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Running title: Location of *Xanthomonas translucens* in pistachio

Location of *Xanthomonas translucens* in pistachio trees

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Abstract

Xanthomonas translucens has been identified as the causal agent of pistachio dieback in Australia. Symptoms include decline, xylem staining, trunk and limb lesions, and excessive exudation of resin. Bacteria were previously isolated from stained wood in 2 year old twigs but little was known about their presence in other parts of the tree. The pattern of staining and location of *X. translucens* were studied following felling and dissection of asymptomatic and diseased trees. Chestnut-coloured smears and specks occurred in the

sapwood of diseased trees and were continuous from the trunk to 1-2 year old twigs. *Xanthomonas translucens* was isolated mainly from young sapwood (stained and unstained) of the main trunk, primary and younger branches and current season growth, less frequently from leaves and bunches, rarely from old, stained heartwood and not from roots and associated soil samples. Bacteria and pathogenic fungi were not found in the inner bark and cortex associated with lesions whereas the stained sapwood underlying the lesions yielded *X. translucens*. Scanning electron microscopy revealed bacteria in the main vessels of the xylem of stained tissue and tyloses in the proximity of colonised tissue. Information on the pattern of staining and location of the bacteria will facilitate pathogen detection, thereby improving the accuracy of disease diagnosis.

Additional keywords

Bacterial disease, xylem, sapwood, scanning electron microscopy

Introduction

Bacterial dieback of pistachio in Australia is characterised by decline (little current season growth), xylem staining, trunk and limb lesions (often covered by superficial, black fungal growth) and excessive exudation of resin (Facelli *et al*. 2002, 2005). Foliar symptoms have not been consistently associated with the disease. The disease was first observed in male trees in 1996 (Taylor and Edwards 2000). To date, it has not been reported in other countries.

Xanthomonas translucens is the only pathogen to be isolated consistently from diseased trees. Isolates have been differentiated in two groups on the basis of physiological, biochemical and molecular tests. Group A has been isolated from several orchards whereas Group B has been found in one orchard at Robinvale in Victoria only (Facelli *et al*. 2005; Marefat *et al*. 2006b).

Xanthomonas translucens has been isolated most consistently from 2 year old twigs with stained xylem and only sporadically from lesions (Taylor and Edwards 2000; Facelli *et al.* 2005). The lesions associated with pistachio dieback appear as cracks in the bark without overgrowth of surrounding tissues, and may or may not be sunken. Although originally called cankers (Edwards and Taylor 1998), they differ in structure from typical bacterial cankers (Swings and Civerolo 1993; Agrios 2005) and it is not known if they play a role in dissemination of the pathogen. *Xanthomonas translucens* was not isolated from root or soil samples in preliminary investigations (Taylor and Edwards 2000) and the presence of the bacterium in other parts of the tree has not been reported.

As bacterial dieback may be confused with other diseases, such as verticillium wilt (caused by *Verticillium dahliae*), which also involve dieback and staining of the wood (Epstein *et al*. 2004), the disease is routinely confirmed by isolation of *X. translucens* onto agar media. If necessary, isolates may then be identified as belonging to *X. translucens* group A or B via a molecular test (Facelli *et al*. 2005; Marefat *et al*. 2006a). Verticillium wilt was common on pistachio in California until the introduction of *Verticillium*-resistant rootstocks (Epstein *et al.* 2004). In Australia, approximately half of the trees in production are grafted or budded on *Verticillium* resistant rootstocks, such as those derived from *Pistacia integerrima* (Epstein *et al.* 2004), and verticillium wilt is not considered a threat to production in this country (Robinson 1998).

The aim of this study was to characterise the patterns of staining associated with bacterial dieback and determine the location of *X. translucens* in the trees. Standard isolation techniques and scanning electron microscopy (SEM) were used to locate *X. translucens* in felled diseased male and female trees. Such information will facilitate pathogen detection and lead to improved disease diagnosis.

Materials and methods

Plant material

Trees were located in a commercial orchard at Kyalite, New South Wales, Australia (34º 58’ S; 143º 29’ E), established in 1985-6. The female trees were *Pistacia vera*, cultivar Sirora, and the male trees were cultivar 11/14 (Maggs 1982). All were grafted on the rootstock Pioneer Gold I (*P. integerrima*). All bacterial strains isolated from this orchard to date belong to *X. translucens* Group A (Facelli *et al.* 2005; Marefat *et al.* 2006b).

Disease assessment

In January 2001, 51 male and 20 female trees were classified into three groups (asymptomatic, moderately diseased and severely diseased) on the basis of plant vigour, number of lesions and presence of resinous exudate. Representative trees were then selected for felling and dissection (Table 1).

Isolation from internal tissues: preliminary study

In a preliminary trial in January 2001, one asymptomatic, four moderately and three severely diseased male trees were felled and trunk cross-sections (10-20 cm thick discs) of the rootstock and scion, primary branches and 2-8 year old branches were examined for the presence of staining. Plant material was stored at 5ºC until processed. For each disc, stained and non-stained tissue from heartwood and young and old sapwood, along one randomly chosen radius, was sampled (Fig. 1). Samples, consisting of 2 x 1 x 5 cm (radial, tangential and axial, respectively) prisms, were surface sterilised with ethanol, flamed, sectioned and plated on sucrose peptone agar (Moffett and Croft, 1983) supplemented with Benlate® fungicide (BSPA) as described by Facelli *et al.* (2005). When possible, the inner bark was peeled from the young xylem with sterile forceps, then inner bark and young sapwood were separately shaved with a sterile knife and shavings cultured on BSPA. Plates were incubated at 28ºC in darkness for up to 10 days. *Xanthomonas translucens* was

identified on the basis of colony and cell morphology on BSPA and nutrient agar, mucoid growth on yeast extract-dextrose-calcium carbonate agar and starch hydrolysis by the iodine-starch reaction (Moffett and Croft 1983). Representative isolates were confirmed as *X. translucens* by gas chromatography of fatty acid methyl esters as described by Facelli *et al*. (2005).

