Final report

Challenges to the Australian pistachio industry – Bacterial dieback and nut quality

PS06002 (31/10/2009)

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PS06002

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This report details results of research into bacterial dieback and nut quality issues challenging the Australian pistachio industry.

Funding sources and support

Horticulture Australia Ltd

Pistachio Growers Association

The University of Adelaide

Victoria Department of Primary Industries

South Australian Research and Development Institute

Collaborations

Macquarie University, Sydney

Central Science Laboratory, York, UK

October 2009

1. Media summary

For the last two decades, the Australian pistachio industry has been affected by dieback, a disease that causes trunk and limb lesions, internal wood staining, decline and dieback. External symptoms usually appear after trees reach maturity and can lead to death. The causal agent is a bacterium belonging to the *Xanthomonas translucens* group, hereafter referred to as *Xtp*. Another significant issue for the Australian pistachio industry is Golden Stain (GS), a quality problem first reported in 2002, characterised by a yellow to brown stain on the surface of the shell. Affecting up to 2.3% of the crop in some years, it renders affected nuts unsuitable for the snack market and increases the sorting costs. Preliminary investigation (HAL PS03003 final report) suggested that GS had physiological causes.

The focus of this research project was to achieve a better understanding of dieback and GS to assist pistachio growers with the management of these issues. For dieback, the needs were to optimise the detection of *Xtp*, to further characterise *Xtp* and the epidemiology of the disease, and to evaluate prevention and management strategies. For GS, the aims were to determine the cause(s) of the condition and use that information to devise a management strategy. The major outcomes of this project are:

- The techniques designed previously to detect *Xtp* were optimised and a more sensitive technique was developed.
- The causal agent of pistachio dieback was further characterised and its taxonomic status clarified; the name *Xanthomonas translucens* pathovar *pistaciae* was proposed and accepted by the international scientific community.
- The establishment of a budwood repository as a source of healthy material for propagation was suspended because no suitable orchard could be identified; *Xtp* was not detected in buds collected from infected trees, which supports budding as a low risk propagation method providing good hygiene is practised.
- A long term field assessment of drastic pruning, in which trees are cut back to secondary or tertiary branches, has shown some success in restoring severely affected trees to productivity; it represents a profitable alternative to replacing a diseased tree with a replant.
- Late harvesting, over-ripe nuts, damage to hulls, ‘early-split’ nuts, and increased storage temperature and time were identified as factors associated with increased incidence of GS; recommendations to help manage these issues were communicated to the growers.

This research will lead to a healthier and more profitable pistachio industry and will facilitate expansion. We recommend further studies to identify from where *Xtp* originated and understand how it causes disease in pistachio, to determine if biological and/or environmental factors play a role in spread and symptom expression of pistachio dieback, and to investigate the effect of nutritional factors on the incidence of GS. Such information would underpin further improvement of management strategies.

2. Technical summary

For the last two decades, the Australian pistachio industry has been affected by dieback. Apparently endemic to Australia, the disease is characterised by trunk and limb lesions, excessive resinous exudates, discolouration of mature xylem, stunted growth and shoot dieback. External symptoms usually appear after trees reach maturity. Affected trees gradually decline, fail to produce marketable nuts and eventually die. The first outbreak of the disease occurred with the onset of commercial production in the mid-late 1990s, killing or rendering unproductive up to 10% of the trees. Another serious outbreak occurred in 2005. The causal agent is a bacterium belonging to the *Xanthomonas translucens* group, hereafter referred to as *Xtp*. Another significant issue for the Australian pistachio industry is Golden Stain (GS), a quality problem, characterised by a yellow to brown stain on the surface of the shell. First reported in 2002, and affecting up to 2.3% of the crop in some years, it renders affected nuts unsuitable for the snack market and increases sorting costs. Preliminary investigation suggested that GS had physiological causes. The aims of this project were to achieve a better understanding of dieback and GS to assist management of these issues, leading to a healthier and more profitable industry. The major outcomes of the project are:

- The techniques designed previously to detect *Xtp* were optimised and a more sensitive technique was developed; three techniques, namely culturing, Polymerase Chain Reaction (PCR) and Real-Time (RT)-PCR are now available to detect *Xtp* in a range of infected material, including infected but still asymptomatic tissues.
- Based on its distinctive pathogenicity to pistachio and on molecular characterisation, *Xtp* has been classified as a new pathovar (pathogenic variety, pv.) of *X. translucens*, namely *X. translucens* pv. *pistaciae*; the genetic characterisation further supports the hypothesis that *Xtp* originated from a grass-infecting *Xanthomonas*.
- Surveys showed that, for the last 3 years, progression of dieback has been slow, and trees newly diagnosed as infected did not show any symptoms; these observations suggested that, during the major outbreaks of the disease, some unknown factor(s) triggered spread and symptom expression.
- *Xtp* was detected at very low concentration in some healthy trees, which confounded the establishment of a budwood repository as a source of healthy material for propagation as no orchard could be considered *Xtp*-free. However, *Xtp* was not detected in buds collected from infected trees, which supports budding as a low risk propagation method providing good hygiene is practised.
- A long-term field assessment of drastic pruning, in which trees are cut back to secondary or tertiary branches, has shown some success in restoring severely affected trees to productivity; the technique has proved to be a profitable alternative to replacing a diseased tree with a replant.
- Late harvesting, over-ripe nuts, damage to hulls, ‘early-split’ nuts, and increased storage temperature and time were identified as factors increasing incidence of GS; no pathogen was consistently associated with GS; recommendations to help manage GS were communicated to pistachio growers.

For dieback, we recommend using diagnostic techniques to identify *Xtp*-infected trees and to check that trees to be used for propagation are *Xtp*-free; budding is likely to be a low risk propagation method if good hygiene is practised for propagation; drastic pruning, which is already being used in some commercial orchards, may be used as part of an integrated

management strategy for pistachio dieback. Future research should identify from where *Xtp* originated, understand how it causes disease in pistachio, and determine if biological and/or environmental factors play a role in spread of pistachio dieback and expression of disease symptoms. To minimise incidence of GS, harvest should be carried out promptly, orchards should be managed to reduce nut damage, and postharvest storage time and temperature should be carefully managed. Future research should investigate the effect of nutritional factors on the incidence of GS.

3. Introduction

Dieback has affected the Australian pistachio industry since the onset of commercial production in the early 1990s. The disease causes decline, stunted growth, and dieback. Affected trees fail to produce marketable nuts and eventually die. The first outbreak occurred in the late 1990s, killing or rendering unproductive up to 10% of the trees in some orchards (Anonymous, 2003). Another serious outbreak was recorded in 2005. Since its first description, knowledge of pistachio dieback and of its causes has improved considerably.

Early investigation (HAL NT99004 final report; Facelli *et al*., 2002 and 2005) showed pistachio dieback to be a unique and complex bacterial disease, caused by strains of *Xanthomonas* similar to *X. translucens*, hereafter referred to as *Xtp.* The disease is associated with internal staining of the heartwood and sapwood of the trunk, branches and twigs (Facelli *et al.*, 2009). Severely diseased trees produce excessive resin and have bark lesions, usually covered by sooty mould. There is no evidence of symptoms on the foliage and the pathogen does not appear to depend on high relative humidity for spread or persistence, in contrast to other bacterial diseases. *Xtp* enters the trees and occupies the vascular tissues, where it blocks the xylem and reduces water conductivity. The bacteria seem to be localised to the vascular tissue active in the year of inoculation, with little or no lateral spread.

