# Phellogen Regeneration in Injured Peach Tree Bark

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ABSTRACT

Injury to peach bark phellogen leads to the generation of new tissues and the re-establishment of meristematic continuity. Two types of tissue changes after wounding were identified and quantified in bark of seven peach clones: (1) cell wall modifications (lignification and suberization) of tissues present at the time of wounding, and (2) generation of the new phellogen and its derivatives. Tissue responses were quantified with a microscope photometer using selective histochemistry and autofluorescence to detect lignin and suberin deposition over time. Suberin continuity was re-established via suberin deposition in a layer of cells, present at the time of wounding, approximately 800 $\mu$m internal to the wound surface. Phellogen continuity was re-established immediately internal to and abutting the suberized tissue. The new phellogen gave rise to suberized phellem which, in its outward expansion, crushed the suberized boundary zone tissue formed earlier. All injured peach clones produced the same sequence of tissue changes, although timing and degree of response varied with clone and time of year.

Key words: Differentiation, impervious tissue, lignin, *Prunus persica* (L.) Batsch, suberin, wounding.

## INTRODUCTION

The histology of nonspecific responses in tree bark varies in complexity depending upon the severity of the stimulus (Mullick, 1977). A shallow injury to the bark, deep enough to injure the phellogen but not deep enough to injure the cambium, triggers processes leading to the generation of new phellogen. Deeper injury to phellogen and cambium, but not deep enough to injure functional xylem tissue, induces generation of new phellogen and cambium. Injuries affecting xylem tissue result in blockage of conductive xylem (compartmentalization *sensu* Shigo, 1979), in addition to generation of new lateral meristems (Mullick, 1977).

According to Mullick, injuries which trigger the generation of new phellogen and cambium have in common the production of non-suberized impervious tissue (NIT) as a prelude to generation of new phellogen. Mullick's non-specific defence response model was developed using histological investigations of conifer bark, and Soo (1977), a student of Mullick, validated Mullick's model with dicotyledonous tree bark.

More recently, certain aspects of Mullick's model have been questioned (Biggs, Merrill and Davis, 1984). Biggs (1984*a*) has shown that suberin occurs in bark of fifteen woody dicotyledonous angiosperms as a thin cell wall lining in tissues which are formed from pre-existing cells prior to the appearance of new phellogen. Furthermore, using Mullick's F-F test (Mullick, 1975), it was shown that the suberized cells constitute a tissue which is impervious to fluid diffusion (Biggs, 1985). A qualitative description of the suberized boundary zone tissue and new phellogen formation has been reported for peach (*Prunus persica* (L.) Batsch) bark mechanically wounded or infected by the peach canker fungus, *Leucostoma persoonii* (Nits.) Hohn. (Biggs, 1984*b*).

Studies of tree bark responses to wounding or infection have been mostly qualitative, relying on the author's descriptions in anatomical or histological terms with few attempts made to quantify tissue changes. The approach of Warren Wilson and Grange (1984)

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is unique in this respect. They assert that quantitative analyses 'may assist in elucidation of the largely unknown mechanisms that control the restoration of tissue patterns' (Warren Wilson and Grange, 1984). In addition, non-specific changes in tissues may be associated with resistance to insect, fungal, or bacterial pathogens (Mullick, 1977). The quantification of the generation of impervious layers and of new phellogen derivatives would give a better understanding of non-specific disease resistance phenomena.

The present study assessed quantitatively cell wall lignification and suberization in impervious boundary tissues formed prior to phellogen generation and determined the rate of suberized phellem production following re-establishment of phellogen continuity.

## MATERIALS AND METHODS

In June, July, and August, three trees (6–10 years old) each of seven peach clones, planted in an experimental orchard, were selected for the wounding of five year old scaffold limb bark tissue. A 4-mm diameter cork borer was used to injure the phellem, phellogen, phelloderm, and cortex to a depth of about 2 mm. The injured tissue was removed with a scalpel. The mean bark thickness (including cortex and phloem) of all clones was approximately 5 mm. Care was taken to avoid injuries deeper than 50 per cent of the total bark thickness in order to prevent interference from tissue changes associated with injury to the vascular cambium. Five wounds, 10 cm apart, were placed in a semi-spiral pattern on one scaffold limb per tree so that one wound was not directly above or below another wound. After 3, 7, 10, 14 and 17 d, the wounded areas were removed with a larger diameter cork borer inserted to the depth of the vascular cambium. Non-wounded tissues were taken at the beginning of the experiment and on each subsequent sample date. Each bark disk was halved through the wound to expose the radial longitudinal surface of the injured tissues. Each sample was immediately fixed in formalin:acetic acid:ethanol (Johansen, 1940) for 7 d, dehydrated in a *t*-butyl alcohol series, embedded in paraffin wax, and sectioned with a rotary microtome at 8 $\mu$m thickness (using the same microtome over the entire study). Ribbons of five serial sections were affixed to glass slides, the paraffin was removed with xylene (two rinses, 5 min each), hydrated in an ethanol series, and coverslips (No. 1 thickness, 22 × 50 mm) were mounted in three drops of glycerol or tissues were stained for 2 min with 0·5 per cent toluidine blue and mounted in three drops of Pro-Texx (Lerner Laboratories, New Haven). Special effort was made to insure that fixation, dehydration, and staining times were identical for all samples.

