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Oolong tea and LR-White resin: a new method of plant sample preparation for transmission electron microscopy

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Summary

Simplifying sample processing, shortening the sample preparation time, and adjusting procedures to suitable for new health and safety regulations, these issues are the current challenges which electron microscopic examinations need to face. In order to resolve these problems, new plant tissue sample processing protocols for transmission electron microscopy should be developed. In the present study, we chose the LR-White resin-assisted processing protocol for the ultrastructural observation of different types of plant tissues. Moreover, we explored Oolong tea extract (OTE) as a substitute for UA in staining ultrathin sections of plant samples. The results revealed that there was no significant difference between the OTE double staining method and the traditional double staining method. Furthermore, in some organelles, such as mitochondria in root cells of tomatoes and chloroplast in leaf cells of watermelons, the OTE double staining method achieved little better results than the traditional double staining method. Therefore, OTE demonstrated good potentials in replacing UA as a counterstain on ultrathin sections. In addition, sample preparation time was significantly shortened and simplified using LR-White resin. This novel protocol reduced the time for preparing plant samples, and hazardous reagents in traditional method (acetone and UA) were also replaced by less toxic ones (ethanol and OTE).

Introduction

Transmission electron microscopy (TEM) is a very useful technology in cell biology for observation of cell ultrastructures and location different molecules at the subcellular level (Watson, 1958; Ferguson *et al.*, 2017). In TEM observations, it is very important to let the samples keep much closer to their native states (Hall & Hawes, 1991; Hawes & Satiat-Jeunemaitre, 2001). From fresh materials to final TEM observations, the specimen preparation procedures are tedious and arduous. While improving sample protocols, three main aspects are taken into account. First, sample processing for TEM can be hard and wasting time, because of the different steps for fixation, dehydration, embedding and polymerization of the materials. Methods that reduce preparation time without loss of quality of the cell ultrastructure are highly desired. Second, fixation is one of the key steps in specimen preparation. A few researchers have attempted to optimize this step by replacing conventional chemical fixation with cryofixation. These methods demonstrated some advantages over conventional ones (Hess, 2007; Kang, 2010; Hurbain & Sachses, 2011), but they are limited by the high cost, low throughput, and potential sample damages during freezing. Therefore, optimizing sample fixation for TEM remains significant. Finally, the use of uranyl acetate (UA) and lead citrate (Pb), two contrasting agents that are essential in nearly all TEM sample preparation methods and routinely used to date, is being increasingly restricted in a lot of countries (Hall & Hawes, 1991; Hayat, 2000; Hawes & Satiat-Jeunemaitre, 2001; Sato *et al.*, 2008; Yamaguchi *et al.*, 2010; Leng *et al.*, 2017). UA, albeit effective in TEM staining, is a water-soluble uranium compound that poses considerable threats to human health and environment. It is also expensive to obtain, transport, and dispose of, and requires a dedicated workspace and careful observance of safety regulations for radioactivity. European regulations for the use of radioactive compounds further complicate the work with UA (Sato *et al.*, 2008; Nakakoshi *et al.*, 2011; Carpentier *et al.*, 2012). Therefore, finding suitable alternatives for UA is extremely important.

Aiming to solve these three problems (optimizing sample preparation protocols, shortening the time needed for specimen preparation, searching safer staining solution for replacing UA as a contrasting agent), we have previously performed

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assays that combined LR-White resin with the use of polyphenolic compounds (Oolong tea extract, OTE) for bacteria and animal tissues (He & Liu, 2015, 2017). In this study, this approach was extended to plant specimens.

Materials and methods

Plant materials

Rapeseed plants (*Brassica napus* L.) were grown in the paddy field of Northwest A&F University (seeded in middle September, 2015, and approximately five floral buds or maturing inflorescences were collected in early April, 2016).

Three true-leaves-old tomato (*Solanum lycopersicum* L.) and watermelon [*Citrullus lanatus* (Thunb.) Matsum et Nakai] seedlings were initially grown in a controlled environment greenhouse at Northwest A&F University. For tomato, the air temperature was maintained at 25 ± 1°C/15 ± 1°C (day/night). A 12-h light/12-h dark photoperiod was imposed with a daylight intensity of 800 $mmol \cdot m^{-2}\ s^{-1}$. As for watermelon, the greenhouse was under the conditions of an average day/night temperature of 26–30°C/16–18°C, a 12-h light and 12-h dark photoperiod, and 50–90% relative humidity.