Studies into the epidemiology of the disease (HAL PS04015 Final report, Facelli *et al.*, 2009) revealed that the bacteria are rarely found on tree surfaces. It is not known how they spread in nature: natural points of entry, including leaves and lenticels, and dispersal via air, rain or irrigation run-off, do not appear to contribute significantly to the spread of the pathogen. Furthermore, no evidence was found to suggest that buds, bark lesions, leaves or nuts are likely sources of inoculum from which infection is able to spread. However, bacteria were recovered from pruning tools and it was further confirmed that *Xtp* can be transmitted via pruning tools between orchard trees and that severely diseased trees may have received multiple re-infections via pruning. Field trials showed that pruning with simultaneous application of bactericide could reduce this spread. Although not registered for use on pistachio, quaternary ammonium-based bactericides protected pruning wounds from infection, when used at high concentrations. Preliminary results suggested that the recovery of severely diseased trees may be possible via drastic and clean pruning to promote growth of new uninfected shoots.

Molecular investigations (HAL NT02007 final report) showed that *Xtp* is closely related to, but distinct from, the wheat pathogen *X. translucens*. Two genetically distinct groups of *Xtp*, namely *Xtp*A and *Xtp*B, were described that differ in their host ranges (Marefat *et al*, 2006b). Based on this knowledge, a specific and reliable Polymerase Chain Reaction (PCR) assay was developed to improve disease diagnosis (Marefat *et al*., 2006a). To date, *Xtp*A has been found to occur in all affected orchards, whereas *Xtp*B has been found in only one, together with *Xtp*A.

Another challenge to the Australian pistachio industry in the recent years has been Golden Stain (GS). It is a quality problem of unknown origin which renders affected nuts unsuitable for the snack market. Preliminary data suggested that it could have a physiological origin.

In this project, investigations were undertaken to (1) optimise the detection of *Xtp*, (2) clarify the origin of *Xtp* and its pathogenicity to pistachio, (3) further characterise the disease cycle and epidemiology, (4) evaluate management and prevention strategies for controlling *Xtp* and (5) determine the cause(s) of GS and propose possible management strategies.

The specific objectives were to:

- Refine the detection of *Xtp* by PCR and develop a quantitative assay (real time (RT)-PCR) to increase sensitivity and to allow detection from a range of infected tissues, including from asymptomatic trees;
- Clarify the taxonomic position of *Xtp* within the *Xanthomonas* genus, and further compare the pathogenicity to pistachio between the two groups;
- Follow up investigations addressing the location of *Xtp* in infected trees and orchards and infection pathways;
- Evaluate drastic pruning as a management option for diseased trees, establish a source of clean propagation material and review the potential of biological control methods;
- Investigate various physiological and pathological factors as possible causes of GS and propose management strategies to control or minimise GS.

4. Detection

Methods for isolation and detection of *Xtp* from infected wood were developed previously, based on culturing the bacteria on semi-selective medium (HAL NT99004 final report; Facelli *et al.*, 2005). Later a PCR assay was designed not only to detect specifically *Xtp* but also distinguish the two groups of the pathogen (HAL NT02007 final report; Marefat *et al.*, 2006a). Here, we optimised and validated the PCR protocol with a range of infected tissues. We also developed an RT-PCR protocol to overcome some limitations of the PCR and to increase sensitivity, particularly to allow detection of *Xtp* in asymptomatic material where the bacterial population is likely to be very small.

4.1. Material and methods

4.1.1. Optimisation and validation of PCR

The PCR protocol developed previously included the following steps: 500-700 mg of woody tissue were soaked overnight in 10 ml of nutrient broth (NB). Bacteria were collected by centrifugation at 5,000 rpm for 5 minute, resuspended in 100 µl of sterile distilled water (SDW) and 5 µl of the resulting suspension were used as templates in the PCR using *Xtp*-specific primers. Polyvinylpyrrolidone (PVP, MWt 44,000) was added to the reaction mixture to neutralise PCR-inhibiting components coming from pistachio. Typical PCR products for *Xtp*A and *Xtp*B were 331 bp and 120 bp, respectively.

