ANATOMICAL PATHOGENESIS OF *PSEUDOMONAS SAVASTANOI* ON OLIVE AND GENESIS OF KNOTS

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SUMMARY

The development of the olive knot disease was studied taking into consideration the anatomical changes in the different parts of inoculated twig tissues, the genesis of knots and the defense reactions of the plant. At the inoculation site, bacterial inoculum invaded the cortical parenchyma, moving into the intercellular spaces and degrading the primary cell walls. The proliferation and advance of the pathogen into the different tissues of the twig was accompanied by the hyperplastic activity of parenchyma cells in affected tissues, resulting in the development of the knot. The knot was made up of hyperplastic cells, numerous cambia, and xylem elements of different origins. Cells with lignified walls surrounded bacterial cavities and the outer surface of the knots was surrounded with new periderm. At later stages of knot development, hyperplastic activities of new tissue masses exerted pressure on the periderm at the surface of the knot and induced fissures that cracked open to the outside exposing bacterial cavities.

Key words: Histopathogenesis, anatomy, *Olea europea,* olive knot, bacterial disease.

INTRODUCTION

The olive tree (*Olea europaea* L.), valued for its fruit, oil, and wood, is a species native to the countries bordering the Mediterranean Sea and is linked to the civilizations of this region since time immemorial. Most of the world's olive trees are still grown in the Mediterranean basin.

In Lebanon, serious losses to the olive and oil industries are caused by peacock eye leaf spot disease, caused by the fungus *Spilocea oleagina,* followed by olive knot caused by *Pseudomonas savastanoi* pv. *savastanoi* [(ex Smith 1908) Gardan *et al*., 1992; Saad and Melkonian, 1992]. Characteristic symptoms of the latter disease are the formation of outgrowths in affected host tissues, called knots. Besides olive the disease attacks oleander (*Nerium oleander* L.), ash (*Fraxinus* spp.), jasmine (*Jasminum* spp.), privet (*Ligustrum* spp.), *Forsythia* spp., *Phillyrea* spp. (Bradbury 1986), myrtle (*Myrtus communis* L.), and buckthorn (*Rhamnus alaternus* L.) (Saad and Melkonian, 1992; Saad and Hanna, 2002).

The present investigation was conducted to study knot genesis and development on young olive twigs, at specific time intervals, after artificial inoculations, and to compare it with the anatomical pathogenesis of *P. savastanoi* on other hosts. Defense reactions of olive in response to infections by *P. savastanoi* were also studied. Results of this study were compared with those of comparable investigations on hosts infected by *P. savastanoi*, i.e. ash (Janse 1982), buckthorn (Temsah *et al.*, 2007a), myrtle (Temsah *et al.*, 2007b), and olive (Surico 1977).

MATERIALS AND METHODS

One-year-old olive seedlings were inoculated with a virulent strain of *P. savastanoi,* isolated from active knots of naturally infected olive twigs collected from North Lebanon. The bacterial isolate was characterized by the LOPAT scheme of tests for grouping green fluorescent pseudomonads (Lelliot and Stead 1987) and its identity confirmed by pathogenecity tests (Saad and Hanna 2002). A type culture of the *P. savastanoi* isolate used in this study was deposited at the Culture Collection of the Istituto Tossine e Micotossine da Parassiti Vegetali, CNR, Bari, Italy.

Bacterial inoculum was prepared in sterile water, from 24 h cultures on nutrient agar (DIFCO), and standardized turbidimetrically to a concentration of 10^7 cfu/ml^{-1}. Rooted olive cuttings were grown in 30 cm pots and their twigs were inoculated as described by Temsah *et al.* (2007b). Plants were then kept in a glasshouse at a temperature of 20-24°C and 70% RH, the optimal conditions for the growth of *P. savastanoi* (Wilson, 1935), for the 55 day duration of the experiment.

The pathological and anatomical features of knot development, on inoculated twigs of olive, were studied

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using histological techniques and light microscopy. Macroscopic observations on knot development and collection of samples for histological studies were done at 1, 3, 6, 9, 12, 15, 30, and 55 days post inoculation (d.p.i.). Diseased tissue samples, collected from inoculated twigs, were fixed, paraffin-embedded, microtome-sectioned, and stained according to the procedures by Jensen (1962) and Ruzin (1999), as detailed by Temsah *et al.* (2007a). Stained sections, 10 µm thick, were mounted in Permount mounting medium and observed with a light microscope.

RESULTS

Macroscopic observations of inoculated olive twigs revealed the appearance of small swellings at the inoculation point 9 d.p.i. These swellings grew to become clearly visible knots by 15 d.p.i.

