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Using Microwave Irradiation to Improve Preservation of Female Nematodes and Gall Tissues for TEM Observations

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Abstract: Microwave irradiation of glutaraldehyde-immersed samples was evaluated for the chemical fixation of 3-week-old galls that resulted from the infection of tomato roots (*Lycopersicon esculentum*) by a root-knot nematode, *Meloidogyne incognita*. Observation by transmission electron microscopy indicated that the best results were obtained when vials containing the intact galls were immersed in buffered glutaraldehyde and irradiated for 10 seconds then allowed to cool for 30 seconds; this procedure was repeated two additional times. Galls that were fixed by this method and subsequently embedded in resin provided thin sections that remained stable in the electron beam so that fine structural details could be evaluated and photographed. Root cortical cells displayed no indication of osmotic stress, which usually results in plasmolysis or displacement of the cytoplasm toward the interior of the cell. All organelles in the giant cells appeared normal and well fixed. Cross sections near the center of the gall showed that the hypodermis of the female was not separated from the cuticle, which in turn was appressed to the outer cell walls of the giant cells. No obvious evidence of shrinkage, distortion, or failure of resin infiltration into the female nematode was apparent. High magnifications of the female nematode indicated that fine structural features of the tissues were also well preserved. Immersion fixation combined with microwave irradiation not only improved fixation of older tissues but enabled preservation of stages and feeding sites that could not be easily obtained by conventional methods.

Key words: electron microscopy, giant cell, *Meloidogyne incognita,* methodology, microwave fixation, nematode.

During the last 10 years, microwave irradiation has been used to improve fixation of many diverse biological samples (Login and Dvorak, 1994). For example, Heumann (1992) showed that microwave irradiation of plant tissues immersed in buffered glutaraldehyde improved preservation of the internal membrane systems and the starch grains of plastids. Benhamon et al. (1991) concluded that the structure of the compound middle lamella of the cell wall was improved after microwave irradiation as opposed to either conventional immersion fixation or conductive heating. In animal tissues, Login and coworkers (Login and Dvorak, 1985, 1988; Login et al., 1986, 1987, 1989) showed that only a few seconds of irradiation of 1-cm^3 cubes of rat liver produced preservation that equaled hours-long immersion fixation in similar glutaraldehyde mixtures.

Recently, Jones and Gwynn (1991) used microwave irradiation to improve fixation of unhatched eggs and second-stage juveniles of *Globodera rostochiensis*. The authors also stated, ''It is also often extremely difficult to obtain well-fixed preparations of the nematode at other stages in its life cycle, such as those that occur inside the host plant, because the root tissue surrounding the animal presents a further barrier to fixatives.'' In our laboratory, transmission electron microscopy (TEM) and scanning electron microscopy (SEM) studies also have been hampered by difficulties in preserving older nematodes and plant galls by conventional chemical immersion techniques. Encouraged by the favorable results that others have obtained using microwave irradiation for chemical fixation of biological tissues, we initiated a study to evaluate the benefits of using this technique to improve tissue preservation and resin infiltration of galls induced by the root-knot nematode.

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Materials and Methods

Nematode cultures: Monoxenic cultures of the root-knot nematode (*Meloidogyne incognita*) on excised roots of tomato (*Lycopersicon esculentum* cv. Rutgers) were maintained on Gamborg's B5 Medium (Gibco BRL, Grand Island, NY). For TEM observation, 3-week-old galls were removed from the cultures and processed by the procedures described below.

