HISTOLOGY OF PATHOGENESIS OF *PSEUDOMONAS SAVASTANOI* ON *MYRTUS COMMUNIS*

M. Temsah, L. Hanna and A.T. Saad

Faculty of Agricultural and Food Sciences, American University of Beirut, P.O. Box 11-0236, Beirut, Lebanon

SUMMARY

Myrtle (*Myrtus communis* L.) is an evergreen shrub widely grown in the Mediterranean region for its aromatic leaves and medicinal uses. The bacterium *Pseudomonas savastanoi* was recently reported to attack this shrub with characteristic symptoms of knot formation on stems and branches leading, in severe cases, to their death. The pathogenesis of *P. savastanoi* on myrtle was studied macroscopically and by light microscopy. Inoculation of myrtle shoots with *P. savastanoi* initiated swellings that developed into knots typical of the disease. Starting from the inoculation wound, the bacteria spread into the intercellular spaces, invading and colonizing host tissues by degrading cell walls. They multiplied in the resulting cavities inducing hypertrophy and hyperplasia in the contiguous cells to form tissue masses that grew into knots. The size of a knot depended on the depth of the inoculation wound and more precisely on the parenchyma tissues involved, including those of the cortex, vascular tissues, and/or pith. At an advanced stage of invasion, the knot was composed of an aggregate of tissue masses within which xylem elements had differentiated. The knot became surrounded by wound periderm. Lignification of the parenchyma cells of the knot finally led to its hardening and death. These findings on myrtle were compared to those reported to be caused by the same pathogen on ash, buckthorn, oleander, and olive.

Key words: Myrtle, *Pseudomonas savastanoi pv. savastanoi*, anatomy, knot.

INTRODUCTION

Pseudomonas savastanoi (ex Smith1908) Gardan *et al.*, 1992 was first shown by Smith to cause the olive knot disease on olive (Smith 1920). Disease symptoms were characterized by the formation of outgrowths called knots on affected host tissues. The bacterium multiplied best at 22-25°C, 70-100% RH, and during active plant growth (Wilson, 1935). Besides olive (*Olea europea* L.), the disease was reported on oleander (*Nerium oleander* L.), ash (*Fraxinus* spp.), jasmine (*Jasminum* spp.), privet (*Ligustrum* spp.), *Forsythia* spp., and *Phillyrea* spp. (Bradbury 1986) and recently on myrtle (*Myrtus communis* L.) (Fig. 1), and buckthorn (*Rhamnus alaternus* L.) (Saad and Melkonian, 1992; Saad and Hanna, 2002). Anatomical studies on the developmental stages of the knots in *Myrtus communis* L., inoculated with *P. savastanoi*, have not been done previously. The objectives of this study were to record disease symptoms and anatomical changes in inoculated young stem tissues of myrtle. The changes in myrtle tissues caused by *P. savastanoi* were compared to those reported on ash (Janse 1982), buckthorn (Temsah *et al.*, 2007), oleander (Wilson 1965), and olive (Surico 1977), by different strains of the same pathogen, originating from the respective hosts. Similarities and differences are discussed.

MATERIALS AND METHODS

A virulent strain of *P. savastanoi* was isolated from active knots of naturally infected myrtle plants collected from the American University of Beirut Campus in Lebanon. The strain was characterized by the LOPAT scheme of tests for grouping green fluorescent pseudomonads (Lelliot and Stead, 1987) to confirm its identity. A type culture of the *P. savastanoi* isolate used in this study was deposited at the Culture Collection of Istituto Tossine e Micotossine da Parassiti Vegetali, CNR, Bari, Italy.

Inoculum was prepared from 24 h cultures of *P. savastanoi* growing on nutrient agar (DIFCO), and standardized turbidimetrically to a concentration of 10^7 cfu ml^{-1} of sterile water.

Myrtle plants, obtained from rooted cuttings, were grown in 30 cm pots. Thirty five young healthy myrtle shoots from 1-year-old plants were inoculated between the third and fourth nodes below the apex by pricking a 5-10 µl aliquot of bacterial inoculum into the shoots with a hypodermic needle. Another set of 35 young

