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Histopathology of scar skin disease of apple.

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HISTOPATHOLOGY OF SCAR SKIN DISEASE OF APPLE

A Thesis Presented

By

Paul Edmund Parker

Submitted to the Graduate School of the
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HISTOPATHOLOGY OF SCAR SKIN DISEASE OF APPLE

A Thesis

By

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March, 1973

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INTRODUCTION

Scar skin, a graft-transmissible disease of apples, causes formation of corky, scar-like patches on the surface of affected fruits. Not only are the fruits made unsightly by this corky development, but their size is reduced drastically and the flesh of the fruit has a decided off flavor. Most studies concerning scar skin have dealt with transmission and host range studies in an attempt to locate susceptible varieties and ones which could serve as good indicators. Histological work has been lacking not only with scar skin but with most viruses of pome fruits and other plants. The few histological studies which have been conducted of virus-infected fruit trees were mostly restricted to studies on the histopathology of stem pitting problems. Scar skin, with its distinct fruit symptoms offers an excellent opportunity to explore the interrelationship between external fruit symptoms and the internal histological changes responsible for them.

It is the purpose of this research to determine the sequence of histological changes which form the basis for the observable external symptoms on scar skin-infected fruit. Information obtained on such an interrelationship

should improve our understanding of the mechanisms by which structural changes are induced by viruses and, consequently, of the nature and behavior of plant viruses.

LITERATURE REVIEW

Scar skin disease of apple was first noticed by Millikan in 1955 as an unusual symptom on Red Delicious fruit in northern Missouri orchards. The symptoms consisted of a corky periderm on the fruit skin similar to that caused by spray damage (Millikan and Martin, 1956). Subsequent observations showed that symptoms commenced with the formation of light, water-soaked blemishes which radiated out from the calyx end. Later, scar tissue developed in the epidermis. This was followed by the development of patches of scar tissue on the sides of the fruit (Millikan, 1957). Scar skin symptoms were also observed on other varieties. On Virginia Crab fruits were reduced in size and blemished with corky areas on the skin. The variety Jonathan also proved susceptible and the fruit showed a reduction in size and a mild mottle. Other varieties showing mild to severe symptoms include Red Gold, Stayman, Rome Beauty, and Stark's Earliest. Lodi, Golden Delicious and Minjon showed no visible symptoms, but there was a decided off flavor in the fruit of Golden Delicious (Millikan and Guengerich, 1960). Because of the susceptibility of Virginia Crab to scar skin disease it has been suggested as an indicator for detecting this fruit disorder (Millikan and Guengerich, 1963).

Certain other viral diseases exhibit symptoms resembling to some extent those of scar skin. Thus, leaf pucker of apple causes the production of dwarfed and distorted fruit, covered with purple blotches and sometimes accompanied by large expanded cracks. These symptoms appear to be influenced by climatic conditions and are inconsistent from year to year. McIntosh infected with the same virus showed russetting and skin depressions (Welsh and Keane, 1959). Fruit russet ring and leaf flecking virus induces depressions on the fruit, sometimes accompanied by complete or incomplete, brown, ring-shaped bands of russeted tissue on McIntosh fruits. A band of yellowish-green tissue that appears water-soaked surrounds the corky dots and lines, the latter often showing cracks reaching the healthy tissue beneath (Agrios, 1965). Star crack disease produces small, corky, star-shaped scars on apple fruit (Posnette, 1963). Apple green crinkle, a disorder which has been reported from various areas throughout the world, causes a distortion to the fruit with a warty character (Posnette, 1963). It is not clear whether all of the above viruses are distinct entities, but whether some of them are related or are the same is still unknown as mechanical transmission, purification and serology studies have not been accomplished with most of these viruses.

Histological studies involving apple viruses have often dealt with stem disorders. Hilborn et al (1965) showed that stem pitting virus caused slow decline of virus-infected apple trees through starvation. Fiber tracheids in the pitted areas were deformed. The pitting resulted from failure of certain cambial initials to differentiate cells normally. Many of the sieve tubes became necrotic or collapsed and the phloem rays were disorganized. Parenchyma cells were larger and more numerous than in healthy specimens and the actual pitting of the wood was due to wedge-shaped protrusion of phloem into the xylem, thus resulting in pits (Hilborn et al, 1965). Gilmer (1962) observed that, on Spy 227 infected with rapid decline, areas of ray and cortical parenchyma cells were hypertrophied. Sieve tubes appeared necrotic and some were collapsed after only ten to twelve weeks following inoculation. Later samples showed an increase in phloem necrosis and in hypertrophic area. Wound cambium often developed circumscribing larger areas of hypertrophy (Gilmer, 1962). In Virginia Crab stem pitting, phloem projected into the xylem while some cambial initials became distorted. Distortion of xylem elements and phloem rays also occurred (Hilborn and Hyland, 1957). In histological studies associated with fruit symptoms, Atkinson and Robbins (1951) observed that the vascular

system of green crinkle-infected apple fruit was distorted and exhibited a green coloration in the tissue. The green coloration sometimes extended as far as the core zone, but was most pronounced below the epidermis. There was no necrotic tissue or pit formation in the cortex or core (Atkinson and Robbins, 1951). Fruits from Virginia Crab infected with stem pitting show fluting of the fruit as a result of depressions in the epidermis. In areas of the cortex the parenchyma cells appeared to be torn (Hilborn et al, 1965).

