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Aleppo pine knot disease: histology of the knots, detection of causal agent and mode of transmission

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Summary. Knot disease of Aleppo pine (*Pinus halepensis* subsp. *halepensis*) occurs in the western range of the host in the Mediterranean region. The disease, originally named Aleppo pine tuberculosis (from tubercle, i.e. knot) by Petri in 1924, causes a hyperplastic growth on the twigs and small branches, with knots that initially are small, green and lignified, and expand to 5–6 cm diameter at maturity. These knots have been associated with various bacterial species. In this study, only one bacterium was isolated from Aleppo pine knots, and this bacterium was constantly associated with the pine weevil *Pissodes castaneus*. The hyperplastic growth started either in the cortex or in the xylem, while the knot was formed in the cortical parenchyma. Bacteria were grouped in zoogloeae in the intercellular spaces or inside lysigenous cavities. Bacterial microcolonies have also been observed in the parenchyma cells. Inoculation of two of the bacterial strains on healthy Aleppo pine twigs did not induce knot formation. Knots did however form when adults of *P. castaneus* were externally contaminated with the bacteria and were then allowed to feed on pine saplings. These latter knots contained the same bacterial isolate as that which had been used to contaminate the weevils. All the isolates examined were tentatively assigned to the genus *Erwinia*. As this bacterium seems to be the causal agent of Aleppo pine knot disease, its complete identification and characterisation is needed.

Key words: bacteria, *Pinus halepensis, Pissodes, Erwinia,* knot anatomy, tuberculosis.

Introduction

Bacteria are less common than fungi as agents of disease in forest and shade trees. However, some bacteria (e.g. *Erwinia amylovora*, *Agrobacterium tumefaciens*, *Pectobacterium numipressuralis*, *Xanthomonas populi*) pose a serious threat to tree health (Tainter and Baker, 1996). A bacterium has also been associated with a disease of conifers originally named Aleppo pine tuberculosis, now Aleppo pine knot disease which affects Aleppo pine (*Pinus halepensis* Miller) throughout Spain, France and western Italy (Lanier *et al.*, 1976; Liguria Forest Service, personal communication) (Fig. 1). Aleppo pine knot is characterised by the formation of knots on the twigs and branches and is known to have caused significant tree mortality in past time (Prillieux, 1889; Petri, 1924). Knots on the twigs and branches appear as hyperplastic excrescences, initially small, round, smooth and green (Fig. 2), becoming lignified upon maturity, when they can reach a diameter of 5–6 cm (Fig. 3). With time they

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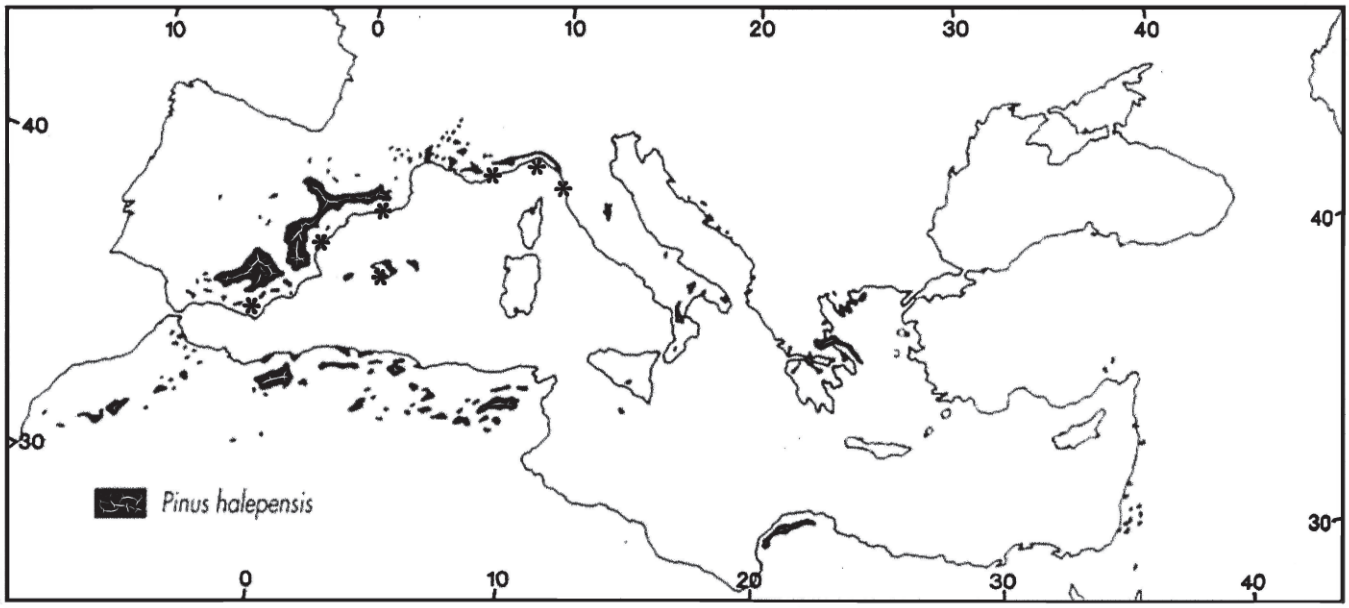

Fig. 1. Occurrence of Aleppo pine knot disease in the Mediterranean region (∗) based on literature data and authors' observations.

undergo degenerative processes, mainly associated with insect and microbial colonisation. (For a detailed description of the disease see Petri, 1924.) A similar disease was reported nearly a century ago for the Swiss stone pine, *Pinus cembra* L., in the Alps (von Tubeuf, 1910, 1911) and more recently also for *Pinus sylvestris* in northern Italy (Forest Service Survey, personal communication).

