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HISTOLOGICAL TECHNIQUE FOR STUDY OF FILBERT POLLINATION AND FERTILIZATION.

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HISTOLOGICAL TECHNIQUE FOR STUDY OF FILBERT POLLINATION AND FERTILIZATION.

Malcolm F. Wharton.

INTRODUCTION.

The filbert (Corylus avellana) is rapidly becoming one of the most promising of horticultural crops for the Willamette Valley due to favorable conditions of soil and climate and a ready market of small supply and large demand. The planting of this nut is advancing as rapidly as the nurseries can furnish the trees and from all indications, the growing of filberts on a commercial scale should mature into a very prominent horticultural enterprise in this locality.

The growing of any crop is always attendant with difficulties of culture of one type or another, and so it is with the filbert. The question of pollination has been the bug-bear of the industry from the very start, as a large percentage of the nuts at first, were empty due to lack of pollination. The pollination of this nut is carried on under the most adverse handicap. Blossoming in the dead of winter, and relying upon the wind for dissemination of its pollen, accompanied by the inherent self-sterility of all varieties and inter-sterility of many varieties, due to the difference in dates and periods of blossoming makes the problem of efficient pollination one

of considerable magnitude and importance.

Excellent work has been accomplished by C. E. Schuster of the Oregon Experiment Station, in working out a combination of varieties to furnish pollination in the commercial orchards. This work was done from the experimental side with little regard paid to the mechanism of pollination within the plant itself. From a practical standpoint, it is invaluable, but we much study the problem from the truly scientific side in order to really under-stand and develop methods that will give the highest type of results.

The problem of studying the pollination and fertilization of the filbert was undertaken by F. J. Rimoldi, at this Station in 1920, and much good work was accomplished. At the very start, however, he encountered difficulty in properly sectioning the young pistils, (ovaries) for microscopic study. As he says in his report of this work: "The extreme hairiness of the bracts and other parts of the female catkins made microtome sectioning of the whole catkin, impossible. The material persisted in tearing out of the paraffin. Celloidin imbedding was next tried but with no greater success. To work out methods for handling such unusual material would probably constitute a problem in itself". Due to this difficulty, it was deemed advisable to further investigate the problem and the writer started work on it in the fall of 1922.

Almost immediately the difficulties mentioned by Rimoldi made themselves evident. The dense hairy covering on the pistils prevented sectioning to such an extent that microscopic study of the progress of fertilization was impossible. As this is really the most important step of the investigation, all attention was directed upon devising means and methods for accomplishing this sectioning. The problem really resolved itself into one of developing histological technique before attempting further work on fertilization study.

Little investigational work has been done from the technical standpoint on the filbert. A review of all available literature at hand reveals the work of Miss Benson (2) and S. Nawaschin (3) to present the entirety of it, outside of the work of Rimoldi.

Miss Benson reports the full fertilization of the filbert in detail but does not give methods of proccedure, and also refers to Nawaschin as authority in this work. Nawaschin in his study of fertilization of Corylus, reports considerable trouble in making of suitable sections for detailed study. He intimates that most of his drawings are reconstructions from a very few sections, often as few as two. Moreover, he studied late stages of development when the ovules had reached a larger size and were much easier sectioned, as was also found by Rimoldi. He also attempted micro-dissection of fresh material, but with little ap-

parent success from the standpoint of exact determination, for he finally returned to his regular histological technique for completion of his study.

Detailed Procedure.

Collections of pistillate buds were made at approximately ten day intervals beginning in the early part of December, as soon as the styles made their appearance. Barcelona and Nottingham varieties were used so as to give a comparison of varietal differences. Buds were brought into the laboratory as soon as collected and divided into two groups: The scales were removed from the first group before placing in the fixing solution, while the second group were sliced through the scales with a scalpel to allow penetration of fixing solution and paraffin. Buds not used immediately were preserved in a solution of Formaldehyde 4 percent in 50 percent alcohol.

General procedure consisted in testing out the effects of different fixing solutions as recommended by Chamberlain (4) in his "Methods of Plant Histology". As soon as the buds were fixed, they were passed through the alcohol series, as requirements of each solution demanded, and were imbedded for sectioning. Different imbedding materials were used including paraffines of different melting points and celloidin. Sectioning was then carried out on the regulation sliding microtome for celloidin, and with the regulation Bausch and Lomb Rotary microscope

for paraffin. Different temperatures while sectioning, and different positions of the sectioning knife were also experimented with.

Various other minor applications of the histological technique will be taken up in its application to the general methods.