Sample preparation for TEM

Plant tissue samples of about 1–3 mm^3 were fixed at 4% glutaraldehyde in phosphate buffered saline (PBS, 0.2 M, pH 6.8) at 4°C for 8 h. After rinsing with PBS (0.1 M, pH 6.8) for 5, 10, 15, 20 and 30 min, respectively, the specimens were post-fixed using 1% osmium tetroxide (OsO_4) in PBS (0.2 M, pH 6.8) at 4°C for 1–2 h. The samples were then rinsed again with PBS (0.1 M, pH 6.8) for 5, 10, 15, 20 and 30 min. The materials were dehydrated for 15 min in a series of ethanol solutions [30%, 50%, 70%, 80%, 90% and 100% (V/V)], and infiltrated overnight in a mixture of LR-White resin (London Resin Company, Reading, U.K.) and ethanol (1:1, V/V), followed by infiltration with pure LR-White resin twice (for 2 h and 1 h, respectively) at room temperature. Then pure LR-White resin was used for embedding and the samples were incubated at 60°C for 48 h. Ultrathin sections of 50–100 nm were obtained using a diamond knife on the Leica EM UC7 ultramicrotome (Leica, Nussloch, Germany), and picked up on 200-mesh formvar-carbon coated grids.

Staining the ultrathin sections with different staining solutions

Sections on the grids were stained by floating grids on single droplets of the staining solution at room temperature following one of the protocols described below:

(1) 3% aqueous solution of UA for 10–15 min.
(2) 0.05% OTE (OTE powder; Suntory, Osaka, Japan) in deionized water or PBS (0.1 M, pH 6.8) for 30 min.
(3) 0.1% OTE in deionized water or PBS (0.1 M, pH 6.8) for 30 min.

After staining, the samples were rinsed with deionized water and dried three times, and then the grids for each sample were divided into two groups. One group was directly subject to observation (single staining), whereas the remaining group was poststained with Pb solution for 8–10 min (double staining).

TEM observations

All sections were examined under a JEM-1230 TEM (JEOL, Tokyo, Japan) at 80 kV, and the whole images were acquired using a side-inserted BioScan Camera (Model 792, Gatan, Pleasanton, Calif., U.S.A.).

Results and analysis

In this study, we used LR-White resin to simplify and improve the sample preparation process. As shown in Table 1, 27 steps, 154 h process using Epon 812 resin was streamlined to include 22 steps requiring only 77.5 h while using LR-White resin as substitute, the whole preparation time nearly reduced by half, but kept similar or even clearer results (Fig. 1, only show the mitochondria results from anthers of rape, others all the same, but data not show).

We performed a comparative analysis (both by contrast and clarity) of the ultrastructure of plant organelles from anthers from rape, roots from tomato and leaves from watermelon, using the traditional (UA) and nontraditional (OTE) methods (all the results showed below were only embedded by LR-White resin). The pictures using new staining method (OTE) appeared analogous or even more legible than the conventional ones (UA), and displayed equal or even more accurate outline of the organelles (Figs. 2–4).

Anthers of rapeseed

As shown in Fig. 2(I), the pictures only stained by single staining solutions (UA or OTE) appeared neither in good contrast which could easily distinguish organelles from cytoplasm nor in nice clarity that yield clear structure of the organelles [Figs. 2 I(D–F)]. Therefore, it was necessary to use double staining methods (UA+Pb or OTE+Pb) [Fig. 2 I(A–C)].

The quality of new staining method was comparable to that obtained with conventional one [Figs. 2 I(A–C)-V(A–C)]. Membranes in organelles [Figs. 2 I(A–C)-V(A-C)] were equally well stained using the conventional and OTE methods. The subcellular compartments, including endoplasmic reticulum (er), mitochondria (m), with their double membrane and inner invaginated cristae (Fig. 2 I), Golgi apparatus (ga) (Fig. 2 II), chloroplasts (c) (Fig. 2 III), nuclear envelope and nuclear contents (Fig. 2 IV), cell wall (cw) and vacuoles (v) (Fig. 2 V), could be clearly recognized. As the results showed, more

Table 1. Comparison the time and preparation process of samples embedded by LR-White and Epon 812 resins.

