MICROSCOPY

Microscopy **62(5)**: 547–553 (2013)
doi: 10.1093/jmicro/dft022

Technical Report

Rapid immunohistochemical diagnosis of tobacco mosaic virus disease by microwave-assisted plant sample preparation

Günther Zellnig, Stefan Möstl and Bernd Zechmann*

Institute of Plant Sciences, University of Graz, Schubertstrasse 51, 8010 Graz, Austria
*To whom correspondence should be addressed. E-mail: bernd.zechmann@uni-graz.at

Abstract Immunoelectron microscopy is a powerful method to diagnose viral diseases and to study the distribution of the viral agent within plant cells and tissues. Nevertheless, current protocols for the immunological detection of viral diseases with transmission electron microscopy (TEM) in plants take between 3 and 6 days and are therefore not suited for rapid diagnosis of virus diseases in plants. In this study, we describe a method that allows rapid cytohistochemical detection of tobacco mosaic virus (TMV) in leaves of tobacco plants.

With the help of microwave irradiation, sample preparation of the leaves was reduced to 90 min. After sample sectioning, virus particles were stained on the sections by immunogold labelling of the viral coat protein, which took 100 min. After investigation with the TEM, a clear visualization of TMV in tobacco cells was achieved altogether in about half a day. Comparison of gold particle density by image analysis revealed that samples prepared with the help of microwave irradiation yielded significantly higher gold particle density as samples prepared conventionally at room temperature.

This study clearly demonstrates that microwave-assisted plant sample preparation in combination with cytohistochemical localization of viral coat protein is well suited for rapid diagnosis of plant virus diseases in altogether about half a day by TEM.

Keywords immunoelectron microscopy, immunogold, microwave-assisted sample preparation, tobacco mosaic virus, transmission electron microscopy

Received 27 February 2013, accepted 20 March 2013; online 10 April 2013

Introduction

Immunoelectron microscopy is often used to diagnose viral diseases and to study the localization of viral particles within plant cells and tissues [1–6]. Sample preparation for such studies is quite time- and labour-consuming as most substances infiltrate the specimen quite slowly, as polymerization of the resin is time-consuming and as most steps have to be performed manually. Fixation, buffer washes, dehydration and infiltration of the sample take up to 2 days, whereas polymerization of the sample takes between 5 h and 3 days [1–9]. After polymerization, the sample has to be sectioned and treated with immunohistochemical methods. Such protocols involve the blocking of the sections, treatment with primary and secondary antibodies and several washes with buffers and distilled water. These steps can take between 4 and 17 h if overnight steps are included [1,2,4,5,8,10,11]. Considering all these different steps, the procedure can take between 3 and 6 days from the harvesting of the sample until the detection of the virus within the plant cell by immunogold labelling.

Recently, we have developed protocols for ultrastructural investigations that reduce plant sample preparation time for transmission electron microscopy (TEM) from 4 days to about 2–4 h [12–14]. Reduction in sample preparation time by microwave irradiation can be attributed to dielectric heating. This causes a rise in temperature within the whole sample, whereas conventional heating increases the temperature at the surface of the sample first and slowly works its way into the sample afterwards. The increase in temperature within the whole sample during microwave-assisted sample preparation enhances and accelerates the diffusion of reagents, protein cross-linking during fixation and the polymerization of the resin [15–17]. Thus, less time is needed for microwave-assisted sample preparation. Even though microwave-assisted sample preparation yields the same ultrastructural results as conventional sample preparation at room temperature (RT) [12,13], it remains unclear whether it alters the antigenicity of the plant sample. In previous studies, we have shown that with the help of microwave irradiation, fixation for plant samples can be reduced from 90 min to about 4 min without the loss of the antigenicity of the sample [10]. Similar results were found by other groups for plant species such as *Allium cepa* [18], tobacco [2,19] and pumpkin [2]. Nevertheless, the use of microwave irradiation during these procedures was limited to fixation and did not reduce sample preparation times for the following procedure. Thus, sample preparation times still took up to 4 days. Such protocols cannot, therefore, be used for rapid diagnosis of plant diseases or for evaluation of the distribution of the viral agents within a cell or plant.