Histological investigations of virus or mycoplasma-infected stone fruits have shown that in western-X-little cherry disease the cortex of diseased pedicels is thicker and larger than normal near the center although not near the base. Smallness of fruit was attributed to reduced cell division. Diseased fruits usually have a wrinkled appearance caused by a thick cuticle of wide epidermal cells, and a layer of injured cells just below the hypodermis. Injured cells were sometimes present in the hypodermis and epidermis (Nichols, 1951). In buckskin disease of cherry the fruit develops a thick leathery surface, the leaf veins swell due to abnormal production of secondary phloem and the sieve tubes become necrotic and later crushed (Schneider, 1945). In peaches infected with buckskin disease, the swollen leaf veins seem to be

due chiefly to enlargement of the vascular bundle by hypertrophy and hyperplasia (Rawlins and Thomas, 1941).

A considerable amount of histopathological work dealing with fruit viruses has been concerned with virus diseases of citrus. In lemon fruits infected with endoxerosis, gummy deposits in the fruits appear to have originated from the middle lamella and the cellulose wall. Phloem appeared to disintegrate first and that was followed by disintegration of bundle sheaths and, finally, of the parenchyma tissues located between the bundles (Bartholomew, 1928). Most other reports on histological changes in virus-infected citrus trees again focused on stem disorders. Webber and Fawcett (1935) found that in psorosis-affected tissues of Citrus sinensis, parenchyma cells in the primary cortex became darker and, later, phellogen was produced subjacent to the darkened cells. Phellogen was more abundant in infected stems than in healthy controls. Production of phelloderm leads to formation of small macroscopic eruptions on the bark surface. Tissues extending to the phellem are sloughed off in scales. Gum deposits are also formed in xylem and, occasionally, in pockets formed in thick abnormal phellogen. Phellogen tissues forming subjacent to darkened epidermal or mesophyll cells are found in leaves exhibiting

corky areas (Webber and Fawcett, 1935). In tristeza disease, a hypertrophy of chromatic (deeply staining cells) and non-chromatic cells appears, followed by hyperplasia and failure of tissues to differentiate normally. As a result, a disorganized mass of soft tissue is produced in place of xylem and phloem. The stem pitting and vein clearing symptoms associated with this disease are both results of the formation of chromatic cells followed by hyperplasia, hypertrophy, and disorganization (Schneider, 1957). Hyperplasia in bundle sheath and mesophyll cells has also been noted in citrus vein enation virus (Hooper and Schneider, 1967). Vein enation virus also induces tumors. One or more vertical strands or primordia adjacent to the protophloem sieve tubes appeared to have darkly staining cytoplasm. These cells ceased elongation, began to divide and produced small masses of cells in the phloem fiber region. As the tumor enlarges, cell divisions become less orderly and the mesophyll and epidermal cells in the area of the tumor increase in number to accommodate the increased amount of tissue. As leaves mature, the tumor cells become less distinguishable from surrounding cells (Hooper and Schneider, 1969).

Although virus-caused fruit symptoms have been little studied histologically, a great deal of work has

been carried out with leaf mosaics of various herbaceous plants. Studies of mosaic-infected plants showed that symptoms are due to hyperplasia of the palisade parenchyma in the lighter pigmented areas, a reduction of chlorophyll content and an increase in trichomes and glandular hairs. Frequently, hypertrophy of palisade tissue occurs in dark green areas of the plant (Dickson, 1921). Cucumber mosaic virus (CMV) causes a hyperplastic effect in yellow-green areas of young cucumber leaves, though in mature leaves the same areas appear hypoplastic. Easter lilies and tulips infected with CMV show necrosis of the mesophyll. Only after severe degradation of the mesophyll cells does the vascular tissue become necrotic. In broad bean infected with the same virus, necrosis proceeds rapidly to vascular tissues (Porter, 1954). Gardner (1925) observed that mosaic-infected tomato fruit exhibited hyperplasia characterized by brownish, translucent blisters of various shapes and sizes, often bordered by a band of translucent tissue. Surface lesions often became hard, dark brown scabs which inhibited symmetrical growth and resulted in malformation of the fruit. He observed that surface blisters are produced by a compact muriform cushion of meristematic cells which have pushed up the epidermis. During the incipient stages of blister formation, necrosis of epidermal cells is visible. Hyperplasia seemed to