The alcohol series used, unless otherwise specified, were of the following strengths of grain alcohol made up to a percentage with distilled water; 5% - 10% - 15% - 25% - 35% - 50% - 65% - 75% - 85% - 90% - 95% - 100%. This graduation was taken as a normal one that with average materials, gives the best condition of the plant cells without plasmolysis or changing of cell conditions or contents.

The Xylol-alcohol series used for clearing the material after passing through the fixing agents and alcohols, was of the following strengths: (1) alcohol 3 parts and xylol 1 part; (2) alcohol 2 parts and xylol 2 parts; (3) alcohol 1 part and xylol 3 parts; and (4) absolute xylol.

Under each test following, ten scaled and ten unscaled Barcelona buds were used,and for varietal comparison, five each of the scaled and unscaled Nottingham buds.

Fixing Solutions Used.

I. Stock Chromo Acetic. This was taken as the standard and the majority of the tests run were fixed with this solution.

Formula.

Chromic Acid ..... 1 gr.

Glacial Acetic Acid ..... 1 c. c.

Water ..... 100 c. c.

Material was treated as follows:

1. Barcelona buds scaled and unscaled were placed in this fixing solution for 24 hours, taken out and washed in running water for another 24 hours, and passed through the alcohol and xylol - alcohol series, leaving in each from 12 - 24 hours. Infiltration was accomplished by keeping material in oven at 54 degrees C for 48 hours, and changing the paraffin frequently until all traces of xylol had disappeared. Material was then imbedded in 52 degrees C paraffin and sectioned on rotary microtome.

2. Same procedure using Nottingham buds.

3. Barcelona buds, scaled and unscaled, with same procedure as in No. 1, differing only in that the material was imbedded in 58 degrees C paraffin.

4. Barcelona buds, scaled and unscaled, procedure same as in No. 1 through 95 percent alcohol. Drain off the alcohol and cover material with ether and leave for 24 hours to remove oils and waxes present. Wash in 95 percent alcohol until all odor of ether is gone, and proceed through xylol - alcohol series and imbed as in No. 1.

5. Barcelona buds, scaled and unscaled, 24 hours in

the fixing solution, washed for 24 hours in running water, and treated with hydrofluoric acid 1-10 strength, for 48 hours, and wash in running water for 24 hours more, then through regular series of alcohol and xylol - alcohols as in No. 1, imbedding in 52 degree C Paraffin as before.

6. Barcelona buds, scaled and unscaled, same procedure as No. 5, using 1-20 hydrofluoric acid instead of 1-10.

7. Barcelona buds,scaled and unscaled, 48 hours in fixing solution, remainder of procedure as No. 1.

8. Nottingham buds, scaled and unscaled, same procedure as No. 7.

9. Barcelona buds, scaled and unscaled, same procedure as No. 1, only difference being 24 hours of paraffin infiltration.

10. Barcelona buds, scaled and unscaled, procedure same as No. 1 up through absolute alcohol, then into solution of ether and absolute alcohol half and half, and from that into 1 percent solution of celloidin in ether for 24 hours. Next imbed in celloidin the consistency of thick syrup and harden in chloroform. The sections are cut out as individual blocks and mounted on wooden blocks in celloidin and stored in chloroform until ready for sectioning.

11. Nottingham buds, scaled and unscaled, same procedure as No. 10.

II. Shaffner's Formula. (Weak Chromo Acetic) Formula.

Chromic Acid ..... 0.3 gr.

Acetic Acid ..... 0.7 gr.

Water ..... 99. c.c.

Material treated as follows:

1. Barcelona buds, scaled and unscaled, placed in fixing solution for 24 hours then washed in running water for 24 hours and run through the alcohol and xylol - alcohol series, leaving material in each solution from 12 - 24 hours. Infiltrate with paraffin in oven at 54 degrees C. for 48 hours and imbed in 52 degree C. paraffin and section.

2. Barcelona buds, scaled and unscaled, same procedure as No. 1 except leave material in fixing solution for 28 hours.

3. Same procedure as No. 1 using Nottingham buds, scaled and unscaled.

III. Pure Alcohol.

Absolute alcohol used straight in this.

1. Barcelona buds, scaled and unscaled, fix for one hour and wash in absolute alcohol for a few minutes and proceed through xylol - alcohol series and remainder of procedure as before.

2. Nottingham buds, scaled and unscaled, same procedure as No. 1.

IV. Carnoy's Fluid.

Formula.

Absolute alcohol ..... 6 parts.

Chloroform ..... 3 parts.

Glacial Acetic Acid ..... 1 part.

