATAACAATCTCCCCTGGAATGCTTGGATCCCATTGCT 3'	RdRp	1325-1805

FIGURE LEGENDS

Figure 1. A. Similarity plot between PepMV-Sp13 (EU) and PepMV-PS5 (CH2). The similarity plot shows the variations of the sequence similarity between both PepMV isolates along the PepMV genome that helped us identify regions with the lowest nucleotide similarity. It also shows the genomic region that was finally chosen for the probes design corresponding to a specific region of the RdRp ORF.

B. Viral probes specificity. Dot-blot molecular hybridization on total RNA extracts from mock and single infected *N. benthamiana* plants. Assays with RdRp ORF1 riboprobes. Riboprobe of the PepMV-Sp13 isolate (PepMV-EU type; EU_505) and riboprobe of the PepMV-PS5 (PepMV-CH2 type; CH_505) were specific to their own isolates and no cross-reaction to counterpart isolates was observed.

Figure 2. Schematic representation of our ISH assay. Mock, mixed and single infected *N. benthamiana* leaves were sectioned in rectangular blocks, paraformaldehyde fixed, paraffin embedded and sectioned; the ISH was performed on consecutive leaf cross-sections of the same sample by using each probe (A or B) in each slide (2 or 1) respectively.

Figure 3. *In situ* hybridization (ISH) on consecutive leaf serial cross-sections of *N. benthamiana* mock and PepMV single infected samples. A, B: Leaf serial cross-sections from mock inoculated plants used as negative control of the ISH showing no target reaction with either the riboprobes EU_505 (**A)** or CH2_505 (**B)**, the uniform blue colour is due to the alcian blue post staining**. C, D:** ISH performed on consecutive leaf serial cross-sections from single EU inoculated plants with either the riboprobe EU_505 (**C**) or CH2_505 (**D**), the viral RNA of the EU isolate is only located in the **C** panel as an extended dark-blue coloured area. **E, F:** ISH performed on consecutive leaf serial cross-sections from single CH2 inoculated plants with either the riboprobe EU_505 (**E**) or CH2_505 (**F**), the viral RNA of the CH2 genotype is only located in the **F** panel as a dark-blue coloured area. In all samples, tissue infected corresponds to spongy mesophyll (M), abaxial and adaxial epidermis (E), palisade parenchyma (Pp) and vascular bundle (Vb) cells. Scale bars are displayed in the images.

Figure 4. *In situ* hybridization (ISH) on consecutive leaf serial cross-sections of PepMV mixed infected *N. benthamiana* leaves. A, B: ISH done on consecutive leaf serial cross-sections from mixed (PepMV-EU+CH2) inoculated plants with either the riboprobes EU_505 (**A**) or CH2_505 (**B**), the viral RNA of both EU and CH2 genotypes are located in the same extended dark-blue coloured area of the leaf sections. The infected tissue corresponds to abaxial and adaxial epidermis, spongy mesophyll, palisade parenchyma and vascular bundle cells**. C, D:** ISH done on consecutive leaf serial cross-sections from mixed (PepMV-EU+CH2) inoculated plants with either the riboprobe EU_505 (**C**) or CH2_505 (**D**), at this high magnification, the viral RNA of both EU and CH2 genotypes are located in a few dark-blue coloured cells (arrowheads) of the leaf together with other non-infected tissue cells or only infected by one of the isolates. The infected cells (arrowheads) correspond to spongy mesophyll, epidermis, palisade parenchyma and bundle sheath cells. Scale bars are displayed in the images.