		LR-White resin		Epon 812 resin	
Preparation process	Step number	Time	Reagent	Time	Reagent
Prefixation	1	8 h	4% glutaraldehyde	8 h	4% glutaraldehyde
Rinse	2	80 min	Phosphate buffer	80 min	Phosphate buffer
Postfixation	3	2 h	1% osmium tetroxide	2 h	1% osmium tetroxide
Rinse	4	80 min	Phosphate buffer	80 min	Phosphate buffer
Dehydration	5	15 min	30% ethanol	15 min	30% ethanol
	6	15 min	50% ethanol	15 min	50% ethanol
	7	15 min	70% ethanol	15 min	70% ethanol
	8	15 min	80% ethanol	15 min	80% ethanol
	9	15 min	90% ethanol	15 min	90% ethanol
	10	15 min	100% ethanol	15 min	100% ethanol
Substitution	11	–	–	30 min	Acetone
Infiltration	12	12 h	LR-White: ethanol (1:1)	2 h	Epon 812: acetone (1:3)
	13	2 h	LR-White	5 h	Epon 812: acetone (1:1)
	14	1 h	LR-White	12 h	Epon 812: acetone (3:1)
	15	–	–	24 h	Epon 812
	16	–	–	24 h	Epon 812
Polymerization	17	48 h	60°C	24 h	30°C
	18	–	–	48 h	60°C
Total time		77 h and 10 min		153 h and 40 min	

effective staining (higher contrast and clear outlines) of the Golgi apparatus (Fig. 2 II), chloroplasts (especially for stroma and grana lamellae) (Fig. 2 III), and nuclear envelope (Fig. 2 IV) was achieved using OTE, compared to conventional staining using UA.

Roots of tomato

Similar to the results from anther cells of rapeseed, all organelles in tomato root cells, including the membrane structure of mitochondria (Fig. 3 I), the Golgi apparatus containing secretory vesicles (Fig. 3 II), the nucleus and plasmodesma, were stained using both conventional and OTE staining methods. However, compared with traditional staining method (UA+Pb) [Figs. 3 I(A)–3 II(A)], staining with 0.05% OTE yielded [Figs. 3 I(B)–3 II(B)] better staining effect, whereas a higher concentration (0.1%) of OTE [Figs. 3 I(C)–3 II(C)] offered equivalent staining results to UA+Pb. Some degree of overstaining, in which the organelles did not sufficiently display in the cytoplasm, was observed using UA+Pb and 0.1% OTE+Pb methods (for example Fig. 3 I).

Leaves of watermelon

As shown in Figure 4, ultrastructural features were correctly stained in the watermelon leaf samples. Micrographs staining by UA+Pb [Figs. 4 I(A)] and OTE+Pb [Figs. 4 I(B-C)] yielded comparable results. Overall, OTE is demonstrated as an acceptable alternative for UA in the plant sample preparation for TEM. Noticeably superior staining results were obtained using OTE+Pb compared to UA+Pb in several aspects. First, membrane structure of the mitochondria and chloroplast stained by OTE+Pb was much clearer than UA+Pb (Fig. 4 I). The internal crest of mitochondria (except the double membrane) stained by OTE+Pb showed higher clarity especially for the ones stained by 0.05% OTE+Pb (we also obtained the same pictures in root cells of tomato, Fig. 3 I). Second, chloroplast samples (including the double membrane), as well as the stroma and grana lamellae, were all more effectively stained using the OTE methods [Figs. 4 I(B-C)] compared to the UA method [Fig. 4 I(A)]. However, the double membrane structures of the nucleus were better stained using the traditional method (Fig. 4 II).

Discussion

For biological specimens, fixation with OsO_4 has been the most widely used technique in TEM (Palade, 1952). OsO_4 acted not only as a fixative, but also as an electron blocking staining reagent. Although post fixation with OsO_4 was essential for high-contrast staining, staining only with OsO_4 did not produce a sufficiently high contrast to satisfactory TEM observations (He & Liu, 2017). The use of single staining solution alone (UA or OTE) also did not produce sufficient increase in contrast of the given samples (He & Liu, 2017) (Fig. 2 I). Hence double electron staining methods that utilize Pb as a

