Study on Paraffin Section Technology of Fruit Tumor Tissues in Cucumber (Cucumis sativus L.)

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Abstract  Fruit tumor is one of the importantly external quality traits affected to the economic value of cucumber fruit. In order to obtain the paraffin section technology and study the cytological mechanism of morphological formation for fruit tumor in cucumber, we used the samples of warty fruit line (S52) and non-warty fruit lines (S42 with big fruit spine and S06 with small fruit spine) as materials, and improved some key links such as dehydration, dyeing and dewaxing etc., based on traditional paraffin section technology. Results demonstrated that increasing ethanol concentration gradient during dehydration can shorten the treatment time of each stage to 30 minutes. Samples were stained with Ehrlich's hematoxylin for three days, which was convenient for subsequent operation. Optimal thickness of the section was 8 μm. After twice pure xylene dewaxing, sections could be examined and selected by microscope, which shortened the time for obtaining high-quality section. High quality sections with complete tissue and clear structure can be obtained by the optimized paraffin section technology, which is an effective method to observe the cytological structure of fruit tumor. This study provides a set of complete and efficient operation steps of paraffin section technology for cucumber fruit tumor, which will provide technical reference for anatomical researches on other organs in cucumber and structural similar characteristics of other plants.

Keywords  Cucumber, Anatomy; Paraffin section; Fruit; Microscopic observation

Introduction  Cucumber (Cucumis sativus L.; 2n=2x=14), which belongs to the Cucurbitaceae, is one of the most important vegetable plants grown widely in the world. It plays an important role in ensuring the annual vegetables supply (Ren et al, 2009; Zhang et al., 2012). Usually, certain cucumber varieties produce warty fruit, while others produce smooth fruit (Zhang et al., 2010; Zhang et al., 2018). Fruit tumor, which is strumae of fruit surface, is a special character of cucumber. Warty fruit is one of the highly valuable external quality traits (spine color, spine number, spine size, fruit colors, dull fruit skin, immature fruit color and ribbed fruit etc.), which are closely related to the economic values of cucumber (Dijkuizen and Staub, 2002; Fazio et al., 2003; Di et al., 2018; Zhang et al., 2018; Xue et al., 2019). Compared with warty fruit varieties, smooth and non-warty fruit varieties have the advantages of less pollution, more convenience in cleaning and packing, higher resistance in transportation and storage etc., and its market price is high than that of the common cucumber (Wang et al., 2007). Therefore, the study of fruit tumor characteristic will promote the quality breeding process of cucumber. In this study, we can understand the cytological mechanism of fruit tumor by obtained the paraffin section technology, which lays a foundation for the study of molecular mechanism of fruit tumor characteristic in cucumber.

Paraffin section is the most common microsection technique, which has been widely used in morphological and anatomical observation of plant materials due to its convenience, easy operation, low cost and permanent preservation (Zhang et al., 2013; Feng et al., 2016; Chen et al., 2017a; Chen et al., 2017b; Wang et al., 2019; Miao et al, 2020). Different plants and organs have different tissue structures, which cannot be applied uniformly. Therefore, it is particularly important to explore the optimization procedure of paraffin section technique for
different plants, organs and tissues. At present, there is no report on the optimization of paraffin section technique for fruit tumor in cucumber. In this study, S52 with fruit tumor and S42 (big fruit spine) / S06 (small fruit spine) without fruit tumor were used as materials. Based on the traditional paraffin section method, the procedure of paraffin section technique was optimized for fruit tumor in cucumber. High quality sections with complete tissue and clear structure can be obtained by the optimized technique, which is an effective method to observe the anatomical structure of fruit tumor. This study provides a set of complete and efficient operation steps of paraffin section for fruit tumor, and a technical reference to the anatomical study of other organs and similarly structural characteristics in cucumber.

1 Results
There are three types for the fruit spine and fruit tumor in cucumber: big spine with warty fruit (Figure 1a), big spine with non-warty fruit (Figure 1b), and small spine with non-warty fruit (Figure 1c). Further cytological observation showed that the fruit spine and fruit tumor formation arise from an increase in cell number caused by cell division (Figure 1a-c, arrow showed). In fact, the fruit tumor was found to be derived from the division of several layers of cells that lie near the fruit spine-base cell in cucumber (Figure 1a, arrow showed).