Previous studies on boundary tissues in tree bark (Biggs, 1984*b*) have shown that selective histochemistry in conjunction with fluorescence microscopy allows for observation of thin suberin linings on the inner wall of impervious cells in the boundary zone. When lignin autofluorescence was selectively quenched with toluidine blue O or phloroglucinol and HCl, the residual autofluorescence was due to suberin and/or associated waxes. When Sudan black B was applied to serial sections, suberin autofluorescence of phellem and inner wall linings was similarly quenched. The technique utilized Leitz filter combination A (340–380 nm excitation, 430 nm suppression) with a BG-12 filter and a ground glass dispersion disk inserted in the incident light pathway.

This technique was used in the present study to examine suberization of extant tissue and new suberized phellogen derivatives. The intensity of autofluorescence of glycerol mounts (lignin and suberin) and toluidine blue mounts (suberin) were measured with a Leitz MPV compact microscope photometer with a stabilized power supply for the 100W HBO mercury lamp and using the multiple plug or core method described by Mendelsohn (1966). The photometer shutter was electronically controlled to measure the autofluorescence intensity of the specimen for 2·5 s. Between measurements, the ultraviolet excitation was excluded by the use of an opaque shutter on the Leitz Ploemopak. Although suberin autofluorescence intensity fades upon exposure to ultraviolet excitation,

preliminary experiments showed no significant loss of autofluorescence during the 2·5 s measurement interval.

The measuring diaphragm of the photometer was adjusted to include a 272 μm diameter area when examined at × 250. This size was chosen because it included the entire boundary zone and new phellem as they appeared at 17 d post-wounding. Five measurements per sample (each serial section) were taken in the phelloderm/cortex region (just beneath the original phellem) approximately 800 μm distal or proximal to the wound surface because these cells were the last to undergo changes leading to imperviousness and phellogen generation, and thus constituted the region where suberin and phellogen continuity were re-established. In non-wounded control tissue or with tissue in the early stage of boundary zone formation (3 or 7 d post-wounding) when well defined boundaries were not apparent, measurements were taken in the phelloderm/cortex region approximately 800 μm internal to the wound margin. Each photometric value for analysis was the average of five observations taken from five serial sections. The experiment was conducted three times on separate peach tree limbs during June, July and August. The data were analyzed with a factorial analysis of variance (five wound ages × seven clones × three wound dates) and separated for interpretation using Duncan's multiple range test. The thickness of the suberized boundary zone and new phellem was measured for days 7 to 17 post-wounding, and the results were grouped across clone. Photographs of the tissue changes and their location as reported in the present study have been published previously (Biggs, 1984*b*).

## RESULTS

In analyses of total autofluorescence, suberin autofluorescence, and total autofluorescence minus suberin autofluorescence (non-suberin autofluorescence), a significant wound date × wound age × clone interaction was noted. In all analyses, wound age accounted for the greatest portion of the observed variation, followed by wound date and clone. For non-suberin (presumably lignin) autofluorescence, wound age accounted for 66·6 per cent of the variation followed by clone (7·2 per cent) and wound date (3·7 per cent). Variation in suberin autofluorescence intensity was accounted for by wound age (67·0 per cent), followed by clone (7·5 per cent) and wound date (6·7 per cent). Although the seven clones fell into three groups with respect to rate and intensity of deposition of autofluorescent compounds, data from all clones combined appeared to best reflect the chronology of events regarding generation of the impervious boundary layer and new phellogen. Data for the effect of wound age and wound date upon the development of non-suberin and suberin autofluorescence for all peach clones combined are presented in Tables 1 and 2, respectively. Interpretation of the significant second order interaction was facilitated by an examination of the significant first order interactions and the corresponding simple effects.