Tree stumps and roots were excavated using a backhoe. Roots less than 5 cm in diameter were sampled from one asymptomatic, two moderately and one severely diseased tree. Roots were washed, cut into 5 cm long sections, surface sterilised in 70% ethanol for 5 min and rinsed in sterile distilled water (SDW). Then, 5-7 mm pieces were excised and placed on sucrose peptone agar (SPA) or incubated in SDW for 12-24 h and serial dilutions (to 10^{-6}) of the suspensions streaked on SPA. Plates were incubated at 28ºC for 7 days. Approximately 1 kg of soil was sampled from each hole and a sub-sample of 1 g damp soil was added to 9 mL SDW. Serial dilutions (to 10^{-7}) were prepared then 0.1 mL aliquots were spread on SPA and incubated as above.

Isolation from internal tissues: main study

In November 2001, moderately diseased male (10) and female (5) trees and asymptomatic male (6) trees were felled, dissected and assessed for staining and *X. translucens*. For commercial reasons, it was not possible to fell productive, asymptomatic female trees. Severely diseased trees were not assessed as it proved difficult to distinguish between staining due to disease and the normal dark colour of the heartwood.

Bacterial isolations were carried out on cross-sections of trunks (rootstock and scion). One symptomatic branch, with stunted growth and/or lesions, from each tree was dissected, taking 5-8 cm long sections from the primary branch up to the current season growth. Prisms of wood were prepared from the trunk and large branches as described for the preliminary study. Segments 5-7 cm long cut from 1 and 2 year old twigs were surface

sterilised in ethanol, flamed and the bark was peeled off using a scalpel. Ten stained and 10 unstained 1 year old twigs were collected from each tree. Due to routine pruning, there were fewer than 10 stained or unstained 2 year old twigs on the chosen branch of most trees, so the number collected from each tree was variable.

Bacterial isolations were also carried out on non-lignified, current season growth as follows. Segments 5-7 cm long were cut from 10 shoots arising from stained twigs and 10 from unstained twigs. Non-lignified tissue was surface sterilised with 1% active chlorine for 5 min and rinsed twice in SDW for up to 5 min. Then 5 x 2 mm thick discs were excised and incubated in SDW for 12-24 h. The resulting suspensions were streaked on BSPA.

Leaves were assessed individually and in bulk. Individual samples comprised one leaf from each of 10 stained twigs and one leaf from each of 10 unstained twigs per tree. Individual leaves were surface sterilised with chlorine as described above. A strip of the lamina (including midrib) was cut into small pieces and these were placed directly on BSPA or incubated in SDW overnight to produce a suspension that was spread on BSPA. Pieces of petioles were treated likewise. Each bulked sample consisted of 20 leaves; two from each of 10 twigs, stained or unstained separately, providing two bulked samples per tree. Bulked leaves were surface sterilised as above. Laminae were excised and blended with 300 mL SDW. Petioles were blended with 100 mL SDW. Serial dilutions (to 10^{-2}) were made from both mixtures and 0.1 mL aliquots were spread on BSPA.

All lesions present on the diseased trees were dissected and examined for stained wood, bacteria and fungi. The following components were sampled; clean bark (without sooty mould), interface between the black sooty mould and the bark, interface between stained and unstained sapwood underlying the lesion, and unstained and stained sapwood underlying the lesion (Fig. 2). Tissues were surface sterilised with ethanol and flamed, then cultured on SPA.

Five moderately diseased and three asymptomatic male trees were uprooted using a backhoe, and roots and soil were sampled and processed as in the preliminary study.

Fisher's exact probability test (Siegel 1956) was used to compare numbers of diseased and asymptomatic male trees with bacteria within each type of tissue, and number of diseased male and female trees with bacteria.

SEM observations of woody tissues

Samples (5 x 5 x 2 mm) from stained and unstained tissue from sections of scion and rootstock from trees felled in the preliminary trial, within each of the three disease categories, were observed by SEM. Specimens were prepared such that stained wood and/or the margin between stained and unstained wood were exposed and patterns of staining on each section were recorded before fixation. Wood was fixed in 4% paraformaldehyde/1.25% glutaraldehyde, critical-point dried, mounted on metal stubs as described by John *et al.* (2004) and coated with carbon and gold. Longitudinal and transverse surfaces were examined for rod shaped bacteria about 0.4 x 1.5 µm in a scanning electron microscope (Philips XL30) at an accelerating voltage of 10 kV.

Isolation from plant surfaces, leaves and bunches

Samples were collected from 10 moderately diseased female trees. Surfaces of lesions, bark and twigs were sampled *in situ* seven times between August 2004 and July 2005, *viz.* in August, September, October, December, March, May and July. Leaves and bunches of nuts were collected as available, between October 2004 and March 2006, and assessed in the laboratory.

One to 2 lesions were sampled from each tree by placing a piece of sterile cotton wool (5 x 5 cm), moistened with SDW, on the lesion surface. Gladwrap® was used to hold the cotton wool in place and prevent desiccation. The cotton wool was applied at 10 am and

removed 2 h later, then soaked in 200 mL SDW for a further 2 h before serial dilutions (to 10^{-2}) of the resulting suspension were made. Aliquots of 0.1 mL were spread in duplicate onto BSPA and incubated as described previously.

One sample was collected from the bark of the trunk, and a primary, secondary and tertiary branch of each tree. SDW (10 mL) was sprayed onto the bark and a sterile funnel used to collect the run-off into a sterile McCartney bottle. Dilutions (10^{-2} and 10^{-4}) of the wash water were made and plated as described above. The same method was used to collect samples from five twigs from each tree, before the wash water and a 10^{-2} dilution were plated.