To optimise this protocol, various changes to the extraction and the PCR itself were assessed for their effect on the consistency and sensitivity of *Xtp* detection. The parameters that were investigated were (1) the time between collection of material and processing, (2) the soaking medium (SDW *vs* NB), (3) the soaking time and temperature (4 h at 28ºC *vs* overnight at room temperature), (4) the resuspension of bacteria after concentration (volume and time) (5) the dilution and volume of bacterial suspension used in the PCR and (6) the number of PCR cycles (35 *vs* 40). All experiments were conducted in triplicate using infected wood collected in the Robinvale orchard, and PCR results were compared with the culturing technique described in NT99004 (HAL final report).

To overcome problems with inconsistency in replicated experiments linked to uneven distribution of bacteria in infected wood, a 'flushing' protocol to extract bacteria from wood samples was assessed in comparison with the 'soaking' protocol described above. Twigs were collected from infected trees and cut in half. For each twig, one half was processed using the 'soaking' protocol. 1 ml of SDW was forced through the other half (3-4 cm long) under vacuum and collected in a sterile Eppendorf tube. An *Xtp*A or *Xtp*B suspension was

flushed through a healthy piece of twig as a positive control for the 'flushing' technique. Resulting suspensions were either spread onto Benlate-Sucrose Peptone Agar (BSPA) or concentrated by centrifugation and used in PCR.

4.1.2. Development of RT-PCR protocol

Note: RT-PCR is a quantitative variant of PCR; the amplification of the template DNA is followed in real time, via the addition of a fluorescent probe in the reaction. RT-PCR results are expressed as Cycle Threshold (Ct) values, which can be related to the initial amount of bacterial DNA in the sample analysed, by comparison with Ct values obtained with a range of known concentrations of the template DNA (or standard). The higher the Ct value is, the less bacterial DNA in the sample. In our conditions, the maximum Ct value was set at 40.

The sequences of the Internal Transcribed Spacer (ITS) of *Xtp*A and *Xtp*B isolates determined previously (HAL NT02007 final report) were used to design a set of primers and a probe suitable for multiplex detection of *Xtp*A and *Xtp*B in one RT-PCR. The reaction conditions were optimised for that particular set using dilutions of *Xtp*A DNA as standard.

The specificity of the primers and probe set was assessed using various PCR conditions against DNA extracted from a collection of 31 *Xanthomonas* strains, including all *X. translucens* pathovars.

The specificity of detection of *Xtp*A *vs Xtp*B was also assessed when the two groups were in a mixture. For this purpose, mixtures containing various proportions of *Xtp*A and *Xtp*B DNA or cells were tested using either simple or multiplex RT-PCR.

4.1.3. Detection thresholds – Comparison of techniques

To compare the detection thresholds of the three protocols, 10-fold serial dilutions of bacterial suspensions, from 10^8 down to 1 CFU/ml, were prepared from pure cultures of either *Xtp*A or *Xtp*B. To take into account potential inhibition by wood components, dilutions were prepared not only in SDW, but also in extracts prepared from wood soaked in SDW, with or without concentration of the extract. Wood for this purpose was obtained from healthy trees. Aliquots of each dilution were spread on SPA and used in PCR and RT-PCR. The experiment was replicated three times.

4.2. Results

4.2.1. Optimisation and validation of PCR

Several of the parameters investigated significantly improved the sensitivity and consistency of PCR. In contrast, 'flushing' proved to be no better than 'soaking' as a means of extracting *Xtp* from pistachio wood. The protocol described in NT02007 (HAL final report) was modified accordingly:

500-700 mg of woody tissues soaked 4 h at 28°C with shaking in 10 ml of SDW; bacteria collected by centrifugation of 1.5 ml of the suspension for 15 minutes at 10,000 rpm; pellets resuspended in 15 µl of SDW and left to resuspend at least overnight; 1-8 µl of bacteria extract to be used as template in PCR with 40 cycles.