Increases in non-suberin (presumably lignin) autofluorescence were observed 7 d following the June wounding and 3 d following wounds initiated in July and August. Lignin deposition tended to level off by 10 d post-wounding, particularly in July and August when 71·4 per cent of the clone × wound date combinations showed no increase in lignin from day 10 to day 17. Lignin deposition in June appeared delayed within the first 7 d post-wounding relative to samples taken in July and August. The rate of increase in lignin deposition (Table 3) was highest between 7 and 10 d post-wounding in 12 of the 21 clone by wound date combinations.

Suberin deposition in boundary zone tissue generally appeared by day 7 or 10, dependent upon clone and time of year. New phellem was generally first observed on day 10 or 14 and cell numbers appeared to increase continually over time. Suberin deposition in boundary zone tissue was initially detected at 7–10 d post-wounding during

TABLE 1. *Effect of wound age and time of year on the development of non-suberin autofluorescence intensity at the site of phellogen generation in mechanically wounded peach bark cortical tissue**

| Wound age (d) | Wound date | | |
|---|---|---|---|
| | 12 June 84 | 10 July 84 | 1 August 84 |
| 3 | 0·7 a† | 1·7 b | 2·9 b |
| 7 | 5·6 b | 10·3 c | 19·2 c |
| 10 | 18·9 c | 15·1 d | 25·8 d |
| 14 | 23·1 d | 16·3 d | 22·6 dc |
| 17 | 24·2 d | 18·9 d | 28·6 d |

* Autofluorescence intensity measured over a circular area with 272 μm diameter, each measured area contained approximately 100 cells. Values represent total autofluorescence intensity of boundary zone (impervious tissue) or boundary zone plus necrophylactic phellem, depending on wound age, minus suberin autofluorescence intensity. Non-wounded bark autofluorescence intensity = 0.

† Different letters in columns within each wound date denote significant differences according to Duncan's new multiple range test ($P = 0{\cdot}05$); 3-d post-wounding values significantly different from 0 are indicated by the letter b.

TABLE 2. *Effect of wound age and time of year on the development of suberin autofluorescence intensity at the site of phellogen generation in mechanically wounded peach bark cortical tissue**

| Wound age (d) | Wound date | | |
|---|---|---|---|
| | 12 June 84 | 10 July 84 | 1 August 84 |
| 3 | 0·0 a† | 0·1 a | 0·2 a |
| 7 | 0·7 ab | 1·4 b | 2·6 b |
| 10 | 2·2 b | 4·0 c | 8·7 c |
| 14 | 6·5 c | 8·7 d | 12·8 d |
| 17 | 14·4 d | 10·8 e | 17·8 e |

* Autofluorescence intensity measured over a circular area with 272 μm diameter, each measured area contained approximately 100 cells. Values represent autofluorescence intensity of boundary zone (impervious tissue) or boundary zone plus necrophylactic phellem depending on wound age. Non-wounded bark autofluorescence intensity = 0 and all values at 3-d post-wounding are not significantly different from 0.

† Different letters in columns within each wound date denote significant differences according to Duncan's new multiple range test ($P = 0{\cdot}05$).

July and August, and at 10–14 d post-wounding during June. However, one clone consistently displayed boundary zone suberin deposition as early as 7 d post-wounding.

The rates of suberin deposition were slower and more erratic than those for lignin (Table 3). In June, suberin deposition rates increased with time to where all clones showed the greatest rates of deposition between 14 and 17 d post-wounding. In July, five of the seven clones achieved their greatest increases between days 10 and 14, while in August four clones exhibited their largest increases in suberin between days 7 and 10 post-wounding.

Mean thickness across the radial longitudinal axis of suberized boundary zone tissue and new phellem is presented in Fig. 1. This shows that the impervious tissue is present prior to the appearance of new phellogen derivatives. Suberized boundary zone tissue

TABLE 3. *Mean (±s.e.) rate of increase per day in lignin and suberin autofluorescence intensity for all peach clones combined wounded in June, July and August**

| Wound date (month) and wound age (d) | Lignin autofluorescence intensity | Suberin autofluorescence intensity |
|---|---|---|
| 12 June 1984 | | |
| 3–7 | 1·3±0·68 | 0·2±0·24 |
| 7–10 | 4·4±0·47 | 0·5±0·30 |
| 10–14 | 1·1±0·80 | 1·0±0·36 |
| 14–17 | 0·3±0·61 | 2·7±0·70 |
| 10 July 1984 | | |
| 3–7 | 2·1±0·82 | 0·3±0·46 |
| 7–10 | 1·6±0·93 | 0·9±0·47 |
| 10–14 | 0·6±0·11 | 1·2±0·24 |
| 14–17 | 0·9±1·14 | 0·7±0·41 |
| 1 August 1984 | | |
| 3–7 | 4·1±1·43 | 0·6±0·52 |
| 7–10 | 2·2±0·88 | 2·0±0·66 |
| 10–14 | −0·7±0·38 | 0·9±0·60 |
| 14–17 | 1·8±1·10 | 1·8±0·70 |

* Each value represents the mean of 105 measurements (five observations, three trees, seven clones).