originate in the first and second subepidermal layers. Hyperplastic activity resulted in firm adhesion between the pericarp and adjacent placental matrix and ovules. Gardner (1925) suggested that the hypertrophy and hyperplasia are a response of various tissues to necrosis. The flattened necrotic plates are the result of pressure by the hyperplastic tissues. Collapsed necrotic regions are almost always accompanied and bordered by closely packed hypertrophied cells or by regions of hyperplastic tissue (Gardner, 1925). Bawden's (1932) work with potato viruses revealed that, in tubers obtained from plants having necrotic tops, the storage parenchyma cells contained darkly colored areas. First the walls of phloem cells became thickened; this was followed by thickening of the walls of storage parenchyma cells, and finally, the phloem bundles collapsed, the contents of the cells disappeared and the cells became suberized. Necrotic areas became surrounded, often incompletely, by moderately developed phellogens. Within large necrotic zones there was often a mass of hard corky cells with starch grains which were usually absent from adjacent tissues (Bawden, 1932).

MATERIALS AND METHODS

Fruits were collected from eight-year-old Red Delicious apple trees grafted on East Malling VII rootstock and growing in the university orchard at Belcher-town, Massachusetts. Some of the trees had been inoculated with scar skin virus on May 18, 1967 and had been showing symptoms since 1968. The scar skin virus used had been obtained from R. C. McCrum of the University of Maine. Fruits were collected each week and were chosen so that they represented the prevailing condition of the fruits at that particular growth stage. Control fruits were secured from uninoculated Red Delicious trees in the same row, though controls were separated from infected ones by at least three healthy trees. Fruits were placed in plastic bags in the field and were immediately transported to the laboratory. After representative fruits were photographed, sections of tissue approximately five mm square were cut from areas of the fruit exhibiting symptoms and were immediately immersed in the fixative solutions.

Cryotome Sectioning

Cryotome sectioning was performed on a Lipshaw model no. 1500 cryotome at an operating temperature of

-20°C. Small cubes (1-2mm) of fresh fruit tissues were placed on the fast freezing stage of the cryotome and were embedded in Ames O.C.T. embedding compound for frozen tissue specimens for ten minutes or until completely frozen. Sectioning was done at either 10 or 20 microns, depending on the particular item to be observed. Pigmentation changes in the tissue could be best observed in sections 20 microns thick. The sections were placed on microscope slides and observed with the microscope under low or high dry power immediately following sectioning.

Fixation of Tissue

Four different fixative solutions were employed at first, so that the one or two solutions in which the samples were fixed most effectively would be used in subsequent work.

In the first method, a solution of formalin-alcohol-acetic acid (FAA) was used, which was composed of 90 parts 50% ethyl alcohol, 5 parts glacial acetic acid, and 5 parts commercial formalin. The fruit tissue sections were placed under vacuum to assure penetration of the tissue by the fixative. The sections were kept in this mixture for 24 hours, after which they were washed in several changes of tap water. The material was then run through a tertiary-butyl alcohol dehydration series

as outlined by Jensen (1962). After the final change of tertiary-butyl alcohol, the sections were placed in a 1:1 mixture of paraffin oil and tertiary-butyl alcohol for 24 hours. The tissue was then infiltrated with Tissuemat of 61°C melting point and kept at between 62°C and 64°C. Three changes of Tissuemat were made over a two-day period after which the sections were poured into molds and the material was allowed to cool and solidify.

In the second and third methods, acrolein or glutaraldehyde were used as fixatives. The sections were placed into vials to which either a 10% acrolein solution or a 3% glutaraldehyde solution was added. After the tissue was infiltrated with the fixative by means of a vacuum, the vials were placed under refrigeration at 0°C for 24 hours. The fixative was then removed and methyl cellosolve was added. Two changes of methyl cellosolve were made in 24 hours at 0°C. The tissue was dehydrated in the following alcohol series: 100% ethyl alcohol, 100% propyl alcohol, 100% butyl alcohol, each solution at 0°C for 24 hours. After the last change of butyl alcohol the vials were brought up to room temperature, drained off, and a 1:1 mixture of paraffin oil and butyl alcohol was added. Embedding was done in the same manner as in the FAA method (Feder and Obrien, 1968).

Crafs fixative was the fourth one employed in this study. Solutions A and B were mixed just before use and the tissue sections were placed in the mixture for 24 hours. A vacuum was also used to achieve good penetration of the fixative into the tissue. After 24 hours the samples were washed in distilled water and run through a butyl alcohol series. Embedding was done in the same way as before.

Sectioning of Fixed Material

Sectioning of the tissue embedded in Tissuemat was done on a Lipshaw model 50 AB microtome with a blade angle of 30°. Thickness of sections was set at 10 microns. The sections obtained were placed on slides coated with Haupts adhesive medium.

