STRUCTURAL AND ULTRA-STRUCTURAL DISORDERS IN *ZIZIPHUS JUJUBA* MILLER FRUITS UNDER FLUORINE STRESS

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SUMMARY: The *Ziziphus jujuba* Miller (Rhamnaceae) fruit has significant health and nutritional benefits. A new fruit disease called jujube black tip disease caused by fluoride pollution has seriously damaged the jujube industry of northern China. A series of experiments were conducted using light microscopy, transmission electron microscopy, and scanning electron microscopy to study the injury process in *Z. jujuba* Miller "Huping" fruits treated by different concentrations of hydrofluoric acid (HF). The results show that Huping jujube fruits could tolerate HF concentrations less than 200 mg/L but suffered varying degrees of damage when exposed to HF concentrations of more than 200 mg/L. Fruit pericarp and pulp sustained greater damage as HF concentration increased. Analysis of cell ultrasturcture from treated plants shows that mitochondrion tolerance to fluorine stress could be used as an index to determine the jujube fruit tolerance to fluorine stress. HF exposure caused gradual injury to fruits. Scanning electron micrographs show that the epicuticular wax was injured by spraying 150 mg/L HF.

Keywords: Cell ultra-structure; Fluorine stress; Peel anatomy; *Ziziphus jujuba* Miller.

INTRODUCTION

The jujube fruit, *Ziziphus jujuba* Miller (Rhamnaceae) has significant health and nutritional benefits. Jujube is a good source of glucosides, amino acids, proteins and minerals. Due to the high market value of jujube fruits, the jujube industry has become one of the main farming industries of northern China.[1] The area of jujube plantations exceeds 400,000 hectares and the yield of jujube fruits is over 600 million kilograms in Shanxi province alone.[2] However, farmers have a problem in their hands with jujube fruit black tip disease. It is proposed that fluorine, a phytotoxin in air, water, soil, and vegetation, released from industrial sources[3] and application of phosphate fertilizers in agriculture[4] is the primary culprit.[5,6] This fruit disease has ravaged jujube orchards in middle and southern regions of Shanxi province over the last decade. In extreme cases, 90% of an orchard can be damaged when exposed to fluoride pollution even though the disease is not caused by a pathogen.[5,6] Hydrogen fluoride emissions have been reported to damage tree fruits, such as peaches,[7] mangoes,[8] and jujube.[5,6] Hydrogen fluoride also damages leaves of many kind of trees[9-14] and has been reported to cause damage to vegetation in China, India, and Japan.[15] Hydrogen fluoride damage to vegetation from peri-urban brick kilns in Asia has become a growing but unrecognized problem.[16]

It has been reported that the absorption rate and accumulation of fluoride in jujube leaves and fruits were positively correlated with the fluoride concentration

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in the atmosphere during the jujube growing period.[6] Expression of several antioxidant enzymes such as peroxidase, superoxide dismutase, and catalase were observed by our team to increase after exposure to HF and then decrease.[17]

To our knowledge, little information is available regarding the effects of excessive exogenous fluorine exposure on jujube cell ultrastructure. We hypothesize that the injury processes of jujube fruits under different concentrations of fluorine can be explained by examining the structural and ultra-structural changes of jujube fruit skin. In order to study the injury processes of jujube fruits, we treated the Huping jujube fruits *in vivo* with different concentrations of fluorine. Then we examined the structural and ultra-structural changes of jujube fruit skin samples using light microscopy, transmission electron microscopy, and scanning electron microscopy.

MATERIALS AND METHODS

Plant material and growing site: Fifteen-year-old *Z. jujube* Miller 'Huping' were grown in the nursery garden of Shanxi Agricultural University, Taigu, Shanxi Province, China. The average level of fluorine contamination at the growing site from air fluorine was 1.21 μg/dm^2/day.[18] Excessive fluorine was supplied by spraying hydrofluoric acid (HF) (Analytical reagent, Tianjin Yongli United Chemical Co. Ltd., PR China) at six different concentrations: 0 (used as control), 150, 200, 250, 300, and 350 mg/L. For each treatment, 5 trees were selected randomly. Jujube fruits were selected during the white ripe season from July to August in 2010 and 2011. In order to ensure consistent results, the samples were examined via microscope only while Huping jujube fruits were continuously treated once a day for 5 continual days with their respective treatments.

Paraffin section method: The top section of fresh jujube fruit were cut into small 3–4 mm^3 blocks of and fixed in a formalin–acetic acid–alcohol solution (F. A. A.: 55% ethanol, glacial acetic acid, 10% formaldehyde; 8:1:1 v/v), embedded in paraffin and sectioned into 6–10 μm thick slices. Sections were stained with Safranine-Fast green (Biological stain, Tianjin Guangfu Fine Chemical Research Institute, PR China) and Teifan-hematoxylin (Biological stain, Tianjin Guangfu Fine Chemical Research Institute, PR China) and mounted by Canada balsam (Chemical pure, Tianjin Guangfu Fine Chemical Research Institute, PR China). The slides were observed and photographed using an Olympus BX61 microscope. Ten fruits were used for replicates.

Scanning electron microscopy (SEM): For SEM, ten fresh jujubes were collected from trees and rapidly fixed in 2.5% glutaraldehyde mixed with phosphate buffer solution at pH of 7.4 at 4ºC for 3 days. The pretreated jujubes samples were then cleaned for 5 min in a 75% ethanol solution in an ultrasonic bath. This was followed by dehydration through a graded ethanol series of 80%, 90%, 95%, and 100% for 5 min, respectively, and two final dehydrations, with 100% ethanol. Samples were dried in a CO_2 critical point dryer, mounted on a holder using electric adhesive tape, sputter-coated with gold, and examined with a JSM-6490LV scanning electron microscope (JEOL, Japan) at an accelerating voltage of 10 kV.

Transmission electron microscopy (TEM): Sample preparation for TEM was similar to that used for SEM. Ten samples of fresh jujube were cut into small 1 mm^3 blocks and pre-fixed in 2.5% glutaraldehyde mixed with phosphate buffer solution at pH of 7.4 at 4ºC for 3 days, then fully washed in 0.1 mol/L, pH 7.4 phosphate buffer, and post-fixed in 1% osmium tetroxide mixed with phosphate buffer solution at pH of 7.4 at 4ºC for 4 hr, and finally cleaned with distilled water. Dehydration was carried out once for 15 min in each of a graded acetone solution series of 50%, 70%, 90%, each of which was diluted using double distilled and deionized water. Samples were rinsed again and dehydrated with 100% acetone three times for 10 min each. Dehydrated tissues were infiltrated with araldite (Histochoice) and embedded in Epon 812. Ultrathin sections were cut using an ultramicrotome (Leica EMUC6) and stained with uranyl acetate and lead citrate, and examined under a JEOL JEM-1400 transmission electron microscope at 80 kV.

RESULTS

Anatomy of pericarp and pulp in Huping jujube fruits in the control samples: The cuticle is covered by epicuticular wax and was 5.7±0.04 μm thick, smooth, and uniform. Epidermis thickness was 66.31±1.27 μm and consisted of 4–5 layers of small, denser, regularly arranged approximate square cells that were mostly stained garnet with the staining agents. The subepidermis was a different thickness and consisted of 1–2 layers irregularly shaped cells that were partly stained red with the staining agents (Figure 1A).