Fixation protocols: All microwave irradiations were performed in a commercial microwave oven (model ML-40, 500 watts, Amana Refrigeration, Amana, IA). Initially, the cool spots and hot spots within the oven were determined with a neon bulb array (Login and Dvorak, 1994). A beaker containing 400 ml of distilled water was placed in the rear corner of the oven to serve as a water load in all of the trials, to prevent overheating of the fixative, which consisted of 3% glutaraldehyde in 0.05M phosphate buffer at pH 6.8 (Login and Dvorak, 1994; Utsunomiya et al., 1991). The following protocols were tested: (i) intact galls were irradiated continuously at the oven's high setting for 30 seconds in a cool spot of the oven, and attempts were then made to cut the galls and remove the female nematodes; (ii) intact galls were irradiated continuously at the high setting for 30 seconds in a cool spot, then kept in the fixative for 1.5 hours; (iii) intact galls were irradiated continuously at the high setting for 30 seconds in a cool spot, then transferred to fresh, room-temperature fixative; (iv) intact galls were irradiated continuously at the high setting for 10 seconds in a cool spot, then transferred to fresh fixative (this procedure was repeated five additional times before proceeding further); (v) intact galls were irradiated continuously for 10 seconds at the high setting in a cool spot and allowed to cool for 30 seconds this procedure was repeated two additional times, then the samples were transferred to fresh, room-temperature fixative; (vi) as a control, intact galls were fixed by immersion for 2 hours in the fixative at room temperature.

In subsequent processing, samples from protocols 3, 4, and 5 remained in glutaraldehyde at room temperature for 2 hours, then were placed in the refrigerator overnight. Following fixation, these samples and those from the remaining protocols were processed as previously described (Endo and Wergin, 1973; Wergin and Endo, 1976). Briefly, fixation was followed by washing in 6 changes of 0.05M phosphate buffer over a 1-hour period. The samples were then postfixed in 2% buffered osmium tetroxide for 2 hours, dehydrated, and infiltrated with a low-viscosity embedding medium (Spurr, 1969). Silver-gray sections of selected areas of the galls were cut on a Reichert/AO Ultracut microtome (Leica, Deerfield, IL) with a Diatome diamond knife (Diatome US, Fort Washington, PA) and mounted on 400-mesh grids. The sections were stained with 4% aqueous uranyl acetate for 10 minutes, then with 3% lead citrate for 5 minutes. Thin sections were viewed in a Hitachi H-7000 transmission electron microscope operating at 75 kV with a 30-µm objective aperture. Images were recorded on electron microscope film.

Results

Six different protocols were used to chemically fix the 3-week-old galls. The best results were obtained with protocol 5, in which vials containing the intact galls immersed in buffered glutaraldehyde were irradiated continuously for 10 seconds in a predetermined cool spot of the oven and allowed to cool for 30 seconds; this procedure was repeated two additional times. In protocol 1, too much mechanical damage was introduced when attempts were made to cut the galls into smaller sections and remove the female nematodes prior to further processing; consequently, these samples were discarded without further processing. The remaining protocols resulted in tissue blocks that exhibited varying degrees of improper infiltration with resin; consequently, thin sections were difficult to obtain from these samples. One to 2-µm-thick sections could occasionally be obtained for light microscopic examination; however, the thin sections from these blocks were generally

fragmented and unstable when observed with the electron beam in the TEM. The section fragments that could be obtained from these blocks occasionally exhibited well-fixed giant cells, but the female nematode usually was not properly infiltrated with resin.

Thin sections: Galls fixed with protocol 5 provided the most consistent, intact thin sections that could be easily recovered and mounted on grids. Furthermore, these sections remained stable in the electron beam during observation so that fine structural details could be evaluated and photographed. The detailed observations on plant and nematode tissues described below were obtained by using this protocol.

Fine-structural detail: Intact sections of an entire block face, measuring about 0.5 mm by 1.0 mm, were easily obtained (Fig. 1). These sections contained cross-sections of the entire gall, including the female nematode, the giant cells, and surrounding vascular and cortical tissues of the root. Tissues of both the female nematode and the plant cells were well infiltrated with resin that appeared fully polymerized.

The giant cells had cytoplasm with small but conspicuous vacuolar elements throughout their lumens, whereas each of the adjacent cortical cells had a single, large vacuole and only a narrow peripheral band of cytoplasm that lay appressed to the cell wall (Fig. 2). The cortical cells displayed no indication of osmotic stress, which usually results in plasmolysis or displacement of the cytoplasmic band toward the interior of the cell. The vacuoles of the cortical cells along with the lumens of the thick-walled xylem elements were generally well infiltrated with resin.