In this study, we have applied microwave irradiation in order to rapidly identify tobacco mosaic virus (TMV) in infected leaves by immunoelectron microscopy. To verify the obtained results, we have compared the achieved labelling results between samples prepared with the help of microwave irradiation and conventional sample preparation at RT. TMV belongs to the genus of Tobamovirus, which is the most prevalent viral pathogen in tobacco plants and responsible for large crop losses every year [20]. Thus, the rapid diagnosis of TMV is of great importance to set measurements that help to constrain the spread of TMV in the field.

Methods

Plant material and virus inoculation

Nicotiana tabacum (L.) cv. Samsun nn, obtained from the German resource centre for biological material (DSMZ, Braunschweig, Germany), were grown in growth chambers with a day and night temperature of 24°C and 20°C, respectively, an illumination of 250 µmol m^{-2} s^{-1}, a photoperiod of 16 h and a humidity of 70%. Plants were kept in pots with soil and were watered adequately. Five-week-old *Nicotiana* plants were inoculated with TMV obtained from the German resource centre for biological material (DSMZ, strain id. for TMV: DSMZ PV-0107, TMV U1). For inoculation, 1 g of TMV-infected plant material (leaves of *N. tabacum* (L.) cv. Samsun nn showing strong symptoms) was homogenized in 1 ml of 0.06 M Sörensen phosphate buffer (pH 7.2) [21]. Then, celite (Sigma-Aldrich GmbH, Vienna, Austria) was applied to the homogenate and the inoculum was rubbed onto the first true leaves of the plants of one plant group. Next, mock inoculation was conducted on control plants by rubbing the buffer with celite onto the first leaves. Two weeks post inoculation, the youngest fully developed leaves (approximately 8 cm long and 5 cm in width) of control and TMV-infected plants (Fig. 1a and b) were harvested 2 h after the onset of daylight. Samples were taken from the centre of the leaves close to the middle vein and prepared for further investigations.

Conventional sample preparation

Sections of leaves (1 mm^2) from control and TMV-infected plants were cut on a modelling wax plate in a drop of 2.5% paraformaldehyde and 0.5% glutaraldehyde dissolved in 0.06 M Sörensen phosphate buffer at pH 7.2. Samples were then transferred into glass vials and fixed for 90 min at RT in the medium. Specimens were rinsed in buffer (4 times at 15 min each) and dehydrated for 20 min each step in a graded series of increasing concentrations of acetone (50, 70 and 90%). Samples were infiltrated with increasing concentrations (30, 50, 70 and 100%) of LR-White resin (London Resin Company Ltd, Berkshire, UK) mixed with 90%-acetone for a minimum of 3 h per step. Samples were left overnight (about 12 h) in 70% LR-White resin diluted with