Corresponding author: A.T. Saad
Fax: +961.1.744460
E-mail: asaad@aub.edu.lb

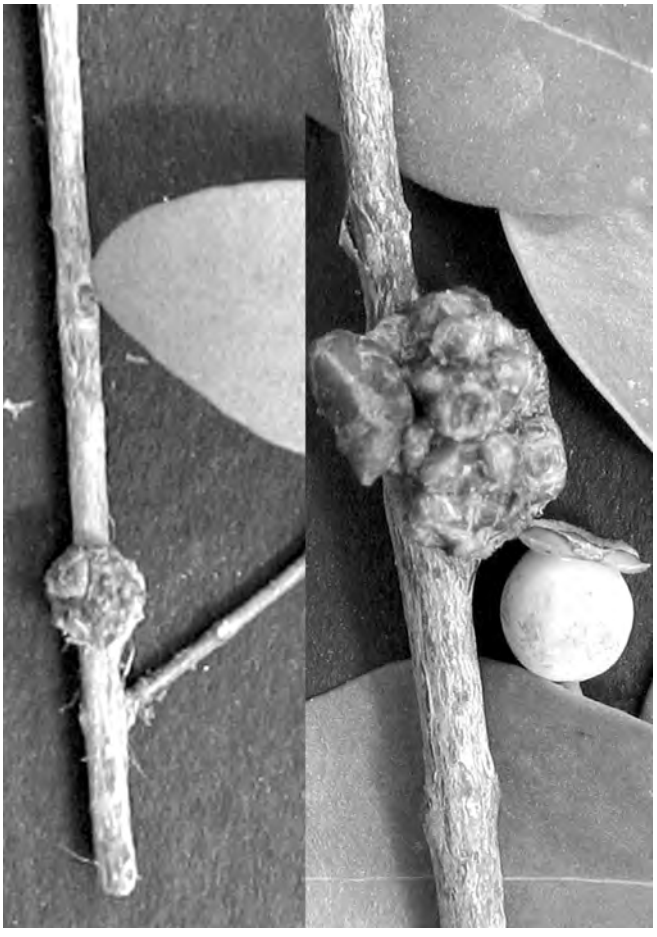

Fig. 1. Symptoms of the olive knot disease on *Myrtus communis*.

shoots was inoculated with 5-10 µl of sterile water as a control. Inoculated plants were kept in a glasshouse with an average temperature of 25°C and 100% relative humidity for the first 72 h. Subsequently, the temperature in the glasshouse ranged from 20 to 24°C for the 60-day duration of the experiment.

Macroscopic observations on knot development and collection of samples for histological studies were made at preinoculation for the healthy tissue, and at 3, 10, 17, 24, 31, 45, and 60 days post inoculation. Cuttings of inoculated shoots, 3 per collection date, at the inoculation sites, were immediately fixed in FAA (formalin, acetic acid, 95% ethanol=1:1:10 v/v). Fixed tissues were washed under tap water and dehydrated in tertiary butyl alcohol (Jensen, 1962), embedded in Paraplast X-tra paraffin embedding medium and sectioned with a manual rotary microtome (Microm HR®). Longitudinal and transverse 10 µm sections were affixed to slides coated with Haupt's adhesive according to Ruzin (1999). Serial sections were stained with safranin-fast green or Gram stain, and mounted in Permount® Fisher mounting medium, for light microscopy examination.

RESULTS

The first seventeen days after inoculation revealed no visible external changes in inoculated shoots. Twenty four days after inoculation, small swellings started to appear at the inoculation sites and by the thirty first day these swellings had increased in size to become clearly visible outgrowths referred to as knots *ca.* 5-7 mm in diameter and up to 3 mm thick (Fig. 1a). By sixty days after inoculation, the knots were multilobular, 1.5 cm in diameter and 1 cm thick (Fig. 1b). Shoots inoculated with sterile water showed only faint scars at the inoculation site with no development of outgrowths or knots.

Microscopic examination of transverse internodal sections of a young healthy shoot of myrtle, between the third and fourth nodes below the apex, concerned the following tissues and layers: (i) epidermis, made up of one layer of cutinized cells and including stomata and leaf hairs; (ii) cortex, made up of several layers of parenchyma cells separated by intercellular spaces, fibers distributed in discontinuous masses, the lumen of which becomes narrower as the stem ages, a periderm differentiating below the fibers, with the growth of the plant, and well-spaced secretory cavities occurring under the epidermis; (iii) vascular tissues, made up of phloem (primary and secondary), cambium, and xylem (primary and secondary), the secondary xylem and phloem, including parenchyma rays, being made up of one or two cell layers; (iv) the pith occupies the center of the stem and is made up of parenchyma cells, some of which contain food reserves (Fig. 2).

Development of disease knots. Three days after inoculation, cross sections of inoculated shoots revealed bacteria in parenchyma cells of the cortex, vascular tissues and pith that were injured by inoculation. The cells around the inoculation wound were plasmolysed and necrotic.

Ten days after inoculation, degradation of portions of parenchyma cell walls (Fig. 3) and increased bacterial cavities were observed in tissues around the inoculation wounds. Hypertrophic activity followed by hyperplasia was observed in adjacent parenchyma cells. Concurrently, bacteria continued to spread throughout parenchyma tissues away from the inoculation wound. Wound periderm differentiated below the necrotic cells around the inoculation wound.

Seventeen days after inoculation, bacteria-filled cavities had enlarged with the continuous degradation of parenchyma cell walls. Initials of hyperplasic tissue masses formed adjacent to the inoculation wound, as a result of incessant hypertrophic and hyperplasic activity in cortex parenchyma cells of the phelloderm, phellogen, rays, and in xylem, phloem, cambium, and the pith (Fig. 4).

