IAWA Journal, Vol. 25 (2), 2004: 165–174

PATHOLOGICAL ANATOMY OF NECTRIA CANKER ON *FRAXINUS MANDSHURICA* VAR. *JAPONICA*

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SUMMARY

The anatomical characteristics of Nectria canker on *Fraxinus mandshurica* var. *japonica* were analyzed. Typical cankers were conspicuous, round to oval, with uniform concentric rings of affected xylem in a target-like structure. Each concentric annual growth ring was wider than the corresponding annual rings lateral to the cankers. The xylem elements were extremely disoriented. The cambial zone became discontinuous and disappeared. An inoculation test with the causal fungus, *Nectria galligena*, produced similar anatomical abnormalities and revealed the process of canker formation. Fewer and narrower vessels were formed, and water conduction took place only in the large vessels of the current year in the cankers.

Key words: *Fraxinus mandshurica* var. *japonica*, *Nectria galligena*, canker, wood anatomy, water conductivity, inoculation.

INTRODUCTION

Nectria canker, a serious perennial fungal disease in broad-leaved trees, is caused by *Nectria galligena* Bres. In North America and Europe, canker formation has been reported in many species, i.a., of *Acer*, *Alnus*, *Betula*, *Fagus*, *Juglans*, *Malus*, *Populus*, *Pyrus*, *Quercus*, *Salix*, *Sorbus* and *Ulmus* (Flack & Swinburne 1977; Sinclair et al. 1987). In Hokkaido (a northern island of Japan), the canker on *Fraxinus mandshurica* var. *japonica* is widely distributed (Sasaki 1979). The infection causes twig blight, deformation of tree shape, and brittleness of the affected part.

Many studies have been conducted on the epidemiology and chemical control of Nectria cankers (e.g., Welch 1934a, b; Swinburne 1971a, b; Flack & Swinburne 1977; Sasaki 1979; Sasaki 1986; Blanchard & Tattar 1997; Cooke 1999). However, little attention has been given to anatomical studies (e.g., Crowdy 1949; Zalasky 1968). Sasaki (1983) performed a brief anatomical study of canker on *F. mandshurica*; however, the mechanism of canker formation and the invasion behavior of the pathogen in the affected tissues are unclear. To clarify these points, this study deals with the precise anatomy of canker on *F. mandshurica*.

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Table 1. Sample collection (natural cankers).

Tree	DBH (cm)	Location	Date of collection	Year
A	30	Bifuka	April 18	1998
B	40	Tomakomai	June 26	1998
C	20	Tomakomai	September 20	1998
D[a]	15	Tomakomai	September 20	1998
E	22	Tomakomai	November 15	2001
F	23	Tomakomai	November 15	2001
G[a]	6	Tomakomai	November 15	2001

[a]) Healthy trees for control.

MATERIALS AND METHODS

Natural cankers

Sampling

Sampling of natural cankers for macroscopic and microscopic observations was performed in the natural forests of Bifuka (northern Hokkaido) and Tomakomai (central Hokkaido) (Table 1).

Macroscopic and microscopic observations

The outside appearance and transverse and radial views of more than 10 cankers were observed with the naked eye or under a dissecting microscope (magnification: ×8–40).

For general observations, samples of various sizes were fixed with FAA (formalin : acetic acid : 50% ethanol = 5 : 5 : 90) for more than one month. Fixed samples were divided into small pieces (approximately 1.5 × 1.5 × 1.5 cm) and then washed under running water for 3–4 hours. They were dehydrated in an ethanol series and embedded in cedukol (Merck). Transverse and radial sections 12–25 μm thick were cut on a sliding microtome. They were stained with 0.3% safranin O aqueous solution or double-stained with 1% safranin O solution in 50% ethanol and 0.1% fast green solution in 95% ethanol. They were then observed under a light microscope.

For detailed observations of the cambial zone and the xylem and phloem, the samples collected from Trees A and C (Table 1) were fixed with 4% glutaraldehyde solution in phosphate buffer and then embedded in epoxy resin. Transverse sections 1 μm thick were cut on an ultramicrotome (EM-ULTRACUT-J). They were stained with 0.3% safranin O aqueous solution and then observed under a light microscope.

Water conduction

The dye injection test was carried out to examine the water-conductive vessels in the branches with cankers in the naturally infected trees.