Staining Procedures

After the paraffin sections, which had been placed on Haupts adhesive on glass slides, had dried thoroughly, they were immersed in xylene for ten minutes to dissolve the paraffin. The slides were then placed in a 1:1 mixture of 100% ethyl alcohol and xylene for five to ten minutes. Subsequently, the slides were placed, for the same amount of time, into 100% ethyl alcohol, 95% ethyl alcohol, 70% ethyl alcohol and 50% ethyl alcohol. The various staining procedures followed this general slide hydration series.

Safranin 0 and fast green.--When the slides were brought down to 50% ethyl alcohol, they were then immersed in a 0.5% solution of safranin 0 made from 1% safranin 0 in 95% ethyl alcohol diluted with an equal volume of distilled water. The sections were left in the safranin solution for two hours, after which the slides were thoroughly washed in running water to eliminate deposits of excess stain. Destaining was then done in acidified 70% ethyl alcohol. The duration of destaining was determined by trial and error so that most of the stain ran freely from the tissue but the tissue still retained the appropriate reddish coloration. The slides were then briefly passed through 95% ethyl alcohol and 100% ethyl alcohol, after which they were counterstained with 0.5% fast green in a mixture of 50% clove oil and 50% absolute ethanol. Staining time with fast green varied from 10 to 20 seconds. The fast green was differentiated by passing the slides through two 15-minute changes of a 1:1:1 mixture of clove oil, xylene, and 100% ethyl alcohol. Following the second passage through the above mixture, three changes of xylene were used to further clear the slides and prepare them for cover slip mounting. Permunt was used for final mounting (Jensen, 1962).

Crystal violet and erythrosin B.--At the end of the hydration series the slides were brought to distilled water and stained in a 1% aqueous solution of crystal violet for 10 minutes. After washing the slides in water they were quickly dehydrated in 95% ethyl alcohol and 100% ethyl alcohol and were then stained in a saturated solution of erythrosin B for three minutes. The slides were then cleared in a 1:1 solution of 100% ethyl alcohol and xylene and subsequently in three changes of xylene (Johansen, 1940).

Hendenlain's iron haematoxylin-orange G.--Deparaffinized sections were brought to water and mordanted in a 3% ferric chloride solution for two hours before being washed again in water. Slides were then stained for two hours in a 0.5% aqueous haematoxylin solution. Haematoxylin was prepared by making a 10% aqueous solution in absolute alcohol. Five ml of this solution was added to 100 ml of distilled water along with a trace of sodium bicarbonate, this producing a solution of deep red color. Following the haematoxylin stain, a 2% ferric chloride solution was used as a destaining agent. The slides were then washed and dehydrated to absolute alcohol. Counterstaining with a stain consisting of 0.5% orange G in clove oil for three minutes resulted in an orange background. Differentiation

was carried out in a 1:1:1 mixture of clove oil, xylene and 100% ethyl alcohol for fifteen minutes. As with other staining sequences, this was followed by three passages through xylene.

A slight variation to the above was also employed using bismarck brown. The procedure is similar to the orange G counterstaining except that, after primary staining and washing, the slides were placed into a 1% aqueous solution of bismarck brown for two hours. Slides were then dehydrated through an alcohol series followed by three changes of xylene.

A tannic acid-ferric chloride-safranin series was used but was soon discontinued due to inferior results.

In all cases the slides were mounted in Permount and the sections covered with the proper size cover slips.

RESULTS

A. External Symptoms

The first external fruit symptoms on scar skin-affected Delicious apples were observed on June 27 as small reddish brown areas, about 0.5-1 mm in diameter, radiating out from the calyx end. When symptoms first appeared, fruit size was about 2-3 cm in diameter. Examination with a stereoscopic microscope revealed raised, pimple-like structures of red pigmentation which seemed to be more abundant along the ridges of the fruit. During the following week, the symptoms became more intense as a result of coalescence of the small pimple areas into reddish blotch areas about 5 mm or more in diameter. Nearly all fruits on scar skin-affected trees were exhibiting similar symptoms at this time. Three weeks after the first appearance of symptoms, when fruits were about 3-4 cm in diameter, small areas of brownish, scar-like tissue appeared within the now enlarged red blotches (Fig. 1). About 30 percent of the surface was covered with the pathological red coloration at this point. Scar areas were more distinct, appearing as necrotic islands surrounded by red pigmented areas. Six weeks after the appearance of symptoms, small fissures developed in the brown, scar-like areas. Areas

Fig. 1 External symptoms of scar skin-affected young Red Delicious apples. Small pimple areas (A) radiating out from the calyx end, later coalesce into larger blotch areas with zones of scar-like material forming in them. Small fissures (B) are also apparent in the scar tissue.

Fig. 2 External symptoms of scar skin-affected mature Red Delicious apple compared to healthy control fruit. Extensive scar tissue with deep cracks covers the surface. Distorted shape and smaller size of the fruit are also apparent.

of scar material increased in size and covered most of the red areas, often forming large, brown, shield-like zones. Small fissures increased in depth and width and eventually developed into cracks about 2-3 mm in depth and 2-3 mm wide (Fig. 2). As the cracks enlarged, various secondary micro-organisms became established in them, invaded the fruit through them, and caused further damage to the fruit. By the middle of September, fruits were discolored and distorted in shape, reduced in size, and covered with large cracks and extensive zones of shield-like scar tissue.