Twenty four days after inoculation, a new and charac-

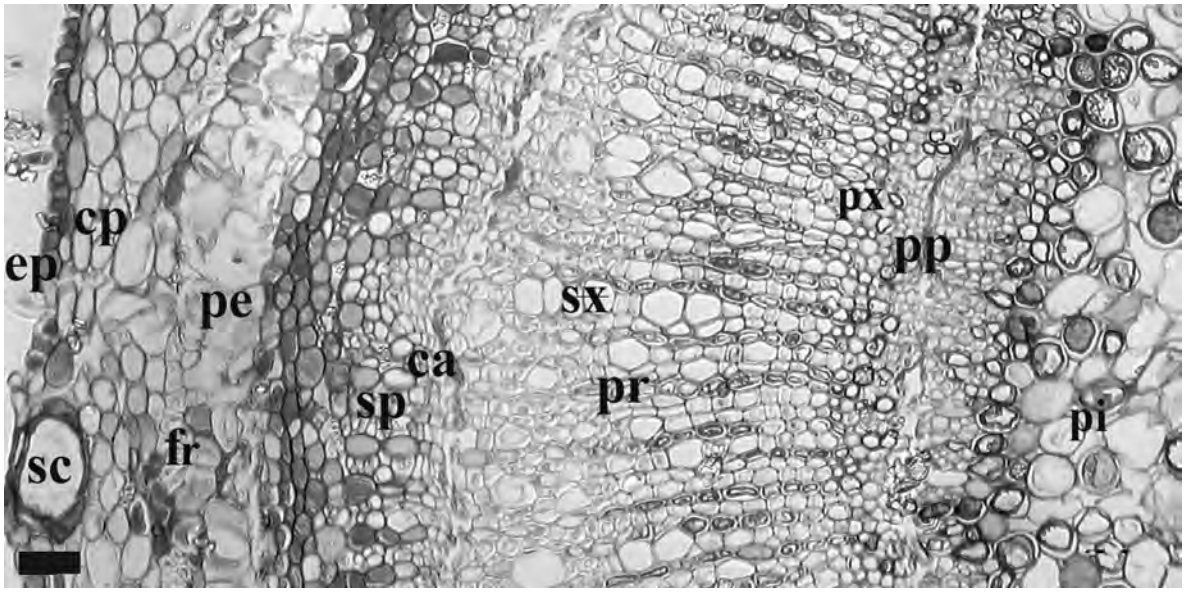

Fig. 2. Cross section of young healthy stem of myrtle, between second and third nodes from top showing, epidermis(ep), secretory cavities (sc), cortical parenchyma (cp), fibres (fr), periderm (pe), secondary phloem (sp), cambium (ca), secondary xylem (sx), parenchyma ray (pr), primary xylem (px), primary xylem parenchyma (pp), pith (pi). Bar = 100 µm.

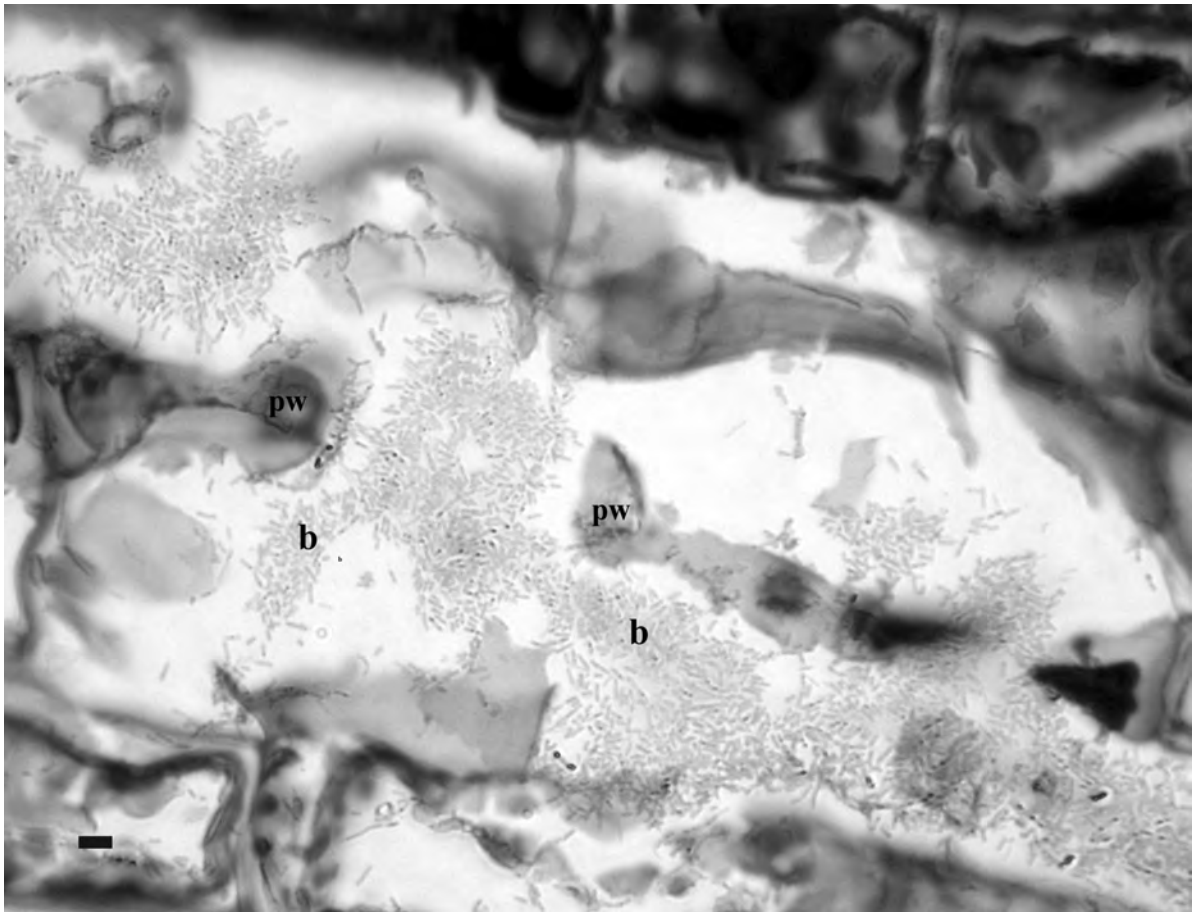

Fig. 3. Transverse section of myrtle stem, 10 days after inoculation, showing bacteria (b) colonizing the tissue after degrading portions of parenchyma cell wall (pw). Bar = 10 µm.