All organelles in the giant cells appeared normal and well fixed. The numerous proplastids exhibited characteristic electron-opaque contents and small vesicles; the mitochondria, which were somewhat more translucent, exhibited well-preserved cristae throughout their lumina (Fig. 3). The remaining cytoplasm contained distinct ribosomes, rough endoplasmic reticulum, and sparsely scattered lipid bodies. In the mitochondria, discrete electron-opaque particles, about 15 nm in diameter, probably represented the mitochondrial ribosomes.

Cross-sections near the center of the gall

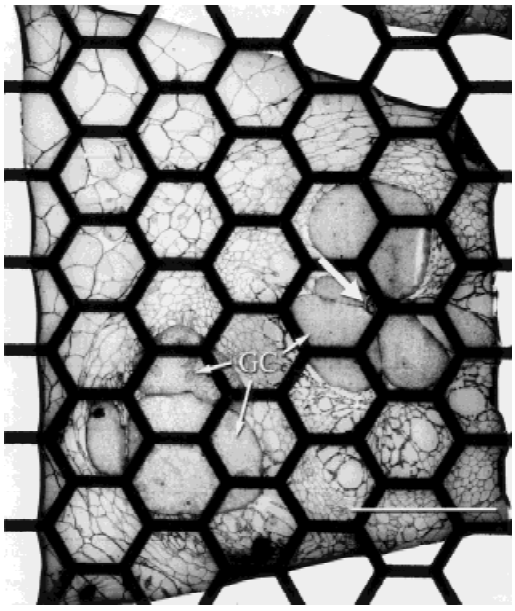

FIG. 1. Effect of microwave irradiation on glutaraldehyde fixation and sectioning of tomato roots infected with *Meloidogyne incognita*. Thin section on nickel grid illustrating the giant cells and surrounding tissues well infiltrated with polymerized resin. Nematode (large arrow) is partially obscured by grid bar. Scale bar = 0.2 mm (GC = giant cells).

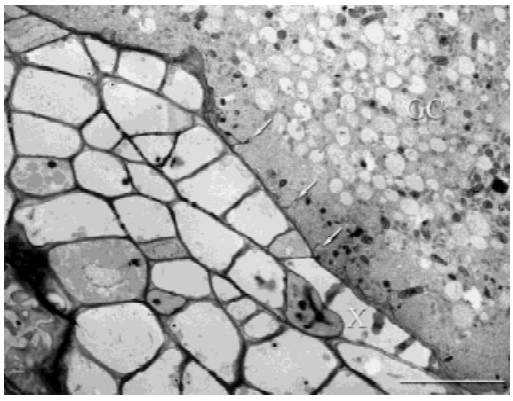

FIG. 2. Preservation of cytoplasmic fine structure by microwave irradiation of glutaraldehyde-fixed galls from tomato roots: portion of a giant cell (GC) bounded by small, numerous cortical cells. The lumen of the giant cell, which exhibits wall ingrowths (arrows), is filled with cytoplasmic organelles. Most cortical cells exhibit a thin band of parietal cytoplasm appressed to the cell walls. Neither plasmolysis nor shrinkage are evident. Scale bar = 10.0 μm (X = Xylem element).

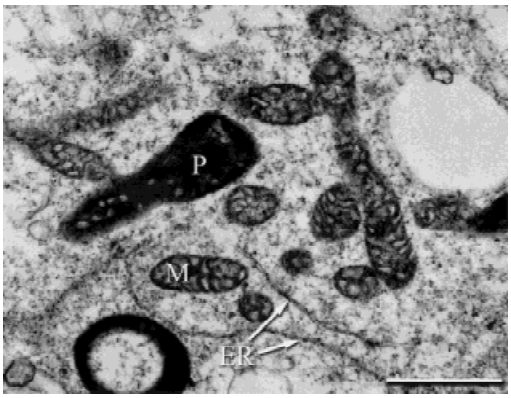

FIG. 3. Preservation of organelle fine structure by microwave irradiation of a glutaraldehyde-fixed giant cell from tomato. Ribosomes are distinct. Membranes of the rough endoplasmic reticulum (ER) are well-defined along with those of the cristae and vesicles found in the mitochondria (M) and proplastids (P), respectively. The matrices of the latter organelles also exhibit electron opaque content. Scale bar = 1.0 µm.