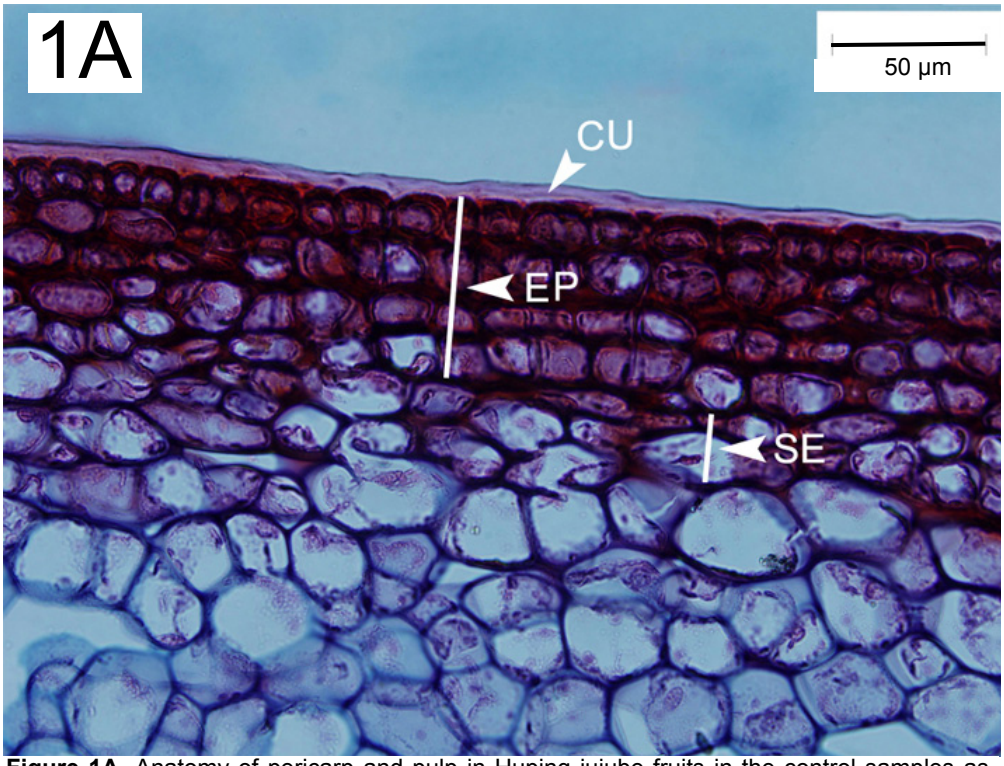


Figure 1A. Anatomy of pericarp and pulp in Huping jujube fruits in the control samples as observed via light microscopy. 1A: Pericarp morphology (200×). Abbreviations: CU, cuticle; EP, epidermis; SE, subepidermis.

Pulp cells varied in shape and were loosely arranged with many empty cavities. Pulp cells were stained green by the staining agents, varied in shape, were loosely arranged, and formed many cavities. Some mucilage cavities stained deep red. Some vascular bundles stained purple and were scattered sporadically among the various shaped cells (Figure 1B).

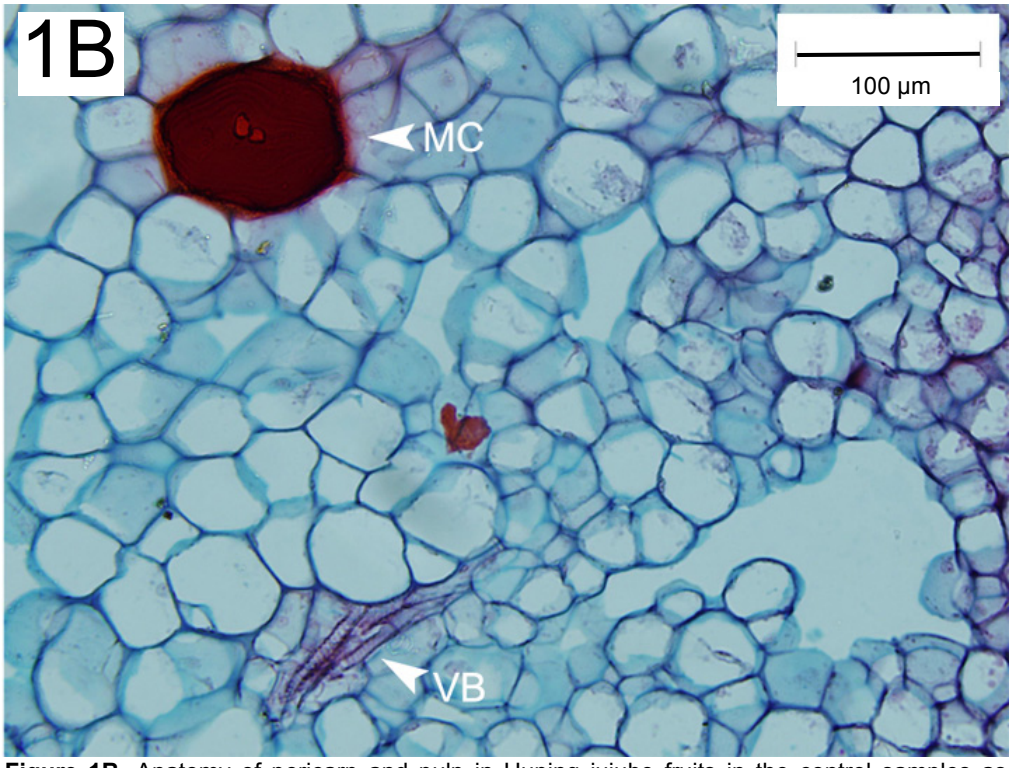
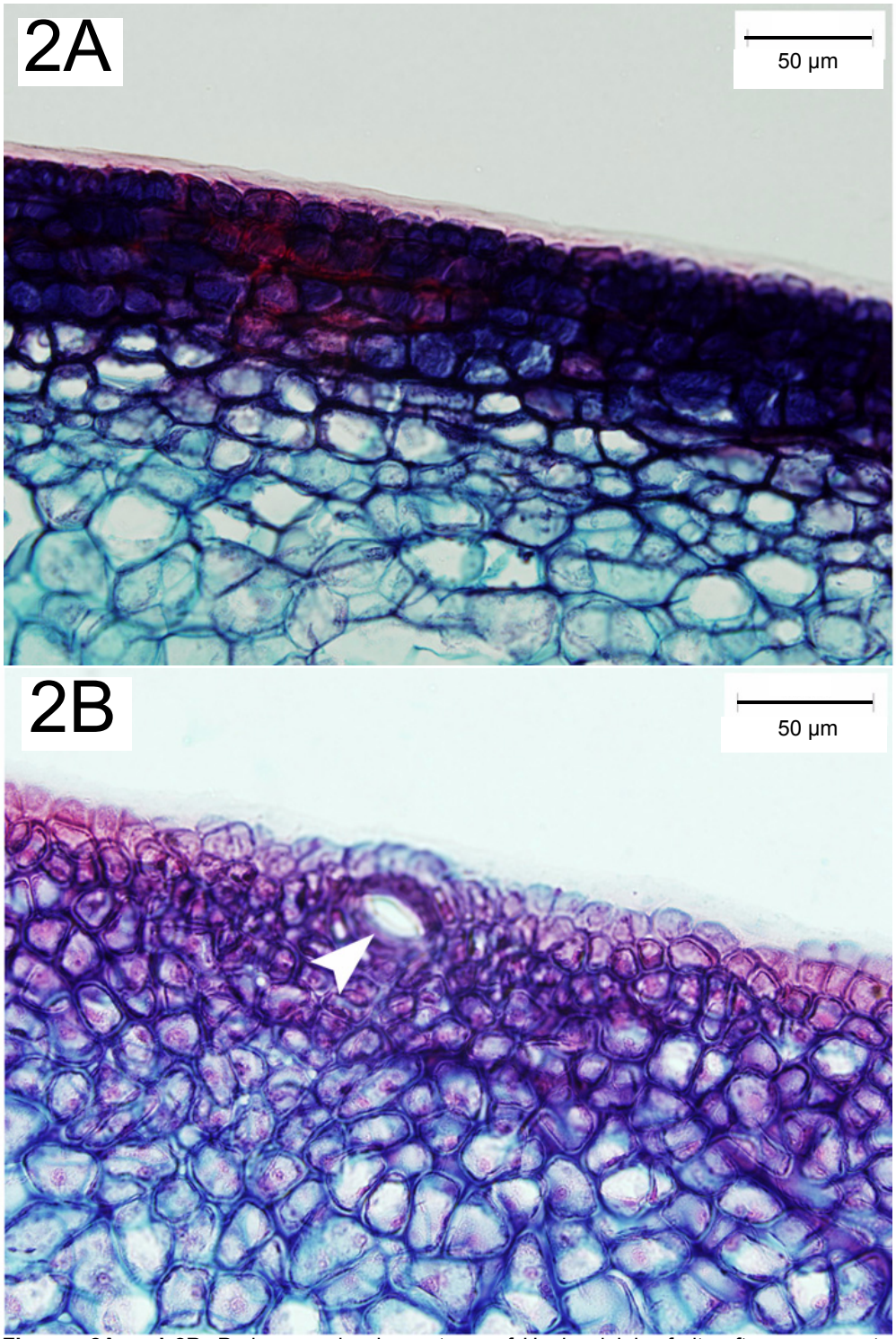
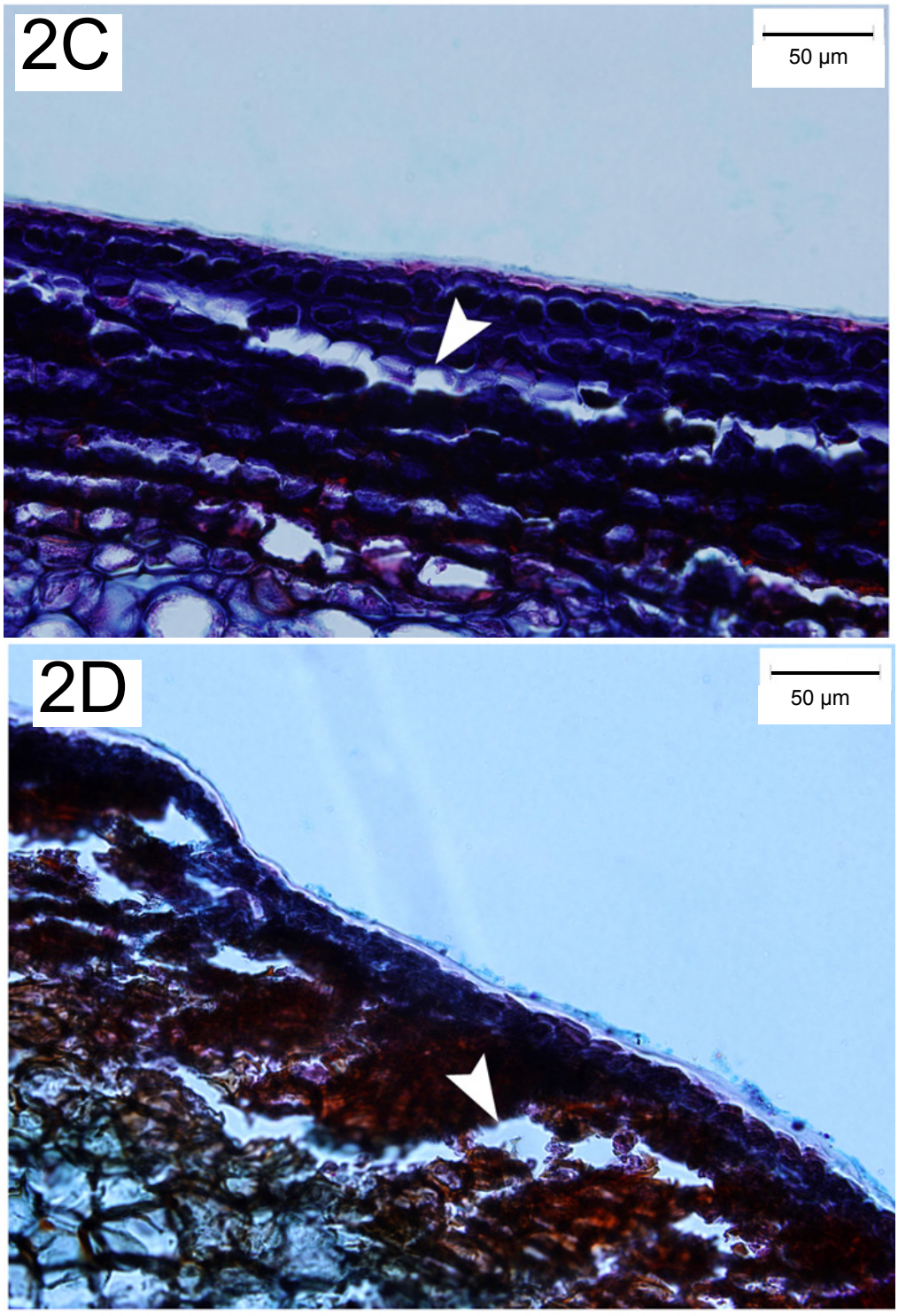