On June 26, 1998, two branches with several cankers were collected from Tree B (Table 1), and their freshly cut bases were soaked in a 0.2% safranin O aqueous solu-

tion. After 90 minutes, the cut bases of the branches were coated with petroleum. After removal of the phloem, the area stained with the dye was examined with the naked eye. Transverse sections (12 μm thick) of the cankers and the lateral and opposite sides of the cankers were cut on a sliding microtome and observed under a light microscope.

Canker formation by inoculation

The inoculation test was performed on July 6 and 7, 1999. Fifty-four *Fraxinus mandshurica* seedlings, 4 to 6 years old, were inoculated with *Nectria galligena* isolated from natural cankers. Eight holes were bored into the xylem with a cork-borer (4.5 mm in diameter) in the stem of each seedling. A disc (4.5 mm in diameter) of potato dextrose agar (PDA: Eiken E-MF21) bearing mycelium of *N. galligena* cut from the margins of an actively growing plate culture was placed into each hole. Plastic film was wrapped around the wounded stems to prevent desiccation. Eighteen control seedlings were treated similarly with PDA discs without mycelium. The samples for macroscopic and microscopic observations were collected from a set of six inoculated and two control seedlings nine times from August 9, 1999 to October 16, 2001 (Table 2). The development of visible alterations, such as swelling, cracking and formation of cankers on the inoculated points, was observed with the naked eye or a dissecting microscope (magnification: ×8–40) during the course of the sampling. The samples were fixed, embedded, dissected (20–25 μm thick), double-stained and observed under a light microscope by the same method used in the case of the natural cankers.

Table 2. Collection schedule of inoculated cankers.

Year	Date	Year	Date	Year	Date
1999	August 9	2000	May 17	2001	May 24
	September 5		August 22		August 29
	October 19		October 31		October 16

Iodine stain

For the observation of starch granules in parenchyma cells in the xylem, the healthy (Tree D, Table 1) and cankerous (both natural and inoculated) samples were stained with iodine and potassium iodine (Miyazaki et al. 2002) and immediately observed under a light microscope.

RESULTS

Natural cankers

Typical cankers had a target shape due to the exposed uniform concentric rings of affected xylem (Fig. 1). On the transverse disks of the cankers, cambial cells already appeared to be dead, and the exposed xylem was discoloured and decayed in many cases (Fig. 2). The affected xylem of the cankers was extremely deformed. The phloem of the cankers was thick, and dead phloem could be broken off easily.