1. Barcelona buds, scaled and unscaled, are fixed for 20 minutes then washed in absolute alcohol and run through xylol-alcohol series and imbed as in other procedure.

2. Same procedure as No. 1 using Nottingham scaled and unscaled buds.

V. Gilson's Fluid.

Formula.

Alcohol (95%) ..... 42 c.c.

Water ..... 60 c.c.

Glacial Acetic Acid ..... 18 c.c.

Nitric Acid (conc.) ..... 2 c.c.

Corrosive Sublimate ..... 11 c.c.
(Sat. Sol. in water.)

1. Barcelona buds, scaled and unscaled, fix for 24 hours then wash in 60 percent alcohol and run through rest of alcohol series and the xylol-alcohol series and imbed as before in 52 degree paraffin.

2. Same procedure as No. 1 using Nottingham buds, scaled and unscaled.

VI. Formalin Alcohol.

Formula.

Commercial Formalin ..... 6 c.c.

Alcohol (50%) ..... 100 c.c.

1. Barcelona buds, scaled and unscaled, fix for one hour and wash in 50 percent alcohol until all odor of formalin is gone. Then run material through regular alcohol series starting at 50 percent alcohol and through the xylol - alcohol imbedding in 52 degree paraffin as before.

2. Same procedure as No. 1 using Nottingham buds both scaled and unscaled.

NOTE: Material may be left in this fixing solution indefinitely without deterioration, until ready for use.

VII. Bouin's Fluid.

Formula.

Commercial Formalin ..... 25 c.c.

Picric Acid (Sat.Sol. in water.).... 75 c.c.

Glacial Acetic Acid ..... 5 c.c.

1. Barcelona buds, scaled and unscaled, fixed for 24 hours then washed in water for 30 minutes and started through alcohol series at 35 percent alcohol. Run through alcohol series and through xylol - alcohol series and imbed in 52 degree paraffin.

2. Nottingham buds scaled and unscaled, using same

procedure as in No. 1.

VIII. Corrosive Sublimate, Picric Acid, Acetic Acid.

Formula.

Corrosive Sublimate .....5 gr.

Glacial Acetic Acid .....5 c.c.

Picric Acid..... 100 c.c.
(Sat. Sol. in 50% alcohol)

1. Barcelona buds, scaled and unscaled, fix for one hour, wash in 50 percent alcohol until starch test with iodine leaves a permanent brown color, then pass through alcohol series. Start in at 50 percent. Pass through xylol - alcohol series and imbed in 52 degree paraffin in usual manner.

2. Nottingham buds, scaled and unscaled, same procedure as No. 1.

IX. Acetic Alcohol.

Formula.

Alsolute Alcohol ..... 2 parts.

Glacial Acetic Acid .....1 part.

1. Barcelona buds, scaled and unscaled, fix for 15 minutes and wash in absolute alcohol and run through regular xylol - alcohol series imbedding in 52 degree C. in regular manner.

2. Nottingham buds, scaled and unscaled, same procedure as No. 1.

Paraffin Sectioning.

Microtome. The regular standard Bausch and Lomb

rotary microtome was used in all paraffin sectioning. The blade was set at different angles and the best results were obtained with a straight edge to the object to be sectioned, with enough tilt to the edge of the knife to clear well after each section was cut.

The knife. Particular care was exercised in sharpening the knife, that the smoothest cutting edge possible might be obtained. After each sharpening, the edge was examined under the microscope to insure a first class cutting edge at all times. The knife was moved in the frame after sectioning each bud, to give an unused edge for every bud. As soon as the blade showed signs of dulling in sectioning a piece of material, it was moved to a new place to insure efficient sectioning apparatus continually.

The material. The material was squared up on the cutting block and sections made to determine the best position. With the bud lying longitudinally parallel with the cutting edge of the knife, seemed to give the best results with the least tearing of the material.

Methods of cutting. From each of the tests, two sections of scaled and unscaled buds were sectioned under normal methods; that is, merely cutting out the material from the block of paraffin and mounting on the cutting block and sectioning at room temperature.

Another method was used consisting of soaking the

imbedded material in water for a time before sectioning. Two scaled and two unscaled buds were taken from each test and carefully shaved of all paraffin with the exception of enough to furnish a base for mounting on the cutting block. They were then placed in a vial of distilled water and left tightly corked at room temperature to determine the effect of the water on the tissue. One scaled and one unscaled bud were removed and sectioned at the end of thirty days while the remaining were left for an additional thirty days before sectioning. In the same manner, one scaled and one unscaled bud from each test were shaved of paraffin and placed in a vial of water and put into an oven of constant temperature of 44 degrees C for a period of ten days and then sectioned.