Figure 1B. Anatomy of pericarp and pulp in Huping jujube fruits in the control samples as observed via light microscopy. 1B: Pulp morphology (100×). Abbreviations: MC, mucilage cavity; VB, vascular bundles.

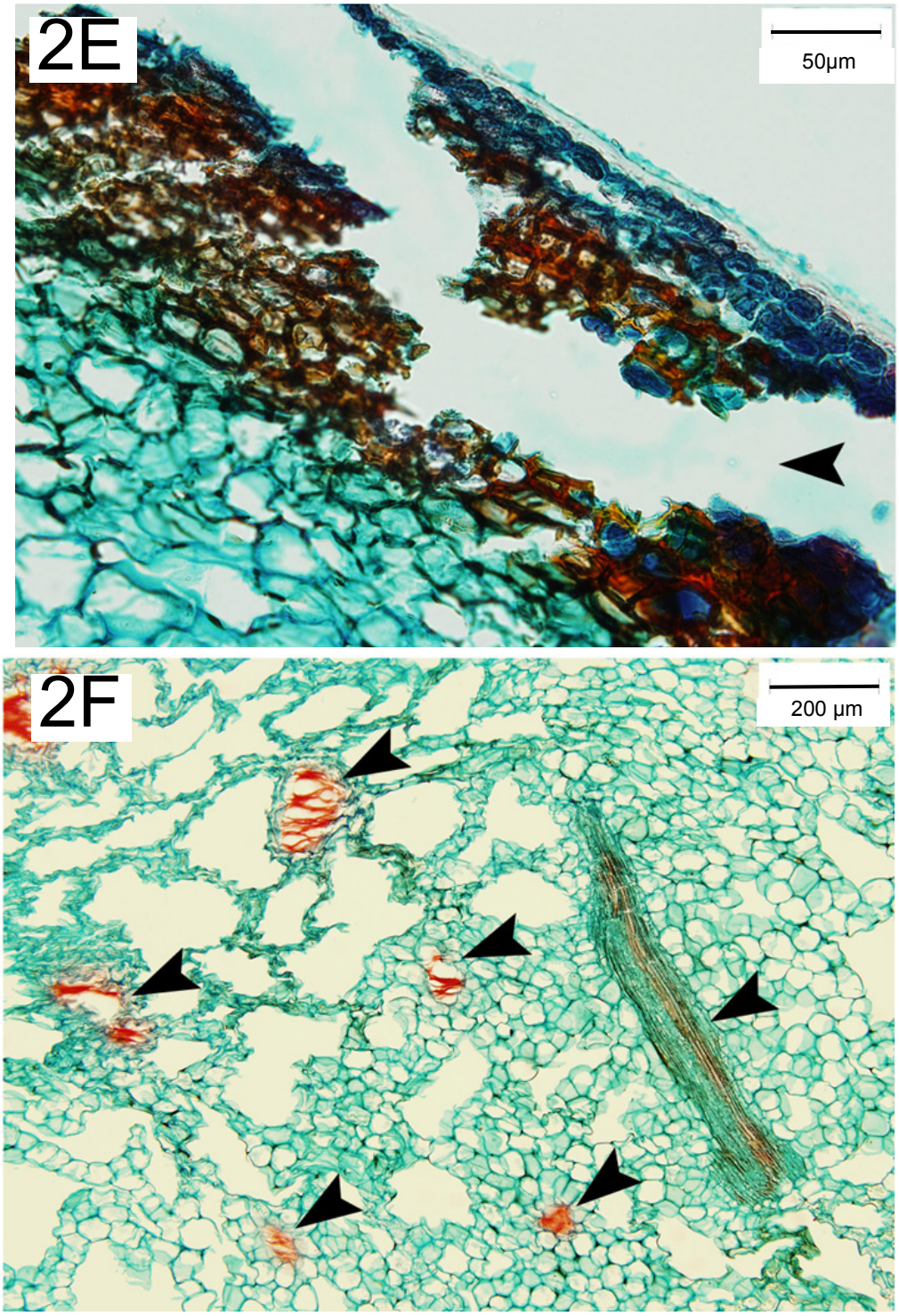
Pericarp and pulp anatomy of Huping jujube fruits under excessive fluorine stress: Pericarp and pulp morphology of Huping jujube in response to 5 days of 150, 200, 250, 300, and 350 mg/L HF treatments are shown in Figure 2. At 150 mg/L HF solution, no obvious morphological differences were observed in comparison to the control (Figures 1A, 1B, and 2A). At 200 mg/L HF solution, the cuticle became non-uniform. Epidermal and subepidermal cell arrangement deformed into curviform; vacuoles appeared in the epidermis (Figure 2B). The shape and arrangement of the pulp cells were similar to the control. At 250 mg/L HF solution, several longitudinal dilacerations of different sizes appeared in the epidermis and subepidermis. Shape and arrangement of the pulp cells were similar to the control (Figure 2C). At 300 mg/L HF solution, epidermal and subepidermal layers dislocated and the entire pericarp tissues became seriously deformed (Figure 2D). Pulp cells were crumpled and deformed with several empty cavities often connecting into a large cavity. The mucilage cavities were deformed badly (Figure 2F). At 350 mg/L HF solution, serious exfoliation appeared in the pericarp tissues (Figure 2E). The morphology and layering of the pulp cells were deformed with exposure to the 300 mg/L HF solution (Figure 2F).