According to the structural characteristics of fruit tumor in cucumber, the study shortens the production time and increases production efficiency of making paraffin section by exploring and improving the paraffin section technology. High quality sections with complete tissue and clear structure can be obtained by the optimized paraffin section technology, which is an effective method to observe the cytological structure of fruit tumor (Figure 1).

![Figure 1 Cytological observation of the fruit tumor and spine S52 line and S42 line, as well as small fruit spine S06 line](image)

**Note:** a: Cytological observation of S52 line (large fruit spine with warty fruit); b: S42 line (large spine with non-warty fruit); c: S06 line (small spine with non-warty fruit); Sp: spine, Spp: spiny pedestal, Tu: tuberculate fruit; Bar=100 μm

1.1 Fixation and dyeing
Fruit tumor including fruit spine and fruit spine base on fresh fruits were collected. The sampling location is shown in Figure 2. All samples collected were fixed for 30min, when immersed in the liquid surface of FAA-fixed solution. Cucumber fruit is rich in water. In order to ensure complete fixation, we poured out the first FAA-fixed solution, and then add new FAA solution to fix for more than 48 hours again. The results showed that the fixed solution changed for more than two times had a good effect on the dehydration of plant samples.

Ehrlich hematoxylin dilute solution of 50% alcohol was used to dyeing whole test materials in the study. Compared with the traditional paraffin section technology, it is more time-saving and more convenient for subsequent operations on embedding, sectioning, dewaxing etc. The results showed that the color of dyeing materials for the whole dyeing process would be light after dehydration, transparence and wax soaking, but it did not affect the quality of production and observation, and could save the time of later dyeing and selection slices.
1.2 Dehydration and transparence

In order to ensure the materials dehydrated completely in the shortest time, we increased seven ethanol concentration gradients (50% - 60% - 70% - 80% - 90% - 95% - 100%), and shortened the induction time of each gradient to 30 minutes. The results showed that dehydration effect was very good. Waxing process in the follow-up can completely penetrate each cell of the material, and the slices were completely expanded and do not fall off. In addition, due to the long dehydration time, we will encounter the situation that all seven concentration gradients cannot be completed at one time. We found that the material can be kept in 70% ethanol for up to 12 hours, and then it can be induced by 70% ethanol for 30 minutes, and then continue the latter concentration gradient, without affecting the production quality. In order to make the material fully transparent, we set four concentration ratios, also shorten the treatment time to 30 minutes, and only induce in pure xylene for 60 minutes; the results showed that the concentration and time of the transparent treatment can achieve the expected effect.

1.3 Waxing and embedding

There are two steps in waxing process. Firstly, wax scraps are gradually added into the mixed solution including the samples in the 42℃ incubators. The smaller wax scraps are, the better waxing is. Constant temperature is required for incubator to avoid large temperature change affecting the embedding quality. Then, the filtered liquid wax is added and the materials are induced at a constant temperature of 60℃. The results show that the materials can be soaked more fully by two steps (wax scraps and liquid wax).

Embedding process is the same as the traditional paraffin section technology, and the whole process is carried out in constant temperature incubator to ensure the embedding quality. In order to ensure the integrity and the best position of the samples in embedding process, the preheated anatomical needle is used to gently adjust the material to the fruit spine upward and the whole material perpendicular to the bottom of the embedding box. The results show that adjusting the sample to the best position is beneficial to the integrity of the materials for subsequent slicing process. In order to prevent the compression of material tissue and the difficulty of unfolding of slice wrinkles caused by high room temperature, the embedded wax blocks were stored in the refrigerator at 4℃.