The causal agent of Aleppo pine knot disease was first reported to be *Bacillus vuillemini* by Trevisan (1889), then *Bacillus pini* Kruse 1896, *Bacterium pini* (Kruse) Chester 1897, and *Aplanobacter pini* (Kruse) by Magrou and Prévot (1948). Petri (1924) isolated what he thought was the causal bacterium from knots on Aleppo pine growing near Livorno (coastal Tuscany, Italy), and identified it as *Pseudomonas pini*. Finally, Eskandari and Ridé (1967) isolated a bacterium assigned to the genus *Corynebacterium* and succeeded in inducing the knots by the artificial inoculation of this bacterium. Nonetheless, the causal agent of Aleppo pine knot disease does not at present figure in the Approved List of Names of Plant Pathogenic Bacteria 1864–1995 (Young *et al.*, 1996, 2004).

Petri (1924) and Dufrenoy (1925) studied the histology of the knots and distinguished between superficial knots and deep knots, depending on the depth in the wood ring where the knot formation started. These researchers further hypothesised that the disease was spread by insects feeding on the knots, such as the moth *Dioryctria sylvestrella* (Ratzeburg) (= *splendidella*) (Lepidoptera, Pyralidae) or the aphid *Eulachnus agilis* (Kaltenbach) (Hemiptera, Aphididae). However, the only evidence for this type of transmission were traces of the rostrum of the aphid which Petri (1924) found in the knots and the cortical parenchyma of young diseased branches.

The aim of the present work was to reconsider histological and aetiological aspects of Aleppo pine knot disease, and to identify the causal bacterium by biochemical, physiological and molecular means. An investigation of the association between knots and various insects, first in field surveys, and then in bioassays under controlled conditions, has enabled the insect vectors of the disease to be identified.

Materials and methods

Plant material

Knots of different size (Fig. 2, 3) were collected in 1999–2002, by clipping off 2–5-year-old twigs from adult Aleppo pine trees (6–15 yrs old) at three sites

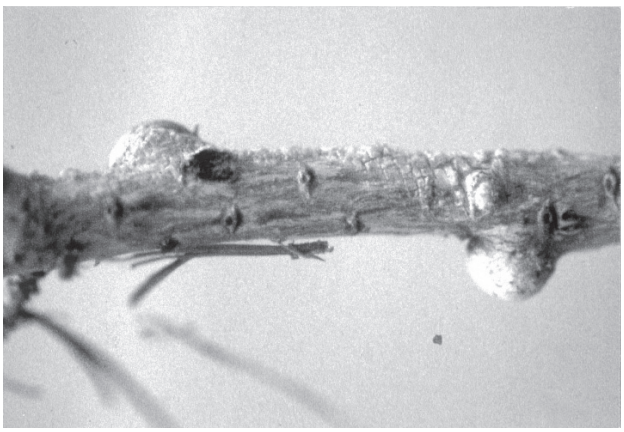

Fig. 2. A 2-year-old Aleppo pine twig with small knots.

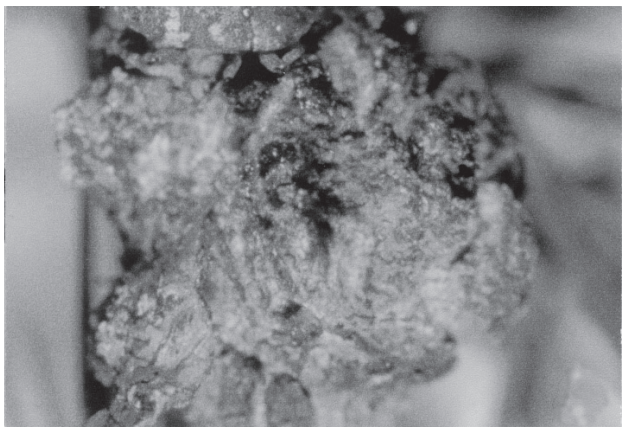

Fig. 3. A 3-year-old Aleppo pine twig with large, lignified and suberised knot.

along the northwestern coast of Italy, where Aleppo pine is native (Lerici, Monte Marcello Natural Park, La Spezia, Italy, 44°05'N 09°54'E, elevation 120 m; Bonassola, La Spezia, Italy, 44°12'N 09°25'E, elevation 150 m; Livorno, Italy, 43°27'N 10°21'E, elevation 60 m).

Knot histology

Twenty knots collected from 2–3-yr-old twigs were used for histological analysis. Knots of 3–4 mm in diameter were fixed, together with a portion of the attached twig, in a 4% formaldehyde solution in distilled water, dehydrated and embedded in Technont 7100 resin (Kulzer). Knots larger than 4 mm in diameter were detached from the twig, cut into smaller fragments, and fixed in a 2% gluteraldehyde solution buffered with PBS 0.15 M at pH 7.4–7.5. These fragments were then washed with the same buffer, dehydrated and embedded in LRWhite resin (Sigma-Aldrich, Milan, Italy).

All the material was sectioned with a microtome Model 2030 (Reichert-Jung, Vienna, Austria) into cross and longitudinal sections 2 μm thick and stained with fuchsin and Astra blue: the fuchsin stained the lignified walls red, and the organules and chromosomes reddish pink; while the Astra-blue stained the pectocellulose walls blue (Kraus *et al.*, 1998). DAPI (4'.6-diamino-2-phenylindole) showed up nuclear DNA (Williamson and Fennel, 1975), fluorescent PAS (Bruni *et al.*, 1973), staining insoluble polysaccharides in green and yellow.

Observations were carried out under a Dialux 22 microscope (Leitz Wetzlar, Wetzlar, Germany).