B. Histological Observations

During the first two weeks of symptom development, no changes appeared in sections of fixed tissues. Although macroscopic symptoms in the form of small red patches were present on the fruit, there was no indication of any histological change when the material was fixed. Because of the possibility that the various chemical agents employed in fixation and dehydration of the tissue sections might be causing obliteration of any histological changes present, fresh tissue was frozen and serial sections were made with a cryotome.

Cryotome sections.--The first histological symptoms in fresh frozen tissue appeared as zones of reddish-pigmented tissue corresponding to the macroscopically visible red

areas on the fruit surface. The cell shapes and sizes in the epidermal, subepidermal and parenchyma tissues of the reddish-pigmented zones were exactly like those of the respective control areas, except for the presence of the red pigmentation. The reddish coloration was localized in the epidermal and hypodermal cell layers. These areas of the red-pigmented tissue soon became necrotic, at first the epidermal layer only, but later necrosis spread to two or three subjacent cell layers. There was a gradual transition from red-colored areas into brown, necrotic areas. In some areas surface necrosis was light and involved only the epidermis, but in other areas surface necrosis was more extensive and resulted in scar tissue formation, while at the same time necrosis advanced into the subepidermal layers.

Fixed tissue symptoms.--Histological observations during the first two weeks of symptom development had detected only changes in pigmentation which was not observable in fixed tissue. By the third week of symptom development, however, two basic types of symptoms could be observed in fixed tissue. These consisted of small protuberances or pimple-like structures, and of thin zones of necrotic epidermal and subepidermal cell layers (Scheme 1).

Histological changes associated with the protuberances involved the reddening of epidermal and subepidermal cell layers. This was followed by a hyperplastic

Scheme 1. HISTOLOGICAL DEVELOPMENT OF INTERNAL SYMPTOMS IN
SCAR SKIN-AFFECTED FRUIT

cell response resulting in the formation of pockets or lense-shaped protuberances. The reddened epidermal and subepidermal cells then became necrotic. Underlying the pocket of hyperplastic cells, phellogen developed circumscribing the pocket. Later, the phellogen formed a more complete periderm layer. Pressures from these enlarging and dividing cells caused the fracturing of the cuticle. Subsequently, the cracks enlarged due to sloughing off of cells lining the walls of the cracks. The enlarging cracks then became the entry points of various secondary microorganisms.

Development of symptoms of the second type, necrotic epidermal and subepidermal cell layers, began with epidermal and subepidermal cells becoming red-pigmented, followed by necrosis of these cells (Scheme 1). Some areas proceeded to develop into "pockets" in the manner described above, but most areas did not develop into protuberances. After necrosis had occurred, phellogen formed subjacent to the necrotic layers. Layers of phellem cells were produced and, when their increased number exerted sufficient pressure, they caused the cuticle to fracture. Fracturing of the cuticle caused the fruit surface to appear as zones of intact cuticle surrounded by areas of exposed corky periderm.

Within the red-pigmented areas, small protuberances of the cuticle developed. Beneath the raised cuticle,

epidermal cells were pushed upward in an almost fan-like shape (Figs. 3,4,&5). Some epidermal cells in the slightly raised area were partially separated from adjacent epidermal cells and were somewhat distorted in shape as compared to normal tissue (Figs. 4,5,&6). Directly subjacent to these epidermal cells a zone of hyperplastic activity occurred. It appeared to originate in the first layer of the hypodermis and occupied the area between the epidermis and hypodermis (Figs. 5,6,&7). Cells within the hyperplastic zone appeared disorganized (Figs. 7&8) and were arranged in pockets, semi-circular or lense-like in shape (Fig. 9). Within the red-pigmented protuberances certain epidermal cells began to become necrotic (Figs. 6,7,8,&9). Epidermal cells later collapsed and this was soon followed by necrosis of underlying hyperplastic cells (Fig. 8). The increased cell division within the pocket areas resulted in the formation of protuberances. Cells within the pocket became more deeply stained with safranin, suggesting suberization of cells and possible lignin deposits. At approximately the same time at which cells became necrotic, a layer of meristematic tissue, probably phellogen, developed subjacent to the zone of necrotic, hyperplastic cells (Figs. 9 &10). The phellogen layer continued to develop until it produced what could be considered a complete periderm (Fig. 11), except that it was not yet exposed to the outside. Associated with the periderm-type layer was a zone of

Fig. 3 Healthy fruit section showing the shape, size and arrangement of cuticle and of epidermal and subepidermal cells. Some epidermal cells (A) are separated from adjacent ones and are partly surrounded by cuticle. Large starch granules (B) are visible in underlying parenchyma layers.