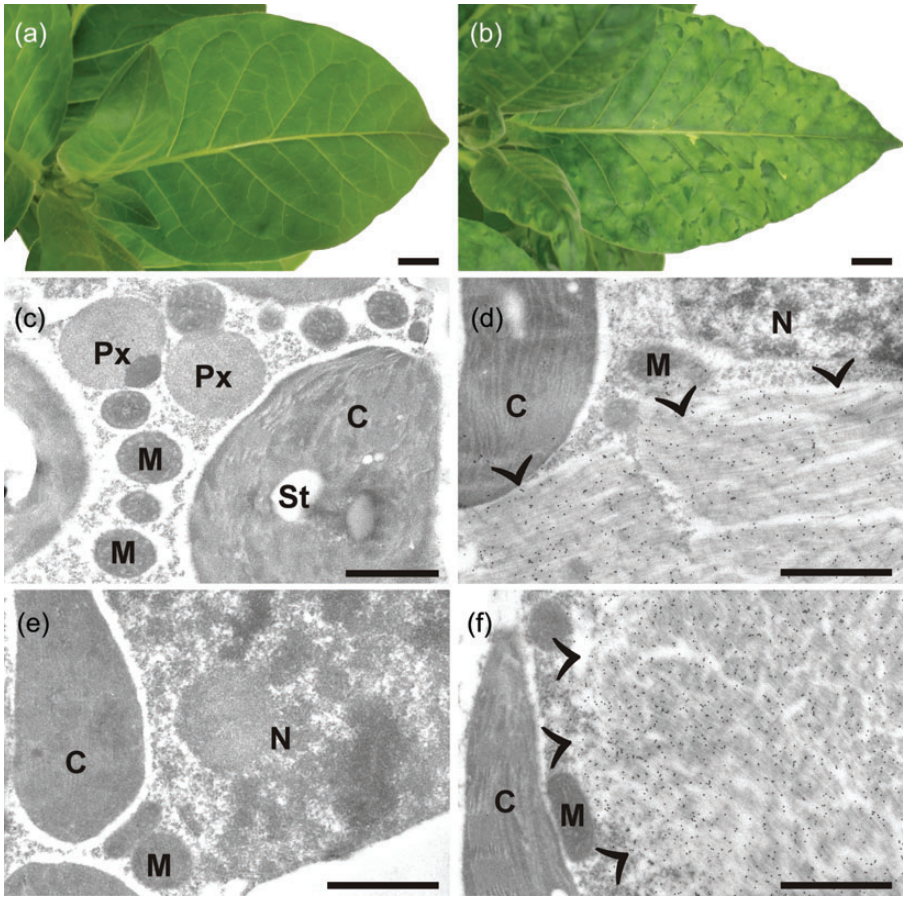

Fig. 1. Images of control and TMV-infected leaves and cells. When compared with the control (a), TMV-infected leaves showed strong symptoms of TMV-disease such as mosaic patterns and dark blisters (b). No ultrastructural differences in the structural preservation could be observed between samples prepared conventionally at RT (c and d) and samples prepared with the help of microwave irradiation (e and f). Control cells (c and e) lack ultrastructural alterations of TMV-disease and do not show immunogold labelling of TMV-coat protein on the sections. TMV-infected samples (d and f) show ultrastructural alterations of TMV such as accumulations of virions in the cytosol (marked by arrowheads). In the latter, gold particles bound to TMV-coat protein were found in large quantities. Higher amounts of gold particles bound to TMV-coat protein could be detected in samples prepared with the help of microwave irradiation (f) compared with samples prepared conventionally at RT (d). C = chloroplasts with and without starch (St), M = mitochondria, N = nuclei, Px = peroxisomes. Scale bar, 1 cm for (a) and (b) and 1 µm for (c)–(f).

90% acetone. Infiltration was continued for 5 h the next day using 100% LR-White resin. Samples were embedded in pure, fresh LR-White resin and polymerized at 50°C for 48 h.

Sample preparation using microwave irradiation

Sample preparation was performed with an automated microwave tissue processor (Leica EM AMW, Leica Microsystems, Vienna, Austria). For sample preparation, small sections of leaves (1 mm^2) from control and TMV-infected plants were cut on a wax plate in a drop of 2.5% paraformaldehyde and 0.5% glutaraldehyde dissolved in 0.06 M Sörensen phosphate buffer at pH 7.2 and transferred immediately into small baskets with a mesh width of approximately 200 µm. Two baskets holding four sections each were filled with TMV-infected plant samples and the remaining two baskets with control samples. These baskets were then transferred into the chamber of the microwave processor which already contained a vial filled with the previously stated fixative solution. Sample preparation was started approximately 2 min after cutting of the samples by starting the programmed protocol (Table 1). Sample preparation time for the different steps was determined and optimized in several preliminary trials. After the samples were infiltrated with pure LR-White resin (London Resin Company Ltd), they were transferred manually into small plastic vials filled with fresh 100% LR-White containing 1 drop (30 mg) of accelerator (London Resin Company