Bacterial isolation, growth and characterisation

Young knots and healthy portions of 2-yr-old twigs with knots were surface-disinfected by immersion in 3% NaOCl for 3 min, washed three times with a sterile saline solution (0.85 g l^{-1}) and peeled. Tissue portions, 2×3 mm, were cut aseptically and transferred to H3 medium (proteose peptone No. 3, 20 g; K_2HPO_4, 1.5 g; $MgSO_4$ $7H_2O$, 1.5 g; glucose, 10 g; agar, 15 g; distilled water, 1 l), prepared following Ridé *et al.* (1967). Petri dishes were incubated at 20°C for 8–12 days, at which time, mucous bacterial masses had developed around the knot portions. A loopful of bacteria was suspended in a drop of sterile distilled water and streaked on Petri dishes containing H3 medium. Bacteria were isolated from single colonies that appeared on the H3 medium. These colonies were circular, slightly convex, with a regular margin and a diameter of about 1–2 mm. Only one type of colony was consistently obtained from all the knots sampled. Isolations were also tried by placing ground-up knot tissue in a drop of sterile saline solution.

Two cultures of the bacterium (APT1 and APT7), out of 20 obtained, were tested for morphological, biochemical and physiological characteristics. *Pantoea agglomerans*, *Pseudomonas syringae* pv. *syringae*, and *Clavibacter michiganensis* subsp. *michiganensis* were used as controls. Cells and flagella were observed by phase-contrast light microscopy and electron microscopy. The tests used

to determine oxidase and catalase activities, potato soft rot ability, pectate degradation in Hildebrand's medium, tobacco hypersensitivity, the mode of utilisation of glucose and the Voges-Proskauer reaction were those described by Dickey and Kelman (1988) and Schroth and Hildebrand (1988). The tests to determine the Gram reaction, fluorescence on King's B medium, hydrolysis of gelatin, casein, starch and aesculin were carried out following the methods of Lelliott and Stead (1987). Growth of the bacterial cultures on H3 in anaerobic jars at 20°C was recorded after 7 and 14 days of incubation in stationary culture. The inocula for biochemical, physiological and pathological tests were prepared from cultures grown for 48–72 h on H3 medium.

Genomic DNA extraction and analysis

Crude lysates of the bacterium (Weidner *et al.*, 1996) were obtained by suspending a small colony grown on a Petri dish with agar in 50 μl lysis buffer (0.05 M NaOH, 0.25% sodium dodecyl sulfate) and heating to 95°C for 15 min. The cell debris was removed by centrifugation at 16,100 *g* for 5 min and the lysate was diluted 1:10 with double-distilled sterile water.

Partial 16S rDNA from genomic DNA was amplified by PCR using oligonucleotide primers annealing to conserved regions of bacterial 16S rRNA genes. The forward primer 63f (5'CAGGCCTAACACATGCAAGTC 3') corresponded to positions 63 to 83 of *E. coli* 16S rRNA (Osborn *et al.*, 2000), and the reverse primer 1389R (5'ACGGGCGGTGTGTACAAG 3') corresponded to the complement of positions 1371 to 1389. The conditions for PCR amplification were as follows: 1 μl of the crude diluted lysate, 2.5 μl of 10× reaction buffer, 0.5 U of *Taq* DNA polymerase, 200 μM of each of the four deoxynucleotides (all from Amersham Biosciences, Dreieich, Germany), and 200 nM of each primer (MWG, Munich, Germany) were combined in a total volume of 25 μl and incubated in a thermal cycler (ICycler Bio-Rad Laboratories, Hercules, CA, USA). The cycling program was as follows: initial denaturation at 94°C for 120 s; 35 rounds of 94°C for 60 s, 54°C for 30 s, 72°C for 150 sec; and a final extension step at 72°C for 300 sec. Amplification products (2 μl lane^{-1}) were visualised after electrophoresis in 0.5× TBE buffer on a 0.8% (w:v) agarose gel containing 0.5 μg ml^{-1} (w:v) ethidium bromide, using a 100 bp DNA molecular ladder (Amersham Biosciences).

For digestion of the 16S ribosomal DNA fragments, the tetranucleotide-recognising enzymes *Alu*I, *Hpa*I, and *Hha*II (MBI, Fermentas, Vilnius, Lithuania) were used. In a typical reaction, 5 μl of a PCR product, containing about 1.5 μg of DNA, was digested with 10 U of an enzyme in reaction buffers, following manufacturer's recommendations. The reaction mixtures (total volume of 25 μl) were incubated at 37°C overnight. Reaction products were resolved by agarose gel (2% w:v) electrophoresis in TBE buffer using a 100 bp DNA molecular ladder (Amersham Biosciences).

Amplified 16S rDNA from three isolates (APT1, APT5, APT7) was sequenced directly on both strands using 63f and 1389r primers on an ABI PRISM 3100 DNA Sequencer (Applied Biosystem, Darmstadt, Germany) and the chemical dye terminator procedure followed Sanger *et al.* (1977). Sequences were edited and aligned with DNASTAR software and were submitted to BLASTn search (Altschul *et al.*, 1990) (National Center for Biotechnology Information; available from: www.ncbi.nlm.nih.gov/BLAST/). The nucleotide sequence of the 16S rRNA gene region that was sequenced in both directions was deposited in the GenBank database under accession number DQ059817.

Insect association with knots

The association between insect damage and presence of knots at branch level was assessed at three sites: Monte Marcello, Bonassola and Livorno. In June 2000, 10 trees (3–7 m tall) were randomly selected at each site, and from each tree 3 branches of 1 m in length, one from each whorl, were collected. Each branch was examined for knots and insect damage. Insect damage was classified according to the extent of occurrence of insect larvae/adults or feeding signs (holes, feces, browsing) on the knots or adjacent twigs. The relative frequency of knot occurrence at branch level on trees with and without insect damage and with different treatments was compared using the χ^2 test.