Fig. 4 The first observable histopathological symptoms in scar skin-affected fruit. Small groups of hyperplastic cells appear under the slightly raised cuticle (A). Epidermal cells are pushed upward.

Fig. 5 Scar skin-affected fruit tissue showing a slight increase in hyperplasia. Small hyperplastic cells directly beneath the epidermis appear to be originating from the hypodermis. Epidermal cells appear disarranged.

Fig. 6 Enlarging zone of hyperplastic tissue beneath the epidermis in scar skin-affected fruit. One epidermal cell has become necrotic.

Fig. 7 Early symptoms of scar skin-affected fruit. Epidermal cells appear distorted and some of them may have divided anticlinally. A few epidermal cells (A) are beginning to show necrosis.

Fig. 8 A further stage in symptom development of scar skin-affected fruit. Some epidermal cells (A) appear necrotic and show signs of collapse.

Fig. 9 The beginning of "pocket" formation in scar skin-affected fruit. Necrosis has spread from necrotic epidermal cells to underlying hyperplastic cells in the "pocket." Phellogen initials have appeared beneath the zone of hyperplastic tissue.

Fig. 10 Early development of a pocket area in scar skin-affected fruit. Epidermal cells (A) are necrotic; underlying subepidermal cells (B) appear necrotic, hyperplastic, and distorted. Periderm is not clearly visible in this young developing area, but a zone of lightly stained cells (C) is evident.

Fig. 11 Developing periderm (A) underlies the necrotic pocket area in scar skin-affected fruit. Red staining cells (B) represent cell suberization and perhaps deposits of lignin. Pocket area is raised above surrounding tissue with the cuticle remaining intact.

Fig. 12 Rupture of the cuticle covering pocket area. Cells surrounding the fissure become sloughed off causing the fissure to enlarge.

lightly stained cells (Figs. 10,11,&12) which circumscribed the pocket area separating the phellogen from the necrotic cells within the pocket area. The periderm walled off the pocket from surrounding tissues and contributed further to outward expansion of the pocket tissues. Cells outside of, but immediately next to, the phellogen appeared rather normal, in that they did not retain safranin as much as did the necrotic cells that lie closer to the center of the pocket area. These cells, however, seemed to retain the safranin red coloration slightly more than did remote healthy cells. During phellogen development the cuticle remained intact, but as successive layers of phellem were produced and internal pressure increased, fissuring of the cuticle occurred (Fig. 12). Fissuring appeared at first as small cracks on the surface of the cuticle over the center of pocket areas. The cracks then deepened until the epidermal layer was reached, at which point the cuticle sometimes tended to peel back from the fissure, exposing underlying cells. The fissures increased in size by the sloughing off of the cells lining the walls of the fissure, until large open cracks formed (Fig. 13). The phellogen and phellem layers continued developing underneath the pocket areas and, when finally complete, the phellem contained from four to six layers of cells. The cracks, which enlarged due to sloughing off of cells, also increased in

Fig. 13 An older pocket area in scar skin-affected fruit. Cells surrounding the crack have been sloughed off exposing phellem cells (A). Note the extensive periderm (B) surrounding the site of the old pocket.

Fig. 14 An old pocket area in infected fruit showing a band of lightly stained cells (arrow) which appear to be the developing periderm.

depth and reached the phellem at various points. Portions of the phellem and adjacent cells surrounding the crack area became compressed into an area of disorganized, crushed cells (Fig. 14). Various secondary fungi appeared to be growing in exposed areas (Fig. 15). Alternaria spores were observed on crushed, necrotic cells surrounding the cracks. Periderm development in areas of secondary invasion appeared extensive, though whether or not the periderm excluded these organisms was not determined.

In red-pigmented cells which did not develop directly into raised pimple areas, necrosis of epidermis occurred (Fig. 16). At first, only the epidermal layer of such red-pigmented areas became necrotic, but later the underlying subepidermal areas also became necrotic. At certain points, zones of hyperplastic cells form pocket-like protuberances in the same manner as described previously for the raised pimple areas in younger fruit. Actually, the earlier and later-forming pockets cannot be distinguished from each other. The areas which do not develop the hyperplastic response develop instead a layer of phellogen which runs parallel to the fruit surface and subjacent to the necrotic epidermal and subepidermal layers (Fig. 17). The amount of periderm produced appeared to depend on the age of the symptoms. Areas which had just become necrotic showed no meristematic activity or only a phellogen layer, while tissue with more advanced symptoms (older necrotic

Fig. 15 Cross section through an advanced crack showing extensive invasion by various fungi. Hyphae and spores (A) of various fungi appear among the necrotic cells. Periderm (B) walls off further invasion of the fruit by fungi.

Fig. 16 Scar skin fruit with epidermal necrosis (A). Periderm layer (B) is beginning to form underneath the necrotic epidermal cells. Cuticle remains intact.