Transverse sections of a healthy olive twig, between the third and fourth node (Fig. 1), showed the following sequence of tissues (exterior to interior): (i) a cutinized epidermis with stomata and star trichomes; (ii) a collenchyma consisting of 3-4 layers of cells; (iii) several layers of cortical parenchyma cells separated by intercellular spaces; (iv) sclerenchyma tissue distributed in discontinuous masses, with fibers becoming thick walled with a reduced lumen as the twigs age; (v) vascular tissues including primary phloem, cylindrical secondary phloem with phloem parenchyma cells, cylindrical secondary xylem made up of vessels, fibers, xylem parenchyma cells and primary xylem; (vi) cambium; and (vii) pith in the center of the twigs. The secondary vascular tissues also included parenchyma rays made up of one or two layers of cells and, as the twig aged, a phellogen that differentiated below the epidermis producing phellem (cork) towards the exterior and phelloderm towards the interior.

During the first 24 h after inoculation the bacteria were present in twig tissues reached by the inoculation needle. No observable anatomical changes were noticed.

Three d.p.i., parenchyma cells around the inoculation wound become necrotic. Adjacent to necrotic cells, hyperplastic parenchyma cell activity was observed in the cortex and the vascular tissues and parenchyma rays leading to the formation of new hyperplastic tissue masses. The cambium developed also in the new tissue masses and small xylem elements (spiral tracheids) differentiated in the hyperplastic masses.

Six d.p.i., bacteria had penetrated the intercellular spaces of cortical parenchyma tissue, degrading cell walls and creating cavities in which they multiplied (Fig. 2). Degradation of cell walls was discontinuous, and cells with complete cell wall degradation became re-

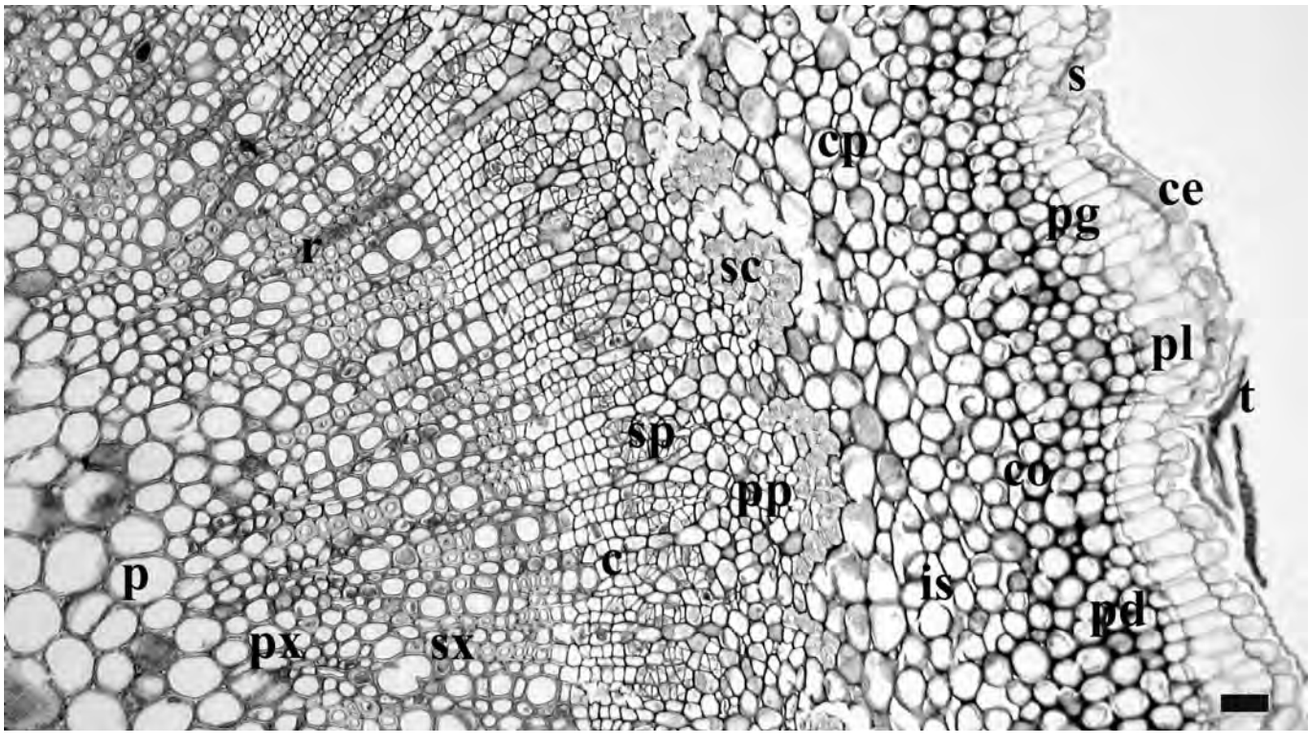

Fig. 1. Cross section of an healthy olive twig of , between the second and third node from the top, showing: cutinized epidermis (ce) with stomata (s) and trichomes (t); periderm consisting of phellem (pl), phellogen (pg), and phelloderm (pd); collenchyma (co), cortical parenchyma (cp), with intercellular spaces (is); sclerenchyma (sc); primary phloem (pp); secondary phloem (sp); parenchyma rays (r); cambium (c); secondary xylem (sx); primary xylem (px); and pith (p). Bar = 100µm.