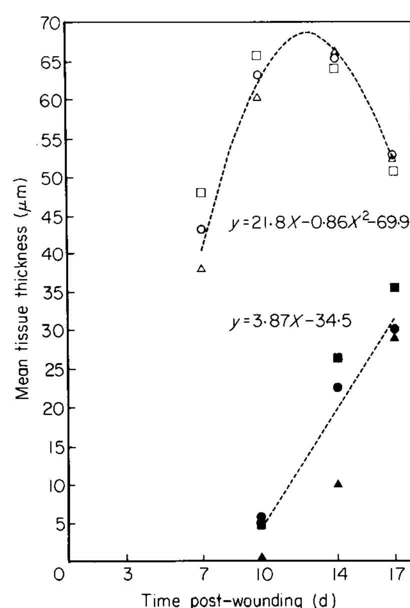


FIG. 1. Relationship between time post-wounding (d) and mean tissue thickness (μm) of suberized tissue formed from cells present at the time of wounding and new phellem. Legend: △, ○, □ indicate impervious tissue thickness in June, July, and August, respectively; ▲, ●, ■ indicate phellem thickness in June, July, and August, respectively.

was first observed at 7 d post-wounding and its thickness increased to its maximum between days 10 and 14. After day 14 boundary zone tissue thickness decreased. Suberized boundary zone tissue thickness as a function of post-wounding time was

described by the polynomial equation $y = 21{\cdot}8x - 0{\cdot}86x^2 - 69{\cdot}9$ ($r = 0{\cdot}56$, $P = 0{\cdot}05$). Suberized phellem was first observed 10 days post-wounding and phellem thickness increased with time. Phellem thickness as a function of post-wounding time was described by the linear regression $y = 3{\cdot}87x - 34{\cdot}5$ ($r = 0{\cdot}89$, $P = 0{\cdot}05$).

Cell numbers in the boundary zone tissue could not be counted after day 10 because the cells appeared to be crushed radially by the outward expansion of new phellem. This was reflected in a plateau followed by a decline in tissue thickness. In peach bark, the suberized boundary zone was usually three to four cells thick by 10 d post-wounding. Phellem cell numbers showed a regular increase with means of 0·5, 2·5 and 3·0 at 10, 14 and 17 d post-wounding, respectively. This increase was reflected as a regular linear increase in tissue thickness. Crushing of the suberized boundary tissues by the outward expansion of new phellem was observed on the tenth day post-wounding in clones exhibiting differentiated phellem.

## DISCUSSION

The present study is the first to successfully quantify suberin in plant tissues using a microscope technique. This approach has two major advantages over conventional staining and light microscopy. First, with respect to wound response in tree bark, conventional staining and light microscopy do not allow reliable detection of suberin formed as thin linings on the walls of cells present at the time of wounding, thus the present technique is more sensitive. Second, when conventional stains do allow detection of suberin (as in suberized phellem), brightfield microscope photometry would not be sensitive enough at the tissue level to discern small differences in stain intensity attributable to one or two new cell layers. This is because of increases in measurement error associated with changes in various other biochemical constituents accompanying cell maturation or tissue necrosis (condensation of tannins, oxidation of vacuolar phenolics, deposition of polysaccharide substances) which influence the penetration of tissues by the light beam. Thus, the dark field of the autofluorescence technique minimizes the error associated with other tissue constituents.

Problems with this technique can be minimized by following standardized procedures in tissue preparation and staining. Phloroglucinol and HCl was the more effective reagent for quenching lignin autofluorescence (Biggs, 1984*a*) although HCl fumes may corrode the microscope objectives. The residual autofluorescence due to suberin may change colour and intensity after prolonged observation under ultraviolet light. Care must be taken to keep the measuring time short through the use of an electrically controlled measuring aperture and an opaque barrier to block the ultraviolet excitation beam prior to measurement.

