Comparison of textural and ultrastructural characteristics of four apple cultivars with different textures during cold storage

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ABSTRACT
Fruit texture is important for the production of quality apples. In this study, the aim was to investigate changes in texture and ultrastructure of apple cultivars during cold storage. The results indicated differences between cultivars and that quality parameters decreased over time. At the ripening stage, compared with 'Qinguan' and 'Ben Davis', 'Fuji' and 'Ruixue' exhibited larger pulp cells and smaller intercellular gaps. Ultrastructural changes in 'Fuji' and 'Ruixue' were small and cell walls remained intact, whereas the tissues of 'Qinguan' and 'Ben Davis' exhibited greater changes and the cell walls decreased. In coarse cultivars, the ultrastructural degradation of 'Qinguan' tissues was slower.

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Introduction
Texture is an important attribute affecting apple quality and is one of the most important qualities affecting consumer preferences. However, apples usually lose density, increase in volume, and become less juicy and more mealy during storage. In addition, the texture of different apple cultivars varies greatly during storage. It is therefore of great importance to understand the ultrastructure of apples with different texture types and to improve their texture and quality. A better understanding of the relationship between the textural and ultrastructural characteristics of apples during cold storage can provide a better understanding of how cultivars with different textures soften and degrade. Many studies have already investigated either the textural differences of different apple cultivars during their development and maturation or changes in texture during storage. However, most of these studies used either a scanning electron microscope (SEM), light microscope (LM), or transmission electron microscope (TEM) to analyze fruit texture, but few studies have incorporated all three methods. Moreover, the ultrastructure of apple cultivars with different texture types during cold storage has been seldom reported. Tong showed that the texture, ultrastructure, and some physiological parameters of the ‘Honeycrisp’ cultivar were significantly different from those of their parents and the ‘Delicious’ cultivar. More recently, Hou reported that the intercellular space of apples was related to their texture by using confocal laser scanning microscopy.

In general, the quality of apple texture is evaluated by hardness, crispness, juiciness, etc. The main factors affecting fruit texture include cell size, shape, cell wall thickness, and strength. It has been reported that the texture of apples is closely related to their ultrastructure.
apples goes through obvious changes during storage and the ultrastructure of different apple cultivars varies significantly.\cite{20,21} The texture of apples is not only related to the ultrastructure but also to the structural characteristics of the fruit. The parameters of a texture profile analysis (TPA) can reflect the structural characteristics of fruit.\cite{22-24}

The present study aimed to use LM, SEM, and TEM to explore the differences in the ultrastructure of different apple cultivars during cold storage. Four apple cultivars with different texture types were selected, namely, ‘Fuji’, ‘Ruixue’, ‘Qinguan’, and ‘Ben Davis’. ‘Fuji’ has a soft and crisp texture, whereas ‘Qinguan’ is characterized by the firmness and coarseness of the matured fruits. ‘Ruixue’ is a new late ripening apple cultivar selected from a cross of ‘Qinfu No. 1’ and ‘Pinklady’.\cite{25} It has a hard and crisp texture, whereas ‘Ben Davis’ is characterized by the softness and coarseness of the matured fruits. Specifically, the objectives of the present study were to: (1) carry out a sensory evaluation and TPA of the pulps of the four apple cultivars during cold storage, (2) to examine the microstructure of apple tissues such as cell size and cell shape using LM during maturity, (3) to examine the cell wall characteristics of the four apple cultivars using SEM and TEM.

**Materials and methods**

**Apple samples**

Apple trees of the four cultivars examined were planted in 2000 at the Experimental Station of Northwest A&F University, Baishui County, Shaanxi Province, China. Apples were harvested at their respective maturity stage determined by sensory evaluation, ground color, and degree of starch clearance in 2017. To ensure the consistency of samples, the appearance and size of the harvested fruits were uniform and were without pests, diseases, or mechanical damage, and were collected from three trees in similar positions. Packaged fruits were immediately transported to the laboratory at the Northwest A&F University. Fruits were divided into two groups, one for experiments with maturing samples and the other for preservation in cold storage (2–4 °C; relative humidity, 80–90%; normal atmosphere).