Figures 2A and 2B. Pericarp and pulp anatomy of Huping jujube fruits after exposure to different levels of fluorine stress and staining. Observed with light microscopy. 2A: Pericarp morphology at 150 mg/L HF treatment (200×); 2B: Pericarp morphology at 200 mg/L HF treatment (200×). Arrows in the figure show the ruptured tissue.

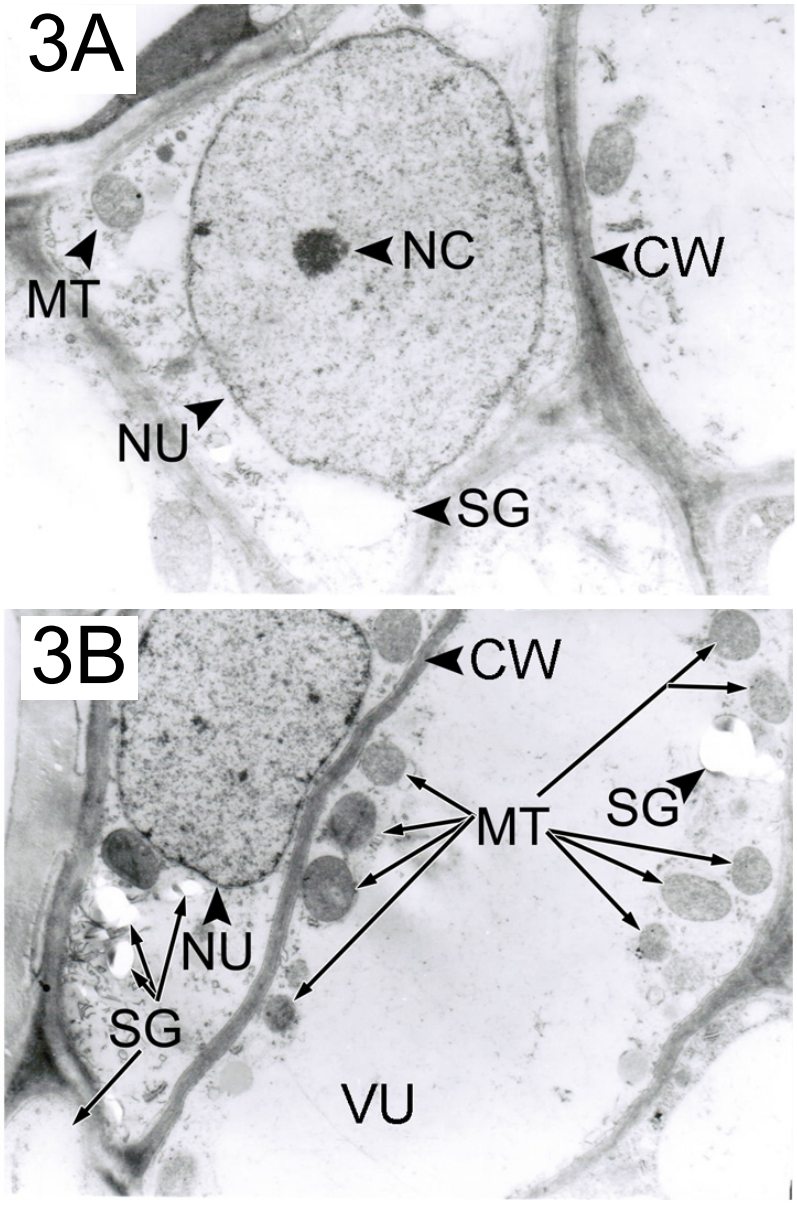


Figures 2C and 2D. Pericarp and pulp anatomy of Huping jujube fruits after exposure to different levels of fluorine stress and staining. Observed with light microscopy. 2C: Pericarp morphology at 250 mg/L HF treatment (200×); 2D: Pericarp morphology at 300 mg/L HF treatment (200×). Arrows in the figure show the ruptured tissue.

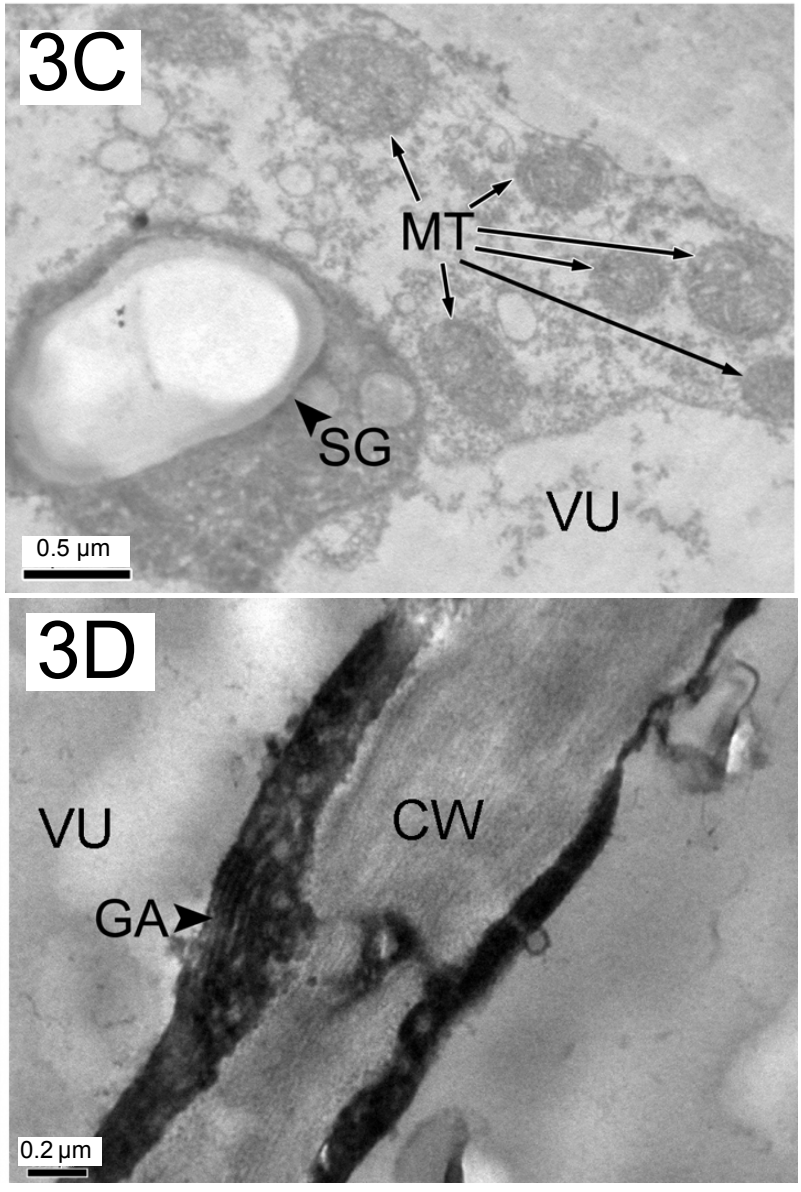


Figures 2E and 2F. Pericarp and pulp anatomy of Huping jujube fruits after exposure to different levels of fluorine stress and staining. Observed with light microscopy. 2E: Pericarp morphology at 350 mg/L HF treatment (200×); 2F: Pulp morphology at 300 mg/L HF treatment (50×). Arrows in the figure show the ruptured tissue.