The last method of paraffin sectioning was by mounting the sections on the cutting block and placing them in an ice bath until they were thoroughly chilled before sectioning. The knife was also placed in the ice and thoroughly chilled, as well, and all during the sectioning, a piece of ice was applied at frequent intervals to the material and the knife to keep them at a constantly low temperature. Several of the buds from the water soak method of thirty days were sectioned in this manner, as well as the buds imbedded in paraffin under normal conditions.

Celloidin Sectioning.

The regulation sliding microtome was used in this, and the same care attended the sharpening and care of the blade as in the paraffin method. The material was fixed on the microtome and sectioning done, so as to furnish a longitudinal section of the bud. Throughout the sectioning, a steady stream of chloroform was kept playing upon the material that it might be kept at maximum hardness.

Microchemical Analysis.

The buds were tested by different methods microchemically to endeavor to ascertain the composition of the hairs which gave them their stiff, wiry character. Tests were run to determine silicon, wax, cutin, suberin, lignin, and cellulose. The results were so arbitrary due to the minute size of the material, that they were discarded as unreliable and for that reason, are not included in this report.

RESULTS.

The normal paraffin sectioning gave the same negative results obtained by Rimoldi and others. The material would section nicely until the hairs were reached, and then would tear out leaving here and there very small fragments of the tissue. Very little difference could be noticed between the different methods of fixing and the effects of the fixing solutions as all samples behaved in

the same manner as to tearing. Different thicknesses of sectioning from five microns up to twenty-five, were used and with no better success. The sections containing the buds with scales attached sectioned beautifully through the scales, but as soon as the hairs were reached, the tearing commenced. This sectioning of the scales shows the technique of imbedding and sectioning to be done properly and shifts all of the cause of tearing upon the hairs. A collection of the torn material under the microscope gives the appearance of a mass of tangled wires, the hairs remaining uncut and appeared as if torn out bodily from the tissue, while the remainder of the tissue was torn to pieces so small as to resemble sawdust.

If any conclusions may be estimated of the effects of the various fixing agents, the following gave apparently the best results, for with these here and there, a section of about one-half the proper size could be obtained: Gilson's Fluid, Pure Alcohol and the series that were fixed with Chromo-Acetic and treated with Hydrofluoric acid, stood out better than the rest.

Water Soak Method. (30 days) Here was found a partial solution to the difficulty. From the first bud sectioned, throughout the entire series, there were obtained many sections that would allow fairly critical microscopic examination. The only difficulty encountered was that sections under 15 microns in thickness would tear

considerably, and were so brittle that in transferring from the surface of the sectioning knife to the slide, they would crack and break badly. It was impossible to make consecutive ribbon sections of the most of the buds, but at least two out of three of the sections could be used for examination. In one or two instances, a ribbon was cut and gave consecutive sections throughout the object. Detailed results under this modification of the paraffin method are:

Chromo-Acetic. All specimens sectioned well. Those treated with hydrofluoric acid 1-10 strength gave the best results with the hydrofluoric acid of weaker concentration next. No varietal differences could be noted between the Barcelona and Nottingham. Very little difference noted in the behavior between the unscaled and scaled buds , but the unscaled gave a few more better sections. Material that was treated only for 24 hours in the infiltration, cut the poorest of all, and under examination, the infiltration was found not thorough at 24 hours.

Shaffner's Formula. All material sectioned well with whatever difference noted in favor of the scaled buds. The material fixed for 48 hours cut the poorest seeming to be in a more brittle condition.

Pure Alcohol. Material from this treatment gave only fair results with no difference between scaled and unscaled, or of the varieties.

Carnoy's Fluid. Material from this sectioned well, and no differences noted between any of the buds.

Gilson's Fluid. This fixative gave by far the best results as it was possible to get ribbon sections of the entire bud with only here and there a poor section. A few individual sections could be obtained as low as 12 microns but the best sections were made at 15 microns. The scaled buds cut by far the best and no varietal differences could be noted.

Formalin Alcohol. All material fixed in this solution cuts in good shape. Impossible to get consecutive sections for any length of ribbon before bad sections broke the ribbon. No varietal difference and the scaled and unscaled cut equally well.

Bouin's Fluid. All material cuts fairly well. The unscaled buds gave the majority of good sections. No varietal difference could be noted.

Corrosive Sublimate, Picric Acid, Acetic Acid. In this fixative, all the material gave very poor results. Only a very few sections could be obtained and these were cracked and scarred so much as to be worthless in comparison with sections from other solutions.