The protocol has been tested with a range of material, which showed that (1) wood samples need to be processed as soon as possible after collection as a delay results in decreased sensitivity of the PCR and (2) the PCR is very sensitive to inhibitors. As described previously, PVP efficiently neutralises PCR-inhibiting components of wood but, if dealing

with severely stained tissues, better results are obtained when the concentrated extract is diluted 1:10 or 1:100 before the PCR. The protocol is not suitable for use with root samples (see section 6.2.1). After the 'soaking' step, 100 µl of the resulting suspension can be spread on BSPA for isolation of *Xtp*, so the two techniques can be run concurrently on the same extract.

4.2.2. Development of RT-PCR protocol

A set of primers and probe was successfully designed to detect both groups of *Xtp* in a multiplex RT-PCR. The protocol was validated on bacterial suspensions prepared from pure cultures and from wood extracts. In all cases, better sensitivity was achieved with *Xtp*B than with *Xtp*A. Preparation of wood extracts as described in section 4.2.1 worked well with most wood samples. However, as for PCR, RT-PCR is sensitive to inhibitors contained in pistachio wood and DNA extraction was necessary to overcome inhibition and/or extraction problems with some materials (roots, pollen, buds).

RT-PCR detected most of the *X. translucens* pathovars and a few other *Xanthomonas* species, but with a much lower sensitivity than *Xtp*. However, this was not considered to be a problem as none of these species and pathovars is present in Australia and/or is known to infect pistachio. When the two groups are in a mixture or at very low concentration of one of the group *vs* the other, cross reactions can be observed between the two groups.

4.2.3. Detection thresholds – Comparison of techniques

The sensitivity of the three techniques was compared by using bacterial suspensions of known concentrations (Table 1). Culturing on semi-selective medium proved to be efficient to detect bacteria down to 10-10^2 cells/ml of *Xtp*A or *Xtp*B, regardless of whether the suspensions were prepared in SDW or wood extracts. In contrast, PCR and RT-PCR revealed different detection thresholds for the two groups of bacteria and were affected by inhibitors contained in pistachio wood. Detection thresholds ranged from 10^4-10^6 and 10^3-10^4 bacteria/ml for *Xtp*A and *Xtp*B, respectively in PCR and from 10^2-10^3 and 10-10^2 bacteria/ml for *Xtp*A and *Xtp*B, respectively, in RT-PCR.

Table 1. Comparison of detection thresholds achieved by culturing, PCR and RT-PCR techniques assessed on *Xtp* suspension in water, wood and concentrated wood extracts

	Detection threshold (in bacteria/ml) by		
	Culturing	PCR	RT-PCR
*Xtp*A / Water	10-10^2	10^4	10^3
*Xtp*A / Wood	10-10^2	10^6	10^2
*Xtp*A / Conc. wood	10-10^2	10^6-10^7	10^3
*Xtp*B / Water	10-10^2	10^3	10
*Xtp*B / Wood	10-10^2	10^3	10
*Xtp*B / Conc. wood	10-10^2	10^4	10^2

Our observations with samples collected from the field suggested, however, that culturing and PCR have a very similar sensitivity, whereas RT-PCR showed greater sensitivity, including with material collected from asymptomatic trees (see section 7.2.3).

5. Origin and pathogenicity

Research conducted in project NT99004 (HAL final report; Facelli *et al*., 2005), and project NT02007 (HAL final report; Marefat *et al*., 2006b) convincingly demonstrated the role of a type of *X. translucens* in pistachio dieback. However, the identification of bacteria pathogenic to pistachio, a dicotyledonous woody host, as *X. translucens*, a species only reported in grasses and a few other monocots (Vauterin *et al*., 1995), raised questions about the origin of *Xtp*. Also, several results suggested that *Xtp*A and *Xtp*B, although both isolated from pistachio, differed in their pathogenicity and aggressiveness (Facelli *et al*., 2005; Marefat *et al*., 2006b). Here, we used phylogenetic analysis and screening of integrons to clarify the taxonomic status of *Xtp*. We also compared *Xtp*A and *Xtp*B for their pathogenicity to pistachio in terms of incidence of infection, after artificial inoculation, and spread in natural conditions.