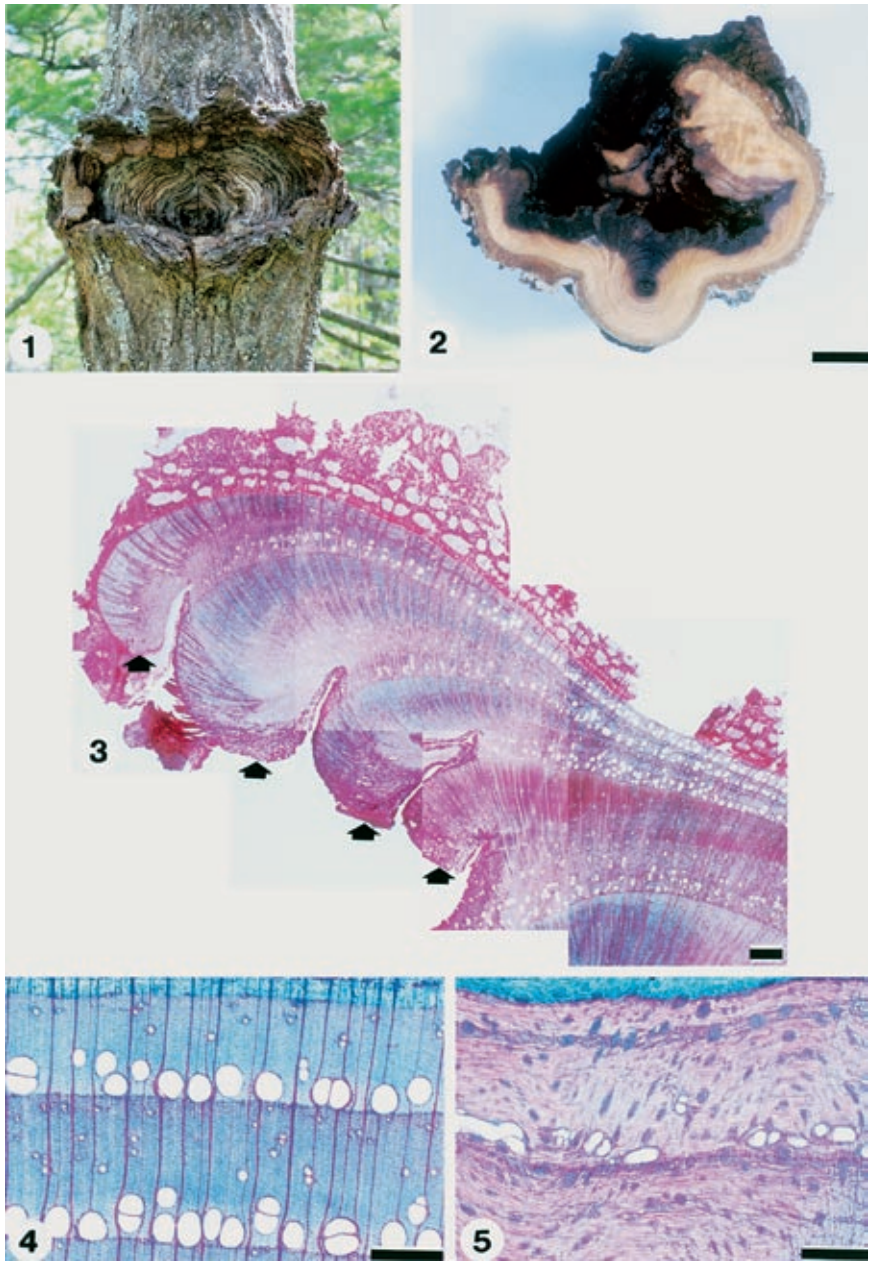

Fig. 1. The target-like structure of a typical canker (photographed 29 May 2002 in Rubeshibe, in its natural environment). — Fig. 2. Transverse view of a typical canker. — Fig. 3. Transverse view of a canker. Arrows indicate the concentric rings of the target-like structure. — Fig. 4. Transverse view of healthy xylem (collected from Tree G, Table 1). — Fig. 5. Transverse view of the irregular orientation of the xylem elements of the canker. — Scale bar for Fig. 2 = 1 cm, for Fig. 3–5 = 500 μm.