showed that the hypodermis of the female nematode had not separated from the cuticle, which in turn was tightly appressed to the outer cell walls of the giant cells (Fig. 4). No obvious evidence of shrinkage, distortion, or failure of resin infiltration was apparent. High magnifications of the female indicated that fine structural features of the tissues were well preserved (Fig. 5). Structures such as thick and thin filaments in the somatic muscle cells were discrete, the mitochondria had an evenly distributed electron-lucent matrix, and the electron-opaque granular content of the sheath cell, which surrounded the amphidial nerve processes, helped to delineate the amphid from the surrounding tissues.

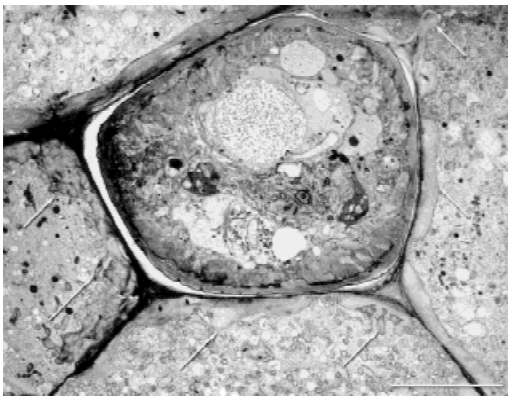

FIG. 4. Preservation of fine-structural features by microwave irradiation of glutaraldehyde-fixed galls from tomato roots. Cross-section near the anterior of the female nematode bounded by several giant cells, which exhibit considerable cell-wall ingrowths (arrows). The nematode is well infiltrated with resin, and very little space is apparent between the hypodermis and the inner cuticular layer of the nematode or between the outer cuticular layer and the outer cell walls of the adjacent giant cells. Scale bar = 10 µm.

DISCUSSION

Investigators have long known that biological membranes act as boundaries to diffusion (Hayat, 1981; Hopwood, 1967); therefore, chemical infiltration with fixatives requires an hour or more to penetrate most tissue samples. Recently, investigators have

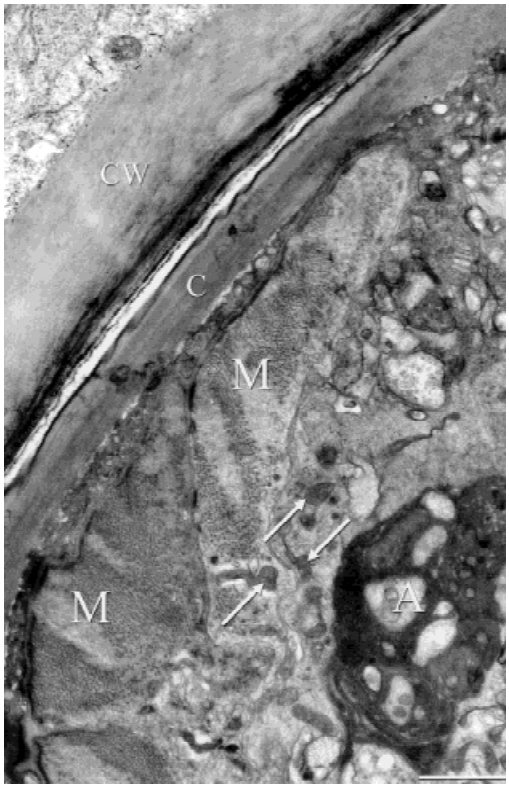

FIG. 5. Preservation of fine-structural features of *Meloidogyne incognita* by microwave irradiation of glutaraldehyde-fixed galls. Some of these features include the somatic muscle cells (M), with well-defined thick and thin fibers; the amphid (A), which is characterized by electron-opaque contents in the sheath cell; and mitochondria (arrows) that have granular matrices and internal cristae. Scale bar = 1.0 µm (C = cuticle; CW = cell wall).