Ultrastructure of Huping jujube fruit cells in the control samples: Cell membranes and cell walls were continuous and integrated. Starch granules were present as aggregated pellets and distributed in the cell cytoplasm. Organelles, such as the nucleus, mitochondria, Golgi apparatus, and vacuole were visible and complete. The number of visible and complete mitochondria varied among the cells. Some cells had a large nucleus and less than ten mitochondria. Other cells had a large vacuole and more than ten mitochondria. No mitochondrion were found in several cells (Figures 3A-3D).

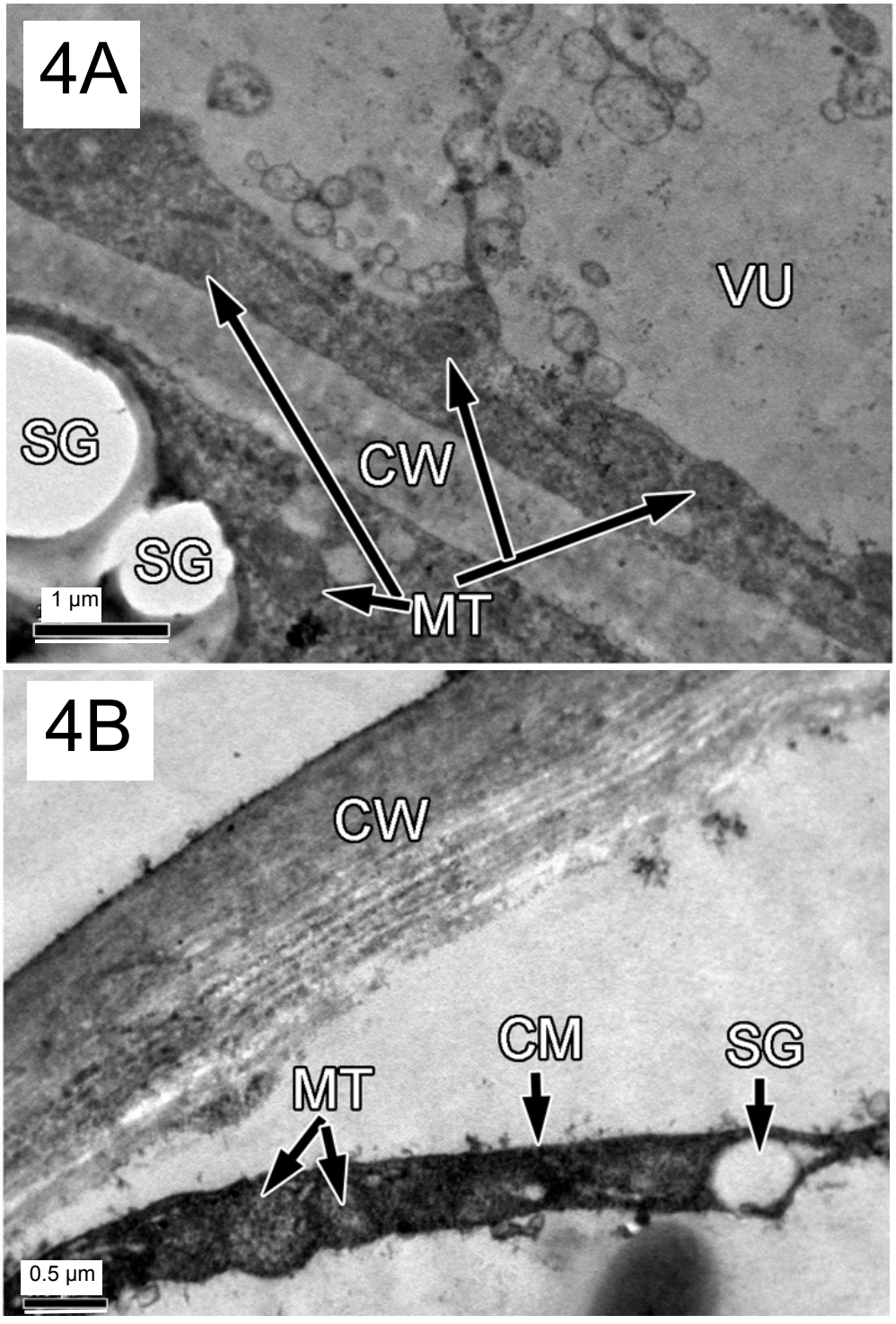


Figures 3A and 3B. Ultrastructure of Huping jujube fruit cells in the control samples observed by TEM. 3A: Huping jujube fruit cell with large nucleus; 3B: Extended Huping jujube fruit cells with a large nucleus or vacuole. Abbreviations: CW, cell wall; MT, mitochondrion; NC, nucleus; NU, nucleolus; SG, starch granule; VU, vacuole.

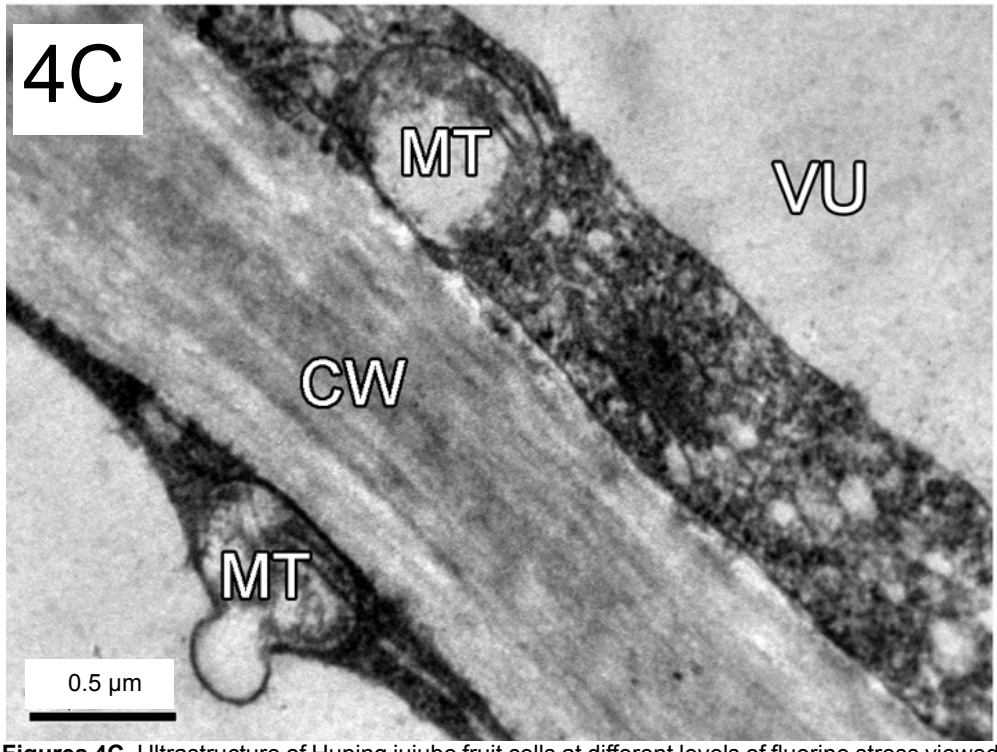


Figures 3C and 3D. Ultrastructure of Huping jujube fruit cells in the control samples observed by TEM. 3C and 3D: Partially enlarged Huping jujube fruit cell with large vacuole. Abbreviations: CW, cell wall; GA, Golgi apparatus; MT, mitochondrion; SG, starch granule; VU, vacuole.

Ultrastructure of Huping jujube fruit cells under excessive fluorine stress: With 150 mg/L HF solution, vacuoles were enlarged and crowded other cellular contents such as starch granules. Vacuoles arranged along the cell membrane. Mitochondria and other organelles were visible (Figure 4A). With 200 mg/L HF solution, plasmolysis caused mitochondria membranes to partially rupture. Other organelles were invisible (Figures 4B and 4C).