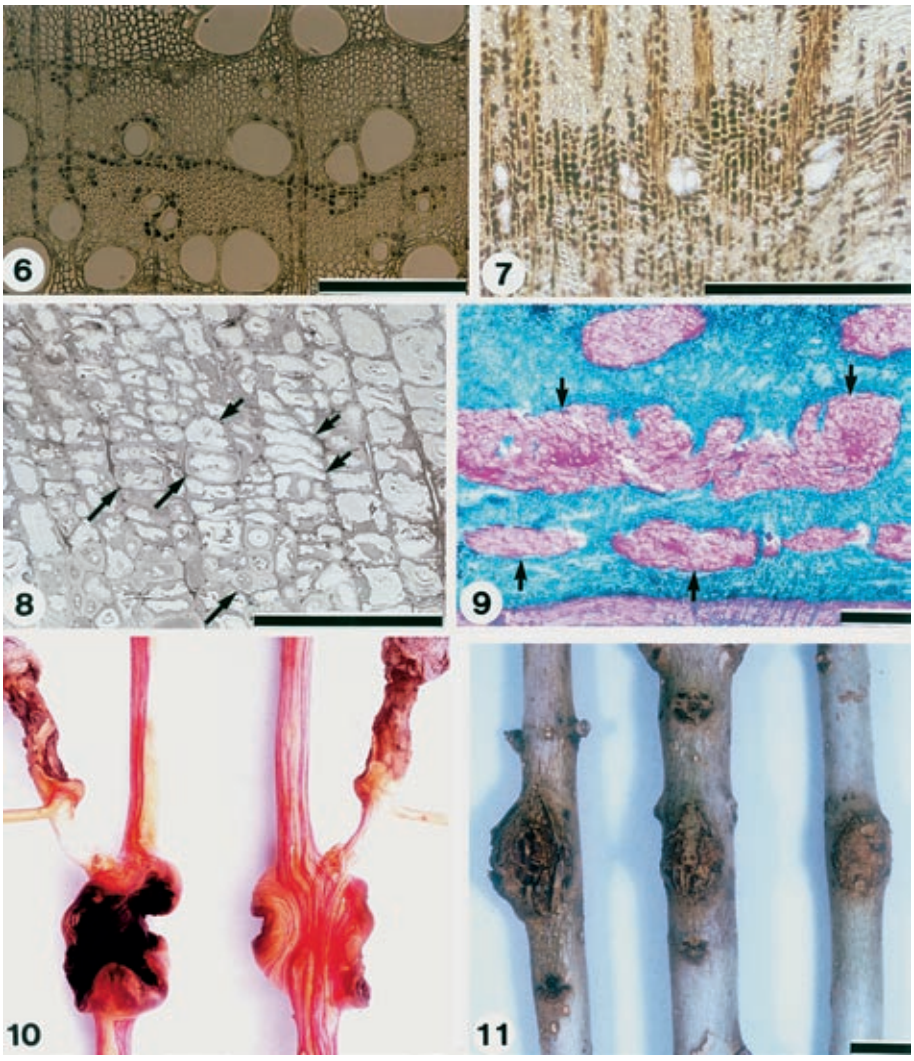

Fig. 6 & 7. Iodine staining. The starch granules in parenchyma cells were stained purple. 6: Transverse view of the healthy xylem. 7: Transverse view of the cankers. Note the large number of axial and ray parenchyma cells with an irregular arrangement. — Fig. 8. Cambial zone of the canker. Note the thick-walled mature cells (long arrows) and the thin-walled immature cells (short arrows). — Fig. 9. Transverse view of the affected phloem. Arrows indicate the dense cluster of sclereids. — Fig. 10. Dye injection test of the branch with canker. Note that the conductive vessels bypass the exposed (necrotic) xylem; left: front view; right: rear view. — Fig. 11. Cankers by inoculation (approximately two years after inoculation). — Scale bar for Fig. 8 = 50 μm; for Fig. 6, 7 & 9 = 500 μm; for Fig. 11 = 1 cm.

The affected xylem had much wider annual growth rings than the corresponding annual rings lateral to the cankers. Formation of each concentric ring in the target-like structures was due to the continuous cambium necrosis (Fig. 2).

The wider annual rings were composed mainly of axial parenchyma cells and wood fibers and contained only a small number of vessels (Fig. 3). The diameter of the large vessels was narrower in the abnormal xylem area than in the healthy xylem (D and G, Table 1) (Fig. 4). The vessel lumina of affected xylem often contained fungal hyphae. An irregular orientation of all xylem elements, such as ray and axial parenchyma cells, vessels and wood fibers, was commonly observed (Fig. 5).

The number of axial parenchyma cells stained with iodine in the affected xylem was obviously abundant compared to the healthy xylem (Fig. 6 & 7). There are four types of axial parenchyma cells in *Fraxinus mandshurica*: scanty paratracheal, vasicentric, terminal and diffuse (Ishida & Ohtani 1989). However, because the parenchyma was disordered in the affected xylem, the four types could no longer be distinguished.

The cambial cells were normally ordered on the opposite side of the cankers (Tree C, Table 1). In the cambial zone of the cankers and adjacent to that area, the arrangement of the cambial cells was disordered (Fig. 8). Thick-walled mature cells were mingled with thin-walled immature cells. Such cambial abnormalities were more obvious as the cankers developed further. It was impossible to trace the cambial zone in the advanced cankers because the cambial zone became discontinuous.

Anatomical abnormalities were also observed in the xylem lateral to the cankers. The width of the annual growth was narrower than that in the healthy xylem (Trees D and G, Table 1), and the small vessels were poorly developed.

In the affected phloem of the cankers, a large number of parenchyma cells and sclereids were commonly observed. It was very difficult to distinguish between ray parenchyma and axial parenchyma cells because the arrangement of the cells was extremely disordered. The sclereids were grouped in dense clusters (Fig. 9).

Water conduction

The conductive vessels, which were stained with the dye, bypassed the exposed (necrotic) xylem of the cankers (Fig. 10).