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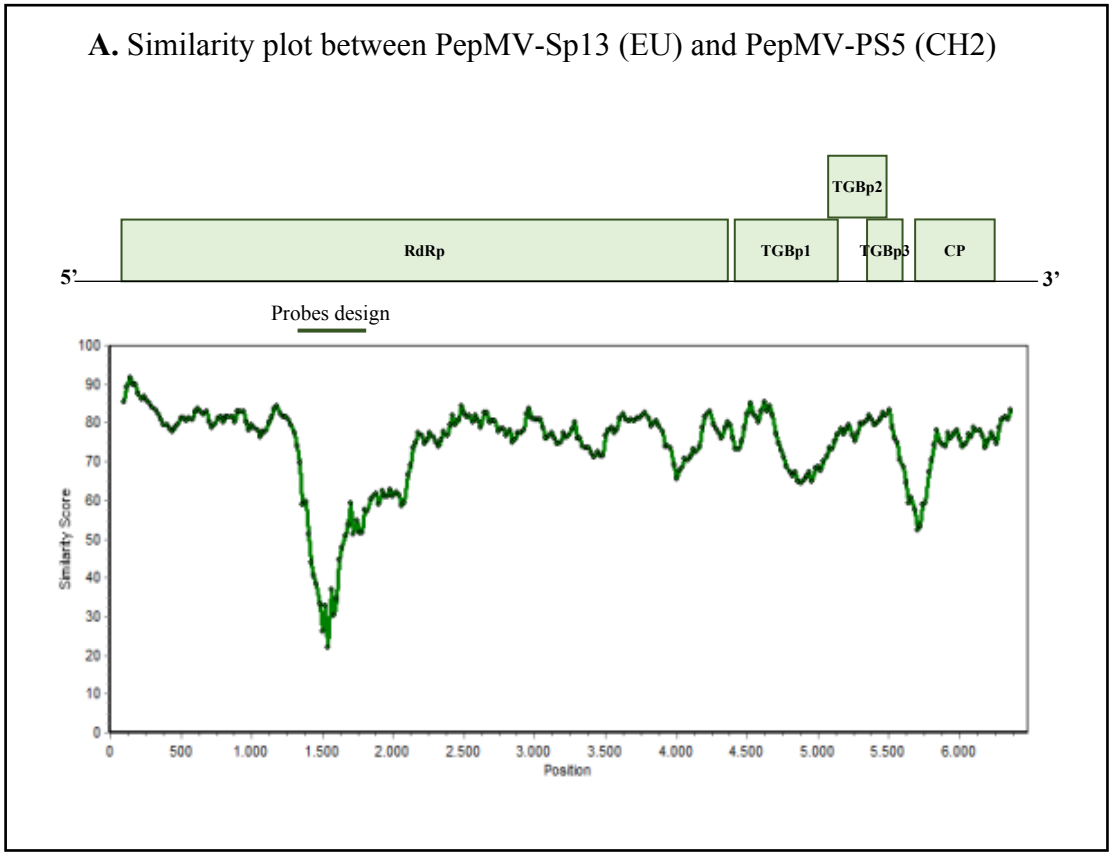
Figure 1. Gómez-Aix et al., 2018