Pathogenicity tests and transmission by insect vectors

Cultures of isolates APT1 and APT7 were grown to log phase in H3 liquid medium, then centrifuged, resuspended in sterile distilled water, centrifuged again and finally resuspended in water immediately prior to inoculation. Three inoculation tests were carried out on 6-year-old potted trees in a tree nurs-

ery near Florence, on the top (youngest) part of the stems, consisting of 2–3-year-old wood growing in near-natural conditions. Each test used a different technique of wounding and bacterial application. In the first test, deep vertical wounds were made by driving a scalpel through the phloem to the xylem at nine randomly selected sites on the younger part of the stem of each tree. Inoculum (10 μl, 10^8 CFU ml^{-1}) was placed in six of the nine wounds with a fine hypodermic syringe; the other three wounds (controls) were inoculated with the same amount of sterile distilled water. Three trees were inoculated with each isolate (6 inoculations per tree). In the second test, the tip of young stems of three other trees were punctured with a needle previously contaminated by dipping in a colony of the bacterium isolate to be assayed, for a total of twenty punctures per tree. Lastly, on three trees per isolate, a small amount of a homogenate of young knots was streaked on superficial wounds made with a scalpel kept nearly parallel to the surface of the tree, for a total of twenty wounds per tree. In the last test, a small amount of alumina type WN-9 was added to the inoculum to adsorb the phenolic substances released by the wounds. Ten control wounds were included in each tree of the second and of the third tests. Each inoculation site was covered for 48 h with Parafilm M and inspected for knots for up to 12 months after inoculation. Pathogenicity tests were performed in the spring of 2001, and repeated in 2002 and 2003.

In 2002 and 2003 bioassays were carried out with the weevil *Pissodes castaneus* (Degeer) (Coleoptera, Curculionidae), which had been associated with Aleppo pine knots in the field. The adults of this species do maturation feeding in the bark and xylem of young twigs by inserting their long rostrum, at the end of which there are powerful mouthparts (Alfaro, 1995). Seven 6-year-old potted Aleppo pine trees (1 m in height) were tested in the same tree nursery as that in which the pathogenicity tests were carried out. Approximately 50 adults of *P. castaneus* were collected from Aleppo pine at Bonassola in June 2001. A few days after collection, two adult weevils were placed on each of two randomly selected 2-year-old branches of each of the potted trees and allowed to feed. On the first branch, we used weevils that had previously been externally contaminated with bacteria by allowing them to walk in a culture dish (isolate APT1). On the second branch we used field-collected weevils of which it was impossible to know whether they were naturally contaminated with the bacteria or not, because molecular probes were not available, and isolation would have required much more material.

In both treatments, the branches were covered with mesh bags for two weeks before removing the weevils and assessing the amount of feeding done by counting the feeding holes. The trees were inspected for knots at monthly intervals thereafter. Any knots that formed were collected, their histology examined, and the causal agent isolated and identified.

Results

Histology

In cross-section the outer cell layers of the knot, periderm and cortex appeared normal. However, there was evidence of compression due to knot formation, sometimes associated with traumatic resin canals. Other parenchyma cells formed the bulk of the knot, which contained bacterial microcolonies (zoogloeae) throughout (Fig. 4 and 5). These cells were divided in irregular directions, and were smaller than those of the isodiametric and thinner-walled parenchyma above them. There were hardly any intercellular spaces, and where they occurred, they were very small and filled with substances that were probably phenolic in nature. The cells surrounding the bacterial colonies (Fig. 5) had a spiral shape with thick walls, sometimes separated by sparse interruptions, and they appeared to be rich in phenols (Fig. 6). In other cases the cytoplasm appeared shrunken, and the nucleus was either degenerated or in division (not shown). Further away from these abnormal areas the parenchyma cells gradually returned to normal, the intercellular spaces increased again in number, and the levels of phenols decreased. At the point of bacterial infection the secondary phloem and the last xylem (wood) ring appeared thinner. Interestingly, in all samples the position of the point of bacterial infection clearly showed that the infection started in the spring of the second year of Aleppo pine shoot growth.

The xylem was damaged by abnormal tissue that had formed (Fig. 7). This abnormal tissue was composed of parenchyma cells with thickened and lignified walls instead of tracheids, and seemed to have origina-

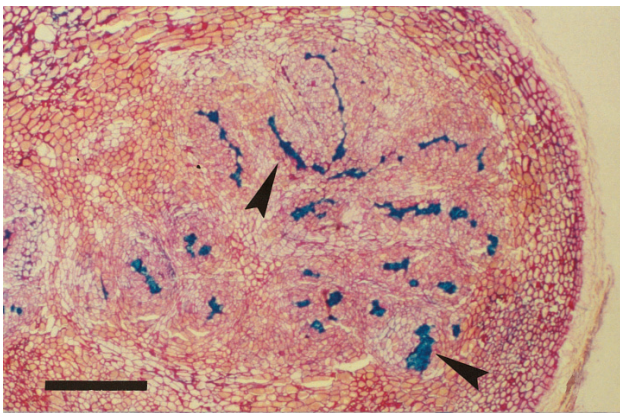

Fig. 4. Cross-section of a knot with bacterial zoogloeae (arrow heads), surrounded by parenchymatic cells. Bar=500 μm (fuchsin-Astra blue).