Leaves and bunches of nuts were sampled monthly between October 2004 and March 2005 except for January. A further leaf sample was collected in May, after harvest. In the 2005-06 growing season, leaf and nut samples were collected from the four trees that yielded bacteria from these materials in 2004-05. Samples were collected fortnightly from October to December 2005 and once in March 2006 at harvest. Nut samples were not collected in December.

Ten intact leaves were collected from each tree. In 2004-05, leaves from individual trees were pooled. The laminae were placed in plastic bags containing 500 mL SDW, sufficient to allow submersion of the large leaves, and gently agitated on an orbital shaker for 10 minutes, ensuring that the petioles protruded above the water at all times. Wash water was plated undiluted except in November and December, when 10^{-2} and 10^{-4} dilutions were plated. In addition, leaves collected in February 2005 were surface sterilised in 1% chlorine for 10 min, rinsed three times in SDW and blended in 250 mL SDW. The resulting slurry and a 10^{-2} dilution were plated. In 2005-06, each leaf was washed separately. After washing, leaves were surface sterilised as above and blended individually with 100 mL SDW. Two undiluted replicates (0.1 mL) of wash water and one of slurry were plated onto BSPA.

Five bunches of nuts were collected from each tree. Bunches were gently agitated in 500 mL SDW on an orbital shaker for 10 minutes, with the cut end of the peduncle protruding above the water. The wash water (0.1 mL) was plated without dilution, one plate per bunch. In October 2004, bunches were pooled and entire bunches were blended, whereas for subsequent samples bunches were washed individually and the nuts and rachi then separated and blended. Undiluted slurry (0.1 mL) was plated onto BSPA. In March 2005, hulls, shells and kernels from each bunch were separated and either washed (shells) or blended with 100 mL SDW. In season 2005-06 entire bunches were washed, surface sterilised and blended, except at harvest when bunches were separated into rachi, hulls, kernels (blended) and shells (washed). For each bunch, the resulting slurry and wash water (0.1 mL of each) were plated without dilution, one plate per sample.

Results

Pattern of staining

Two patterns of staining were evident, *viz*. chestnut brown specks or a denser chestnut brown smear in the cross-sections of the scions of diseased trees. The stain was located mainly in the sapwood, within annual rings, with little or no spread across rings (Fig. 1). The inner bark was not stained. The staining was continuous from the trunk to the upper part of the selected branch, including 1-2 year old twigs. Cross sections of current season shoots or petioles did not have staining. The heartwood was dark brown/black in most of the trees, diseased or asymptomatic. It was difficult to distinguish chestnut specks within the dark heartwood and, in severely diseased trees, where extensively discoloured sapwood merged with the heartwood. Rootstock sections were almost free from stain; only a few specks were found in a few trees. Roots did not have internal staining. Lesions were

formed where the stain reached the outer parts of the sapwood. Staining in the sapwood underlying lesions was localised within growth rings (Fig. 2).

Isolation from internal tissues: preliminary study

In the preliminary trial, *X. translucens* was recovered from young sapwood, generally unstained, and from stained and unstained old sapwood from the main trunk and limbs of severely and moderately diseased scions. Bacteria were not isolated from inner bark. The bacterium was found in the young sapwood (slightly stained) of the rootstock of only one severely diseased tree (Table 2), and in chestnut-coloured specks of the limb of the single asymptomatic tree sampled. *Xanthomonas translucens* was not detected in root and soil samples, but plates were rapidly overgrown by soil-inhabiting fungi and bacteria (data not shown).

Isolation from internal tissues: main study

Xanthomonas translucens was isolated from the main trunk and primary branches of all diseased male and female trees and from 3-4 year old branches of most of these trees (Table 3). For male trees, bacteria were more frequent in diseased male trees than in asymptomatic trees and in the young and old sapwood than in the heartwood ($P < 0.05$). Overall, there were no significant differences between young and old sapwood and stained or unstained wood samples from diseased trees ($P > 0.05$). Likewise, there were no differences between diseased male and female trees ($P > 0.05$) (Table 3).

Most diseased male and female trees yielded bacteria from stained or unstained 1-year-old twigs and from current season shoots. With respect to unstained 1 year old twigs, *X. translucens* was isolated more frequently ($P < 0.001$) from diseased than from asymptomatic male trees. *X. translucens* was isolated from only one asymptomatic male tree, from one 1 year old unstained twig and from one current season shoot from an

unstained twig (Table 4). All diseased male trees carried bacteria in stained 2 year old twigs (Table 4). Likewise, *X. translucens* was isolated from 1 and 2 year old twigs and current season growth from most diseased female trees.

Xanthomonas translucens was isolated from leaves (petioles and laminae) and current season shoots removed from stained or unstained twigs on diseased male trees with equal frequency ($P > 0.05$) (Table 4). It was detected more frequently in bulked leaves from diseased male trees than asymptomatic male or diseased female trees ($P < 0.05$).

Bacteria and pathogenic fungi were not found in the inner bark and cortex associated with lesions whereas the stained young sapwood underlying each lesion yielded *X. translucens* (data not shown). Root and soil samples were overgrown by soil microorganisms.

SEM observations

In the main trunk, rod-shaped bacteria, about 0.4 x 1.4 µm, were located in and between the large xylem vessels in stained areas (specks or smears) (Figure 3a). The presence of bacteria was associated with gumming and a mesh like lattice on the vessel walls in which the bacteria were embedded (Figure 3b). Tyloses, but no bacteria, were observed in vessels from unstained areas adjacent to stained tissue (Figure 4). Bacteria were not observed in unstained sapwood or in heartwood. Bacteria and tyloses were not observed in the xylem of asymptomatic trees.