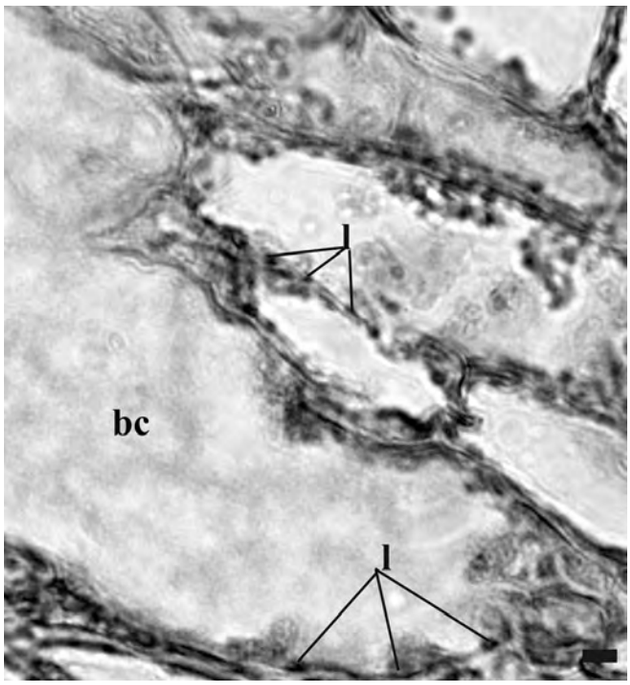

Fig. 2. Longitudinal section of an olive twig 6 d.p.i., showing lignin (l) deposits on the inside of primary cell walls of parenchyma cells around a bacterial cavity (bc). Bar = 10µm.

duced to a protoplasmic mass. Lignin deposits, as revealed by the safranin stain, appeared on the cell walls of parenchyma cells around bacterial cavities and injured tissues. At this stage collenchyma cell walls were not degraded. Coenocytic tetranuclear cell division activity occurred in the cortical collenchyma cells (Fig. 3A) and octonuclear cell division activity in the cortical parenchyma cells (Fig. 3B). When the inoculation wound reached the vascular tissues, parenchyma ray cells underwent periclinal division and dedifferentiated into cambium. Nine d.p.i. the same activities continued, resulting in increased size of new tissue masses and in more numerous bacterial cavities.

Twelve d.p.i., the bacteria continued to spread and invade the different tissues of the twig and, while advancing, they degraded the primary cell walls of parenchyma cells of the cortex, vascular tissues and pith, forming additional bacterial cavities. Within these cavities, portions of undegraded middle lamella were observed, drawing the contour of degraded cells (Fig. 4). Collenchyma cells in contact with bacterial cavities were also degraded. Octonuclear coenocytic divisions of pith cells (Fig. 3B) and differentiation of parenchyma cells into xylem elements were observed, adjacent to bacterial cavities, Cell walls of parenchyma cells around bacterial cavities became lignified. New cambia, independent of the cambium (Fig. 5) were produced by periclinal division of dedifferentiated