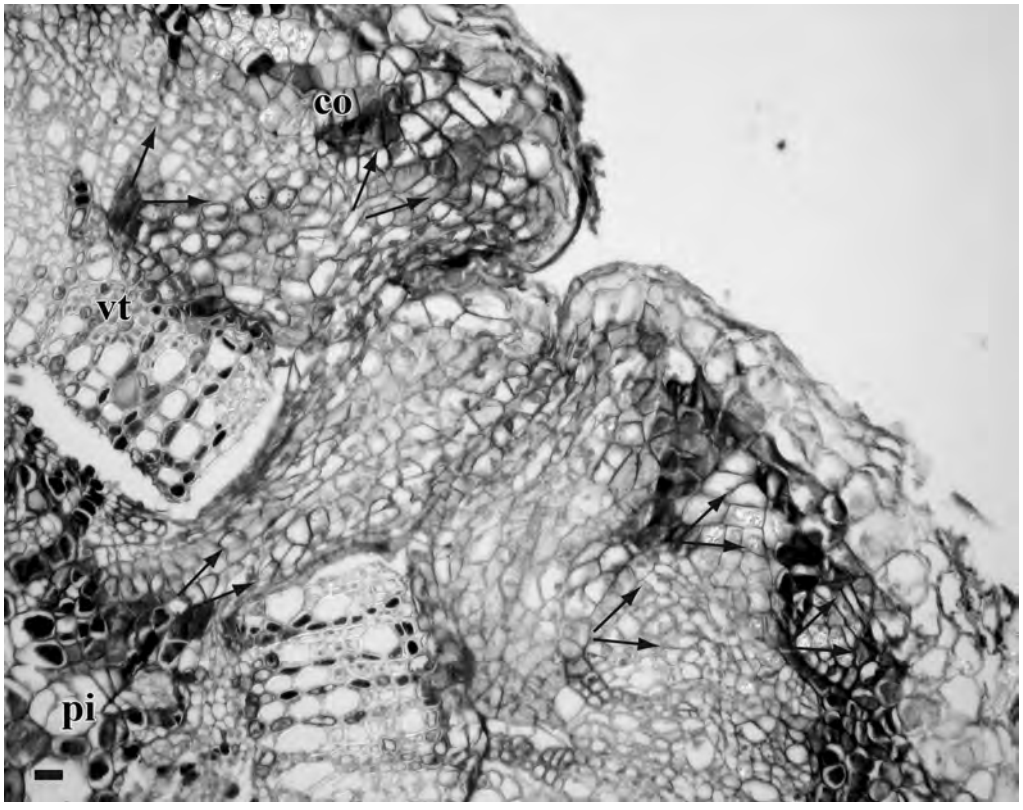

Fig. 4. Transverse section of myrtle stem, 17 days after inoculation, showing hyperplasic masses originating from different parenchyma cells derived from cortex (co), vascular tissue (vt) and pith (pi). Bar = 50 µm.

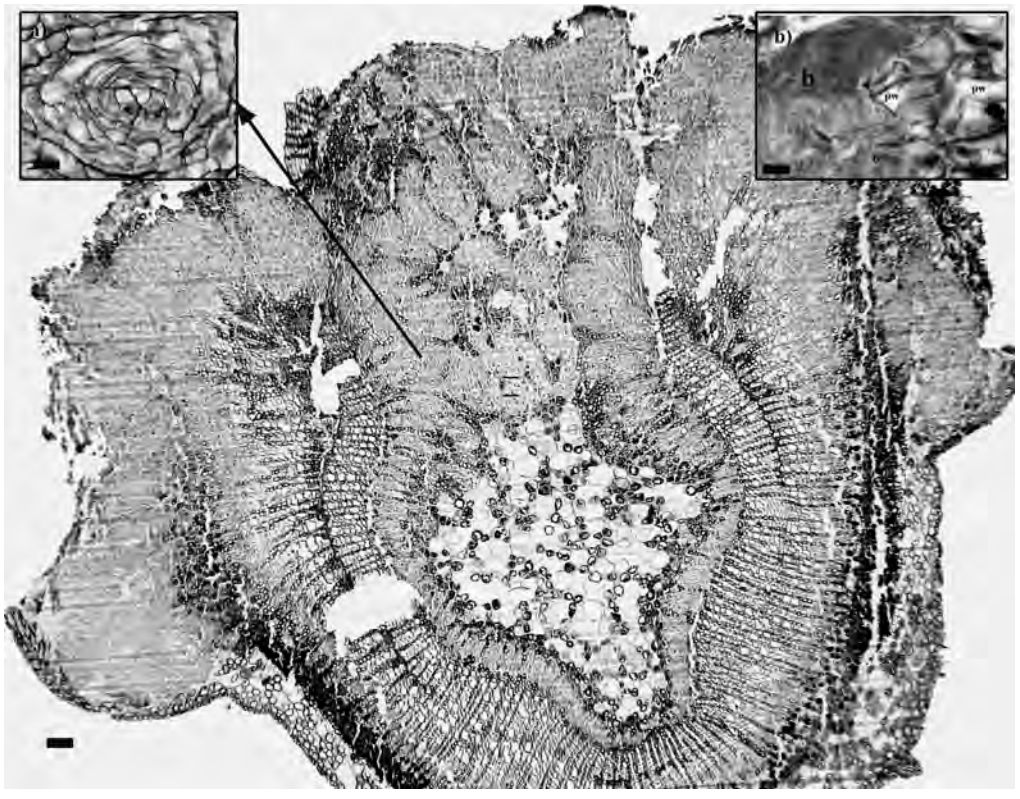

Fig. 5. Transverse section of myrtle stem, 24 days after inoculation, showing development of the knot. Bar = 200 µm. Insert a. Characteristic division of the primary xylem parenchyma with anticlinal cellular division starting in a meristematic initial cell and expanding anticlockwise. Bar = 10 µm. Insert b. Degradation of cell walls around a bacterial cavity. Bar = 10 µm.