found that microwave-assisted fixation occurs in seconds. As a result, rapid cellular events such as vesicle formation can now be analyzed (Eggli et al., 1991; Mizuhira and Hasegawa, 1990). In nematology, infiltration with aldehydes has been commonly used for nearly 30 years to chemically fix nematode tissues for TEM observation. However, in many species the cuticle is almost impermeable to fixatives (Jones and Gwynn, 1991); consequently, investigations of forms such as eggs, cysts, and intact galls, where the nematode is surrounded by plant tissues, have had limited success. Jones and Gwynn (1991) demonstrated that microwave irradiation could be used to improve fixation of unhatched nematodes and second-stage juveniles of *G. rostochiensis,* and suggested that this method could also be used to improve fixation of other stages of the life cycle, especially those that occur in the host plant. Our results indicate that microwave fixation does indeed improve fixation as well as resin infiltration in young females that are embedded in plant tissue. Thin sections through the giant cells reveal ultrastructural details present in the female nematode and the giant cells that are comparable to those that have been described for much younger stages of infection (Endo and Wergin, 1973).

The actual physico-chemical mechanisms involved in microwave-assisted chemical fixation are unknown (Login and Dvorak, 1994), partly arising from the difficulty in separating thermal and nonthermal effects of microwave irradiation. Several investigators have shown that microwaves preserve biological specimens by inactivating thermolabile enzymes (Ikarashi et al., 1984, 1985; Medina et al., 1975; Stavinoha, 1983; Theodorsson et al., 1990) and by denaturing proteins (Hopwood et al., 1984, 1988). However, microwave irradiation also generates heat, which (i) decreases the time of penetration of the fixative into the tissues, (ii) increases the rate of the chemical reactions that occur between the cellular components and the fixative (Hayat, 1970), and (iii) enhances the fluidity of membrane lipids (Hayat, 1989). In our experiments, the best results were obtained when a 10-second microwave interval was followed by a 30-second cooling period, then repeating this procedure two additional times. This procedure was designed to minimize heating of the sample; consequently, the improved results that we obtained may be attributed more to the non-thermal effects of microwave irradiation, such as inactivating enzymes and denaturing proteins.

Many variables must be considered when chemical fixation is combined with microwave irradiation. Temperature, time, oven power, sample size, specimen containers, and water loads must be standardized to achieve reproducible results. Numerous studies of irradiation times ranging from milliseconds to as long as 30 minutes have led to the recommendation that less than 30 seconds is most desirable (Login and Dvorak, 1994). Household microwave ovens operate only on full power, even though they may offer a selection of power settings (Login and Dvorak, 1994). Therefore, selection of a 20-second time period at 50% power causes an oven to cycle on and off, actually delivering 10 seconds of full power and 10 seconds of no power. The size of the specimen and the composition of its container also must be considered. Microwave energy will penetrate only 1 to 2 cm into tissues that have a high water content (Hahn et al., 1980; Johnson and Guy, 1972; Kok and Boon, 1992; Lin, 1986). Plastic vials or metal-free glass, which is transparent to microwave energy, ensure maximum penetration. Finally, adding a container with 200 to 500 ml of water to domestic microwaves during the irradiation period has been associated with improved reproducibility of results (Utsunomiya et al., 1991). This water load serves to warm up the magnetron and power supply and is the principal absorber of microwave energy, thus helping to prevent the biological sample from overheating (Login and Dvorak, 1985). We attempted to standardize a domestic microwave oven in this study; however, laboratory microwave ovens, which are considerably more expensive, have variable power settings and may be easier to calibrate for optimal results.

Immersion fixation combined with microwave irradiation not only improves fixation of older tissues but also enables preservation of nematode stages and feeding sites that cannot be obtained easily by conventional methods. In other biological tissues, investigators have shown that this procedure preserves enzymatic activity. Consequently, it may also serve as a procedure for gaining additional physiological and biochemical insights into events that occur in the female nematode and the giant cells and into resistant and susceptible reactions that occur within host plants.

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