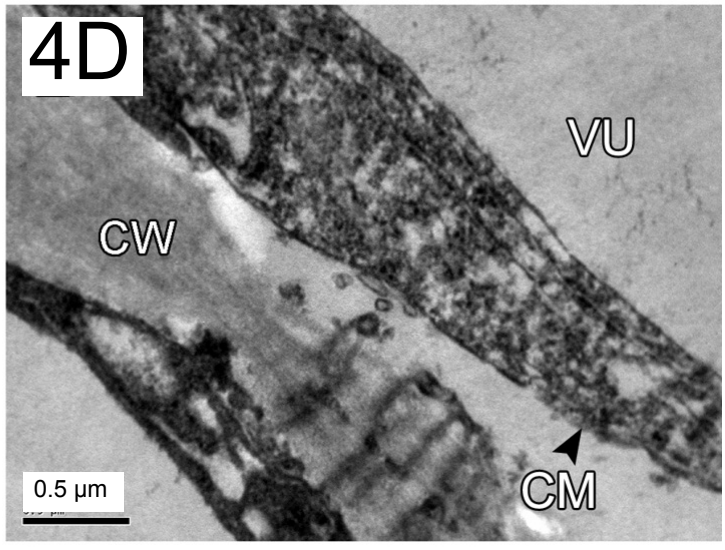


Figures 4A and 4B. Ultrastructure of Huping jujube fruit cells at different levels of fluorine stress viewed with TEM. 4A: cell ultrastructure with 150 mg/L HF treatment; 4B: cell ultrastructure with 200 mg/L HF treatment. Abbreviations: CM, cell membrane; CW, cell wall; MT, mitochondrion; SG, starch granule; VU, vacuole.



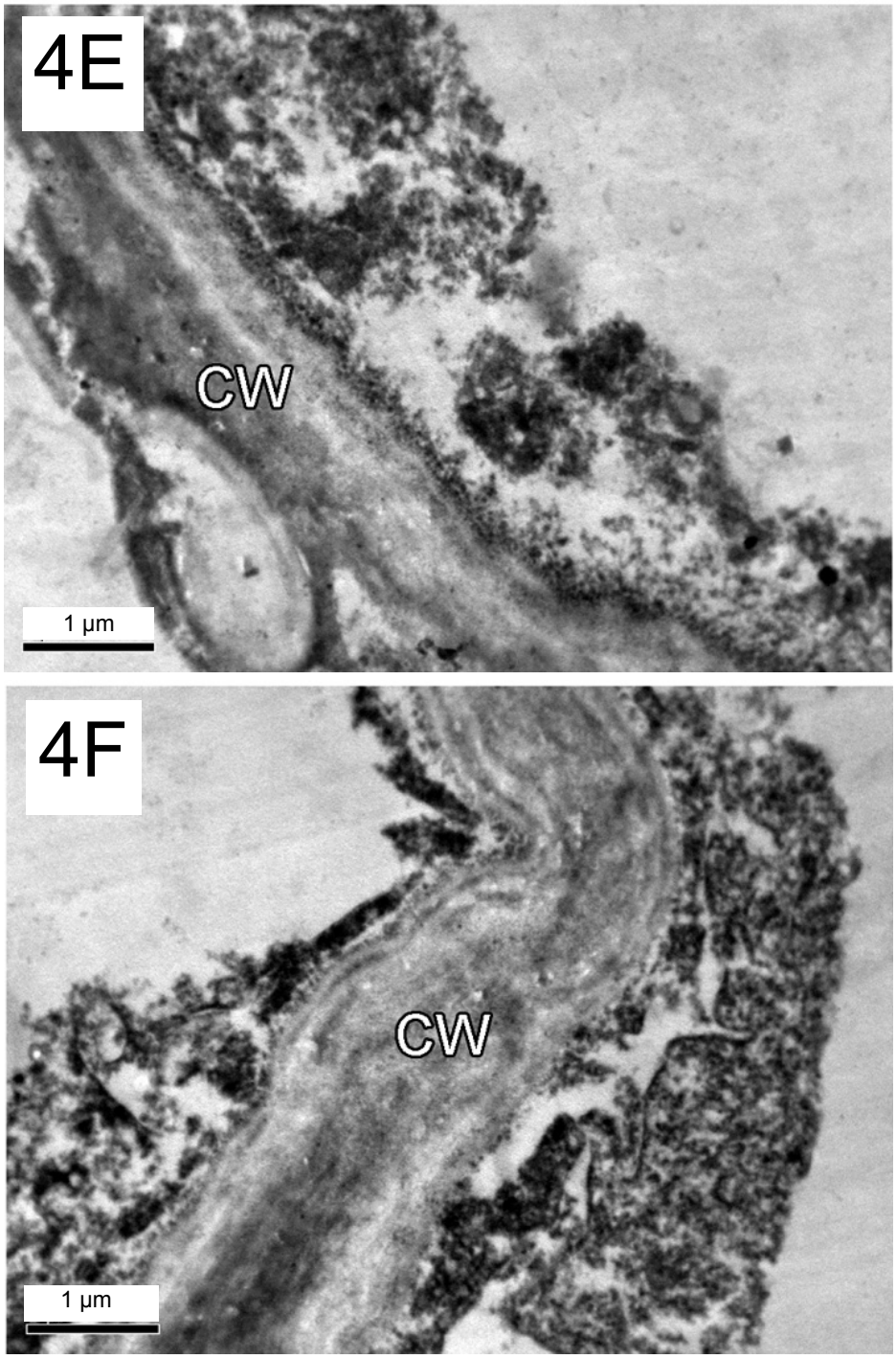
Figures 4C. Ultrastructure of Huping jujube fruit cells at different levels of fluorine stress viewed with TEM. 4C: cell ultrastructure with 200 mg/L HF treatment. Abbreviations: CM, cell membrane; CW, cell wall; MT, mitochondrion; SG, starch granule; VU, vacuole.

With 250 mg/L HF solution, plasmolysis was prominent. Organelles were clearly damaged and fragments mixed together. It was difficult to identify any organelles (Figure 4D).



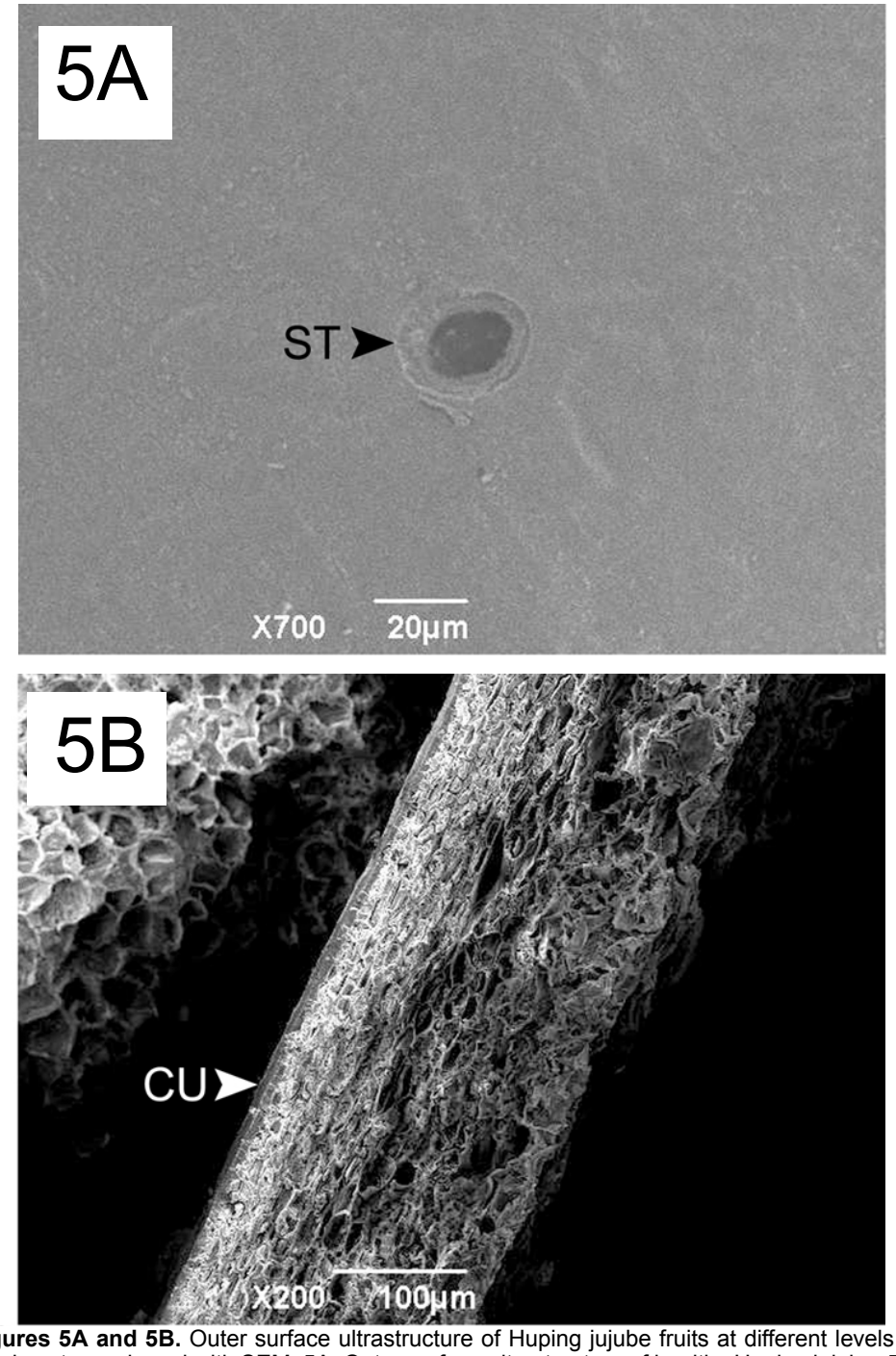
Figures 4D. Ultrastructure of Huping jujube fruit cells at different levels of fluorine stress viewed with TEM. 4D: cell ultrastructure with 250 mg/L HF treatment. Abbreviations: CM, cell membrane; CW, cell wall; VU, vacuole.