teristic division of primary xylem parenchyma was observed, whereby anticlinal cell division started in a meristematic initial cell and expanded anticlockwise to add to the size of the knot. The newly formed cells elongated steadily as they extended away from the central spot (Fig. 5, insert a). Hyperplasic primary xylem parenchyma, together with the expanding hyperplasic tissue masses that were initiated seventeen days after inoculation, surrounded the inoculation wound to form a hyperplasic mass. Other tissue masses originating from proliferation of the above mentioned cells underwent hypertrophy and hyperplasia adding to the tissue mass originating from the primary xylem parenchyma to form the knot (Fig. 5). At this stage, plant cell walls, contiguous to bacterial cavities appeared very lightly stained and completely degraded (Fig. 5, insert b).

Thirty-one days after inoculation, stem growth was accompanied by formation of periderm which caused crushing and disappearance of the cortical parenchyma in certain sections. For this reason, in the inoculated plants, as of this date, cortical parenchyma participated only partially in the development of the knot. In addition, new xylem elements were observed in the growing knot. This xylem had two possible origins; either secondary xylem (Fig. 6) that continued to develop in the hyperplasic mass or newly formed tracheids (Fig. 6, insert) that differentiated in all the hyperplasic masses.

Forty-five days after inoculation, the knot developed outward and nearly surrounded the whole stem. The knot, by this time, was formed by aggregation of a multitude of hyperplasic masses. These hyperplasic masses adhered to each other by parenchyma cells derived principally from the division of secondary parenchyma and parenchyma rays (Fig. 7). Each hyperplasic mass consisted entirely of tracheids deposited concentrically, often around a bacterial cavity (Fig. 7, insert), and separated by parenchyma cells. Moreover, secondary xylem continued to develop in some of the masses. The aggregate of hyperplasic masses gave a characteristic multilocular shape (Fig. 7) around the inoculation site. The surface of the knot was covered by wound periderm.

By sixty days after inoculation, lignin had been deposited on the walls of all parenchyma cells in the knot. Bacterial cavities opened to the outside through fissures formed by the cracking of lignified parenchyma tissues (Fig. 8).

In control plants, inoculated with sterile water, necrotic cells were observed around the inoculation