**Light microscopy and transmission electron microscopy**

The apple tissues were cut into small cuboids of 5 mm × 2 mm × 1 mm and wrapped in bovine serum and then fixed in 4% glutaraldehyde solution at 4 °C for 12 h. Samples were then washed in phosphate buffered saline (PBS, pH 6.8) four times and post-fixed in 1% osmium tetroxide for 2 h. Samples were washed with PBS buffer five times and then dehydrated with 2 ml ethanol solution using a gradient of ethanol concentrations. The samples were then infiltrated, embedded, and polymerized in LR white resin (London Resin Company, Reading, UK) at 55 °C for 48 h. Semi-thin sections (20 μm thick) were cut with an ultramicrotome (Leica RM2265, Germany) and were viewed under the LM (Olympus BX51, Japan). Finally, Image J was used to measure and analyze the morphological characters of cells in the section. Then, ultra-thin sections (70 nm) were cut with an ultramicrotome (Leica UCT, Germany), dyed with uranyl acetate and lead citrate, and observed with the TEM (HT-7700, Hitachi, Japan and JEM 1230, JEOL, Japan).

**Scanning electron microscope**

The apple tissues were cut longitudinally into a thin strip. A long strip of pulp was split into two halves, and the fracture surface was trimmed into a cuboid of 5 mm × 3 mm × 2 mm. Samples were fixed in 4% glutaraldehyde for 10 h, washed four times, dehydrated using a gradient of ethanol concentrations, and replaced with isooamyl acetate once for 15 min. The samples were then placed in the EMITECH K-850 carbon dioxide critical point dryer for drying, sprayed with gold, and photographed under the SEM (JSM-6360LV, JEOL, Japan).
**Instrumental texture profile analysis**

TPA of four apple cultivars was performed using a texture analyzer (TMS-pilot, Food Technology Corporation, America). TPA was performed as described Gao et al.\cite{26} and Yang et al.\cite{27} with modification. Apples were cut longitudinally with a blade and perforated with a 10-mm-diameter perforator. The tissue was then cut with a cylinder with a height of 10 mm. Each sample was measured at six points and had three duplicates. The parameters of the determinations were: force induction range = 250 N, deformation 100 = 30%, test speed = 60 mm/min, input starting force = 0.2 N. Three textural representative parameters, namely, hardness, gumminess, and chewiness, were determined from each curve.

**Sensory evaluation**

The sensory evaluation method used in this study is modified from the literature.\cite{7,28} The sensory group consisted of 10 specialists trained and repositioned specific texture attributes using reference criteria and selected test samples. The apples were cut into wedges of the same size, and the experts tasted only the flesh. Each team member scored the pulp based on five texture descriptors by consensus: crispness, hardness, coarseness, juiciness, and mealiness. Samples were scored for texture attributes using 150 mm unstructured line scales, where 0 = absent and 150 = extreme.

**Data analysis**

The results of the experiment described above were analyzed in the SPSS statistics v20 software and compared with a Student’s t-test. All data were obtained from at least three independent experiments, and the means are presented herein. Image J software was used to measure morphological parameters of pulp cells.

**Results and discussion**

**Textural parameters and sensory evaluation**

In the present study, three typical textural parameters (hardness, gumminess, and chewiness) of the four cultivars were measured by TPA and compared, and apparent differences were found among the cultivars (Figure 1). The results showed that the variation trends of the three parameters were similar among the different cultivars. The three texture parameters of ‘Ruixue’ were the largest during the three periods, and those of ‘Fuji’ and ‘Qinguan’ were the second largest, and the differences between them were relatively small, whereas those of ‘Ben Davis’ were always the smallest. These results are in agreement with previous studies that found that the regional apple cultivars showed different structural characteristics among them by using the TPA method.\cite{24}