With 300 or 350 mg/L HF solution, the cell walls became seriously deformed. Damaged cellular contents were arranged along the deformed cell wall (Figure 4E, 4F).



Figures 4E and 4F. Ultrastructure of Huping jujube fruit cells at different levels of fluorine stress viewed with TEM. 4E: cell ultrastructure with 300 mg/L HF treatment; 4F: cell ultrastructure with 350 mg/L HF treatment. Abbreviation: CW, cell wall.

Outer surface ultrastructure of Huping jujube fruits in the control samples: The outer surface of healthy Huping jujube fruits was smooth and flat, without any wrinkles. The smooth and uniform cuticle is covered with a uniform epicuticular wax. Stomata are non-uniformly distributed in the cuticle (Figures 5A-5C).



Figures 5A and 5B. Outer surface ultrastructure of Huping jujube fruits at different levels of fluorine stress viewed with SEM. 5A: Outer surface ultrastructure of healthy Huping jujube; 5B: Peel cross-section ultrastructure of healthy Huping jujube. Abbreviations: CU, cuticle;. ST, stoma.

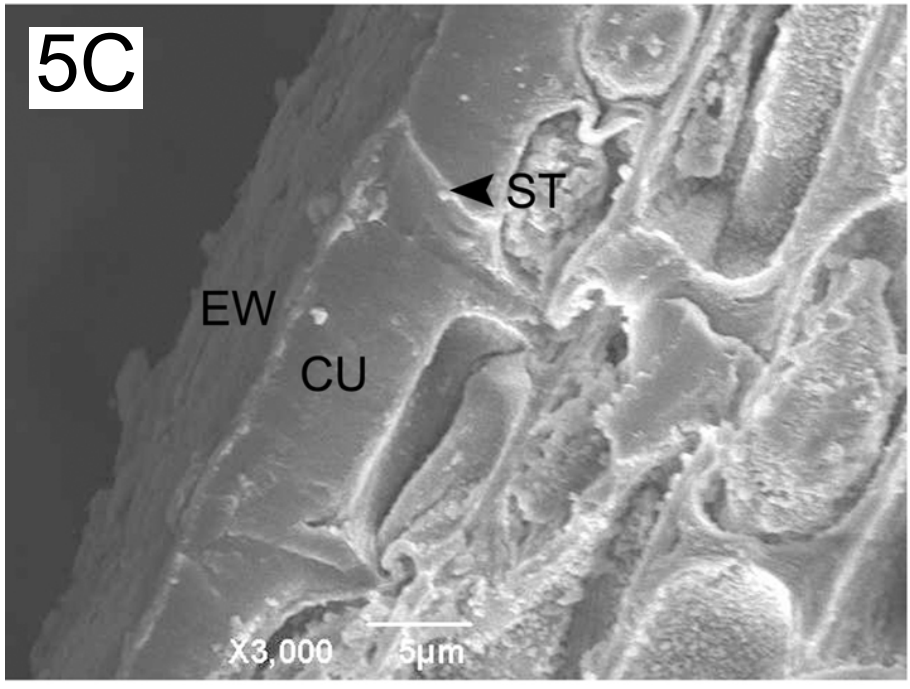


Figure 5C. Outer surface ultrastructure of Huping jujube fruits at different levels of fluorine stress viewed with SEM. 5C: Cuticle cross-section ultrastructure of healthy Huping jujube. Abbreviations: CU, cuticle; EW, epicuticular wax; ST, stoma.

Outer surface ultrastructure of Huping jujube fruits under excessive fluorine stress: At 150 mg/L HF solution, the outer surface of Huping jujube fruits became rough and developed slight sags and crests (Figure 5D).

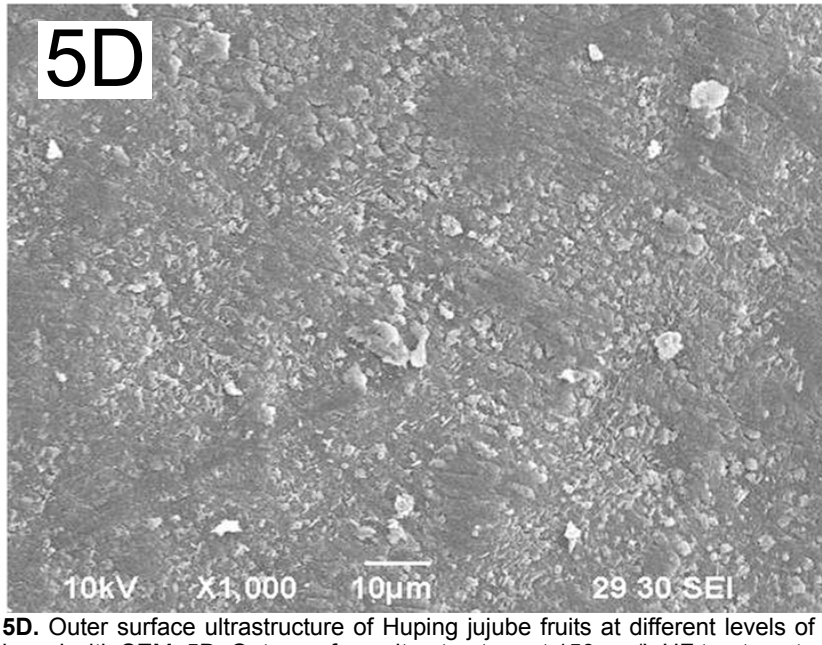
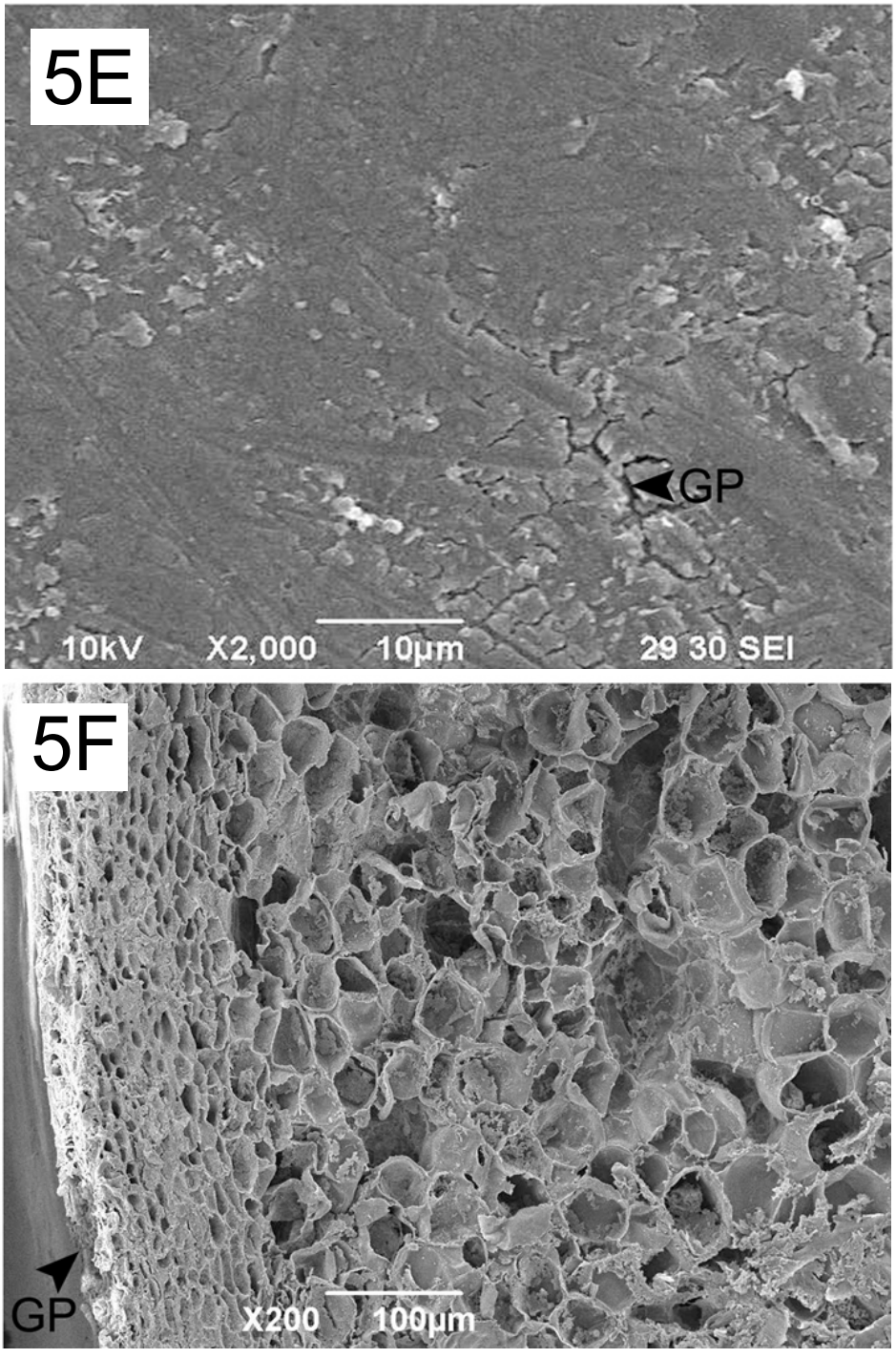