Table 1. Protocol for sample preparation using microwave irradiation

Vial number	Step	Duration (min)	Max. temp. (°C)	Reagent	Microwave mode	Max. power (W)
1	1	4	37	2.5% paraformaldehyde and 0.5% glutaraldehyde in phosphate buffer	Continuous	15
	2	4	20		Continuous	0
	3	4	37		Continuous	15
	4	4	20		Continuous	0
2	5	1	37	Phosphate buffer	Slope	20
3	6	1	37	Phosphate buffer	Pulse	15
4	7	1	37	Phosphate buffer	Slope	20
5	8	1	37	Phosphate buffer	Continuous	15
6	9	3	37	50% Acetone	Slope	20
7	10	3	37	70% Acetone	Slope	20
8	11	3	37	90% Acetone	Slope	20
9	12	5	37	LR-White:90% Acetone = 1:2	Continuous	10
10	13	5	40	LR-White:90% Acetone = 1:1	Continuous	10
11	14	5	45	LR-White:90% Acetone = 2:1	Continuous	10
12	15	5	50	100% LR-White	Continuous	12
13	16	5	50	100% LR-White	Continuous	12
14	17	5	50	100% LR-White	Continuous	12
Total time		59				

Vial number represents the order in which the vial was loaded into the carousel of the processor. The Step column shows the actual process. Max. temp. is the maximum temperature reached in the vial before the microwave irradiation was turned off. The reagents loaded in the vials are shown in the fifth column. The Microwave mode column shows the microwave irradiation setting: Continuous = rapid temperature increase, holding the set temperature; Slope = gentle temperature increase, final temperature reached at the end; Pulsed = Rapid temperature increase, power turned off until the temperature dropped 5°C, power resumed to reach temperature. The last column shows the maximum power of the microwave irradiation.

Ltd) per 10 ml of LR-White. Polymerization of LR-White containing the samples was performed under anaerobic conditions for a maximum of 30 min on ice to avoid excess heating of the sample.

Immunocytohistochemistry

Immunogold labelling of TMV-coat protein was performed with ultrathin sections on coated nickel grids with the automated immunogold labelling system Leica EM IGL (Leica, Microsystems, Vienna, Austria). The ideal dilutions and incubation times of the primary (anti-TMV U1 rabbit IgG; DSMZ, Braunschweig, Germany) and 10 nm gold-conjugated secondary antibody (goat anti rabbit IgG; British BioCell International, Cardiff, UK) were determined in preliminary studies by evaluating the labelling density after a series of labelling experiments. The final dilution of primary and secondary antibodies used in this study showed a minimum of background labelling outside the sample with a maximum of specific labelling in the sample. The sections were blocked for 15 min with 2% bovine serum albumin (BSA, Sigma-Aldrich, St Louis, MO, USA) in phosphate buffered saline (PBS, pH 7.2) and then treated with the primary antibody against TMV-coat protein diluted 1:500 in PBS containing 1% BSA for 30 min at RT. After a short rinse in PBS (3 times 5 min each), samples were incubated with a 10 nm gold-conjugated secondary antibody diluted 1:100 in PBS for 30 min at RT. After a short wash in distilled water (2 times 5 min), labelled grids were post stained with uranyl acetate (2% dissolved in aqua bidest) for 15 s and investigated in a Philips CM10 transmission electron microscope. Several negative controls were made with sections of TMV-infected leaves to support the specificity of the immunogold procedure. Negative controls were treated with (i) preimmune serum instead of the primary antibody, (ii) gold-conjugated secondary antibody (goat anti-rabbit IgG) without prior incubation of the section with the primary antibody or (iii) non-specific secondary antibody (goat anti rat IgG). These negative controls did not contain gold particles bound to TMV (Fig. 2), indicating that the obtained signal in TMV-infected samples (Fig. 1) specifically visualized the presence of TMV.