Given the complex nature of the statistical interaction between wound age, wound date, and clone, and the large percentage of variation explained by wound age, further discussion of wound date and clone influences will be in rather general terms. The influence of clone in this study was limited to a well defined separation of the seven clones into three groups that could be judged qualitatively as relatively rapid, moderate, or slow in their capacity to set boundaries and generate new phellogen. The importance of these parameters as they relate to resistance to fungal wound pathogens is currently being investigated. Preliminary results demonstrated that rapid wound response may be associated with resistance to wound pathogens (Biggs and Miles, 1985).

In the present study, a significant seasonal effect was observed. The importance of wound date appeared to be limited to significant differences in the rate and intensity of autofluorescent material in June relative to August, with the August response appearing most rapid. Seasonal effects in wound response rates in tree bark have been reported

(Mullick, 1977) and may be related to temperature, water availability, plant nutritional status, or other edaphic factors.

Tissue changes in peach tree bark following wounding could be divided into two distinct types: (1) cell hypertrophy and cell wall lignification and suberization in tissues present at the time of wounding, giving rise to an impervious boundary of non-living cells; (2) subsequent differentiation of a new phellogen immediately internal to and abutting the boundary zone. This sequence of tissue changes was originally described for coniferous tree bark by Mullick (1975) and for woody dicots by Soo (1977). These authors described the restoration of phellogen internal to a non-suberized impervious tissue. In contrast, the present study indicated that the phellogen was formed internal to a ligno-suberized zone. More sensitive techniques than those used by Mullick and Soo were necessary to detect suberin deposition in impervious tissue (Biggs, 1984*a*, 1985), although the sequence of tissue changes, in general, was identical to that described by Mullick (1975).

The present study is the first to describe, in quantitative terms, formation of ligno-suberized boundary zone tissue and generation of new phellogen derivatives. The ligno-suberized boundary, formed from cells present at the time of wounding, was usually observed within 7 d post-wounding. New phellogen and its derivatives formed within 10 d immediately internal to the ligno-suberized tissue which became crushed due to the outward expansion of the new periderm (Fig. 1). The ligno-suberized boundary appeared to be a prerequisite for complete differentiation of new phellogen.

The processes recorded in this study are related to the three phases of tissue regeneration described quantitatively by Warren Wilson and Grange (1984). A discussion of the lag phase is particularly relevant in that the ligno-suberized boundary was formed during this period. The lag phase appeared different for different clones lasting usually between 7 and 10 d depending on clone and its interaction with time of year. As described in previous work concerning tree bark response to wounding (Mullick, 1977; Soo, 1977; Biggs, 1984*b*) very few, if any, cell divisions occur prior to the re-establishment of imperviousness. It is known that during the lag period cells are active metabolically and ultrastructurally (Lipetz, 1970; Yeoman and Aitchison, 1973). Processes associated with cell wall lignification and suberization appear particularly active in tree bark.

The role of suberin in controlling the separation of the lag phase from the division phase has not been discussed previously. Bloch (1952) suggested that the induction of activity associated with wound response could be due to diffusing substances from injured cells (hormones or decomposition products) and, in studies of vascular cambium regeneration, Warren Wilson (1978) posed the auxin/sucrose gradient hypothesis. The timing and extent of suberin and lignin deposition could influence biochemical gradients and could explain why, if autolysis products or hormones are diffusible, cell activity is restricted to shallow layers of cells.

Cell division was observed in the present study and in previous studies (Mullick, 1977; Biggs, 1984*b*) after tissue imperviousness had developed, although indications of dedifferentiation were apparent prior to boundary zone suberization. Early cell divisions resulted in formation of the new phellogen so that, in peach bark, the division phase and the differentiation phase were difficult to distinguish. This difficulty may be peculiar to secondary tissues since these phases are quite distinct in herbaceous plants and tissue cultures (Warren Wilson and Grange, 1984; Yeoman, Dyer and Robertson, 1965).

In tree bark, as in herbaceous stems (Warren Wilson and Grange, 1984), the sequence and location of tissues associated with generation of new phellogen could be predicted by 7 d post-wounding even though complete re-establishment of phellogen continuity took 17 d or longer with some clones. Cell wall histochemistry was used to locate tissues

involved in pattern definition in tree bark undergoing phellogen generation, whereas in herbaceous stems regions of relatively active cell division were used to determine patterns for both lateral meristems.

The phellogen wound model in dicotyledonous tree bark could be useful in future studies on the role of diffusible morphogenetic substances involved in pattern forming mechanisms. The timing and quantity of suberin and/or lignin deposition in the boundary zone may exert a controlling influence over formation of gradients in tree bark wounds.

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