Figure 5D. Outer surface ultrastructure of Huping jujube fruits at different levels of fluorine stress viewed with SEM. 5D: Outer surface ultrastructure at 150 mg/L HF treatment.

At more than 200 mg/L HF solution, several gaps appeared on the peel and the sags and crests became worse (Figure 5E-F).



Figures 5E and 5F. Outer surface ultrastructure of Huping jujube fruits at different levels of fluorine stress viewed with SEM. 5E: Outer surface ultrastructure after treatment with more than 200 mg/L HF; 5F: Peel cross-section ultrastructure after exposure to more than 200 mg/L HF. Abbreviation: GP, gap.

DISCUSSION

Pericarp and pulp morphology of Huping jujube fruits under fluorine stress: The pericarp cuticle and epidermal thickness were approximately 5.7±0.04 and 66.31±1.27 μm, respectively. These results differed from Li[19] and Xin.[20] Different sampling locations on the fruit and the degree of ripening may have caused these differences. Different sampling locations and degree of ripening can affect the measured thickness of jujube pericarp.[20] The epidermal and subepidermal cells are arranged closely and difficult to distinguish clearly. They can only be distinguished by cell shape and differently colored stains. Different degrees of dyeing can make differences between epidermal and subepidermal difficult to measure. Pulp cells were arranged loosely and formed many cavities which is consistent with previous studies.[20-22]

Spraying fluoride solution at different concentrations could induce the symptoms of jujube black tip disease. As higher concentrations of HF solution were applied, deformation of jujube fruit pericarp and pulp paralleled the symptoms of different grades of the induced jujube black tip disease.[23] Jujube fruits tolerated excessive fluorine at concentrations less than 200 mg/L because no obvious changes of pericarp and pulp morphology were detected at 150 mg/L HF solution. However, structural and ultra-structural changes were detected when concentrations of 200 mg/L HF solution or higher were applied.

Ultrastructure of Huping jujube fruit cells under fluorine stress: Vacuoles were enlarged and crowded other cellular contents along the cell membrane at 150 mg/L HF solution. The diseased jujube fruit exhibited signs of senescence, similar to those seen in healthy mid- and late-stage jujube fruits.[24] This phenomenon has also been observed in peach fruits,[7,25] mango,[8] grapes,[26] and tomatoes.[27,28] That is to say, the same phenomenon occurs regardless of the ripening process in drupe or berry fruits. Jujube fruit cells are still able to function despite symptoms of cellular senescence. Plasmolysis occurred and organelles were invisible except for ruptured mitochondria membranes in fruits treated with 200 mg/L HF solution. This implies that dehydration was initiated in the fruit cells. Most organelles were severely injured at 200 mg/L HF solution and mitochondria were seen to have a higher tolerance to fluorine stress than other organelles. The Golgi apparatus is a major site of carbohydrate synthesis.[29] Carbohydrate accumulation may be influenced when the Golgi apparatus is severely injured and fruit sugar accumulation may be affected. On the other hand, the outer mitochondrial membrane contains large numbers of porins that bind special signaling proteins to actively move them across the membrane.[30] The cytochrome complex is localized to the perimitochondrial space[30] and the inner mitochondrial membrane contains proteins required for oxidative phosphorylation, ATP synthesis, and transport proteins.[29] Signal protein transport and electron transport chain function may be adversely affected along with energy metabolism of jujube fruit cells when the mitochondrial membrane ruptured at 200 mg/L HF stress. At 250 mg/L HF, severe plasmolysis indicates further dehydration of fruit cells, which corresponds to the symptoms of induced jujube black tip disease. It was reported that shrinkage on the top of jujube fruits was visible to naked eye.[23] Organelles were damaged

evidently and could not be found by TEM. Severe dehydration of jujube fruit cells results in abnormal physiological and metabolic processes. Cell walls were seriously deformed after exposure to over 300 mg/L HF solution. The cell wall functions with the cell membrane and serves as an additional layer of protection from mechanical and chemical stress.[29] The damage by fluorine stress to jujube fruit cells was too severe for the cell wall to function properly. The cell wall has sufficient tensile strength to withstand internal osmotic pressures several times greater than atmospheric pressure that result from differences in solute concentration between the cell interior and external water. The fluorine stress exceeded the tensile strength of jujube fruit cell walls when HF solution concentration was more than 300 mg/L. Mitochondrial structure showed that this organelle can tolerate fluorine stress. The damage by fluorine stress to mitochondria is a gradual process.

Outer surface ultrastructure of Huping jujube fruits: The outer surface of the Huping jujube fruits was rough with slight sags and crests at 150 mg/L HF solution. Epicuticular wax was damaged by fluorine stress although the pericarp and pulp morphology did not have obvious damage compared to the control. Epicuticular wax mainly consists of straight-chain aliphatic hydrocarbons with a variety of substituted groups which decrease surface wetting and moisture loss.[31] Epicuticular wax forms crystalline projections from the plant surface, which enhance their water repellency and reflect ultraviolet radiation.[32] The damaged epicuticular wax may aggravate fruit tissue dehydration and make the fruit cuticle susceptible to ultraviolet light. Fruit cuticles are protective, hydrophobic, waxy coverings produced by the epidermal cells of fruit and resist ultraviolet light as well.[33,34] Ultraviolet light may damage jujube fruit peels after damage to the cuticle caused by HF.

CONCLUSION

HF exposure caused gradual injury to Huping jujube fruits. The jujube fruits could tolerate HF concentrations less than 200 mg/L but suffered varying degrees of damage when exposed to HF concentrations of more than 200 mg/L. Analysis of cell ultrastructure from treated plants shows that mitochondrion tolerance to fluorine stress could be used as an index to determine the jujube fruit tolerance to fluorine stress. Scanning electron micrographs shows that the epicuticular wax was injured by spraying 150 mg/L HF.

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