1.4 Slicing, gluing and unfolding

Sections thickness directly affects the observation quality on cell tissue. Results show that the 12 μm wax-band is easy to overlap and fragile (Figure 3c), the 8 μm and 10 μm wax bands are continuous, smooth and uniform in thickness (Figure 3b, a), which are better slice thickness. After slice observation, results showed that the slice thickness of 8 μm tissue is clearer. The procedures of gluing and unfolding processes are the same as that of the traditional paraffin section technology. Protein adhesive reagent used in the study could reduce the amount of material falling off, and the stable 42℃ constant temperature platform could ensure the quality of the unfolding process.
1.5 Dewaxing and dyeing

There are some problems in dewaxing and re-dyeing, such as tedious operation, easy stripping and reagent toxicity. To find the target sections faster and more accurately, we inspected the sections under a microscope after twice pure xylene dewaxing. Non-target sections were removed, and only the target sections were dewaxed and dyed subsequently, which greatly shortened the processing time of obtaining the target sections. In addition, we used the whole dyeing method to dye the materials in the early stage of Paraffin section process, the color of the materials becomes lighter by dehydration, transparency, waxing immersion, dewaxing etc. In order to make the sections color more clearly, it is necessary to use Ehrlich hematoxylin dilute solution to re-dye for 10 minutes, and to separate the color in 4% ammonium ferric alum (each section). The sections were detected under the microscope for the second time to remove the non-target sections. Through the optimization of dewaxing and dyeing, unnecessary work can be reduced and operation time can be shortened.

1.6 Sealing and photographing

Neutral balsam can be used for sealing, and the size of cover glass can be selected according to the size of the material. The operation time should be as short as possible to avoid tissue oxidation. After sealing, the sections are dried in the natural environment, and then observed and photographed under the microscope.

2 Conclusion and Discussion

According to structural characteristics of fruit tumor in cucumber, based on the traditional paraffin section technology method, we optimized the important processes such as dehydration, dyeing and dewaxing etc., and explored the whole paraffin section technology procedure for fruit tumor in the study (Figure 4). High quality sections with complete tissue and clear structure can be obtained by the optimized technique shortened the time for obtaining high-quality section and improved the operation efficiency.

Different tissues of different plant materials have different dehydration methods and time. Dehydration time is too short, sample tissues are not dehydrated completely, and it is easy to form little holes; dehydration time is too long, the subsequent unfolding slices are incomplete, and it is easy to fold (Chen et al., 2017a). According to the structural characteristics of cucumber fruit tumor with more water content, in order to obtain the best dehydration method and time, we increased seven ethanol concentration gradients and correspondingly shortened the dehydration time to 30 minutes for each treatment based on the traditional paraffin section method. As a result, the materials dehydrated thoroughly and were not easy to shrink. The optimization method for dehydration process is the same as some plant tissues, but dehydration time of different tissues in different plants is different, so it is necessary to explore and optimize the best paraffin section technology procedure for the different plant tissues.

Tissue dyeing is usually carried out after dewaxing in traditional paraffin section technology (Zhang et al., 2013; Feng et al., 2016; Chen et al., 2017b; Wang et al., 2019; Miao et al., 2020). In this study, in order to obtain the paraffin sections with complete structure of fruit tumors and fruit spines, the dyeing method was adjusted, and the whole samples were directly dyed by Ehrlich hematoxylin after FAA fixation. The advantages of the whole
The dyeing method lie in dyeing first, embedding later, sectioning and unfolding, only two times of xylene dewaxing is needed, that is, the preliminary microscopic examination can be carried out, the non-target sections can be removed, the target sections with better quality can be obtained in the shortest time, and the tedious operation of dewaxing and dyeing link can be shortened. Based on the advantages of the whole dyeing method, it has been applied to paraffin section technology of plant materials such as petioles, stems, anthers, female flowers and root tips and so on (Zhang et al., 2013; Wang et al., 2019; Feng et al., 2016; Shi et al., 2020). However, there are also some problems in the whole dyeing method, such as different plants have different coloring abilities and the dyeing results are lighter; after dyeing, the color becomes lighter after dehydration, transparency, dewaxing and other technical processes; there are dyeing particles in dyed tissues and cells that affect the production quality (Feng et al., 2016). Disadvantages of poor section quality caused by the whole dyeing method were avoided by optimizing the treatment methods and time of dehydration, transparency, dewaxing, and adding re-dyeing and color-separation in dewaxing.

By optimizing the paraffin section technology, this study provides a set of complete and efficient operation steps of paraffin section for fruit tumor, and a technical reference to the anatomical study of other organs and structural similar characteristics in cucumber.