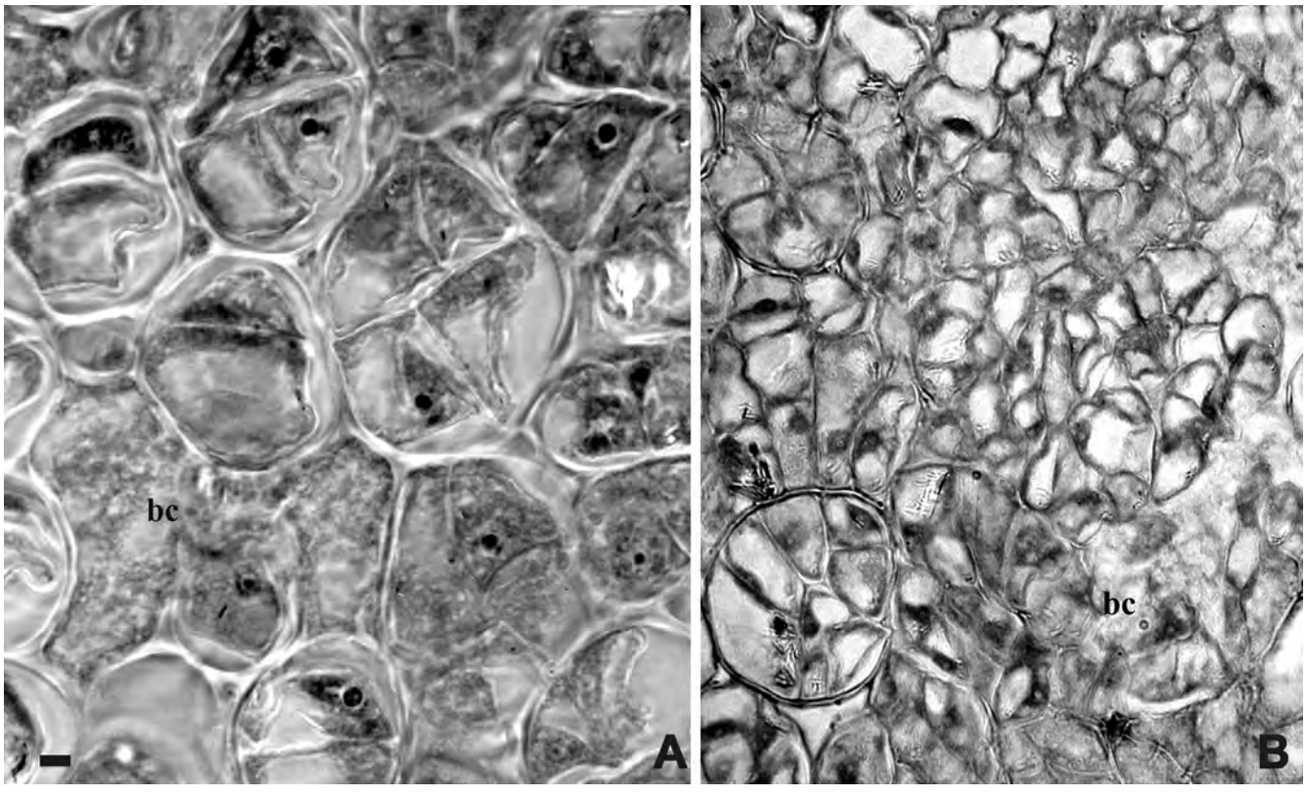

Fig. 3. Transverse section of an olive twig, showing (A) tetranuclear coenocytic division of cortical collenchyma cells, 6 d.p.i. and (B) octonuclear coenocytic division of pith cells 12 d.p.i. bc = bacterial cavity. Bar = 10µm.

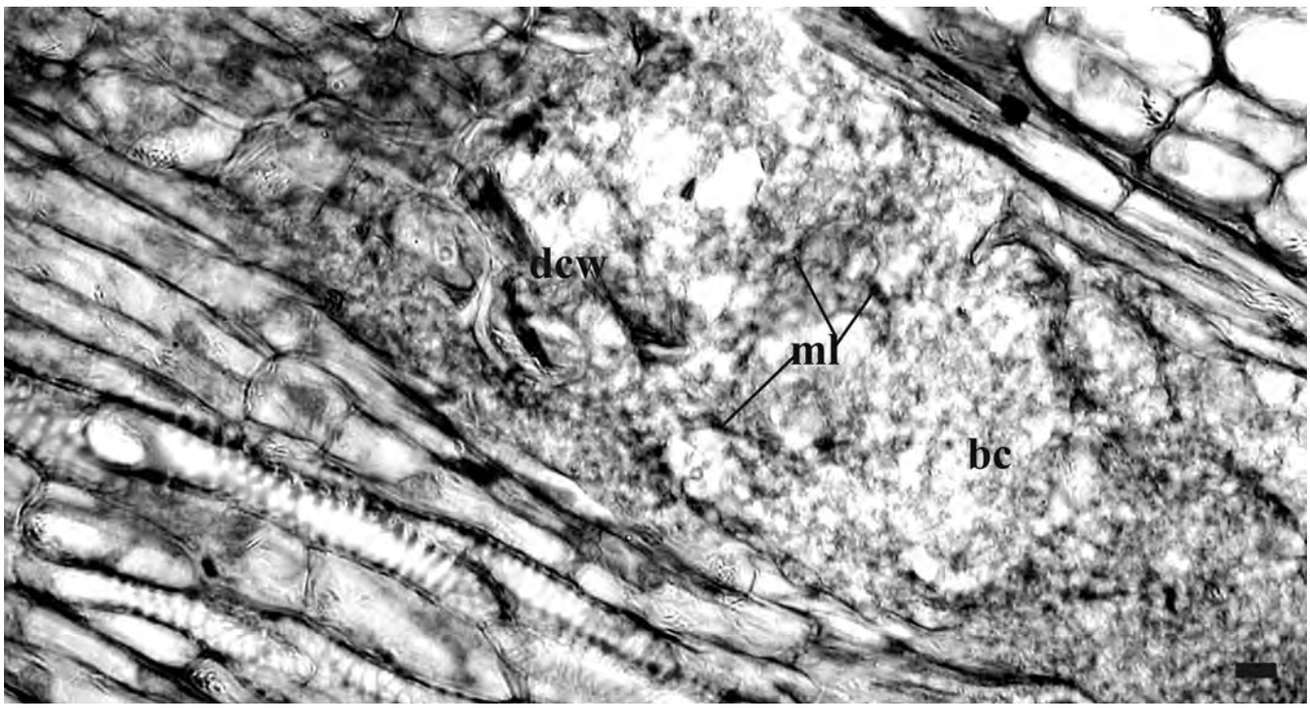

Fig. 4. Longitudinal section of an olive twig, 12 d.p.i., showing degraded cell walls (dcw) and portions of middle lamella (ml) within bacterial cavities (bc). Bar = 10µm.

parenchyma cells of the cortex, phloem, xylem, and parenchyma ray cells. New cambia were also produced by dedifferentiated cells of new tissue masses. The new cambia produced xylem elements, some of which were not centripetally oriented, in contrast to those of the xylem of the twig (Fig. 5, insert).