Isolation from plant surfaces, leaves and bunches

Xanthomonas translucens was not isolated from suspensions obtained by washing lesions, bark or twigs. In growing season 2004-05, *X. translucens* was isolated from leaf washes from three trees, from two trees in October and from a different tree in November 2004 (Table 5). The only time that a slurry of blended leaves was cultured in 2004-05, *X.*

translucens was isolated from the pooled leaves from one tree in February (20 cfu per mL), but not from the leaf wash from that sample. In growing season 2005-06, *X. translucens* was detected in leaf washings from three of the four trees sampled in October and November, when one leaf from each of three trees yielded *X. translucens,* with populations ranging from 10 to 500 cfu/mL. The bacterium was isolated from blended leaves from mid-October until late December, with populations ranging from 10 cfu/mL to those too numerous to count. Only two of these isolations were from leaves where *X. translucens* had been found in wash water.

In growing season 2004-05, *X. translucens* was isolated from bunch washings from the same tree (tree 3) in October and November and from blended bunches at all sampling times, whereas detection in trees 1 and 2 was sporadic (Table 5). In season 2005-06, *X. translucens* was isolated from the surface of one bunch on 12 October (10 cfu/mL) and from three blended bunches from a different tree on 27 October, at which time populations were too numerous to count. *X. translucens* was isolated from the washed rachis, shells, blended hulls and kernels of ripe nuts from one bunch sampled from tree 3 at harvest in March 2005, but not from these tissues in March 2006.

Discussion

Xanthomonas translucens was detected in all tree parts assessed, with the exception of the roots, and was isolated frequently from discoloured sapwood in diseased trees. The chestnut-coloured stain typical of bacterial dieback occurred as specks or smears in the sapwood. In the scion, these symptoms were continuous from the graft union to the twigs which gave rise to new shoots. New shoots and leaves were free from staining. However, bacteria were also isolated from unstained wood and not all samples of stained wood yielded bacteria. These characteristics indicate a vascular disease (Agrios 2005). The characteristic brown heartwood with a regular border (Rosengarten 2004) in the scion of

diseased and asymptomatic trees (Fig. 1) did not appear to be associated with bacterial dieback.

The following pattern of staining may allow bacterial dieback to be distinguished from other diseases, such as verticillium wilt: chestnut-coloured staining of the sapwood mainly localized within growth rings, occupying the entire sapwood only in severely diseased trees; rootstocks with occasional specks of chestnut stain and roots free from staining. In contrast, verticillium wilt, which also produces sapwood staining, termed "blackened xylem" in pistachio (Teviotdale *et al.* 2002), manifests as an irregular pigmented central region in both rootstock and scion (Epstein *et al.* 2004) and staining in roots (Teviotdale *et al.* 2002). *Verticillium dahliae* was not isolated from the chestnut-stained sapwood typical of bacterial dieback (Facelli *et al.* 2005). However, confusion between bacterial dieback and verticillium wilt may arise in severely diseased trees where dark, discoloured sapwood merges with the heartwood and the chestnut stain characteristic of bacterial dieback is difficult to distinguish. In comparison, staining in walnut deep bark canker (caused by *Brenneria rubrifaciens*, syn. *Erwinia rubrifaciens*) is localised mainly in the phloem and seldom in the new xylem (Schaad and Wilson 1970; Teviotdale *et al.* 2002). In fire blight of apple (caused by *Erwinia amylovora*), staining is associated with cankers, or with xylem and pith in relatively resistant cultivars (Teviotdale *et al.* 2002).

The presence of *X. translucens* in asymptomatic tissue (unstained sapwood, current season shoots, leaves and bunches) of diseased and asymptomatic trees may indicate recent colonisation and that staining is a consequence of the host response to the accumulation of bacteria. Lateral movement and egress of bacteria onto surfaces is thought to be caused by the pressure created by expanding masses of *X. camprestris* pv. *pruni* in plum stems and fruit (Du Plessis 1990) and peach leaves (Miles *et al.* 1977). It is possible then, that the large areas of staining in severely diseased pistachio trees are caused by masses of *X. translucens* that cross xylem vessels and invade young tissue. This may explain also the

formation of the lesions, which coincided with areas of sapwood where the staining had reached young xylem. Pressure may rupture the cambium and bark, allowing phloem contents to reach the surface and provide a substrate for sooty mould. However, to date, *X. translucens* has not been recovered from bark and the resinous exudate appears to be bacteriostatic (Sedgley *et al*. 2004).

Masses of gum, mesh-like material and bacteria occupied the same stained woody tissue. Likewise, bacteria are associated with gumming and fibrillar material in fire blight of pome fruit (Suhayda and Goodman 1981), black spot of peach, caused by *X. campestris* pv. *pruni* (Du Plessis 1990; Miles *et al.* 1977) and Pierce's disease of grape, caused by *Xylella fastidiosa* (Stevenson *et al.* 2004). Gumming is generally attributed to extracellular polysaccharides produced by the bacteria (Suhayda and Goodman 1981). Tyloses, such as were found in unstained areas close to stained and heavily colonised parts of the xylem, are formed in xylem vessels in response to injury, stress or invasion by insects or pathogens (Agrios 2005). Although bacteria were not observed in unstained tissue, from which they were isolated sporadically, this may reflect the small amounts of tissue that could be examined by SEM at any one time.

Typically, xanthomonads produce symptoms primarily on leaves, or leaves are the entry points for the bacteria (Rudolph 1993; Swings and Civerolo 1993). Although *X. translucens* was isolated from leaves in this study, particularly from male trees in 2001, there was no evidence of leaf lesions or other foliar symptoms on any of the trees. That bacteria were recovered from leaves of more diseased male than female trees may indicate a difference in susceptibility between the sexes or a role for leaves in the disease cycle. Pistachio growers observed symptoms of bacterial dieback initially on mature male trees and the observations reported here support the idea that the disease may have spread from male to the female trees. This needs further investigation.