3 Materials and Methods

3.1 Plant materials
All plants were planted in the greenhouse of the farm at Shanghai Jiaotong University, using a unified substrates and fertilization management. It is the most clear and accurate judgment on fruit tumor during 7-10 days after female flowers anthesis (Zhang et al., 2012). Therefore, within 7-10 days after flowering, the fruit tumor with fruit spine and spine base from the S52 line and fruit spine with fruit pericarp from S42 line (big fruit spine without tumor) / S06 line (small fruit spine without tumor) were sampled.

3.2 Fixation and Dyeing
Fresh materials were sampled and immediately put into FAA-fixed solution (100% Ethanol: Formaldehyde: Acetic acid =10:2:1), the volume of which was 3-5 times of that of the material, gently shaken for several times, and fix the material in 30 minutes after sinking the material under the liquid surface. Then pour the fixing liquid and add the fresh the FAA-fixed solution for fixing more than 48h. In order to ensure the fixation effect, seal the bottle mouth with a sealing film and put it at room temperature store for reserve. When the temperature is too high in summer, it can be stored in 4℃ refrigerators. For dyeing, poured out the FAA-fixed solution, added 50% alcohol for 1h, and dyed by Ehrlich hematoxylin for 3d with shaking gently for 1-2 times a day.

3.3 Dehydration and transparency
The fixed samples were taken out from the Ehrlich hematoxylin dyeing solution which may be recycled, and were induced twice separately by 50% alcohol at room temperature for 30min, and then were induced sequentially by 60% - 70% - 80% - 90% - 95% - 100% - 100% at room temperature for 30min. Then, the samples were induced by 1/3 xylene + 2/3 anhydrous ethanol, 1/2 xylene + 1/2 anhydrous ethanol, 2/3 xylene + 1/3 anhydrous ethanol (volume ratio) for 30min respectively, and induced by pure xylene at room temperature for 60min, and repeated twice. Finally, half of pure xylene solution was poured out and half of tiny wax chips were added, and the mixture above steps was overnight at room temperature.

3.4 Waxing and embedding
Put the samples in mixed solution of xylene and wax scraps into 42℃ incubators for 60 min, gradually add tiny wax scraps to keep waxing at 42℃ temperature for 60 min each time, gently shake the mixture for 1-2 times until it is saturated. Then, discard the mixed solution as much as possible, and add the melted pure paraffin to induce the samples at 60℃ temperature for 4 h at least, repeat the above a step for 6 times. The paraffin-soaked samples were poured into the folded paper boxes placed on the 42℃ temperature table, and then gently moved to the appropriate location using the anatomical needle preheated in the 60℃ incubator. After that, put the folded paper
boxes including the samples into the prepared cold-water slot for rapid cooling. After embedding, the wax blocks were stored in the 4°C refrigerators.

**Paraffin Section Procedure of Fruit Tumor tissues in Cucumber**

**Fixation and Storage**
Fresh materials were sampled and immediately put into FAA-fixed solution, gently shaken for several times, and fix in 30 minutes after sinking under the liquid surface. Then pour out fixing liquid and add the fresh FAA-fixed solution for fixing more than 4h. Seal bottle mouth with a sealing film, and put it at room temperature store. When the temperature is too high in summer, it can be stored in 4°C refrigerator.

**Dyeing**
Pour out FAA-fixed solution, add 70% alcohol for 1h, and dye by hematoxylin for 3d with shaking gently for 1-2 times a day; the volume of which was 15-20 times of that of materials.

**Dehydration**
Fixed samples were taken out from Ehrlich hematoxylin dyeing solution which may be recycled, and were induced twice separately by 50% alcohol at room temperature for 10min, and then were induced sequentially by 60% - 70% - 80% - 90% - 95% - 100% - 100% at room temperature for 30min (if all seven concentration gradients cannot be completed at one time, materials can be kept in 70% ethanol for up to 12 hours).

**Transparency**
Samples were induced by 1/3 xylene + 2/3 anhydrous ethanol, 1/2 xylene + 1/2 anhydrous ethanol, 2/3 xylene + 1/3 xylene + 1/3 anhydrous ethanol (volume ratio) for 30min respectively, and induced by pure xylene at room temperature for 60min, and repeated twice. Finally, half of pure xylene solution was poured out and half of tiny wax slabs were added, and the mixture above steps was overnight at room temperature.