Fifteen d.p.i., the knot continued to develop because of the hyperplastic activity of parenchyma cells of different tissues, cortex and phloem in particular. New cambia became more abundant.

Thirty d.p.i., the cambium extended towards the external side of the knot, producing secondary xylem and phloem tissues close to the knot surface (Fig. 6). Other xylem elements were also produced from the new cambia. Among the new tissue masses, sectors of original twig tissues were still visible within the growing knot tissues. The impregnation of lignin in the cell walls of the pith cells resulted in halting hyperplastic activities in the pith. Xylem elements were distributed haphazardly in islets, in linear arrangement or concentrically around bacterial cavities, where they were particularly abundant. Xylem elements were also abundant close to the surface of the knot which was covered by periderm originating from the new phellogen, which dedifferentiated from cells of new tissue masses. The thickness of cork cell layers increased in contact with bacterial cavities. Bacterial cavities that had grown in size, opened at the surface of the knot, as a result of cork cracking.

Fifty five d.p.i., the knot increased in size to about 2.0

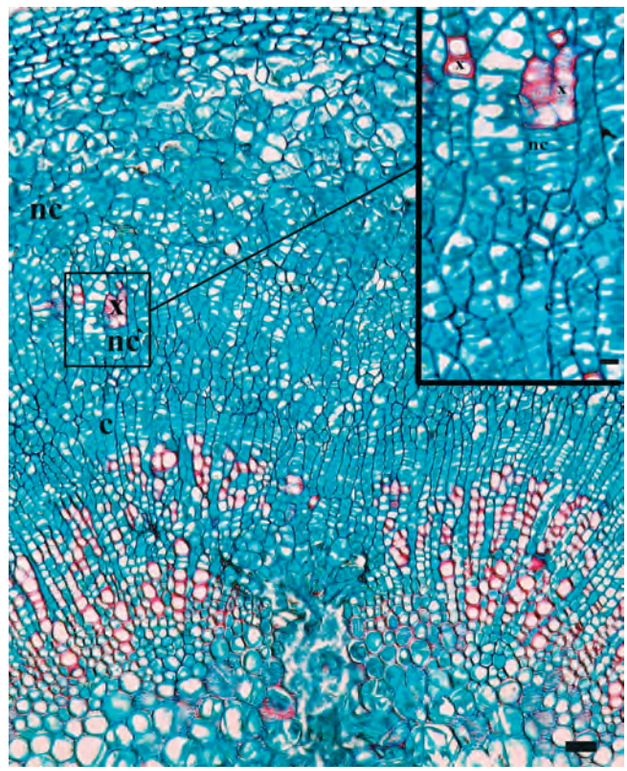

Fig. 5. Transverse section of an olive twig 12 d.p.i., showing: cambium(c); differentiated new cambia (nc), in cortical parenchyma and phloem parenchyma, secondary xylem (sx); and centrifugally oriented xylem elements (x) (insert). Bar = 10µm.