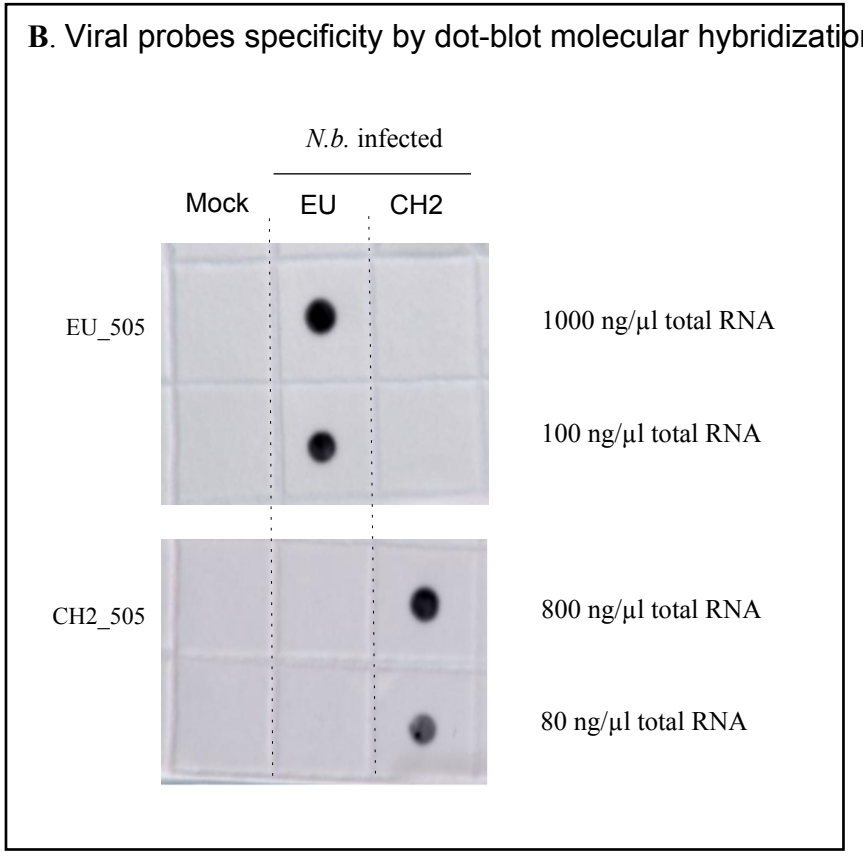


Figure 2. Gómez-Aix et al., 2018

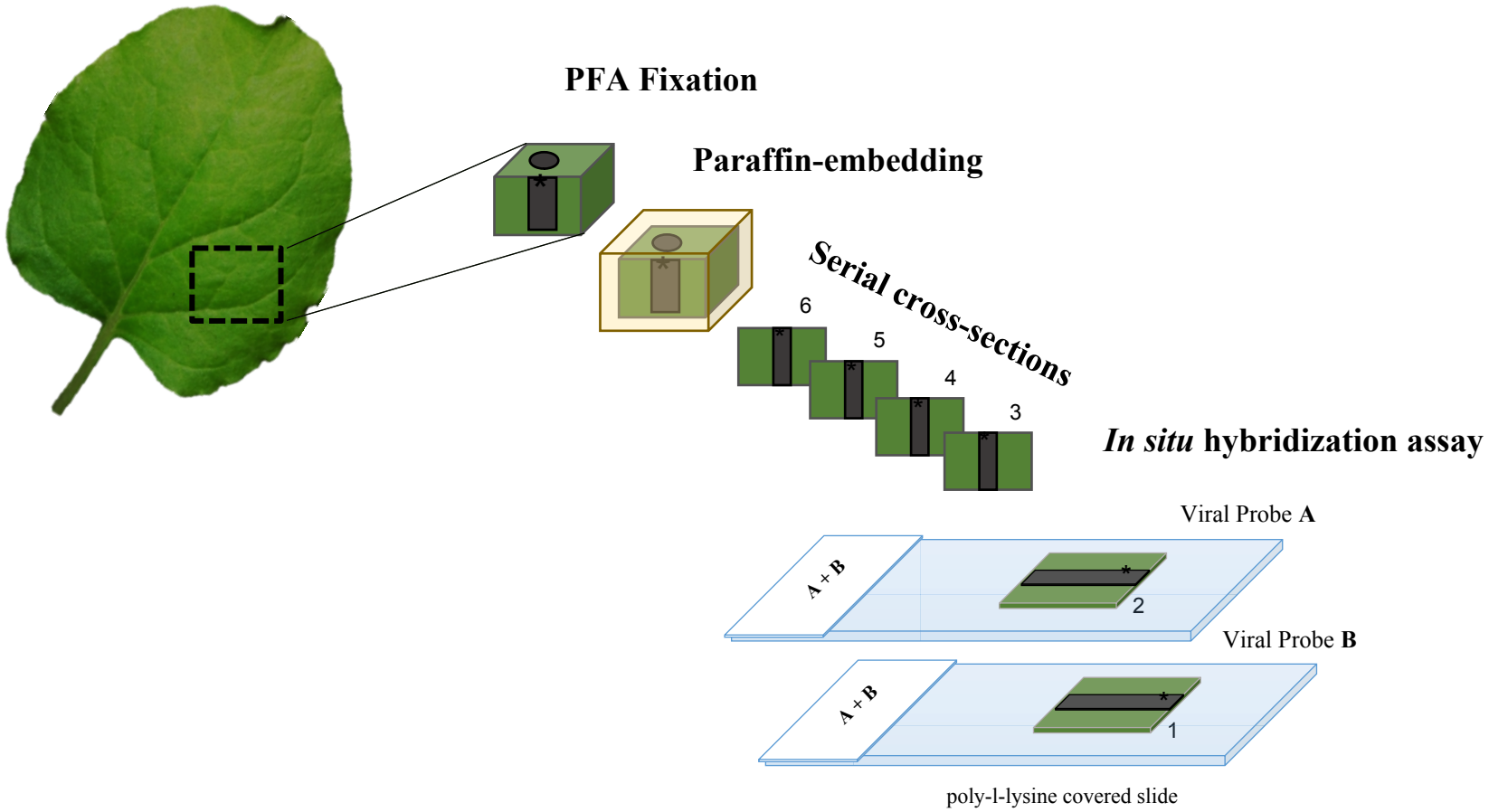