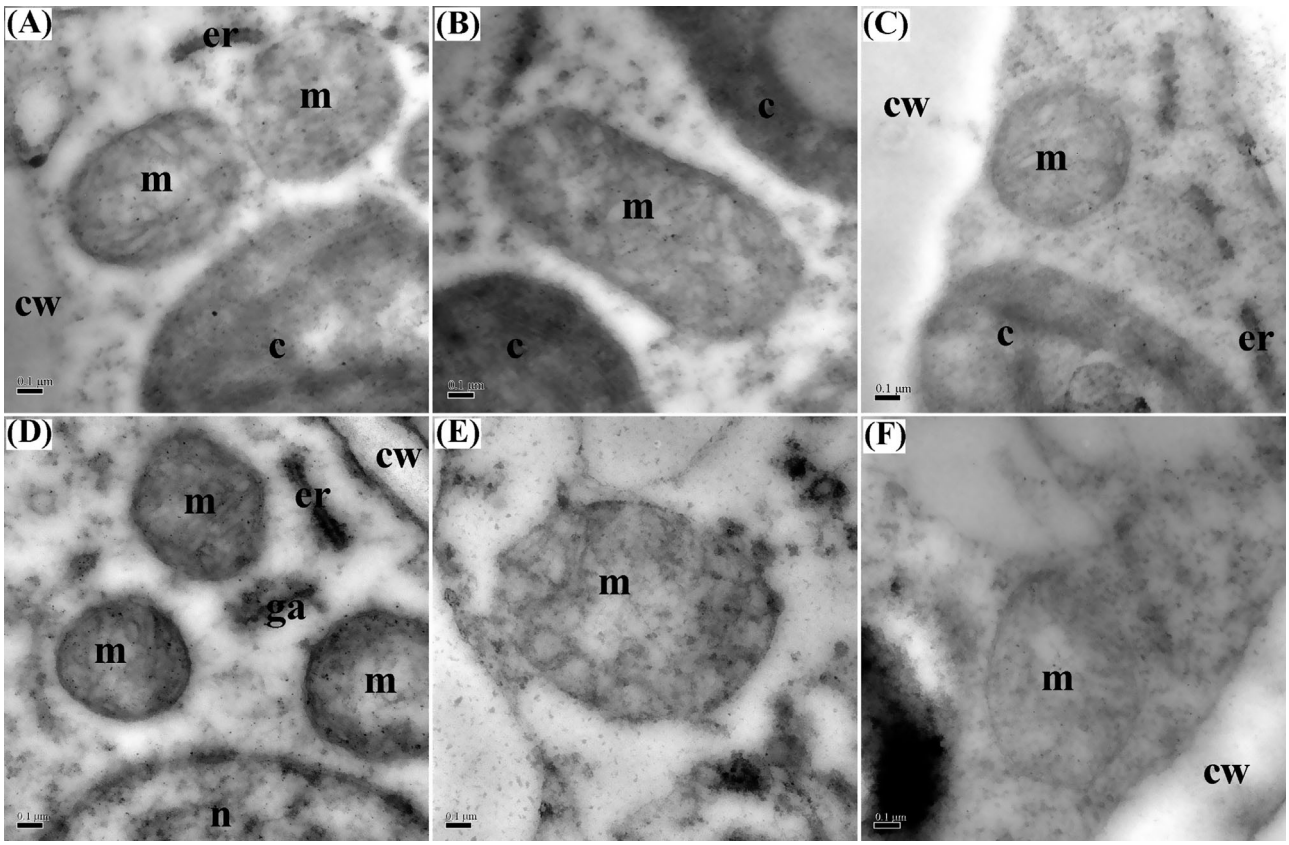

Fig. 1. Comparison of routine method embedded by Epon 812 resin and new one embedded by LR-White resin on mitochondria ultrastructure in rapeseed anthers. (A)–(C) embedded by Epon 812 resin; (D)–(F) embedded by LR-White resin. (A) and (D) stained by U+P; (B) and (E) stained by 0.05% O+P; (C) and (F) stained by 0.1% O+P. n = nucleus, ga = Golgi apparatus, m = mitochondria, cw = cell wall, c = chloroplast, er = endoplasmic reticulum. Scale bars = 100 nm.

post staining reagent was necessary to provide high contrast enhancement [Figs. 2(A–C)-4(A–C)].