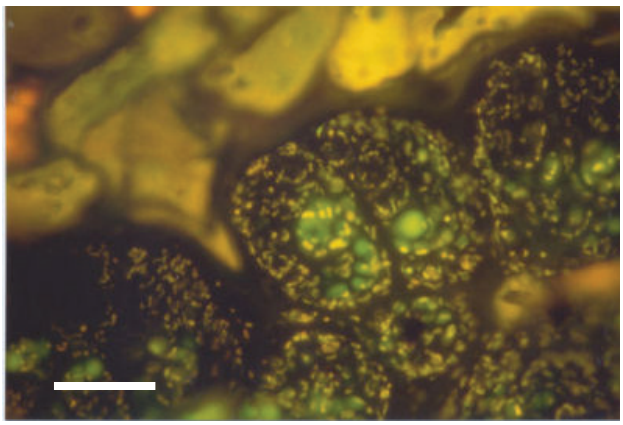

Fig. 5. Bacterial cells organized in zoogleae. Bar = 10 μm (fluorescent PAS).

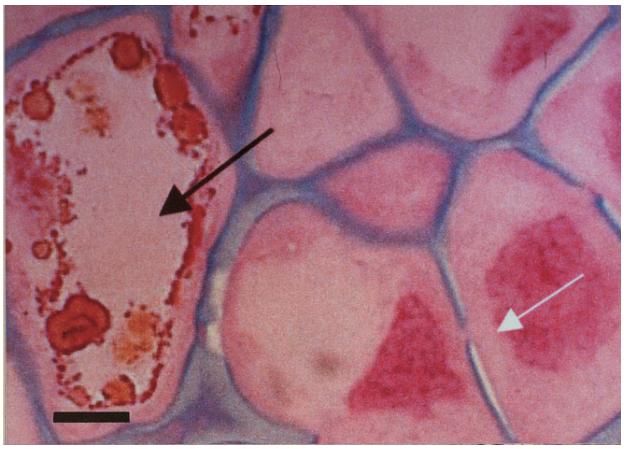

Fig. 6. Knot parenchyma cells with substances of probable phenolic nature (black arrow), and cells with interruptions in the cell walls (white arrow). Bar=10 μm (fuchsin-Astra blue).

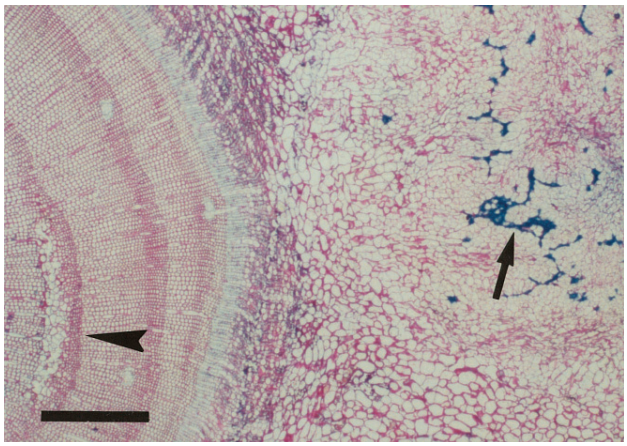

Fig. 7. Cross-section of a 5-year-old Aleppo pine twig with a superficial knot. Note the disorganisation of the second-year wood ring (arrow head) and the bacterial zoogloeae in the knot parenchyma (arrow). Bar=500 μm (fuchsin-Astra blue).

ted in the proliferating radial parenchyma cells (Fig. 8 and 9). Tangential longitudinal sections also showed short and deformed tracheids above and below the mass of the parenchymatic cells (data not shown).

The knots were always associated with a disorganisation of the xylem, but there were differences between superficial knots and deep knots. In superficial knots the disorganised xylem affected only the wood ring of the second growth year and the mass of cells making up the knot grew out of the cells of the cortex only (Fig. 7). In deep knots, on the other hand, bacterial aggregates did not appear in the wood ring until the following (i.e. third) year (Fig. 8). These bacterial aggregates were surrounded by spirally arranged parenchyma cells which disrupted the continuity of the xylem ring (Fig. 8). In the superficial knots, on the other hand, the disorganised xylem affected only the wood ring of the second growth year, and the mass of cells making up the knot grew only out of the cells of cortex (Fig. 7). In both cases, however, as the knot aged, the groups of parenchyma cells became de-differentiated, and meristem tissue (vascular cambium) (Fig. 9) was formed that produced tracheids oriented in various

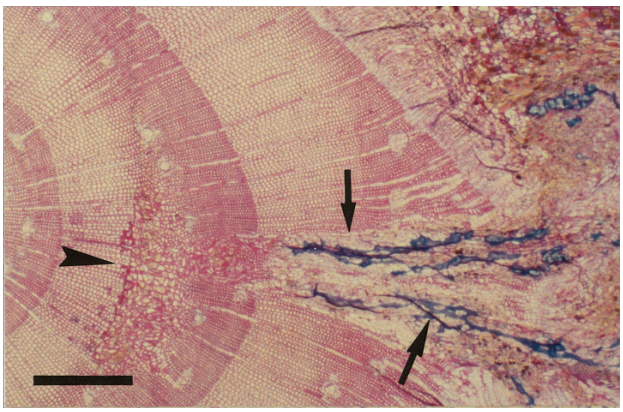

Fig. 8. Cross-section of an Aleppo pine twig with a deep knot. The disorganisation of the second-year wood ring (arrow head), the presence of bacteria and the interruption in the 3rd xylem ring (arrows) are clearly visible. Bar=500 μm (fuchsin-Astra blue).