Sampling of leaves and bunches at monthly intervals during the growing season in 2004 to 2006 resulted in sporadic isolation of *X. translucens*. The bacterium was recovered from leaves of four and bunches of three female trees at various times but there was no obvious pattern of recovery except for one tree (designated tree 3). For this tree, *X. translucens* was detected on or in bunches at each sampling time in season 2004/05 but on only one occasion in 2005/06, and from leaves only once in 2004/05 but at five consecutive sampling times in 2005/06. Again, the leaves and bunches were asymptomatic, even when the bacterial population was deemed too numerous to count.

The characterisation of staining and location of the bacteria associated with bacterial dieback will facilitate the collection of samples for diagnosis employing PCR (Marefat *et al.* 2006b). However, the sporadic distribution of *X. translucens* in the wood will need to be taken into account in the development of a sampling strategy that minimises the risk of 'false negatives'. As yet, we have not detected the bacteria on the surface of woody tissues and they appear to be infrequent on and in the leaves and bunches of diseased female trees. Further research is required to elucidate the natural means of transmission and the disease cycle in general.

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Table 1. Pistachio tree condition categories and number of trees felled in the preliminary and main studies

Disease indicators	Asymptomatic	Moderately diseased	Severely diseased
Vigour	Vigorous current season growth on whole canopy	50% of branches with stunted current season growth	No current season growth
Lesions	No trunk lesions	1-2 trunk lesions	More than 3 trunk lesions
Resinous exudate	No exudate	Some dribbles of dry or wet exudate	Abundant dry or wet exudate
Trees sampled in Jan 2001	1 male	4 males	3 males
Trees sampled in Nov 2001	6 males	10 males and 5 females	0

Table 2. Presence or absence of *Xanthomonas translucens* detected in each of eight male pistachio trees felled and dissected in January 2001

Tissue sampled		Tree condition							
		A[A]	Moderately diseased				Severely diseased		
Rootstock	interface[B]	n/a	-	-	-	n/a	n/a	n/a	n/a
	young sapwood	-	-	+[C]	-	-	-	-	-
Main trunk	unstained sapwood	-	-	-	+	+	n/a	n/a	n/a
	specks	-	-	-	+	+	+	-	+
	smear	n/a	n/a	-	n/a	n/a	+	n/a	n/a
	young sapwood	-	+	+[C]	-	+	+	+[C]	+[C]
Branch	unstained sapwood	-	-	n/a	-	-	+	+	n/a
	specks	+	+	+	-	+	+	+	-
	smear	n/a	-	n/a	n/a	+	n/a	+	-
	young sapwood	-	+	+[C]	-	n/a	-	n/a	+
Twigs	entire	-	+	-	-	+	-	+	-

[A]Asymptomatic.

[B]Interface between heartwood (dark) and sapwood (not stained).

[C]Some staining.

+ = *X. translucens* detected.

- = *X. translucens* not detected.

n/a = tissue not available for testing.

Table 3. Presence of *Xanthomonas translucens* in woody tissue of pistachio trees felled in November 2001, expressed as number of trees with bacteria of the total assessed

Type of tissue	Diseased males (10 trees)[B]	Asymptomatic males (6 trees)	Diseased females (5 trees)
Main trunk			
Total - All parts (p < 0.05)[A]	**10/10a**	**2/6b**	**5/5a**
inner bark	8/10a (14)[C]	1/6	5/5
young sapwood	10/10a (11)	1/6	5/5
old sapwood	9/10a (75)	1/6	4/5
heartwood	2/10b (50)	0/6	2/5
Primary branch			
Total – All parts (p < 0.001)[A]	**10/10a**	**0/6b**	**5/5a**
inner bark + young sapwood	8/10a (25)		5/5
old sapwood	9/10a (36)		3/5
3-4-old year branch			
Total – All parts (p < 0.05)[A]	**8/10a**	**2/6b**	**5/5a**
inner bark	6/10a (0)	1/6	3/5
young sapwood	8/10a (33)	2/6	4/5
old sapwood	5/10a (40)	1/6	3/5
Rootstock			
Total – All parts (p < 0.01)[A]	**7/10a**	**0/6b**	**3/5a**
inner bark	3/10a (0)		0/5
young sapwood	6/10a (17)		2/5
old sapwood	3/10a (0)		1/5

[A]Data for diseased and asymptomatic males and diseased females and males were compared using Fisher's exact probability test (Siegel 1956). There were no asymptomatic controls for female trees. Shared letter (in bold) indicates no significant differences between diseased male and female trees and asymptomatic male trees considering all parts sampled (P indicated in table). (ns) not significant.

[B]Shared letter within each column and within each tree part indicates no significant differences between samples from different tissues, P < 0.05, Fisher's exact probability test (Siegel 1956).

[C]Numbers in brackets are positive stained samples expressed as percentage of the total number of positive samples (from stained and unstained tissue).