OTE contains polyphenolic compounds, which exhibit similar properties to tannic acid (TA). TA can easily penetrate the cell, and provide good contrast with sharply defined cellular structures in TEM images, especially in membranes (Kajikawa *et al.*, 1975). TA reacts with osmium in membranes, and facilitates lead and uranium binding (Hall & Hawes, 1991). However, TA does not penetrate deeply into the tissue (Sato *et al.*, 2003) and is rarely used without UA (Carpentier *et al.*, 2012). OTE is reportedly made up of smaller polyphenols than TA, hence is expected to function better as a membrane contrasting agent (Nakahara *et al.*, 1993; Sato *et al.*, 2003, 2008). In our research, the results also well supported this conclusion [Figs. 2(A–C)-4(A–C)]. OTE can be performed as a staining solution to obtain a high contrast, making it a suitable substitute for UA. OTE is expected to be a superior contrasting agent to TA, due in part to its smaller size (2 kDa), which facilitates its penetration in membranes (Nakahara *et al.*, 1993; Sato *et al.*, 2003, 2008). However, commercial TA reagents consist of even smaller compounds (1.7 kDa), rendering the above mentioned report invalid. We believe that the superior performance of OTE was a result of its multiple tannin components in various sizes and types (Sang *et al.*, 2011).

The clearest results were obtained using 0.05% OTE [Figs. 2(B), 3(B) and 4(B)]. Higher concentrations led to overstaining of the cytoplasm in consideration of the subcellular components (Fig. 3). This reduced the contrast between cytoplasm and organelles. However, 0.1% OTE previously demonstrated better performance than 0.05% OTE in bacterial cells and animal tissues ultrastructure observations (He & Liu, 2015, 2017). This difference could be due to different electron adsorption capacities in different samples.

Previous studies demonstrated that the nature of the staining solution could affect the staining effect in ultrathin sections. In animal tissues (Sato *et al.*, 2003; He & Liu, 2017) and bacterial cells (He & Liu, 2015), OTE was dissolved in a specific buffer. In this research, however, the nature of the OTE solvents (i.e. water or PBS) did not affect the quality of TEM results (data not shown), which is consistent with the