According to the results of the sensory evaluation, there were significant differences in the texture of the four apple cultivars during cold storage (Table 1). These results are consistent with the basic texture characteristics of the four cultivars at maturity stage. ‘Fuji’ had a soft and crisp texture and ‘Ruixue’ had a hard and crisp texture, while ‘Qinguan’ had a hard and tough texture and ‘Ben Davis’ had a soft and tough texture. After two months of cold storage, the hardness and crispness of ‘Fuji’ decreased slightly, whereas there was little change in the texture of ‘Ruixue’. The hardness of ‘Qinguan’ decreased slightly, and it became slightly mealy, and ‘Ben Davis’ acted similarly. After four months of cold storage, the hardness and crispness of ‘Fuji’ decreased significantly, but the texture was still crisp. The hardness and crispness of ‘Ruixue’ decreased slightly. The hardness of ‘Qinguan’ decreased greatly and the texture became mealy, while ‘Qinguan’ became very soft and mealy.

Brummel\cite{29} observed that the hardness and juiciness of different cultivars were different. Seppä et al.\cite{5} showed that the texture of different apple cultivars varies greatly during storage and that the texture change of the new cultivar ‘Y9330’ was very small. Through comparing the results of the...
sensory evaluation and the TPA measurements, we found that there was a correlation between them. The sensory texture and TPA parameters of the four apple cultivars decreased gradually during cold storage. Especially, the sensory texture of ‘Ruixue’ that remained hard and crisp throughout the whole storage period. The textural parameters of this cultivar were the largest, and they changed the least. The sensory texture of ‘Ben Davis’ was the worst and it quickly changed to mealiness; its texture parameters were smaller than the other cultivars, and they decreased more rapidly. These results are in agreement with the results of previous studies [20], that found that the sensory evaluation of fruit quality was closely related to the mechanical properties of the fruits.

**Light microscopy of the tissues of the four cultivars**

The cell morphology of the apple tissues was almost unchanged after reaching maturity, so the microstructures of the four apple cultivars in the mature period were compared using LM. The
morphological indices of microstructure were calculated using the Image J software. It was found that there were obvious differences among the four cultivars (Figure 2; Table 2). The cell areas of the crisp apple cultivars ‘Fuji’ (Figure 2a) and ‘Ruixue’ (Figure 2b) were significantly larger than those of the coarse apple cultivars ‘Qinguan’ (Figure 2c) and ‘Ben Davis’ (Figure 2d). Among them, the cell areas of ‘Fuji’ and ‘Ruixue’ were similar and the area of the pulp cells of ‘Qinguan’ was larger than that of ‘Ben Davis’. The cell perimeter, length, width, and cell area were similar (Table 2). ‘Fuji’ and ‘Ruixue’ had a large aspect ratio, whereas ‘Qinguan’ and ‘Ben Davis’ had a small aspect ratio (Table 2). In cell roundness, ‘Qinguan’ showed the most round cells and ‘Fuji’ and ‘Ben Davis’ had the smallest roundness (Table 2).

These results are in accordance with previous studies\cite{7,30} that found that the texture of apple cultivars with large cells was crisper. Contrary to the literature\cite{31}, tissues containing large cells were observed to have a higher susceptibility to mealiness. The texture of tissues is related not only to the size of individual cells but also to the shape and spatial arrangement of cells.\cite{32} By comparing the aspect ratio and roundness parameters of the pulp cells, it was found that the texture (hardness and crispness) and the shape of pulp were not significantly correlated. This contradicts the results of a previous study\cite{19} that found that the harder ‘Scifresh’ were larger and more angular than ‘Royal Gala’. By comparing the porosity of the tissues of the four cultivars, it was found that there was a close correlation between pulp texture and cell gap. The cell gap of the crisp ‘Fuji’ and ‘Ruixue’ was significantly smaller than that of the coarse ‘Qinguan’ and ‘Ben Davis’. Moreover, in cultivars with similar crispness, the harder cultivars had smaller cell gaps, such as ‘Ruixue’ compared with ‘Fuji’, and ‘Qinguan’ compared with ‘Ben Davis’. Indeed, Hou\cite{15} showed that there was a negative correlation between the intercellular space and the firmness of apples. Tong\cite{14} found that apples had large cells, small gaps, and large intercellular contact areas, which may lead to a higher swelling pressure and help to increase the firmness of the fruit. However, Toivonen and Brummel\cite{33} suggested that tissues with smaller cells had a greater area of cell-to-cell contact and a lower amount of intercellular spaces. Therefore, the relationship between cell size and intercellular space may not be the same in different fruits. These findings suggest that there are correlations between cell size, intercellular space, and texture in the four apple cultivars examined.