Table 4. Presence of *Xanthomonas translucens* in non-lignified tissue and young twigs of pistachio trees felled in November 2001, expressed as number of trees with bacteria of the total assessed

Type of tissue	Diseased males[F]	Asymptomatic males[G]	Diseased females[H]
Leaves - Bulk[A]			
Lamina or petioles on stained twigs	8/10a	(n/a)	1/5 (*)
lamina	6/10		1/5
petioles	7/10		1/5
Lamina or petioles on unstained twigs	6/10a	0/6 (*)	0/5 (*)
lamina	5/10		
petioles	3/10		
Leaves – Individual[B]			
Lamina or petioles on stained twigs	7/10a	(n/a)	0/5 (*)
laminae	0/10		
petioles	7/10		
Lamina or petioles on unstained twigs	4/10a	0/6 (ns)	1/5 (ns)
laminae	1/10		0/5
petioles	3/10		1/5
Current season shoots[C]			
On stained twigs (bacteria in 2-6 shoots/tree)	8/10a	(n/a)	3/5 (ns)
On unstained twigs (bacteria in 1-4 shoots/tree)	6/10a	1/6 (ns)	3/5 (ns)
1-year twig[D]			
Stained (bacteria in 4-10 twigs /tree)	9/10a	(n/a)	5/5 (ns)
Unstained (bacteria in 2-10 twigs/tree)	9/10a	1/6 (**)	3/5 (ns)
2-year twig[E]			
Stained (bacteria in 1-8 twigs/tree)	10/10a	(n/a)	5/5 (ns)
Unstained (bacteria in 0-1 twig/tree)	3/5a	0/6 (ns)	4/5 (ns)

[A]Each bulked sample comprised 20 leaves, two from each of 10 twigs, stained or unstained.

[B]Individual samples were one leaf from each of 10 twigs (stained or unstained) per tree.

[C]20 shoots per tree, 10 from stained and 10 from unstained twigs.

[D]10 stained and 10 unstained twigs.

[E]Fewer than 10 stained and unstained 2-year-old twigs, number varied from tree to tree.

[F]Shared letter indicates no differences between samples from stained and unstained twigs within each type of tissue, $P < 0.05$, Fisher's exact probability test (Siegel 1956).

[G](*); (**) Significant differences between diseased and asymptomatic males at $P < 0.05$ and 0.001 respectively. (ns) not significant, Fisher's exact probability test (Siegel 1956).

[H](*); (**) Significant differences between diseased males and females at $P < 0.05$ and 0.001 respectively. (ns) not significant, Fisher's exact probability test (Siegel 1956).

(n/a) not available

Table 5. Isolation of *Xanthomonas translucens* from wash water and from blended tissue of leaves and bunches detached from four pistachio trees, expressed as number of samples with bacteria of the total assessed

Sample date	Tree 1				Tree 2				Tree 3				Tree 4			
	Wash		Blend		Wash		Blend		Wash		Blend		Wash		Blend	
	Leaf	Bunch	Leaf	Bunch	Leaf	Bunch	Leaf	Bunch	Leaf	Bunch	Leaf	Bunch	Leaf	Bunch	Leaf	Bunch
27/10/04[A]	0/1	0/1	n/a[B]	0/1	0/1	0/1	n/a	0/1	1/1	1/1	n/a	1/1	1/1	0/1	n/a	0/1
25/11/04	1/1	0/5	n/a	1/5	0/1	0/5	n/a	0/5	0/1	2/5	n/a	Rachis 2/5 Nut 1/5	0/1	0/5	n/a	0/5
20/12/04	0/1	0/5	n/a	0/5	0/1	0/5	n/a	2/5	0/1	0/5	n/a	Rachis 4/5 Nut 2/5	0/1	0/5	n/a	0/5
9/2/05	0/1	0/5	0/5	1/5	0/1	0/5	0/1	0/5	0/1	0/5	1/1	Rachis 1/5	0/1	0/5	0/1	0/5
24/3/05	0/1	0/5	n/a	0/5	0/1	0/5	n/a	0/5	0/1	Rachis 1/5[C] Shell 1/5[C]	n/a	Hull 1/5[C] Kernel 1/5[C]	0/1	0/5	n/a	0/5
18/5/05	0/1	n/a	n/a	n/a	0/1	n/a	n/a	n/a	0/1	n/a	n/a	n/a	0/1	n/a	n/a	n/a
12/10/05	0/10	1/5	2/10	0/5	1/10	0/5	0/10	0/5	0/10	0/5	0/10	0/5	0/10	0/5	0/10	0/5
27/10/05	1/10	0/5	0/10	0/5	0/10	0/5	0/10	0/5	0/10	0/5	1/10	3/5	0/10	0/5	1/10	0/5
9/11/05	0/10	0/5	0/10	0/5	0/10	0/5	0/10	0/5	1/10	0/5	4/10	0/5	0/10	0/5	0/10	0/5
23/11/05	0/10	0/5	0/10	0/5	1/10	0/5	0/10	0/5	1/10	0/5	1/10	0/5	0/10	0/5	0/10	0/5
9/12/05	0/10	n/a	1/10	n/a	0/10	n/a	0/10	n/a	0/10	n/a	1/10	n/a	0/10	n/a	0/10	n/a
20/12/05	0/10	n/a	0/10	n/a	0/10	n/a	0/10	n/a	0/10	n/a	1/10	n/a	0/10	n/a	0/10	n/a
8/3/06[D]	0/10	0/5	0/10	0/5	0/10	0/5	0/10	0/5	0/10	0/5	0/10	0/5	0/10	0/5	0/10	0/5

[A] Leaves from each tree were pooled between October 2004 and May 2005.

[B] Not applicable.

[C] Nuts from same bunch.

[D] Rachi washed and blended, hulls and kernels blended separately, shells washed.

Figure legends

Fig. 1. Cross-section of the trunk of a moderately diseased male pistachio tree.

A = annual ring, sapwood

B = chestnut-coloured specks

C = chestnut-coloured smear

D = heartwood staining

Fig. 2. Dissected lesion from the branch of a moderately diseased male pistachio tree.

Fig. 3. Rod-shaped bacteria and lattice-like deposit in xylem in stained pistachio wood viewed by scanning electron microscopy: (*a*) bacteria (circled) in stained young sapwood from the trunk of a severely diseased male tree; (*b*) bacteria and deposit (arrowed) in stained 2-year old xylem from the trunk of a moderately diseased male tree.

Fig. 4. Xylem vessels with tyloses (arrows) in unstained tissue immediately adjacent to stained tissue from a twig from a severely diseased male tree, viewed by scanning electron microscopy.