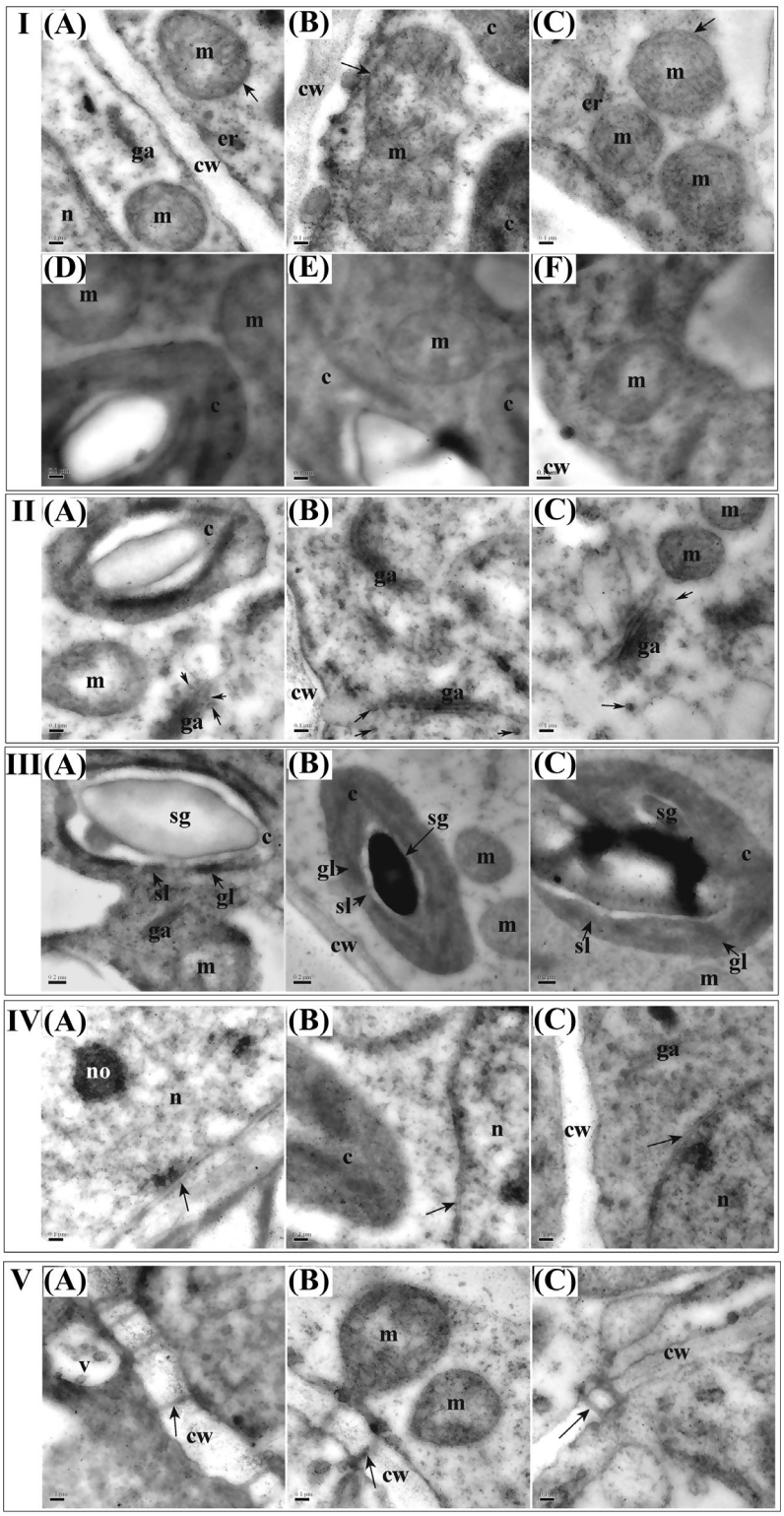

Fig. 2. Comparison of routine electron staining method (UA+Pb) and OTE staining method (OTE+Pb) on some ultrastructures in rapeseed anthers. (A) U+P; (B) 0.05% O+P; (C) 0.1% O+P; (D) U; (E) 0.05% O; (F) 0.1% O. (I) The places where the arrows pointed indicate the double membrane structure of the mitochondria; (II) Arrows indicate secretory vesicles of Golgi apparatus; (IV) The places where the arrows pointed indicate the double membrane structure of the nucleus; (V) The places where the arrows pointed indicate the plasmodesma. n = nucleus, ga = Golgi apparatus, m = mitochondria, cw = cell wall, c = chloroplast, er = endoplasmic reticulum, sg = starch grains, sl = stroma lamellae, gl = grana lamellae, no = nucleolus, v = vesicle. Scale bars = 100 nm.