**Waxing**
Put samples in mixed solution of xylene and wax scraps into 42°C incubator for 60min, gradually add tiny wax scraps to keep waxing at 42°C temperature for 60min each time, gently shake the mixture for 1-2 times until it is saturated. Then, discard the mixed solution as much as possible, and add melted pure paraffin to induce samples at 60°C temperature for 4h at least, repeat the above step for 6 times.

**Embedding**
Paraffin soaked samples were poured into folded paper boxes placed on the 42°C temperature platform, and then gently moved to appropriate location using anatomical needle preheated in 60°C incubator. After that, put folded paper boxes including samples into prepared cold water slot for rapid cooling. After embedding, wax blocks were stored in the 4°C refrigerator.

**Slicing gluing and unfolding**
Embedded wax blocks were stuck on the small wood block fixed on paraffin slicer. The wax belts were continuously sliced with a thickness of 5 μm, and put on the white paper. Then, cut off the appropriate length from the continuous wax belts, drop a drop of protein adhesive on the slide, wipe it with little finger, add a drop of distilled water to spread it evenly, and carefully spread the wax belts on the middle of slides with tweezers or dissecting needle. All slides were placed on 42°C temperature platform for 1-2 days.

**Dewaxing and dyeing**
Place slides into different solution dyeing tanks by following order and time: xylene 20min, xylene for 15min, 1/2 xylene + 1/2 anhydrous ethanol for 3min, anhydrous ethanol for 3min, 92% ethanol for 3min, 90% ethanol for 3min, 85% ethanol for 3min, 85% ethanol for 3min, 60% ethanol for 3min, 50% ethanol for 3min, distilled water for 6min, hematoxylin for 10min, water gently rinse for 15-20min, 4% ammonium ferric alum to color for each slide combining microscopic test, distilled water for 6min, 20% ethanol for 30s, 60% ethanol for 30s, 70% ethanol for 30s, 85% ethanol for 30s, 90% ethanol for 30s, 95% ethanol for 30s, 100% ethanol for 1min, 100% ethanol for 1min, xylene for 3min, and xylene 10-30min.

**Sealing**
Final slides were sealed with neutral gum, dry at room temperature, and then observed and taken photos under microscope.

Figure 4 Paraffin section Procedure of fruit tumor tissues in cucumber
3.5 Slicing, gluing and unfolding
The embedded wax blocks were trimmed by a single-sided blade into trapezoid blocks and stuck on the small wood block fixed on the Leica RM2126 paraffin slicer. The wax belts were continuously sliced with a thickness of 8 μm, and put on the white paper. Then, cut off the appropriate length from the continuous wax belts, drop a drop of protein adhesive tablet (egg white 25ml + glycerin 25ml + sodium salicylate 0.5g) on the slide, wipe it with little finger, add a drop of distilled water to spread it evenly, and carefully spread the wax belts on the middle of slide with tweezers or dissecting needle. All slides were placed on 42°C temperature platform for 1-2 days.

3.6 Dewaxing and dyeing
Place slides into different solution dyeing tanks by following order and time: xylene 20min-xylene for 15min, 1/2 xylene: 1/2 anhydrous ethanol for 3min, anhydrous ethanol for 3min, 95% ethanol for 3min, 90% ethanol for 3min, 85% ethanol for 3min, 75% ethanol for 3min, 60% ethanol for 3min, 50% ethanol for 3min, distilled water for 6min, hematoxylin for 10min, water gently rinse for 15-20min, 4% ammonium ferric alum to color for each slide combining microscopic test, distilled water for 6min, 50% ethanol for 30s, 60% ethanol for 30s, 70% ethanol for 30s, 85% ethanol for 30s, 90% ethanol for 30s, 95% ethanol for 30s, 100% ethanol for 1min, 100% ethanol for 1min, xylene for 3min, and xylene 10-30min.

3.7 Sealing and photographing
Final slides were sealed with neutral gum, dry at room temperature, and then observed and taken photos under Olympus BX51 (Japan) microscope.

Authors’ contributions
Conceived and designed the experiments: ZWW. Performed the experiments: GDJ CY BWM. Analyzed the experiment data: ZWW PJS. Contributed reagents / materials / analysis tools: PJS. Organized the manuscript: ZWW. All authors read and agree to the final contents.

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