**Table 2.** The flesh cell morphological index of the four apple cultivars.

| Cultivar   | Area/μm² | Perimeter/μm  | Width/μm | Length/μm | Aspect | Roundness | Porosity/% |
|------------|----------|---------------|----------|-----------|--------|-----------|------------|
| ‘Fuji’     | 20405.13c| 389.89c       | 186.77c  | 127.42bc  | 1.39c  | 0.70bc   | 0.18b      |
| ‘Ruixue’   | 20230.32c| 555.31c       | 186.90c  | 137.34c   | 1.30bc | 0.77bc   | 0.13a      |
| ‘Qinguan’  | 13564.71b| 449.46b       | 149.36b  | 119.11b   | 1.18a  | 0.81c    | 0.23c      |
| ‘Ben Davis’| 10411.72a| 389.89a       | 130.06a  | 103.02a   | 1.26ab | 0.69b    | 0.39d      |

different normal letters stand for significant difference among accessions at 0.05 level for the same index in the same column.

**Figure 2.** Light micrographs of the tissues of the four apple cultivars.

**Tissue fractures**

Fractures in the tissues of the four apple cultivars with different textures were observed during cold storage by SEM (Figure 3). At maturity, the crisp cultivars ‘Fuji’ and ‘Ruixue’ had larger pulp cells,
flat fracture surfaces, clean cell surfaces (Fig. 3A and B), smaller cell gaps, and closer arrangement of cells (Fig. 3A and B), while the coarse cultivars ‘Qinguan’ and ‘Ben Davis’ had small pulp cells, extremely uneven fracture surfaces (Fig. 3C and D), rough cell surfaces, and irregular cellular arrangement. The cell space was large, and the arrangement of cells was loose (Fig. 3C and D). These results are in accordance with previous research.\[6\] According to Allan-Wojtas et al.\[7\], the fracture surfaces of apple cultivars with better texture were neater and cleaner. Compared with other cultivars with similar crispness, the cells of the harder cultivars were arranged more closely and had smaller intercellular spaces. For instance, the cells of ‘Ruixue’ were more closely arranged than those of ‘Fuji’, and those of ‘Qinguan’ were more closely aligned than those of ‘Ben Davis’.

These results are consistent with the porosity obtained from the semi-thin slices under LM (Table 1). After two months of cold storage, the fractures in the tissues of the four cultivars changed by varying degrees. Compared with the pulp cells at maturity, the intercellular gaps between the

\textbf{Figure 3.} Scanning electron micrographs of the tissue of four apple cultivars.
cells of ‘Fuji’, ‘Ruixue’, and ‘Qinguan’ were slightly increased (Fig. 3A₃, B₃, and C₃), while the cells of ‘Ben Davis’ had collapsed and the pattern of cell fractures had changed (Fig. 3D₃). The rupture pattern of the tissues in the three cultivars ‘Fuji’, ‘Ruixue’, and ‘Qinguan’ was mainly in the form of cellular rupturing, while ‘Ben Davis’ exhibited separated cells. As Ng et al.⁸ showed, the fractures in ‘Scifresh’ tissues were also in the form of cellular rupturing rather than the cell-to-cell-separation observed in ‘Royal Gala’. The fracture pattern of ‘Ben Davis’ changed, which was related to the changes in texture parameters and sensory texture. During this period, ‘Ben Davis’ became mealy and its texture parameters decreased rapidly (Figure 1). These results confirm the findings of Glenn and Poovaiah.³⁴