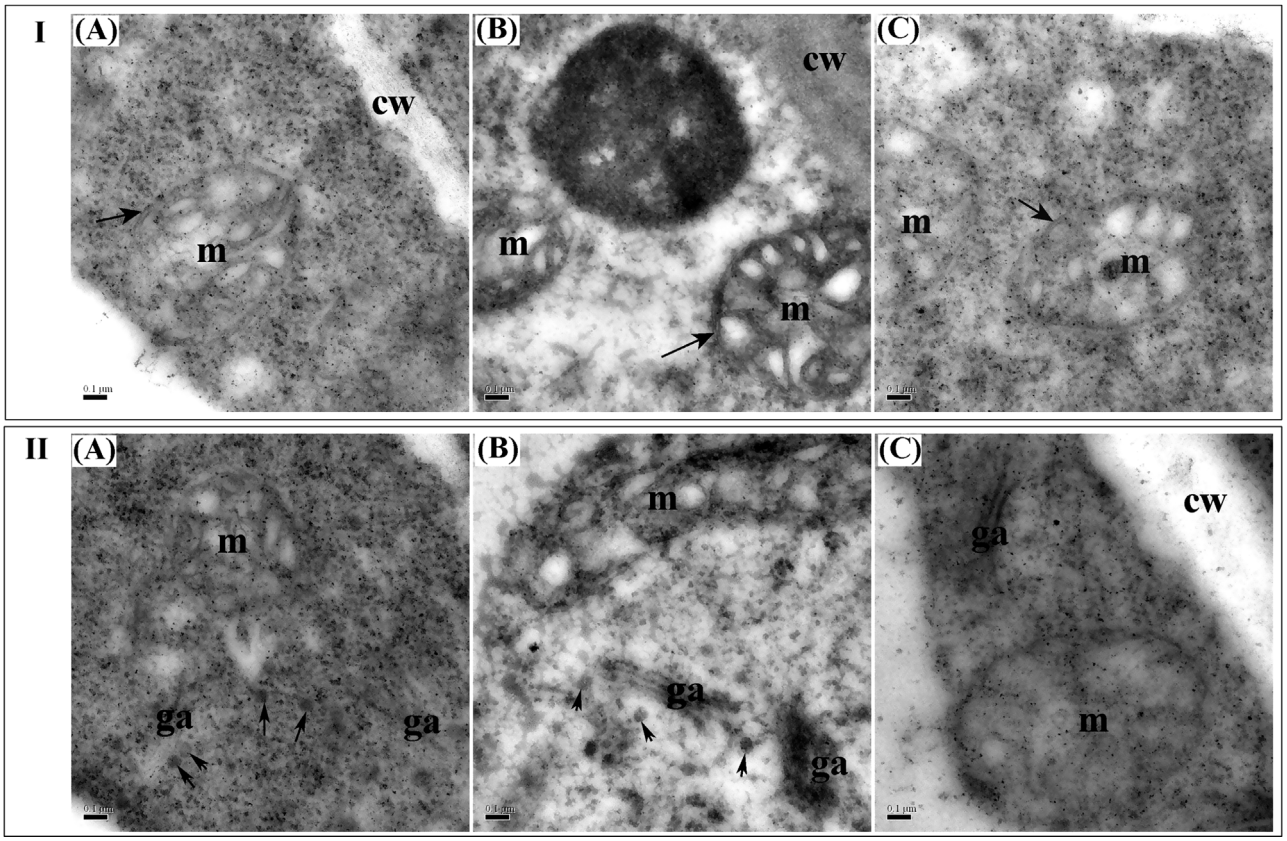

Fig. 3. Comparison of routine electron staining method (UA+Pb) and OTE staining method (OTE+Pb) on some ultrastructures in root cells of tomato. (A) U+P; (B) 0.05% O+P; (C) 0.1% O+P. (I) The places where the arrows pointed indicate the double membrane structure of the mitochondria; (II) Arrows indicate secretory vesicles of Golgi apparatus. m = mitochondria, ga = Golgi apparatus, cw = cell wall. Scale bars = 100 nm.

observations by Carpentier *et al.* (2012) This may be because of the different types of samples, since the samples in Carpentier's (2012) and our studies were derived from plant tissues, whereas in Sato's (2003) and our previous works (He & Liu, 2015, 2017), the samples were of animal or bacterial origins. But it is noteworthy that a freshly prepared OTE staining solution is essential to achieve successful staining, as is confirmed in all of our studies.

In general, the structures could be clearly seen in the sections stained by OTE+Pb [Figs. 2(B–C), 3(B–C) and 4(B–C)], and the quality of staining was comparable to that of traditional double staining [Figs. 2(A), 3(A) and 4(A)]. Some noticeable differences were found between the OTE method and the UA method. For example, in mitochondria, Golgi apparatus, and chloroplast, OTE method yielded better results than UA method (Figs. 2 II-III, 3, 4 I), particularly for stroma and grana lamellae in chloroplast (Figs. 2 III, 4 I) (not only in terms of contrast, but also in clarity).

Rapeseed anther, tomato root and watermelon leaf tissues were prepared following the both protocols described in Table 1. The LR-White resin-assisted processing protocol allowed for easy and rapid visualization of all the ultrastructures in all of the examined samples. This LR-White resin based protocol for plant tissues, which took less than 78 h in total, yielded highly informative results. The subcellular features were indeed comparable to those obtained with conventional procedure running nearly 7d (Fig. 1). LR-White resin has lower viscosity and higher permeability than Epon 812 resin, which is frequently used for preparing ultrathin samples. This is particularly advantageous in plant samples, since they comprise of a cell wall and vacuoles that obstruct high viscosity resins from permeating into the cells (Brewis *et al.*, 2017). LR-White resin is also a low temperature embedding medium that is used in immunolabeling (Newman *et al.*, 1982, 1983; Boassa, 2015; Yamashita, 2016; Hurbain *et al.*, 2017). In our research, we used it as a room temperature embedding medium under the same condition as the Epon 812 resin, thus no additional or specialized equipment was needed (Nicolas & Bassot, 1993).

In summary, in this study OTE was found to be a very suitable substitute for UA as an electron staining agent in TEM, in terms of safety, availability, ease of use, stability, and image quality. The proposed new method combining LR-White resin with OTE could lead to easier, safer, and more efficient TEM sample preparing processes.