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Fig. 1

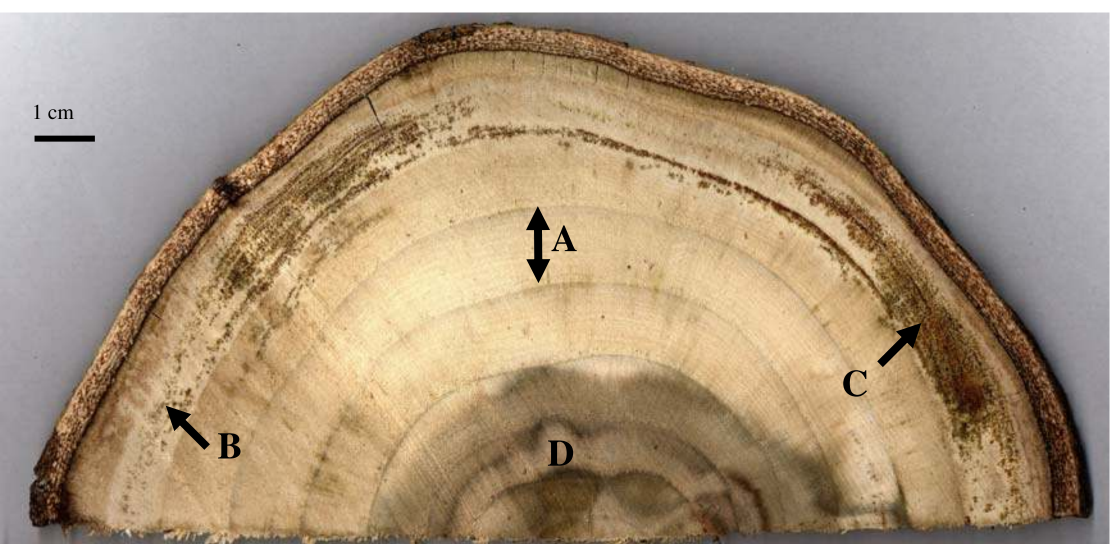


Fig. 2

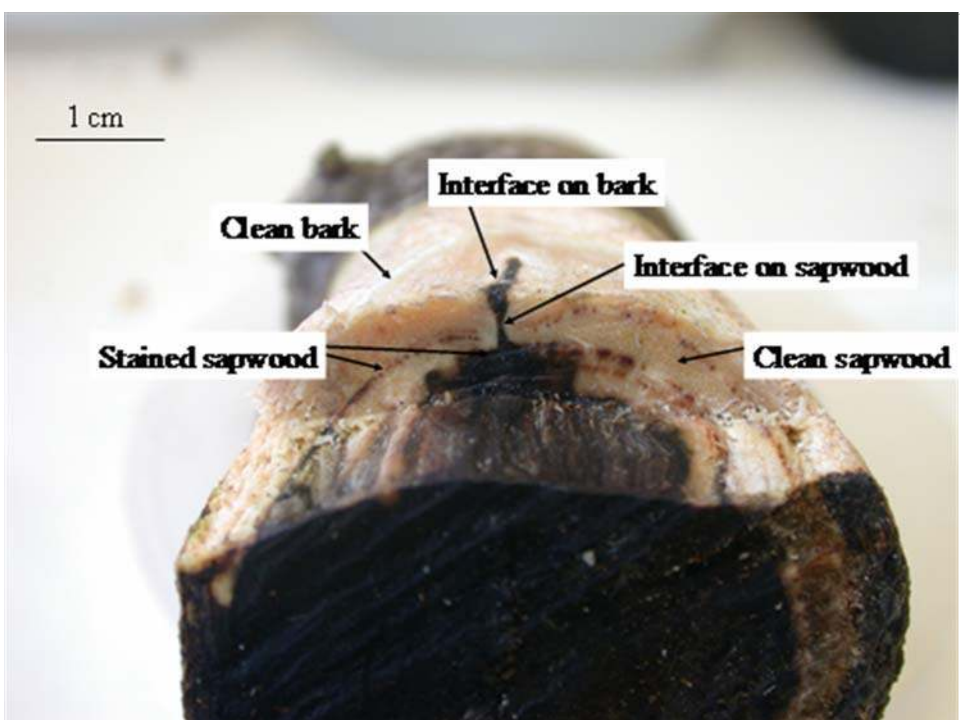
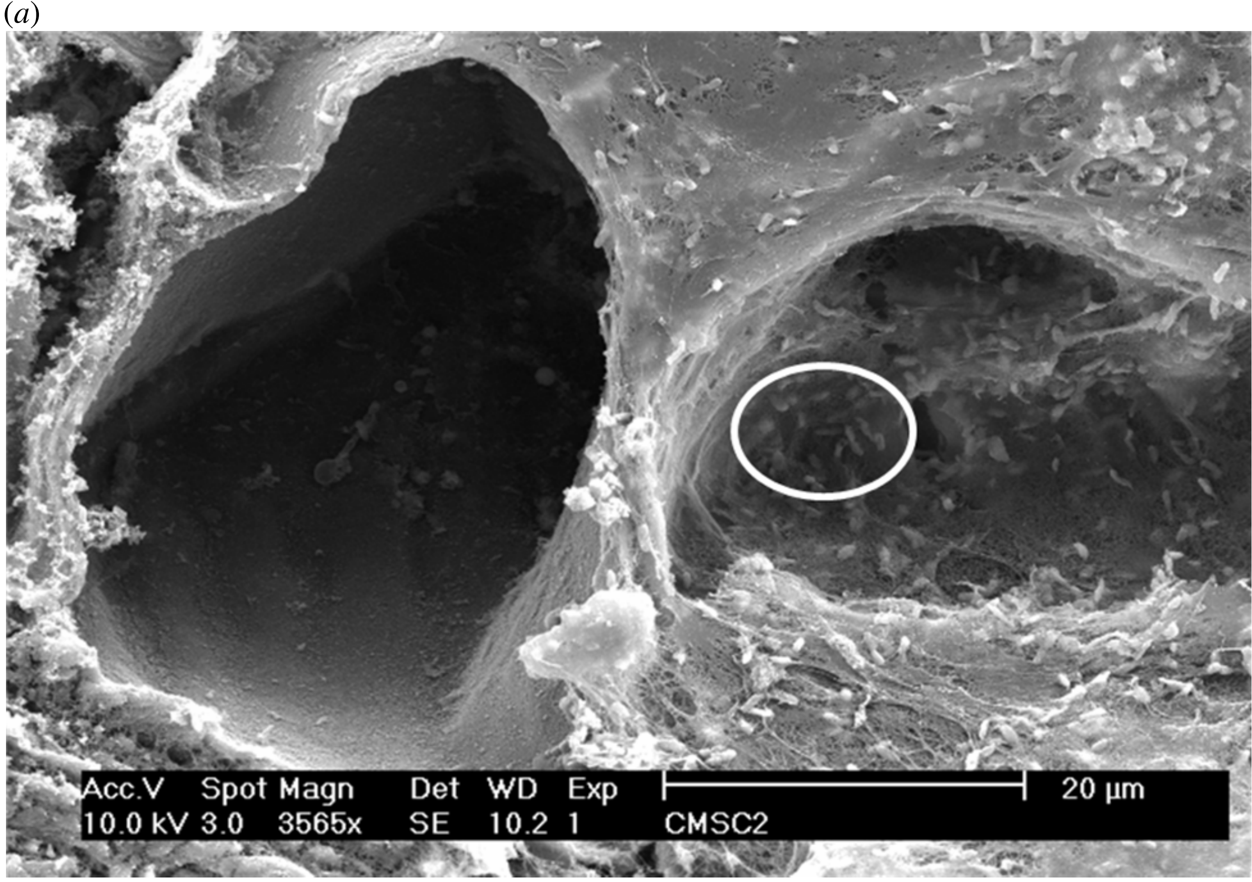