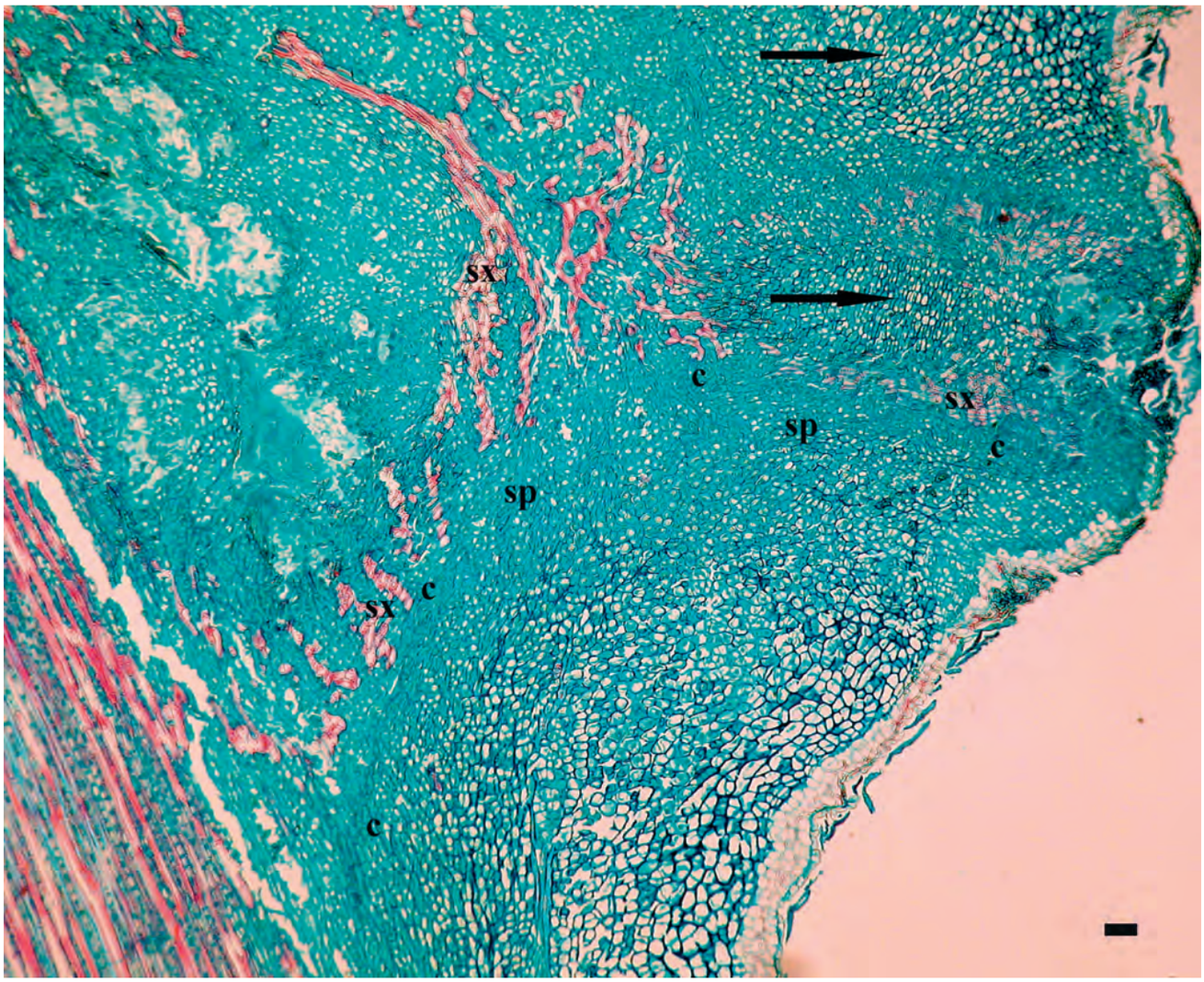

Fig. 6. Transverse section of an olive twig 30 d.p.i, showing induced development of cambium producing secondary xylem (sx) and secondary phloem (sp) towards the knot surface, arrow showing original stem tissues. Bar = 200µm.

by 2.5 cm. At this stage, hyperplastic activity of phelloderm and cortical parenchyma, in particular, contributed to the increase in the size of the knot. Hyperplastic cells of dedifferentiated phelloderm contributed to the production of new cambia. New phellogen differentiated producing cork towards the exterior below preexisting periderm (Fig.7). Fissures arose as a result of the pressure created from continued hyperplastic activities. As the fissures widened the periderm broke open exposing superficial and deep cavities filled with bacteria (Fig. 8).

In the controls (olive twigs inoculated with water), some hyperplastic cell activity was noticed, limitedly to the area surrounding the inoculation wound. Twelve d.p.i. mitotic cell activity ceased with a complete closure of the wound. Thirty d.p.i., the pith of control twigs became entirely lignified. Normally, in healthy non-inoculated olive stems, the pith gets entirely lignified from about thirty days onwards.

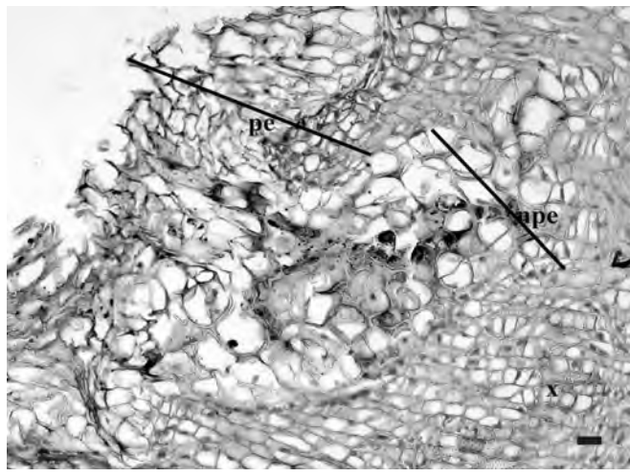

Fig. 7. Transverse section of an olive twig 30 d.p.i., showing new periderm (npe), differentiated below preexisting periderm (pe). Bar = 50µm.