Acetic Alcohol. Sectioning here was met with only fair success. A great deal of tearing was in evidence and extreme care had to be maintained at all times to get any entire sections. No difference noted between scaled and

unscaled or varieties.

Ice Bath Method. A great deal of difficulty was encountered in keeping the material on the cutting block under the chilling. The paraffin persisted in cracking from the face of the block and releasing the material. Sections of 20 microns and over could be obtained in a few cases, but only with the utmost care and with some attendant cracking of the section after removing from the surface of the knife. It could not be recommended as too few sections can be obtained to make it worth while in comparison with the water soak method.

As a test of the effect of this after the water soak treatment, a bud from each of the following solutions was used after being in the water for 30 days: Chromo Acetic, (treated with Hydrofluoric Acid); Carnoy's Fluid and Gilson's Fluid. These were selected as they gave the best results under normal sectioning after the soaking in water. Under the iced chilling, they became brittle and behaved in the same manner as the material that had not been soaked. A great deal of tearing out of the tissue was present and only in one or two cases was a single section obtained.

Celloidin Method. Only negative results were obtained under this as the celloidin does not have the body or rigidity to hold the buds firmly enough under sectioning. Tearing out of the material was encountered from the very

start, and after a few sections, the entire bud gave way and tore out of the celloidin block.

NOTE: The time limit is not up as yet on the material in the water soak under constant temperature of 44 degrees C for ten days, or the other material under water soak for 60 days at room temperature. As soon as this is up and the sectioning performed, the results will be added to this paper as an appendix.

SUMMARY.

1. The use of various fixing agents showed no differential effect on the tearing of the filbert tissue under microtome sectioning, either from paraffin or celloidin.

2. The angle of the microtome knife and the position of the bud on the cutting block will not prevent tearing of tissue.

3. Whether the scales are removed from the bud before fixing and imbedding, or it is carried through in its entirety, has little effect upon sectioning, except in the case of one or two of the fixing solutions under the water soak method.

4. Normal histological methods of sectioning with paraffin imbedding, are of no avail for sectioning filbert pistils.

5. Using a modification of the paraffin method

consisting of soaking the imbedded material in water for thirty days was discovered, to give the best results yet obtained. Consecutive and satisfactory sections of 15 microns were obtained under many of the fixing agents.

6. Gilson's Fluid gives by far the best results under this water soak method as consecutive ribbon sections of the entire bud, were obtained. Material fixed in Chromo-Acetic, and treated with Hydrofluoric acid,gave next best results under this method. Material fixed with Carnoy's Fluid and the Weak Chromo Acetic give promise,as many good sections were obtained from them.

7. Ice chilling of imbedded material, and cutting while chilled, will not give workable results as the material tears fully as badly as under the normal method. It is extremely difficult to hold the material on the sectioning block with this method as the paraffin in very brittle and splits from the block.

8. Ice chilled material that had undergone the water soak method, gave very poor results as it seemed excessively brittle and tore fully as badly as the normal material.

9. The celloidin method gave the poorest results of all as entire bud tears out of the imbedding block when sectioning is attempted.

10. The modification of paraffin method, by soaking in water, will no doubt prove valuable in sectioning other material having similar difficulties due to hairiness as encountered in the filbert.

CONCLUSIONS.

It is the writer's belief that the most satisfactory method of sectioning filbert pistils, so far ascertained, may be summarized from the work done above, as follows: Fix unscaled buds in Gilson's Fluid for 24 hours and wash in 60 percent alcohol, then pass material through solutions as follows: 65%, 75%, 85%, 90%, 95% and absolute alcohol, and a series of (1) xylol 1 part, alcohol 3 parts; (2) xylol 2 parts, alcohol 2 parts; (3) xylol 3 parts, alcohol 1 part, and (4) absolute xylol, leaving in each from 12-24 hours. Infiltration may be done with 52 degrees C paraffin over a period of 48 hours, keeping material in a constant temperature of 54 degrees C and changing paraffin at frequent intervals. At the end of this period, imbed in 52 degrees C paraffin. When cooled, cut buds from the paraffin, shave off all excess and place in stoppered vial of distilled water for 30 days at room temperature. Sectioning should be done on rotary microtome with bud lying in a parallel position to the cutting edge of the knife.

The above method is recommended for trial with material presenting similar obstinacy to sectioning as, has the filbert. It is believed that the water soak method will prove of value in histological studies of other plants as it seems to soften the tissue to a marked degree after imbedding.

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