The transverse sections showed that only the large vessels formed in the current year were stained in the cankers. In the xylem of the lateral and opposite sides of the cankers, the large vessels formed in the current year and the small vessels formed in the previous year and two years earlier were stained.

Canker formation by inoculation

Approximately one month after inoculation, swelling became obvious in the vicinity of the inoculation points. The outer bark became ruptured, and the inoculation points continued to swell every year. Most of the inoculation points became noticeable cankers with necrotic tissues (Fig. 11). On the other hand, no pathogenic reactions (only small necroses) were observed in the control inoculations.

One month after inoculation, anatomical alterations of the affected xylem, such as active cell division with fewer vessels, were observed. Irregular growth of xylem con-

tinued every year. The irregular orientation of the xylem cells and the abundant axial parenchyma cells that were stained with iodine became obvious. Two years after inoculation, wider annual growth with fewer and smaller vessels (corresponding to concentric rings of target-like structures) was clearly observed (Fig. 12).

In the control inoculations, traumatic callus-like cells appeared in the xylem formed in 1999. However, no anatomical abnormalities were observed in the xylem formed in 2000 and 2001 (Fig. 13).

DISCUSSION

Characteristic concentric rings of this canker seem to arise from yearly necrosis of phloem and cambial cells in the vicinity of the canker. As a result of the necrosis, the outer bark and phloem at the margins of the canker are removed, and one ring of affected xylem appears annually. Repetition of such yearly events results in the development of the concentric pattern of exposed xylem (Fig. 14). If no such yearly necrosis occurred, the exposed xylem of the canker would be covered by outer bark and phloem before long.

The developmental process of this canker suggests that the ability of the pathogen to extend into the tissues is weak, although it can survive in a limited zone for a long period. Necrosis was restricted to tissues in the vicinity of the canker. No apparent anatomical abnormalities were found on the opposite side of the canker or on parts longitudinally distant from the canker. It is likely that the pathogen can live in phloem, xylem and cambial cells surrounding the canker.

Exposed and necrotic areas in the central parts of cankers and necrotic phloem were also observed in bacterial canker on *Maackia amurensis* var. *buergeri* (Sakamoto 1999). However, conspicuous target-like structures were not formed because there was no abnormal xylem formation in this case.

The narrower annual rings on the lateral side of the cankers indicate that most of the photosynthate to produce new xylem cells was consumed on the canker side.

From the functional point of view, vessel formation is quite important. Fewer and narrower vessels result in greatly reduced water conductivity in the cankers because the theoretical conductivity in capillaries is proportional to the fourth power of their diameters (Zimmermann 1983). Hence, the water conductivity of the cankers was greatly decreased. Although both the large and small vessels on the lateral and opposite sides of the cankers were conductive, the branches and stems with numerous or large cankers could not have conducted enough water at the beginning of the growth season until the large vessels of the current year had developed. The decrease in water conductivity in the cankers in early spring became one of the reasons for the debility or dieback of the seriously affected trees.

Differentiation of narrower vessels caused by mechanical wounding has been reported in some hardwoods (Bauch et al. 1980; Kuroda & Shimaji 1985; Kuroda 1986; Lev-Yadun & Aloni 1993). In the case of *Fraxinus mandshurica*, fewer and narrower vessels were also observed in the vicinity of the wound caused by frost cracks (Sano 1996). These facts suggest that differentiation of fewer and narrower vessels is a common phenomenon occurring in wounded xylem and is not a characteristic feature of this canker.