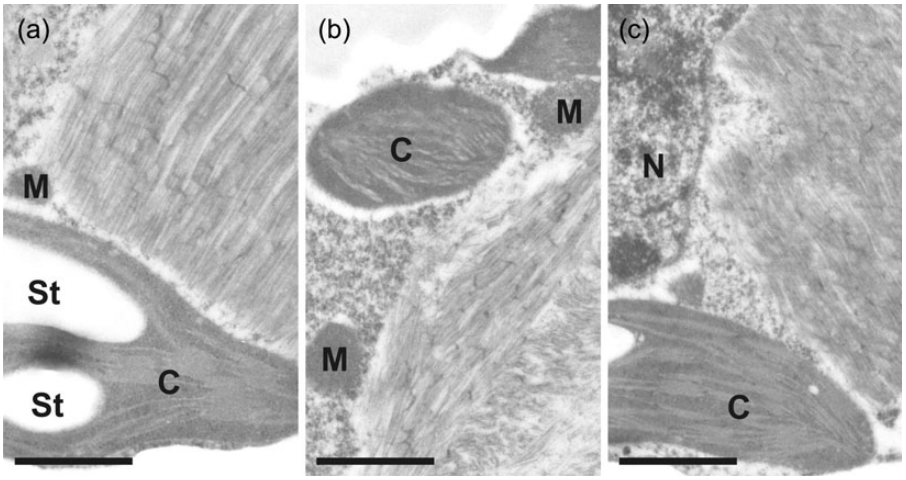

Fig. 2. TEM micrographs of TMV-infected leaf cells on sections treated as negative controls. Transmission electron micrographs of parts of TMV-infected cells after microwave-assisted (a) and conventional sample preparation at room temperature (b and c) treated as negative controls. Gold particles were absent when cells were treated with pre-immune serum instead of the primary antibody (a), after the omission of the primary antibody (b) and after the treatment with an unspecific secondary gold-conjugated antibody (c). C = chloroplasts with and without starch (St), M = mitochondria, N = nucleus. Scale bar, 1 µm.

Results and discussion

The results of this study clearly demonstrate that microwave-assisted plant sample preparation does not alter the ultrastructure and antigenicity of the plant sample. Samples prepared conventionally showed ultrastructure (Fig. 1c and d) similar to that of samples prepared with the help of microwave irradiation (Fig. 1e and f). These results are similar to those of other studies where microwave irradiation was used for the fixation of the plant sample without altering the ultrastructure and antigenicity of the plant sample despite a reduction of sample fixation time [2,10,18,19]. Nevertheless, the use of microwave irradiation in these studies was limited to the fixation process but was not used during the other steps of sample preparation. Thus, besides reduction of fixation time, no further reduction in sample preparation time in comparison with conventional sample preparation at RT was achieved. In this study, sample preparation time was reduced from about 3 days to 90 min (Table 2). Fixation could be reduced from 90 to 16 min with the microwave device used in this study, which is similar to the fixation time of 20 min that has been achieved using another microwave device for plant sample preparation [18]. Nevertheless, shorter fixation times (between 17 s and 4 min) could be achieved with other microwave devices for similar procedures [2,10,19], indicating that sample fixation time strongly depends on the microwave device.

Even though the ultrastructure in this study was not as well preserved in comparison with sample preparation protocols where osmium tetroxide was additionally used as post-fixative [12,13], all plant organelles could be clearly identified in all samples independent of the sample preparation method (Fig. 1c–f). Although osmium tetroxide could not be used in this study as it altered the antigenicity of the sample, fine structures such as thylakoids in chloroplasts and cristae in mitochondria were visible (Fig. 1e and f). In TMV-infected samples, large areas of virions accumulating in the cytosol were visible (Fig. 1d and f), which are typical ultrastructural alterations induced by this viral agent in tobacco [13,22,23]. Cytohistochemical labelling of TMV-coat protein identified these virions as TMV as gold particle labelling could be detected in these areas (Fig. 1d and f). Gold particle density in these areas was higher in samples prepared with the help

Table 2. Plant sample preparation times

Step	Microwave	Conventional
Fixation	16 min	90 min
Buffer washes	4 min	60 min
Dehydration	9 min	60 min
Infiltration	30 min	23 h
Polymerization	30 min	48 h
Total time	89 min (1 h and 29 min)	4470 min (74 h and 30 min)

Comparison of time needed for the different steps between microwave-assisted (Microwave) and conventional sample preparation at RT (Conventional).