Fig. 17 Extensive periderm development underlying necrotic epidermal and subepidermal layers. Two to three layers of phellem (A) are present.

zones) had a phellem of from one to six layers (Fig. 18). In all these stages, the cuticle remained intact, even though a thick periderm existed below the necrotic epidermis. The developing periderm was not restricted to the areas beneath the heavily necrotic portion of the epidermis and subepidermis, but extended outward, underneath an area of normal epidermal cells and, finally, disappeared. The phellem varied in thickness from one cell at the periphery of necrosis to four to six cells at the center of necrosis. The thickness of the developing periderm decreased as the epidermal layer under which it spread became more normal-looking. In all areas of phellogen development some safranin was always retained by the cells directly above the periderm, although in some cases the stain seemed to be present intercellularly rather than within the cells. When four to six layers of phellem had been produced, breaking of the cuticle occurred, apparently due to pressure from the developing periderm. As the phellem increased in thickness, small fissures occurred at scattered points on the cuticle. The fissures increased in size due to sloughing off of cells and, possibly, due to expansion of the underlying periderm. Islands of unbroken cuticle remained on the fruit surface surrounded by areas of exposed phellem cells (Fig. 19). Extensive areas of corky periderm developed underneath the intact cuticle and the exposed phellem areas.

Fig. 18 A section from scar skin-affected fruit showing a pocket area on the left (A) with periderm subjacent to it. A band of periderm (B) continues outward underlying necrotic epidermal and subepidermal cells.

Fig. 19 In old scar skin-affected fruits with epidermal necrosis, the cuticle fractures due to excessive pressure from the developing periderm beneath the necrotic epidermis. Following the fracturing of the cuticle in necrotic epidermal areas, the fruit surface appears as zones of exposed phellem cells surrounding areas of intact cuticle.

DISCUSSION

The study of symptom development of scar skin-affected apple fruit necessitated the sectioning of both fresh frozen tissue and of fixed, embedded and stained tissue. The use of acrolein as a fixative appeared to give the best results although it did not penetrate young fruit tissues quickly. Cryotome sectioning of fresh frozen tissue was necessary for observing changes in pigmentation, which could not be observed in the fixed tissue.

Cryotome sections revealed the presence of red pigments, presumably anthocyanins, in epidermal and sub-epidermal cell layers. This coloration always preceded hyperplasia or epidermal necrosis. Why this happened is not known, but it is probably related to the red coloration commonly appearing around the point of various types of local injury. For example, red pigments, anthocyanins, were produced on the leaves of Galeopsis at the point of feeding of larvae of leaf-boring insects (Onslow, 1925). Insect caused galling in which hyperplasia occurs, has also been observed to induce production of red pigments in the area surrounding the galls (Onslow, 1925). Various plant diseases also induce red coloration at or around the point of infection. Leaf-blotch of bean caused by Cercospora cruenta induces a pronounced reddish border surrounding the

points of infection (Miles, 1917). Reddening has also been observed in several virus disorders, e.g. in red node disease of bean in which reddish concentric rings form on the pods (Holmes, 1964). Many mycoplasma disorders also induce a red coloration, e.g. the X disease of cherry in which the entire foliage takes on a reddish hue (Parker and Palmiter, 1938). Thus, the reddish area which precede hyperplasia and necrosis in scar skin disease are not in any way uncommon. Onslow (1925) suggested that red coloration around points of injury and infection might possibly be due to accumulations of sugars since sugars are necessary for formation of anthocyanins. Watson and Watson (1951) have shown sugar accumulations in leaves of beet associated with infection by beet viruses, but whether the red pigmentation in scar skin is in any way related to an accumulation of sugars remains to be seen.

Hyperplasia similar to that observed in scar skin of apple was noted in TMV infected fruits by Gardner (1925). Gardner suggests the hyperplasia was probably a response to epidermal necrosis in TMV-infected tomato. In scar skin, however, hyperplasia appears not to be directly associated with necrosis. Even in older pockets the hyperplastic (meristematic) tissue is deeper in the fruit and is separate from the necrotic tissue, suggesting that hyperplasia is caused by some continuous stimulus supplied by the infecting

agent rather than by the necrotic tissue. It is true, however, that in older tissue, in which the epidermis has become necrotic, it becomes more difficult to separate hyperplasia from necrosis. In the case of hyperplasia following necrosis of the epidermis, hyperplasia may well be a response to the appearance of the necrotic epidermis. Hyperplasia has also been reported with other diseases such as in tristeza of citrus (Schneider, 1957) and melanose of citrus (Bach and Wolf, 1928).