According to Smedt et al. [31], there were some common cell wall characteristics in mealy tissues, such as the degree of cell separation being higher. After four months of cold storage, the fracture surface of the pulp cells changed more significantly. The intercellular gaps increased by varying degrees compared to the tissues after two months of cold storage. The fractures in the tissues of the crisp cultivars ‘Fuji’ and ‘Ruixue’ maintained patterns of cellular rupturing, while those of the coarse cultivars ‘Qinguan’ and ‘Ben Davis’ were transformed into patterns dominated by cellular separation at the middle lamella. Comparing the two crisp cultivars, it was found that the pulp cellular gaps of the harder textured ‘Ruixue’ were smaller and the cells more closely arranged than those of ‘Fuji’. In addition, the fractures of the harder textured ‘Qinguan’ were formed by cellular ruptures, while the fractures of ‘Ben Davis’ were formed by the separation between cells. During the whole storage period, the fractures of the crisp cultivars were consistently formed through cellular rupturing and the intercellular gaps became gradually larger. However, the fractures of the coarse cultivars gradually changed from those formed by cell rupturing to those formed by the separation between cells, and the transformation frequency and degree of the harder textured ‘Qinguan’ were lower than those of ‘Ben Davis’ during cold storage. Harker and Hallett [35] showed that in newly harvested kiwifruits, the fractures were formed by cell rupturing, but were formed by the separation between cells after 2 weeks of preservation.

The results presented herein suggest that the fracture surfaces of tissues with different textures were different not only at the ripening stage but also in the process of cold storage. The fracture surfaces of the crisp cultivars were consistently formed by cell rupturing, while the fracture surfaces of the coarse cultivars changed from those formed by cell rupturing to those formed by the separation between cells during storage. Moreover, between the two coarse cultivars, the change in the type of fracture was relatively late in the harder cultivar. The differences in the fractures between ‘Qinguan’ and ‘Ben Davis’ during cold storage were largely attributed to changes in the texture parameters of apples, whereby the texture parameters of ‘Ben Davis’ were the lowest, but they changed the most during cold storage.

**Cell wall of the four apple cultivars**

Plant cell wall structure is an important factor in determining fruit texture. In order to understand the changes in the cell walls of the four apple cultivars during cold storage, tissues were observed by TEM (Figure 4). The cell walls of the pulp cells were observed to consist of the middle lamella and the primary cell wall. The middle lamella was mainly composed of pectin, while the primary cell wall has been reported to be composed of cellulose molecules, arranged into thin hair-like strands called microfibrils. The microfibrils are arranged together with other components such as hemicellulose, glycosans, and pectin which link them together and help to strengthen the cell wall. At the ripening stage, the cell wall structures of the tissues of the four apple cultivars were intact, exhibiting a light-dark-light structure and being close to the plasma membrane. However, there were some differences in the cell wall structures among the four cultivars. The thickness and electron density of the middle lamella of the cell wall in the crisp cultivars ‘Fuji’ (Fig. 3E₁) and ‘Ruixue’ (Fig. 3F₁) were larger than those of the coarse cultivars ‘Qinguan’ (Fig. 3G₁) and ‘Ben Davis’ (Fig. 3H₁). In addition, the microfilaments of the cell walls of the crisp cultivars were closely arranged and lighter in color (Fig. 3E₁ and F₁). These results were partially consistent with those presented by Cybulska et al. [9],
which showed that cultivars with thicker cellular fibers exhibited a crisper, harder, and juicier texture. The differences in the cellulose microfibrils of 'Fuji' and 'Ruixue', which had different levels of hardness, were not obvious. According to Forster et al.\[37\], changes in fruit texture were the result of the degradation of cell wall polysaccharides (including cellulose, hemicellulose, and pectin).