Table 3. Labelling density of TMV-coat protein

	Microwave	Conventional
Number of gold particles	127 ± 7	88 ± 4***

Data are means with standard errors and document the amount of gold particles per μm^2 bound to TMV-coat protein in areas of virions accumulating in the cytosol of TMV-infected tobacco leaves. Significant differences were calculated by using the Mann–Whitney *U*-test.
***Significance at the 0.001 levels of confidence ($n > 50$).

of microwave irradiation (Fig. 1f) compared with samples prepared conventionally at RT (Fig. 1d, Table 3). An improved antigenicity of samples fixed with the help of microwave irradiation has also been described by other authors [15,18,24–26]. Considering these results, this study clearly demonstrates that the described protocol allows the detection of TMV within infected leaves within about half a day after the harvesting of the sample. As other comparable protocols described in the literature for plant samples usually need between 3 and 6 days [1,2,4,5,8,10,11], this method represents the fastest protocol currently available in the literature for immunoelectron microscopy of plant samples. As most steps were performed automatically, this procedure does not only mean a massive reduction in sample preparation time but also saves a lot of labour and time that can be used otherwise. For example, it is possible to perform negative staining of viral particles and the determination of their size during microwave-assisted plant sample preparation. Additionally, it is possible to perform microwave-assisted sample preparation for ultrastructural analysis as described recently [12–15] during immunogold labelling. This would allow a combined ultrastructural and cytohistochemical diagnosis of plant virus diseases within about a single day.

Conclusions

Summing up, the results of this study demonstrate that microwave-assisted plant sample preparation decreases plant sample preparation time for TEM from 3 to 6 days needed for conventional sample preparation at RT to 90 min without negatively affecting the antigenicity of the sample. In combination with the cytohistochemical localization of viral coat protein, this method is well suited for the rapid diagnosis of plant virus diseases in about half a day by TEM.

Funding

This project was supported by the Austrian Science Fund (P20619-B16 and P22988-B16 to B.Z.). Funding to pay the Open Access publication charges for this article was provided by Austrian Science Fund (FWF).

References

1 Abouzid A M, Barth A, and Jeske H (1998) Immunogold labeling of the abutilon mosaic virus in ultrathin sections of epoxy resin embedded leaf tissue. *J. Ultra. Mol. Struct. Res.* **99**: 38–47.

2 Riedel D, Lesemann D E, and Maiss E (1998) Ultrastructural localization of nonstructural and coat proteins of 19 potyviruses using antisera to bacterially expressed proteins of plum pox potyvirus. *Arch. Virol.* **143**: 2133–2158.

3 Vellios E, Duncan G, Brown D, and MacFarlane S (2002) Immunogold localization of tobravirus 2b nematode transmission helper protein associated with virus particles. *Virology* **300**: 118–124.

4 Hamacher J, Wettern M, and Schulz M (2003) Ubiquitination of TMV coat protein aggregates in infected tobacco leaves. *J. Phytopathol.* **151**: 652–659.

5 Medina V, Pinner M S, Bedford I D, Achon M A, Gemeno C, and Markham P G (2006) Immunolocalization of tomato yellow leaf curl sardinia virus in natural host plants and its vector *Bemisia tabaci*. *J. Plant Pathol.* **88**: 299–308.

6 Isogai M, Nakamura T, Ishii K, Watanabe M, Yamagishi N, and Yoshikawa N (2011) Histochemical detection of blueberry latent virus in highbush blueberry plant. *J. Gen. Plant Pathol.* **77**: 304–306.

7 Wells B (1985) Low temperature box and tissue handling device for embedding biological tissue for immunostaining in electron microscopy. *Micron Microsc. Acta* **16**: 49–53.