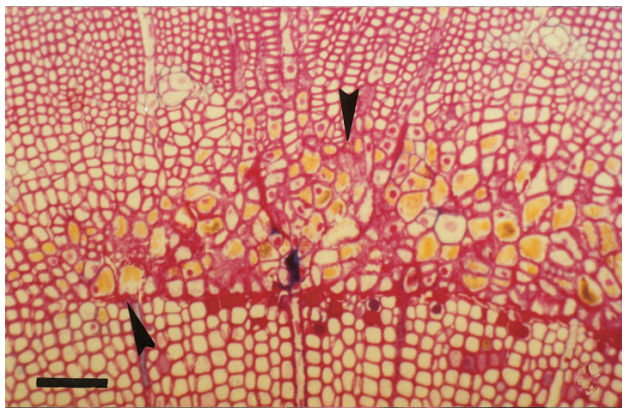

Fig. 9. Cross-section of the 2nd year growth ring of an Aleppo pine twig showing abnormal xylem formed by parenchymal cells with thickened and lignified walls (arrow heads). Bar=100 μm (fuchsin-Astra blue).

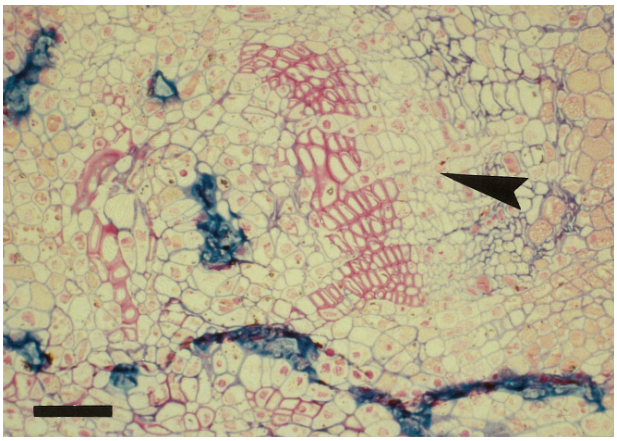

Fig. 10. Cross-section of parenchyma cells inside the knot with meristematic tissue, of vascular-cambium type (arrow head), close to infected areas, and producing tracheids. Bar=100 μm (fuchsin-Astra blue).

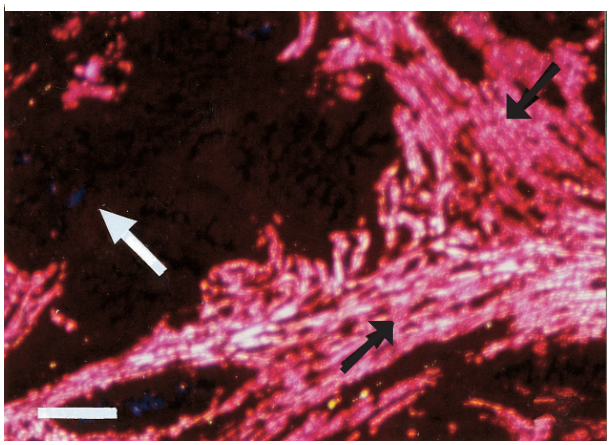

Fig. 11. Cross-section of old knots in which the tracheids (black arrows) tend to surround infected areas (white arrow). Bar=100 μm (fuchsin-Astra blue, observed with polarised light).

directions. These woody elements tended to surround the infected areas, giving rise to structures very similar to wood nodules (Fig. 11). In the older knots these wood nodules became larger and then coalesced, forming a single, irregularly shaped wood nucleus at the centre of the excrescence (Fig. 11). There never however was any direct continuity established between the vascular tissues of the twig bearing the knot and those of the knot itself.

Bacterial cells were never seen in the wood elements, phloem, or resin ducts of the wood or the cortex. The bacteria were always aggregated in masses of varying size, and enclosed in a slimy material possibly of a polysaccharide nature, as shown in Figure 4 and 6. These aggregates normally occurred in the intercellular spaces and in the spaces occupied by the middle lamella, but the bacteria also invaded areas previously occupied by the host cells (Fig. 5 and 6). Lastly, bacteria, singly or in small groups, surrounded or not by slimy material, were occasionally seen inside the parenchyma cells of undamaged knots where they were apparently still alive (Fig. 12).

Agent characterisation

The bacterium was isolated only from knots of Aleppo pine when the H3 dishes were seeded with small pieces of knot tissues. Isolations done by placing ground-up knot tissue in a drop of sterile saline solution were almost never successful.

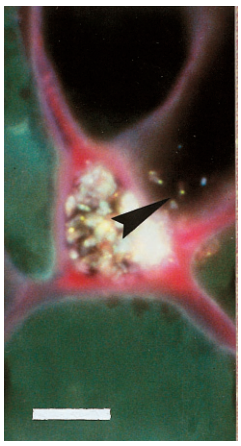

Fig. 12. Single bacteria inside the parenchyma cell of a knot (arrow). Bar=10 μm (DAPI).

The two isolates used for bacterial characterisation always gave the same outcome in the tests performed. The bacterium was gram negative, oxidase negative, catalase positive, facultatively anaerobic, and fermentative as determined by the oxidation-fermentative test. The cells were rod-shaped, did not form spores, and were motile by means of peritrichous flagella. The Aleppo pine bacteria did not induce a hypersensitive response in tobacco leaves and did not macerate potato tuber slices. Casein, gelatin, starch and aesculin were not hydrolysed within 12 days of inoculation. The methyl red test and the Voges-Proskauer reaction were positive. None of the isolates produced polygalacturonase or pectate lyase when they were tested on pectate gels at pH 5 and 8. The bacterium grew at 18–23°C (optimum 20–22°C). Based on all these characteristics, the Aleppo pine bacterium was tentatively assigned to the genus *Erwinia*. Colonies on H3 supplemented with 0.1% glycerol survived for 2–4 months at 4°C, but for only about 30 days at room temperature (22–24°C).