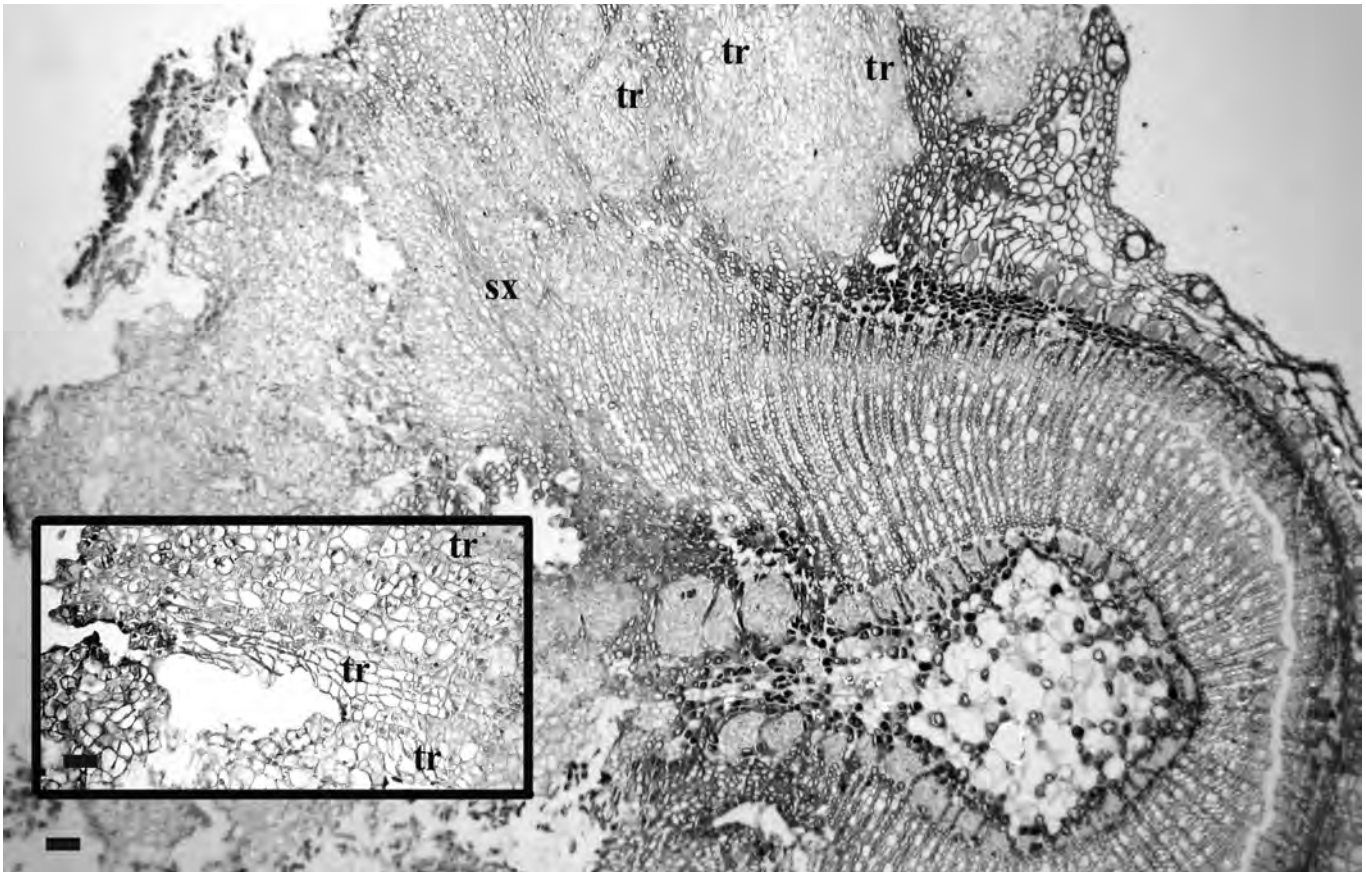

Fig. 6. Transverse section of myrtle stem, 31 days after inoculation, showing tracheid elements (tr) and secondary xylem (sx) in the knot. Bar = 200 µm.

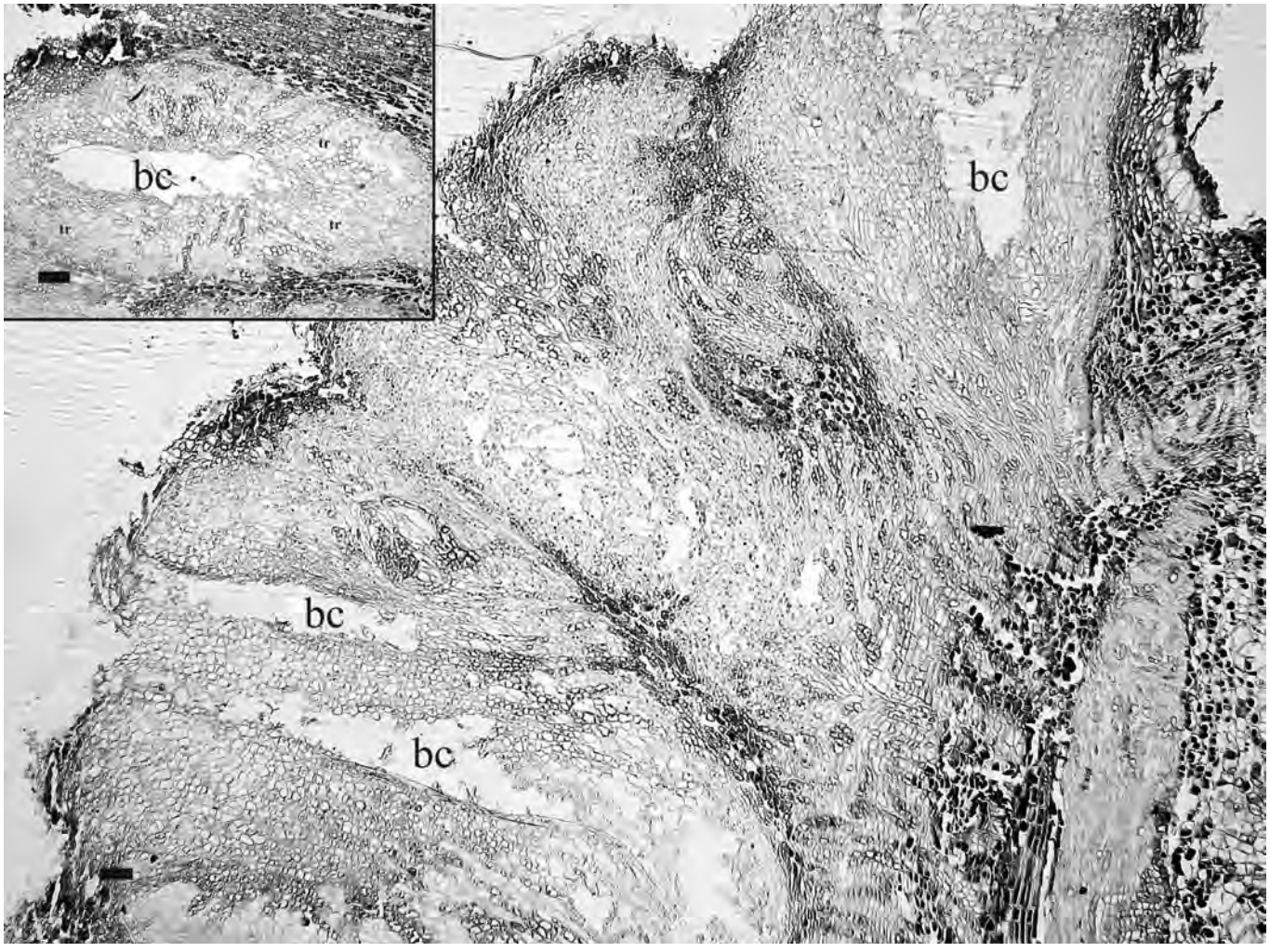

Fig. 7. Transverse section of myrtle stem, 45 days after inoculation, showing a knot that developed following a deep inoculation wound; the knot is formed by the fusion of many tissue masses. Bar = 200 µm. Insert A hyperplasic tissue mass with xylem elements occupying a bacterial cavity (bc). Bar = 100 µm.