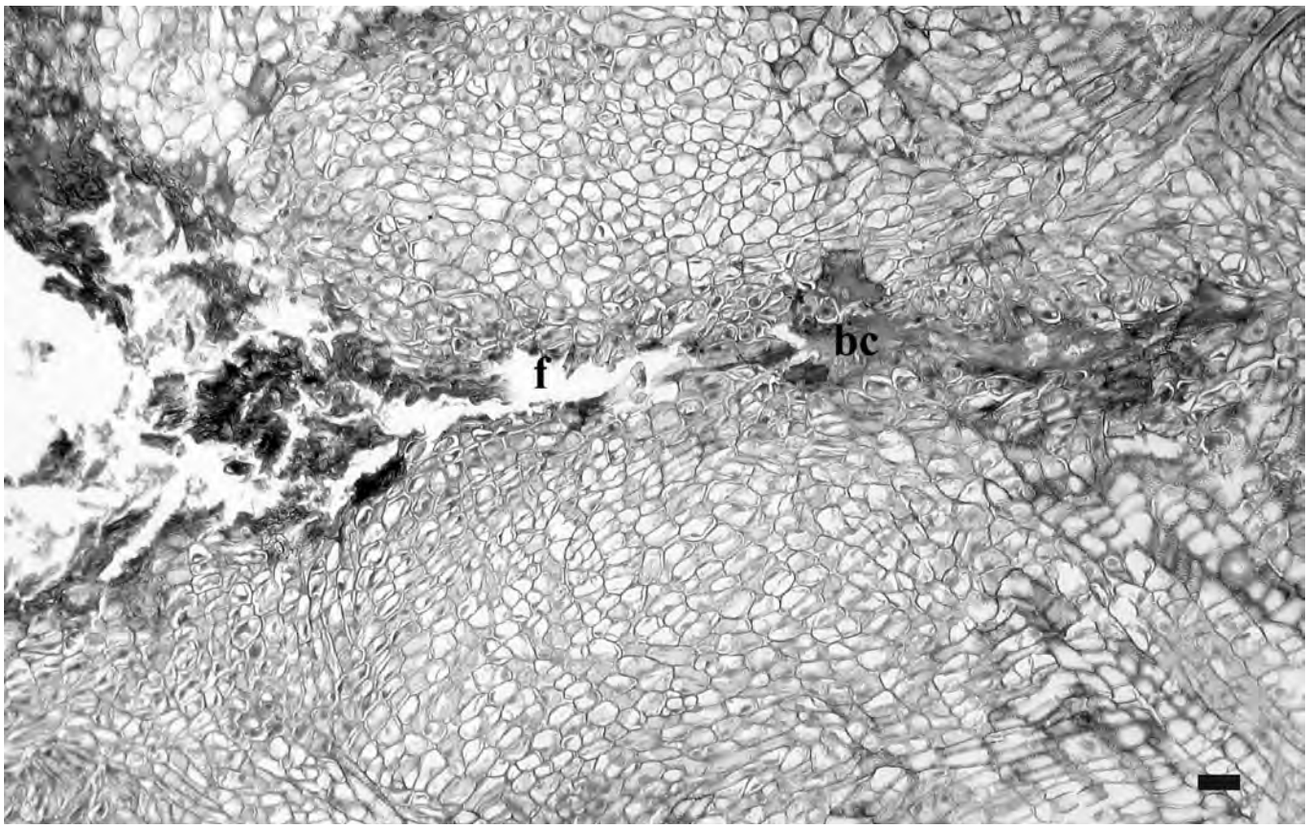

Fig. 8. Transverse section of an olive twig 55 d.p.i., showing a fissure (f) in the knot, and broken periderm (bp). Bar = 100µm.

DISCUSSION

This study focused on the genesis of knot formation on olive, anatomical changes in the different parts of infected twig tissues, as well as possible defense reactions of the plant in response to infection by *Pseudomonas savastanoi*.

The findings were compared with those of similar studies on olive (Surico 1977), ash (Janse 1982), buckthorn (Temsah *et.al.*, 2007a), and myrtle (Temsah *et.al.*, 2007b).

Host invasion by the pathogen. Invasion of inoculated tissues by *Pseudomonas savastanoi* starts with the ingress of bacterial inoculum into the intercellular spaces of cortical parenchyma, leading to degradation of cell walls and to the formation cavities in which they multiply and then spread to parenchyma cells of other tissues. This mode of bacterial invasion of host tissues was also reported to occur in the other hosts of *P. savastanoi* including ash (Janse 1982), buckthorn (Temsah *et al.*, 2007a) and myrtle (Temsah *et al.*, 2007b).