8 Prod'homme D, Le Panse S, Drugeon G, and Jupin I (2001) Detection and subcellular localization of the turnip yellow mosaic virus 66K replication protein in infected cells. *Virology* **281**: 88–101.

9 Isogai M, Uyeda I, and Lee B C (1998) Detection and assignment of proteins encoded by rice black streaked dwarf fijivirus S7, S8, S9 and S10. *J. Gen. Virol.* **79**: 1487–1494.

10 Zechmann B and Müller M (2010) Subcellular compartmentation of glutathione in dicotyledonous plants. *Protoplasma* **246**: 15–24.

11 Zechmann B, Stumpe M, and Mauch F (2011) Immunocytochemical determination of the subcellular distribution of ascorbate in plants. *Planta* **233**: 1–12.

12 Zechmann B and Zellnig G (2009) Microwave-assisted rapid plant sample preparation for transmission electron microscopy. *J. Microsc.* **233**: 258–268.

13 Zechmann B and Zellnig G (2009) Rapid diagnosis of plant virus diseases by transmission electron microscopy. *J. Virol. Methods* **162**: 163–169.

14 Zechmann B, Graggaber G, and Zellnig G (2011) Microwave assisted rapid diagnosis of plant virus diseases by transmission electron microscopy. *J. Vis. Exp.* **56**: e2950: DOI: 10.3791/2950.

15 Leong A S Y and Sormunen R T (1998) Microwave procedures for electron microscopy and resin-embedded sections. *Micron* **29**: 397–409.

16 Kok L P and Boon M E (2003) *Microwaves for the Art of Microscopy* (Coulomb Press, Leyden, Leiden).

17 De la Hoz A, Diaz-Ortiz A, and Moreno A (2005) Microwaves in organic synthesis. Thermal and non-thermal microwave effects. *Chem. Soc. Rev.* **34**: 164–178.

18 Lería F, Marco R, and Medina F J (2004) Structural and antigenic preservation of plant samples by microwave-enhanced fixation, using dedicated hardware, minimizing heat-related effects. *Microsc. Res. Tech.* **65**: 86–100.

19 Benhamou N, Noel S, Grenier J, and Asselin A (1991) Microwave energy fixation of plant tissue: an alternative approach that provides

excellent preservation of ultrastructure and antigenicity. *J. Electron Microsc. Tech.* **17**: 81–94.

20 Scholthof K B G (2004) Tobacco mosaic virus: a model system for plant biology. *Annu. Rev. Phytopathol.* **42**: 13–34.

21 Sörensen S P L (1909) Über die Messung und die Bedeutung der Wasserstoffionenkonzentration bei enzymatischen Prozessen. *Biochem. Zeit.* **21**: 131–304.

22 Milne R G (1966) Multiplication of tobacco mosaic virus in tobacco leaf palisade cells. *Virology* **28**: 527–532.

23 Reunov A V, Gnutova I V, and Lapshina L A (2006) Effect of tobacco mosaic virus strains on the ultrastructure of tobacco leaf parenchymal cells. *Biol. Bull.* **33**: 409–415.

24 Jamur M C, Feraco C D, Lunardi L O, Siraganian R P, and Oliver C (1995) Microwave fixation improves antigenicity of glutaraldehyde-sensitive antigens while preserving ultrastructural detail. *J. Histochem. Cytochem.* **43**: 307–311.

25 Login G R, Ku T C, and Dvorak A M (1995) Rapid primary microwave-aldehyde and microwave-osmium fixation: improved detection of rat parotid acinar cell secretory granule α-amylase using a post-embedding immunogold ultrastructural morphometric analysis. *J. Histochem. Cytochem.* **43**: 515–523.

26 Ruijter E T, Miller G J, Aalders T W, van de Kaa C A, Schalken J A, Debruyne F M, the BIOMED-II MPC study group, and Boon M E (1997) Rapid microwave-stimulated fixation of entire prostatectomy specimens. *J. Pathol.* **183**: 369–375.