Necrosis occurs in all affected areas in scar skin-affected fruit. Outer cells in pocket areas become necrotic while hyperplasia is still taking place in deeper cell layers. Epidermal cells are the first to become necrotic, while the underlying hyperplastic cells become necrotic as the fruit matures. In areas which do not develop into raised pockets, necrosis of epidermal cells follows the initial reddening of epidermal cells. As the fruits mature necrosis proceeds into underlying subepidermal layers. Necrosis is a commonly observed plant response to viral infection. Many viruses such as TMV produce areas of local necrosis, the so-called local lesions, upon mechanical inoculation of the leaves of susceptible hosts. In certain hosts, TMV produces both necrosis and systemic mosaic symptoms (Smith, 1960). In numerous virus-host combinations, necrotic areas appear on the stem, leaves or

fruit of the host following systemic infection, e.g. turnip mosaic on cabbage, potato virus Y on potatoes, tobacco ringspot on watermelon, early browning disease of peas, and potato yellow dwarf (Holmes, 1964). The appearance of necrosis may be linked to increases in polyphenoloxidase and peroxidase activity. Quinones, the product of polyphenoloxidase action on phenolic compounds, may promote the breakdown of protoplasmic structure with the observable result being cell death (Farkas et al., 1959). Production of various quinones and phenolics is not necessarily specific for any disease. Gill (1965) noted that, in bean infected with either rust or TNV, there were greater amounts of fluorescent metabolites, i.e., phenolics, than in controls. It is possible, therefore, that brown coloration and necrosis, which follow the red pigmentation in scar skin, could be due to increased levels of phenolics and, subsequently, quinones in the area affected by the virus.

Periderm development, which occurred after hyperplasia and/or necrosis were initiated, is also typical of many other plant diseases as well as of other forms of mechanical damage. Melanose disease of citrus, caused by Phomopsis citri, induces formation of cork layers circumscribing the black spots produced on leaves, stems, and fruits (Bach and Wolf, 1928). Periderm formation has not

been reported as commonly in connection with virus disorders. Cork formation has been reported in psorosis in citrus (Webber and Fawcett, 1935) and russetting (formation of corky cells) of the fruit was observed in certain virus diseases of apple (Posnette, 1963). Periderm formation, of course, is of common occurrence on apple fruits. Various types of injury to the epidermis can result in periderm formation. "Frost ring," a russetting on the fruit caused by cold injury, results in small concentric rings of corky cells. Spray damage also causes a corky russet on the fruit surface (Smock and Nuebert, 1950). In pear, there is a slight production of cork in fruits infected with stony pit virus (Smith, 1960). Thus, periderm formation in scar skin disease of apple appears to be part of the generalized response of apple fruits to cell injury and epidermal cell death.

The hyperplastic response on parts of the fruit brings about a swelling effect and an uplifting of the cuticle. The cuticle, however, remains intact even though subjacent epidermal cells are necrotic and suberized. Retention of safranin stain by the cells in the pocket indicates cell suberization and possible lignin deposits, according to Johansen (1940). A somewhat similar arrangement of leathery necrotic epidermis under an intact cuticle has been noticed in the buckskin disease of cherry

(Schneider, 1945). In the scar skin disease of apple, the cuticle appears to be quite flexible, fracturing of the cuticle occurring only after extensive periderm formation.

In scar skin, pocket formation and necrosis of epidermal cells appear not to be associated with any other fruit tissue. No connection was observed between pocket formation and vascular tissues. Thus, scar skin symptoms appear to be restricted to epidermal and adjacent sub-epidermal cells.

SUMMARY

The development of external and internal symptoms in scar skin disease, a graft-transmissible disease of apple, was studied. Sections of tissue were taken from healthy and from Red Delicious apples exhibiting typical scar skin symptoms. The tissue was fixed, sectioned and stained by various methods. The most uniform staining of the tissue was obtained with safranin counterstained with fast green.

Early symptoms consisted of raised, red, pimple-like areas. Red coloration in pimple areas preceded the appearance of brown necrotic scar tissue which later replaced the red pigmented tissue. Red pigmentation was present in both the epidermal and subepidermal layers. The initial reddening of epidermal cells was followed by necrosis. Necrosis later spread to adjacent epidermal cells and subepidermal layers. At certain points of the fruit surface the tissue appeared raised due to hyperplastic activity beneath the epidermis. This resulted in the formation of "pockets" of hyperplastic and, later, necrotic cells, underlined by a phellogen and periderm. Pressure from these hyperplastic tissues and the periderm, which circumscribed the necrotic and hyperplastic areas,

caused fracturing of the cuticle. The resulting fissures increased in size due to sloughing off of cells lining the walls of the fissures. The enlarged fissures subsequently allowed entry of various microorganisms into the necrotic areas of the fruit.

Pocket formation occurred only at scattered points of the affected tissues, the remaining scar tissue consisting primarily of necrotic epidermal cells. The subepidermal layers underlying the necrotic epidermis became necrotic as the fruit matured. Beneath the necrotic epidermal and subepidermal areas several layers of periderm formed resulting in increased pressure on the cuticle. Towards the end of the growing season, the cuticle ruptured and the fruit surface appeared as islands of intact cuticle surrounded by zones of exposed phellem cells.

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