Figure 3. Gómez-Aix et al., 2018

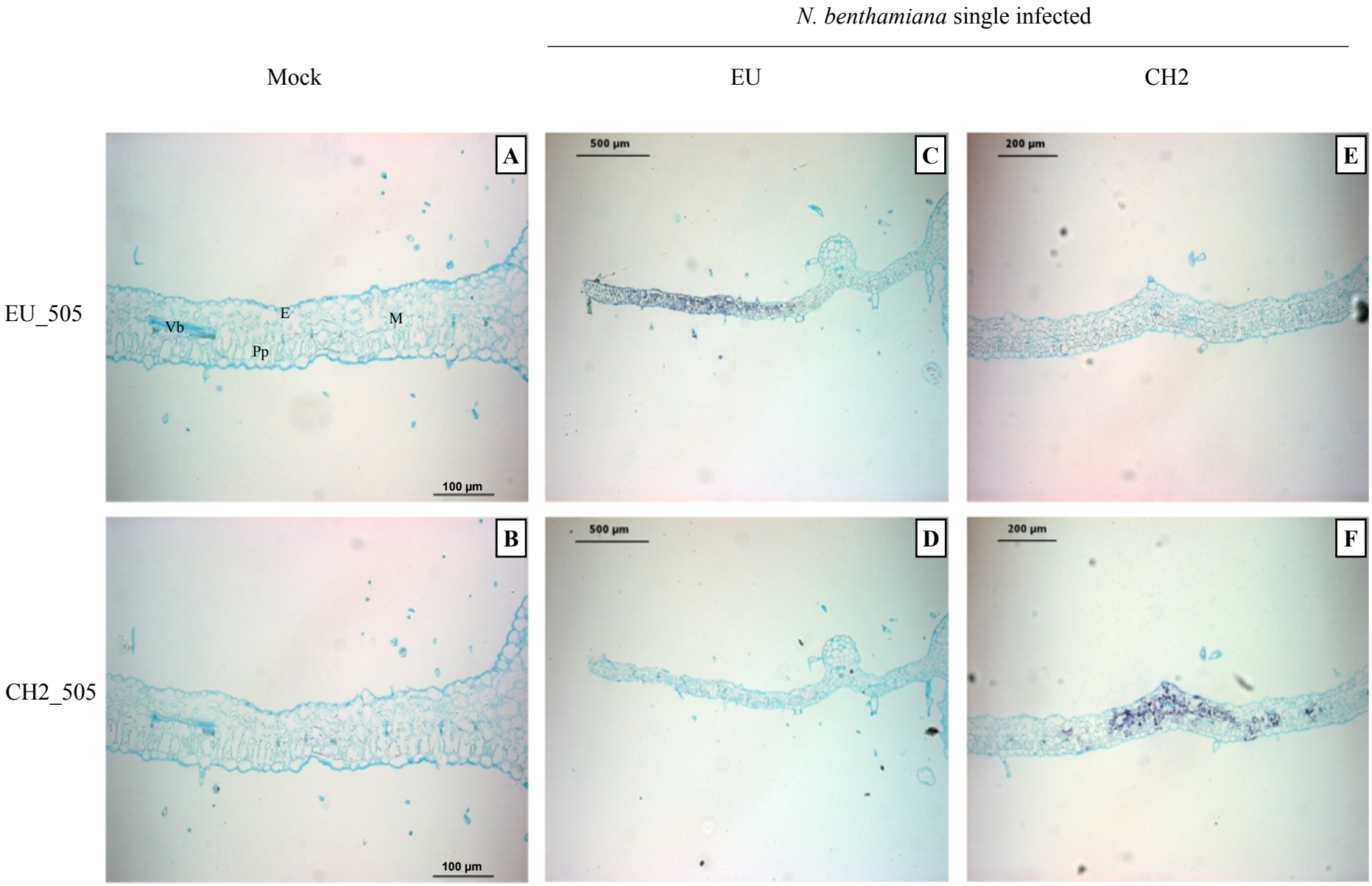


Figure 4. Gómez-Aix et al., 2018