Fig. 3

(*a*)



(*b*)

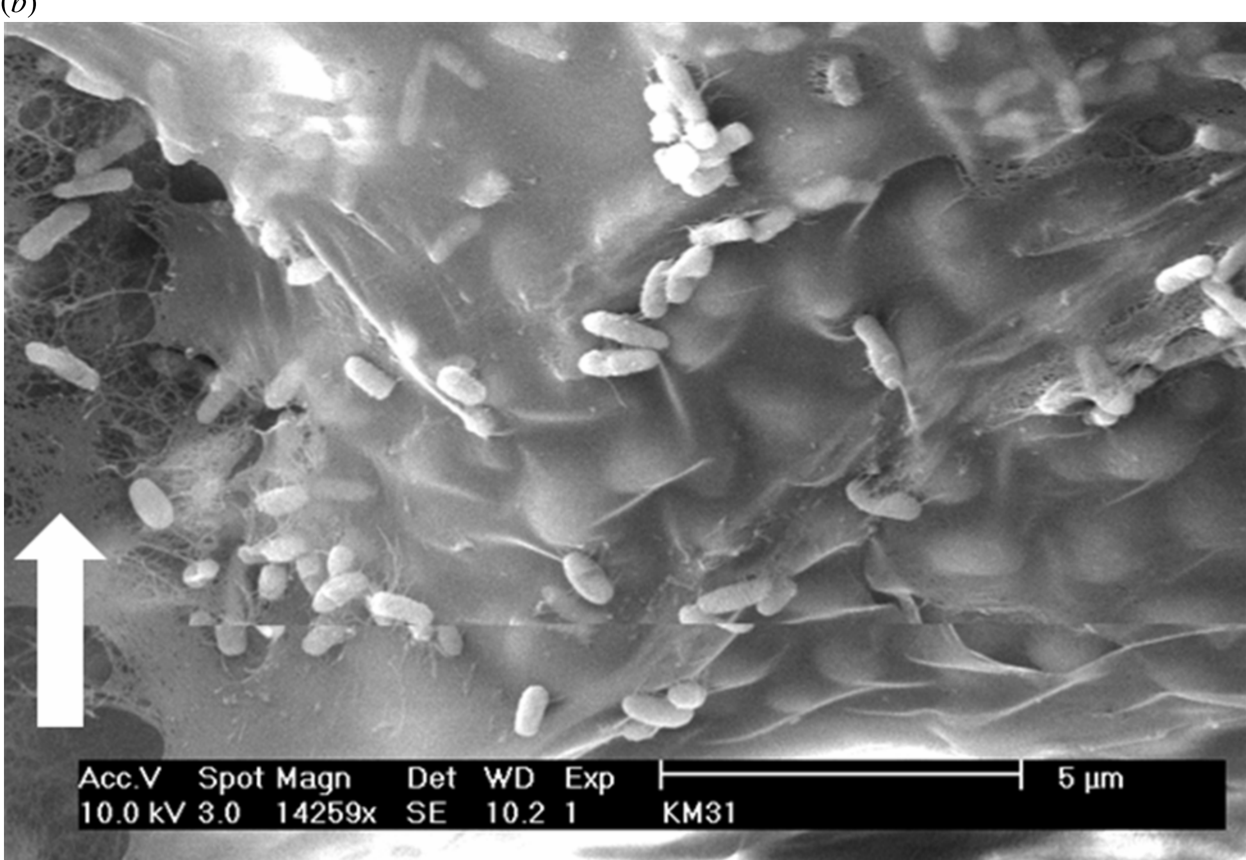


Fig. 4