These results suggest that the main reason for the difference in texture between crisp and coarse cultivars at maturity is the difference between the middle lamella and cellulose microfilaments. After two months of cold storage, the cell wall structures of the four cultivars were degraded by fruit senescence. The cell wall structures of the crisp cultivars 'Fuji' and 'Ruixue' degraded more slowly than those of the coarse cultivars 'Qinguan' and 'Ben Davis'. The middle lamella in 'Ben Davis' tissues was completely degraded, but that of 'Fuji', 'Ruixue', and 'Qinguan' was still observed. The tissues of 'Fuji' and 'Ruixue' retained a dense middle lamella compared with those of 'Qinguan'. The microfilaments of 'Fuji' and 'Ruixue' tissues were obviously more closely arranged and colored more deeply, whereas the microfilaments of the 'Qinguan' and 'Ben Davis' tissues were arranged loosely and became lighter in color. These results are consistent with the observed ultrastructure using SEM. Compared with the mature stage, the middle lamella of 'Ben Davis' was completely degraded. This phenomenon is consistent with the transformation of the pulp cells from cell rupture to separation between cells. The cause of the interrelation between the two phenomena needs to be further studied.

After 4 months of cold storage, the dark areas of the tissues of the four apple cultivars greatly decreased, and cell wall integrity was damaged. 'Fuji' and 'Ruixue' retained a clear middle lamella, whereas the middle lamella of 'Qinguan' and 'Ben Davis' almost completely disappeared. In addition, the microfilaments of 'Ben Davis' disappeared completely, whereas 'Qinguan' retained a small number of microfilaments. 'Fuji' and 'Ruixue' had tighter microfilaments and darker colors than

Figure 4. Transmission electron micrographs of the cell walls of the four apple cultivars.
'Qinguan' and 'Ben Davis. These results are in accordance with Tong et al.\textsuperscript{[14]}, who showed that the cell walls of 'Honeycrisp' remained intact after storage for 6 months, while the cell walls of 'Macoun' and 'Honey Gold' were degraded. Bennett and Labavitch\textsuperscript{[38]} observed that the cell wall degradation was the main cause of fruit softening and loss of quality. These results showed that the middle lamella and cellulosic microfilaments of the crisp cultivars degraded more slowly than those of the coarse cultivars during cold storage, and that cell wall structures were still relatively complete after 4 months of cold storage. This phenomenon is consistent with observations of ultrastructure using SEM, during which it was observed that the tissues of the crisp cultivars are consistently fractured by cell rupturing, whereas the fractures of the coarse cultivars during storage change from being caused by cell rupture to separation between cells. In addition, although the textures of both 'Qinguan' and 'Ben Davis' were rough, the middle lamella and cellulose microfilaments of the harder 'Qinguan' were slower to degrade and the rate of transition from cell-rupture fractures to separation-between-cells fractures was also slower than that of 'Ben Davis' during storage.

**Conclusion**

The ultrastructure and texture characteristics of four apple cultivars with different texture types were observed at maturity to be different during cold storage. At maturity, the crisp cultivars had larger pulp cells, smaller intercellular gaps, and a more compact arrangement of cells, whereas the coarse cultivars had smaller pulp cells and larger intercellular gaps. With the gradual changes in texture characteristics, the ultrastructure of tissues also changed significantly during cold storage. The fractures of the crisp cultivars were consistently formed by cell rupturing, and the fractures were neat and clean, while the fractures of the coarse cultivars gradually changed from being formed by cell rupturing to separation between cells during storage, and the fractures were folded and uneven at maturity. In addition, the degradation of the middle lamella and cellular microfilaments in the crisp cultivars was observed to be slower than that in the coarse cultivars during cold storage. After four months of cold storage, the cell wall content in the tissues of the coarse cultivars were completely degraded. The changes in the tissue fracture surfaces, cell wall content, sensory evaluation, and TPA parameters during cold storage in the apple cultivars with different texture types were correlated.

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