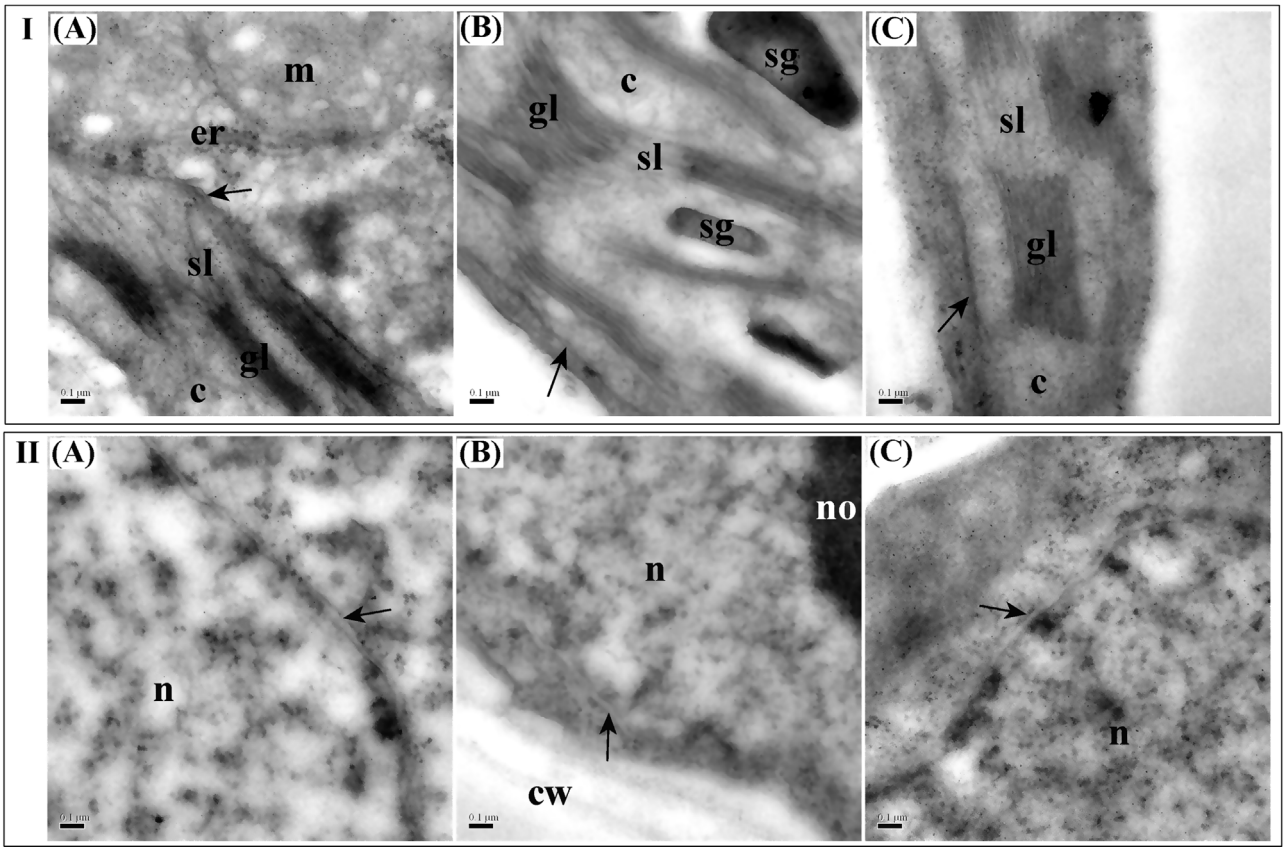

Fig. 4. Comparison of routine electron staining method (UA+Pb) and OTE staining method (OTE+Pb) on some ultrastructures in leaf cells of watermelon. (A) U+P; (B) 0.05% O+P; (C) 0.1% O+P. (I) The places where the arrows pointed indicate the dual-mode structure of the chloroplast; (II) The places where the arrows pointed indicate the double membrane structure of the nucleus. c = chloroplast, sl = stroma lamellae, gl = grana lamellae, m = mitochondria, er = endoplasmic reticulum, sg = starch grains, n = nucleus, no = nucleolus, cw = cell wall. Scale bars = 100 nm.

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