Genesis and origin of knots Three days after inoculation hyperplastic activities of parenchyma cells adjacent to bacterial cavities formed initials of tissue masses that developed into knots. During the early stages of invasion, hyperplastic activities of parenchyma cells of the cortex, xylem, phloem and rays participated in the formation of the knot. Six d.p.i, hyperplastic activities continued with tetranuclear coenocytic division of collenchyma cells and octonuclear divisions of cortical parenchyma and pith cells. Octonuclear cell division was not reported to occur in ash (Janse 1982), buckthorn (Temsah *et al.*, 2007a), or myrtle (Temsah *et al.*, 2007b).

In olive twigs, pith cells adjacent to bacterial cavities became lignified and all subsequent hyperplastic activities in the pith tissue ceased. This differs from what was observed in buckthorn (Temsah *et al.*, 2007a) and myrtle (Temsah *et al.*, 2007b) where the size of the knot was bigger when the inoculation wound was deep, involving hyperplastic activities of ray parenchyma and pith. Development of the knot in olive was primarily due to the hyperplastic activities of the cortex (phelloderm and cortical parenchyma) and phloem parenchyma. In contrast to what occurs in olive, the size of the knots in myrtle and in buckthorn was restricted when knots originated from the parenchyma cells of the cortex.

In olive, phloem parenchyma cells had an important role in the formation of the knot, whereas in buckthorn, knot development was primarily from ray parenchyma cells (Temsah *et al.*, 2007a) and in myrtle, it was mainly

due to the hyperplastic activity of primary xylem parenchyma (Temsah *et al.*, 2007b). According to Surico (1977), knot formation in olive was limited to the hyperplastic activities of preexisting or neoformed cambium cells. However, our observations showed that genesis of the knot in olive was triggered by the hyperplastic activity of parenchyma cells and cambium. Proliferation of hyperplastic cells and hypertrophy of tissues denote the involvement of growth regulators. In fact, the *P. savastanoi* strains used in this study produced indole acetic acid (IAA) *in vitro* (Saad and Hanna 2002), in agreement with the findings by Surico (1985) who reported the involvement of IAA and cytokinins in knot formation in olive.

Histological structure of knots. The knots of olive were composed of hyperplastic cells, numerous cambia, xylem elements and periderm. In buckthorn, knots had a similar composition but with a lower frequency of cambia (Temsah *et al.*, 2007a) while in myrtle knots were composed of irregular hyperplastic masses made up mainly of xylem elements (tracheids) around large bacterial cavities (Temsah *et al.*, 2007b). The new cambia in olive presently observed may originate from: (i) the preexisting cambium, which continues to develop in the knot and produces secondary xylem and secondary phloem; (ii) dedifferentiated parenchyma cells; and (iii) dedifferentiated knot cells. Surico (1977) reported that the new cambia in the knot derive solely from the activity of the preexisting cambium.

Xylem elements of the knots were disorderly. In olives, they appeared as early as 3 d.p.i, (this study) or 5 d.p.i. (Surico 1977) but much later (31 d.p.i.) in myrtle (Temsah *et al.*, 2007b) and in buckthorn (54 d.p.i) (Temsah *et al.*, 2007a). At later stages of knot development, xylem elements in olive knots were in a linear arrangement, in islets, and concentric around bacterial cavities. In buckthorn, xylem elements were in islets and linear arrangements only (Temsah *et al.*, 2007a) and in myrtle, they were arranged mainly concentrically around bacterial cavities (Temsah *et al.*, 2007b). The differentiation of xylem elements suggests the involvement of growth regulators produced by the bacteria as reported before (Wilson 1965; Saad and Hanna 2002; Best *et al.*, 2004).

Bacterial cavities were restricted in size as a result of their becoming surrounded with lignified cells in olive, and formation of periderm in buckthorn (Temsah *et al.*, 2007a). However, in myrtle such phenomena were not observed and, consequently, bacterial cavities were much larger in size and the frequency of xylem elements was much higher than in the other hosts. This difference in olive and buckthorn versus myrtle explains the higher frequency of hyperplastic cell masses occurring in olive and buckthorn in comparison to those found in myrtle. In all *P. savastanoi* hosts studied, the knots were covered with periderm.

Plant defense reactions. Olive twig tissues inoculated with *P. savastanoi* exhibited defense reactions that varied according to the stage of disease development. Lignin deposits in cells around bacterial cavities in olives and periderm formation around bacterial cavities in buckthorn limited the advance of the pathogen. In myrtle, no such defense phenomena were observed (Temsah *et al.*, 2007b). New periderm was found to differentiate at the surface of the knots in olive, forming a barrier that impaired the entrance of other pathogens and saprophytes. Periderm formation around the knot was also reported to occur in buckthorn and myrtle (Temsah *et al.*, 2007a, 2007b).

Deterioration and decline of the knots in olive resulted from the pressure exerted by the activity of the hyperplastic masses towards the surface, which cracked the periderm, producing fissures and exposing bacterial cavities to the outside, which favours the dissemination of the pathogen. By contrast, deterioration and decline of the knots in myrtle originate from the lignification of knot cells (Temsah *et al.*, 2007b).

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