wound, with supplementary layers of suberized cells of wound periderm differentiated below the necrotic cells. Hyperplasic activity was weak and limited around the inoculation wound. Forty-five to sixty days after inoculation, the inoculation wound was entirely closed internally by callus tissue, and surrounded outside by several layers of suberized cells of wound periderm underneath necrotic cells.

Inoculation wounds with bacterial inoculum when limited to the cortex resulted in smaller sized knots. The proliferating tissue masses forming the knots originated from the cortex parenchyma, secondary parenchyma and the phellogen. Consequently, 2 or 3 hyperplasic masses developed externally around an inoculation wound (Fig. 9). Moreover inoculation wounds that reached the pith resulted in hypertrophy and hyperplasia of pith cells, forming tissue masses. The pith tissue masses, however, were of smaller size and were outgrown by the tissue masses that grew from parenchyma cells of other tissues.

DISCUSSION

Our results helped to clarify the different stages of disease progress from inoculation of stem tissues until development of the mature knot and its subsequent death.

During the early stages of invasion in myrtle, the bacteria spread into host tissues from the inoculation wound through the intercellular spaces. This is the same mode of spread as observed in ash (Janse 1982), buckthorn (Temsah *et al*., 2007), oleander (Wilson 1965) and olive (Surico 1977).

The bacteria invaded adjacent parenchyma cells in myrtle, degrading portions of the adjacent cell walls, and continued to multiply and colonize infected tissues degrading further cell walls and leading to separation and death of cells. Complete degradation of cell walls occurred later than seventeen days after inoculation upon accumulation of large masses of bacteria, while in

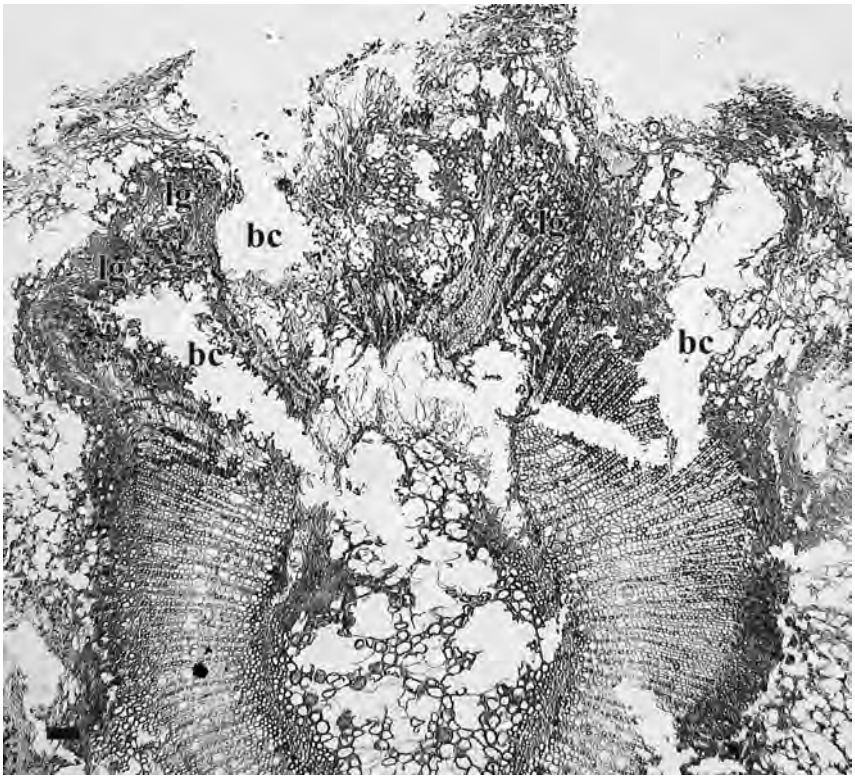

Fig. 8. Transverse section of myrtle stem, 60 days after inoculation, showing deposition of lignin (lg) on the walls of knot parenchyma cells, and bacterial cavities exposed to the outside through fissures. Bar = 200 µm.