Molecular analysis of bacterial isolates

We analysed a total of 28 bacterial isolates, 20 from knots of Aleppo pine in the field and eight from knots that formed on the potted trees used for the transmission test. Amplified ribosomal DNA restriction analysis (ARDRA) (Vaneechoutte *et al.*, 1992) grouped the isolates by their *Alu*I, *Hpa*II, and *Hha*I restriction patterns. Each pattern was compared with all the others, and this allowed a single ARDRA profile, or haplotype, to be determined, corresponding to a single bacterial species.

The 16S rRNA gene partial sequence (1299 bp) of three isolates was determined and examined for similarity with deposited sequences using the BLAST N program available through the National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/). The database search showed that the partial 16S rRNA gene sequence of our isolate had a 98% identity with the corresponding gene sequences of several strains of *E. amylovora* and with one strain of the marine bacterium *Margalefia venezuelensis* sp. nov. (AY702662). The sequence also showed a 97% identity with one strain each of the following bacterial species: *Pantoea agglomerans* (AM184264), *Pantoea stewartii* (AY642383), *Erwinia* sp. (Co9929) isolated from galls on *Conzattia multiflora* and *Leclercia adecarboxylata* (AJ276393).

That the Aleppo pine knot bacterium belonged to the genus Erwinia was further confirmed by analyses performed at the DSMZ (German Collection of Micro-organisms and Cell Cultures), Braunschweig, Germany.

Pathogenicity tests and relationship with associated insects

In the inoculation tests, the isolates APT1 and APT7 and the tissue homogenate did not cause any knots to form in any of the years the tests were performed. Tissue reactions did not differ between inoculated and control wounds, nor between the wounding techniques employed. The addition of a phenol-adsorbing compound did not change the outcome of the inoculation in any way.

The feeding holes of *P. castaneus* were associated to a significant degree with the knots on the branches in two of the three sites (Table 1), where the density of the knots was fairly high. At the third site (Livorno), the incidence of the disease was too low (<1% of branches with knots) to permit statistical testing. Other insects, such as colonies of *Cinara* sp. (Hemiptera Aphididae) and larvae of *Dioryctria* sp. (Lepidoptera Pyralidae), were occasionally found on twigs and in the knots, respectively. However, their numbers were also not high enough for statistical testing. During inspection of the branches, some adults of *P. castaneus* were observed feeding on both twigs and knots. Examination of ten of the feeding holes showed that the wounds made by the weevil were somewhat similar in shape to the wounds that occurred at the base of most knots (Fig. 13).

In the tree contamination experiment, only those trees on which weevils had been placed showed any

Table 1. Association between insect damage (feeding holes of *Pissodes castaneus*) and knots on branches of *Pinus halepensis* randomly sampled at the sampling sites of Bonassola and Lerici. A total of 100 branches from 10 trees were inspected at each site.

Pissodes feeding holes	Knots			
	Bonassola		Lerici	
	Presence	Absence	Presence	Absence
Presence	62	19	23	17
Absence	8	11	6	53
χ^2 independence test, P	8.7 <0.01		25.8 <0.01	

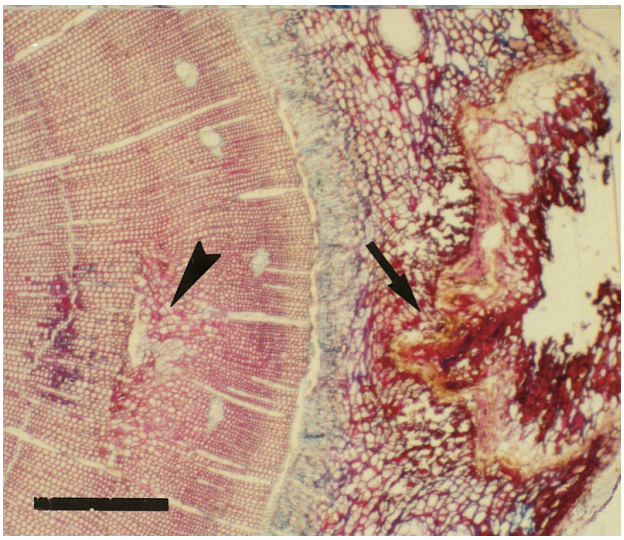

Fig. 13. Two-year-old twig with a wound caused by a feeding hole of *Pissodes* (arrow). Note the abnormal xylem (arrow). Bar=500 μm (fuchsin-Astra blue).On the outer bark, the hole excavated by the weevil at the time of feeding it is still visible.

feeding holes (mean ±SD=32.4±12.5 per twig). More than 30 knots were detected on three out of the seven potted trees about 1.5 years after the experiment, and these knots occurred only on twigs where weevils previously contaminated with bacteria had fed. Bacteria were subsequently isolated from these knots and identified by the same molecular markers as above. All belonged to the strain that had been used to contaminate the weevils. Examination of ten of the knots on the potted trees revealed that a wound caused by a weevil was always associated with a knot.