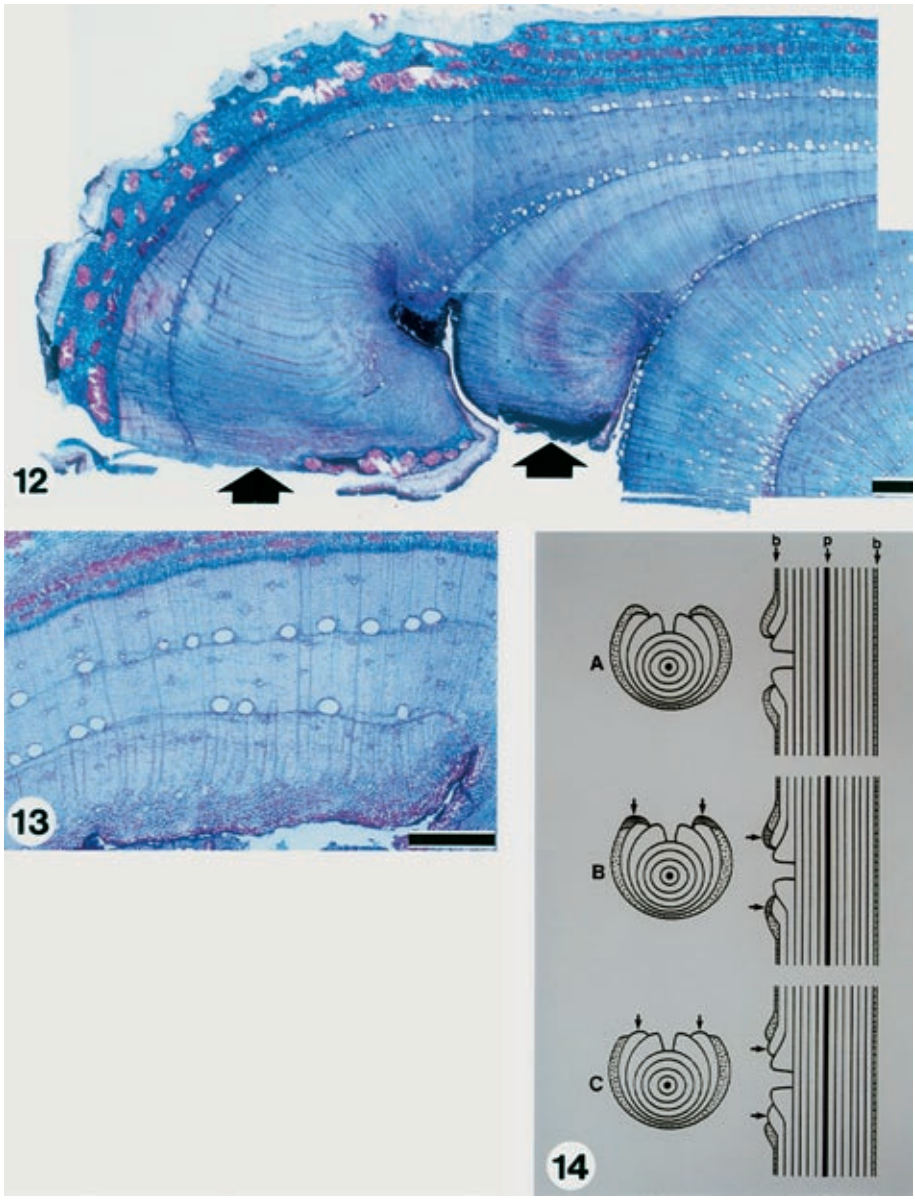

Fig. 12. Transverse view of the canker approximately two years after inoculation. Arrows indicate the concentric rings of the target-like structure. — Fig. 13. Transverse view of a control inoculation (approximately two years after inoculation). Scale bar for 12 & 13 = 500 µm. — Fig. 14. Diagrams of the formation of the target-like structure. – A: Wide growth increments of the xylem and phloem are formed simultaneously. – B: The edges of the phloem become necrotic (arrows). – C: The edges of the wide growth zones of the xylem become exposed and visible (arrows); b = outer bark and phloem; p = pith.

The results of the present study clarify the structure and anatomical characteristics of Nectria canker on *F. mandshurica*. However, the physiological aspects due to anatomical alterations remain unknown. In the case of bacterial gall disease of oleander (*Nerium oleander* L.), auxin (indole-3-acetic acid) production by the causal bacterium is the important factor in producing gall symptoms (Smidt & Kosuge 1978). Aloni (1991) reported that vessel size in the ring-porous tree *Melia azedarach* L. was altered by the concentration of applied NAA (α-naphthalene acetic acid). The application of ethrel, an ethylene-releasing compound, to *Ulmus americana* L. seedlings greatly changed their stem anatomy, such as the thickness of the phloem, the number and diameter of vessels and the ray width and size of individual ray cells (Yamamoto et al. 1987). Hence, the stimulation of plant hormones may play an important role in anatomical alterations of this canker. Further physiological studies should be undertaken to clarify the detailed mechanism of canker formation.

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