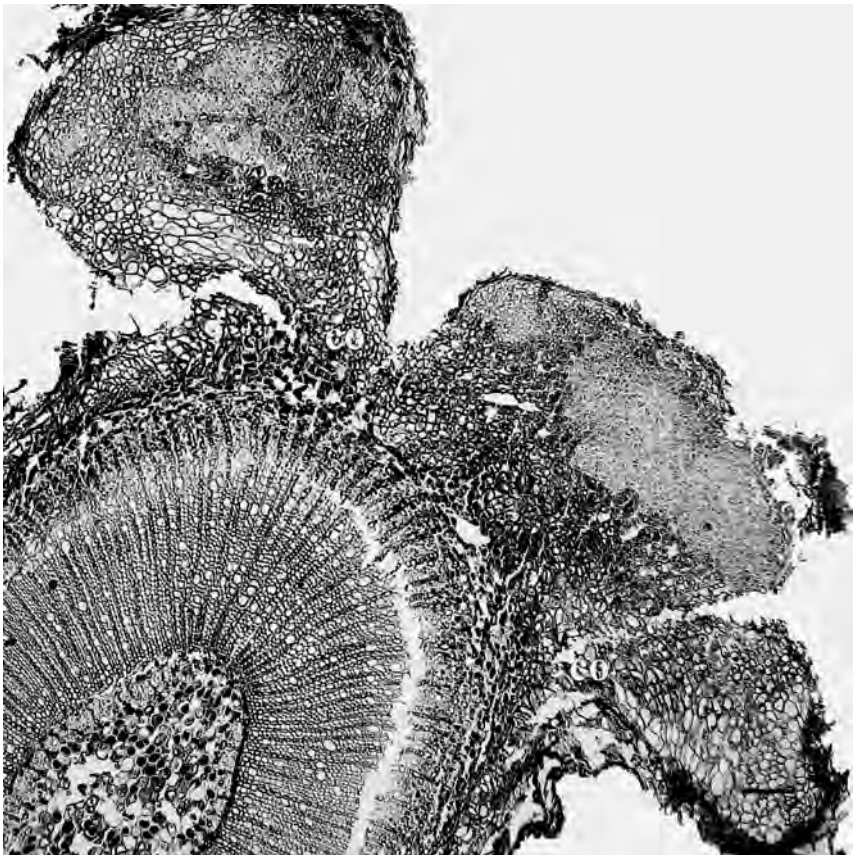

Fig. 9. Transverse section of myrtle stem, 45 days after inoculation, showing a knot that developed after a shallow inoculation wound that reached the cortex (co). In this case the knot is formed of 2 or 3 hyperplasic masses of limited size. Bar = 200 µm.

buckthorn, cell walls were reported to be completely degraded six days after inoculation (Temsah *et al.*, 2007), and early after inoculation in ash (Janse, 1982) and in oleander (Wilson, 1965). Irregular rupture of the cell walls in myrtle suggests enzymatic degrading activities by the bacteria. Moreover, the larger quantities of bacteria required to completely degrade the cell walls in myrtle allude to the higher quantities of enzymes required (Walton, 1994). The enzymatic degradation of cell walls by *Pseudomonas savastanoi* was also suggested as a mode of infection by *P. savastanoi* on buckthorn (Temsah *et al.*, 2007), and ash (Janse, 1982), while Magie (1963) reported that *P. savastanoi* from oleander did produce degrading enzymes *in vitro*.

The knot becomes evident in myrtle four weeks after inoculation compared to fifteen days after inoculation in buckthorn, oleander and olive (Wilson, 1965; Surico 1977; Saad and Hanna, 2002). Hypertrophy and hyperplasia leading to knot formation and subsequent differentiation of xylem in the knot indicates an increase in the amount of auxins. This same phenomenon was reported on oleander by Wilson and Magie (1964) and on olive by Surico *et al.* (1985) who indicated that differentiation of xylem in the knots is also related to a high level of IAA production by the pathogen. Furthermore, the large number of tracheids in the knot in myrtle indicates the production of higher amounts of IAA by the bacterium than in other hosts. In fact, strains of *P. savastanoi* from myrtle did produce higher amounts of IAA *in vitro* as compared to those from ash, buckthorn, oleander and olive (Saad and Hanna, 2002). Numerous small fragments of xylem in the knots usually developed close to bacterial cavities, the source of IAA (Aloni and Zimmerman, 1984; Aloni, 1987). Moreover, the secondary xylem linked with the axial xylem of the stem continued developing into the knot, thus contributing to its growth (Cornelia and Aloni, 2000).

The size of the knot depends on the depth of the inoculation wound and type of tissues reached. Similar findings were reported on buckthorn (Temsah *et al.*, 2007).

The characteristic meristematic divisions of the primary xylem parenchyma observed in myrtle was not reported to occur in the other hosts of *P. savastanoi* and can be linked to the presence of bacteria in the primary xylem. One cell of primary xylem parenchyma, in contact with the primary xylem, dedifferentiates and acquires the capacity of anticlinal division and expanding in an anticlockwise manner, not observed in hyperplasic cells of other types of tissues.

The fact that, in myrtle, no periderm was observed to form around the bacterial cavities allowed the growth of a hyperplasic tissue mass around each bacterial cavity and thus gave the characteristic rosette shape of the knot. In contrast, periderm formation was observed to limit bacterial spread and activity around the bacterial cavities in ash (Janse 1982), buckthorn (Temsah *et al.*, 2007), olive (Surico 1977), and oleander (Wilson 1965).

The defense mechanisms of the plant to limit the size of the knot and bacterial invasion was manifested by a series of reactions that come in succession depending on the progress of invasion by the pathogen. The necrotic cells, resulting from the inoculation wound, and the wound periderm differentiated below the necrotic cells limit the advance of the pathogen. Furthermore, the several layers of suberized cells of the wound periderm forming around the knot prevent its enlargement and attack by other external organisms. Such defense reactions were also reported to occur in buckthorn, olive and oleander, the other hosts of *Pseudomonas savastanoi*, however, at differing phases of knot formation.

Finally, lignin deposits on the walls of knot parenchyma cells in myrtle lead to halting the growth of the knot and its hardening. This was not reported to occur in the other hosts of *P. savastanoi*.

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