Discussion

Although the causal agent has not yet been identified to species level, the results further the understanding of Aleppo pine knot disease. Earlier studies (Petri, 1924; Dufrenoy, 1925; Eskandari and Ridé, 1967) each assigned the causal agent to a different genus: *Bacillus, Pseudomonas,* or *Corynebacterium,* this last genus being very different from the first two. In our study, it proved impossible to assign the bacterium to an already known species, although an affinity with the genus *Erwinia* was established. One of the species of this genus, *Erwinia herbicola* (*Pantoea agglomerans*), contains at least three pathovars that cause diseases characterised by knots similar to those caused by the Aleppo pine knot bacterium: pv. *gypsophilae* (Brown, 1932), pv. *betae* (Burr *et al.*, 1991), and pv. *milletiae* (Opgenorth *et al.*, 1994). A strain of *E. herbicola* has also been associated with knots on Douglas-fir (DeYoung *et al.*, 1998) and olive (Marchi *et al.*, 2006). Unlike these last two bacteria, however, the bacterium of Aleppo pine knots was very difficult to isolate (bacterial masses developed only when pieces of knot tissues were seeded on agar dishes) and to grow on common bacterial media (i.e. nutrient sucrose agar, King's B); they quickly died in artificial culture at temperatures above 20–22°C, and they also apparently caused knots only when in association with *P. castaneus*. We came to this conclusion by carrying out three types of pathogenicity tests: 1. by placing an isolate of the putative causal bacterium on wounds made on young branches and stems; 2. by taking homogenised tissue from existing knots and placing it on somewhat larger wounds made in the stems with a scalpel; and 3. by comparing feeding by weevils externally contaminated with the bacterium, and

feeding by weevils not so contaminated. The first two types of inoculation tests never gave positive results although they were repeated several times, but the tests with the contaminated weevils produced knots in those parts of the tree where the weevils had fed. No knots were produced on any twigs where uncontaminated weevils had fed. These findings therefore indicated that: 1. the isolated bacterium reproduced the symptoms of Aleppo pine knot disease; and 2. infection with the bacterium occurred only if bacterium-contaminated weevils were feeding on the twigs, and only during the second year of bacterial growth. Our conclusions therefore contradict the results reported by Eskandari and Ridé (1967) who succeeded in reproducing the typical Aleppo pine knots when they inoculated on Aleppo pine the bacterium they had originally isolated from that tree. While we are at present unable to explain this discrepancy, we are continuing the task of identifying the causal agent of Aleppo pine knot disease and determining how it is transmitted by the insect vectors.

The histological examination of diseased twigs showed that the initial damage was similar to the damage caused to Aleppo pine by the pine scale *Matsucoccus josephi* Bodenheimer & Harpaz (Lipschitz and Mendel, 1987, 1989). The reaction to this type of damage by the tree appeared to be a normal response of many conifers to natural as well as artificial wounds (Kučera, 1972; Lev Yadun and Aloni, 1991). All twig sections with feeding holes exhibited abnormal xylem formed by the parenchyma cells, with thickened and lignified walls and curved tracheids. When there were knots as well as feeding holes, the abnormal xylem was accompanied by a reduction in the thickness of the second-year wood ring. These findings suggest that the bacterium entered the host tissues through a passage made by a feeding insect, as already postulated long ago by Vuillemin (1888), Petri (1924) and Dufrenoy (1925). By contrast, the other two pine-feeding insects mentioned by those authors, the moth *Dioryctria sylvestrella* and the aphid *Eulachnus agilis*, do not seem to lead to any infection. It is hypothesised that the knot bacteria need the specific type of wound made by *P. castaneus* to initiate knot formation. The fact that insects and knots occur together at branch level in the field, and also that typical *Pissodes* wounds occur in wild knots (Battisti, unpublished results) support this hypothesis. Transmission of bacteria under natural conditions will then occur when weevils feed on the knots, become contaminated with the bacteria from the knots, and then move on to other parts of the twigs for further feeding. Such a mode of transmission is all the more likely as weevils have a relatively long lifespan (up to 1 year). *Pissodes* weevils are known to be vectors of other micro-organisms on pines, e.g. *Pissodes piniphilus* and *Endocronartium pini* in Finland (Pappinen and Weissenberg, 1994, 1996), and are known to feed on conifer bark and phloem, e.g. *Pissodes strobi* in North America (Manville *et al.*, 2002).

Numerous histological observations suggested that whether the knot was deep or superficial depended on the depth at which the bacteria were introduced, and this in turn probably depended on which part of the long rostrum of the weevil was contaminated with the bacteria. If the bacteria were introduced only superficially, in the cortex, knots developed only on the parenchyma. In contrast, if the bacteria were vectored more deeply into the xylem, they caused the parenchyma cells to proliferate and the wood ring to become interrupted. After this the bacteria moved towards the cortex, where they triggered knot formation. This explanation of the origin of the knots is confirmed by the fact that large bacterial aggregates were found only in the cortex, and possibly in the area separating the wood rings, but never in any other tree tissue. Moreover, the cambium of the twig never participated in knot formation, since there was no connection between the vessels of the knot and the vessels of the twig on which the knot was formed.

The formation of wood inside the knot and the subsequent isolation of the bacterial aggregates within spherical structures can be viewed as a mechanical defense response by the tree. Such a response becomes more conspicuous as the knot ages, although it is sometimes seen in very small knots as well. The differentiation of wood elements in knot tissue is also known from other systems, for example it is found in knots caused by Pseudomonas savastanoi on olive trees. In this case, however, the wood nodules do not contain bacterial aggregates (Surico, 1977). The occurrence of bacteria inside the host tissues was consistent with the findings of Petri (1924) and Dufrenoy (1925). Bacteria thrive on the pectins that form the middle lamella and part of the matrix of the cell wall. Specific stains of the wall revealed that it was differentially affected by the bacteria: some

parts of the walls became thicker, but wall thickening was occasionally interrupted. It is likely that through such interruptions in the cell wall, or the collapse of the fibrils, the bacterial cells occasionally invade the host cells, eventually killing them and thus causing the infected area to increase in size (lysigenous colonies; Petri, 1924). The mode used by bacteria to invade the host cells is still unclear, as is the question of whether the death of the parenchyma cells precedes or follows invasion by the bacterium. It seems somewhat more likely that, contrary to the conclusions of Petri (1924) and Dufrenoy (1925), the bacteria invade the living parenchyma cells and then kill them. This matter is of considerable interest and deserves further study.

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