5.1. Material and methods

5.1.1. Phylogenetic analysis

Parkinson *et al*. (2007 and 2009) showed that comparison of sequence of the 'house-keeping' gene *gyrB* yielded a phylogeny of the genus *Xanthomonas* that distinguishes most species and pathovars. A collaboration was established with N. Parkinson (CSL, York, UK) to sequence the *gyrB* gene of *Xtp* and determine its relatedness to other *Xanthomonas*, particularly *X. translucens* pathovars. The *gyrB* gene of two isolates in each group of the pistachio pathogen was amplified by PCR using primers previously shown to amplify *gyrB* from a large number of *Xanthomonas* species and pathovars (Parkinson *et al*., 2007 and 2009). PCR products were purified and sequenced. Sequences of the *Xtp* isolates were aligned, using the program Clustal W (http://www.ebi.ac.uk/clustalw), with those of 35 strains of *Xanthomonas,* including nine of the 10 pathovars of *X. translucens*. The alignment was used to draw a phylogeny of the genus *Xanthomonas*, including *Xtp*.

5.1.2. Integron screening

Gillings *et al.* (2005) have shown that integrons have contributed to genome diversity among *Xanthomonas* and suggested that they may have played a role in adaptation of *Xanthomonas* to various hosts. The presence of such components in the genome of *Xtp* could help explain its ability to infect and cause symptoms in pistachio trees, and help to elucidate its origin. A collaboration was set up with M. Gillings (Macquarie University, Sydney) to investigate this hypothesis.

To take into account the genetic diversity previously described among *Xtp* (HAL NT02007 final report and Marefat *et al*., 2006b), 15 isolates of *Xtp*A and three of *Xtp*B were screened for integrons. *X. translucens* pv. *translucens* (DAR 35705) isolated from wheat in Australia and *X. oryzae* pv. *oryzae* (DAR 61713) were used as references. Chromosomal DNA was extracted from each isolate as described by Gillings *et al*. (2005). A first PCR was conducted and PCR products were sequenced to ascertain the presence of an integron in the genome of *Xtp* isolates. A second PCR, with another set of primers, was used to compare the integron-associated genes between isolates. Primers and protocols were as described by Gillings *et al*. (2005).

Some *X. translucens* isolated from grasses collected in pistachio orchards in previous projects (HAL NT99004 and PS04015 final reports) were also screened for integrons and compared with *Xtp* and other *Xanthomonas* species. Eighteen *X. translucens*-like bacteria

isolated either in winter (11 isolates) or in summer (seven isolates) were tested. As for *Xtp*, DNA was extracted from each isolate and the two PCRs described above were conducted to check the presence of an integron and to compare integron-associated genes between isolates.

5.1.3. Comparison of Xtp*A and* Xtp*B for their pathogenicity to pistachio*

a- Incidence of infection after artificial inoculation

To compare the ability to initiate infection between *Xtp*A and *Xtp*B, an experiment was initiated in December 2004. Trees were inoculated by applying 100 µl of a suspension containing 10^7 *Xtp* cells/ml on the surface of a pruned twig, as described in NT99004 (HAL final report). Control trees received 100 µl of SDW. 27, 20 and 20 trees were inoculated with *Xtp*A, *Xtp*B and SDW, respectively. Some trees were destructively sampled in August 2005. The rest of the trees were sampled in March and August 2007. Assessment was by culturing at all sampling times and also by PCR in March and August 2007.

b- Spread in natural conditions

Robinvale is the only orchard in which both *Xtp*A and *Xtp*B have been found. In Stage 4 of that orchard, after the 2005 outbreak, *Xtp*A had been isolated from 15 trees and *Xtp*B from two trees (Appendix 2). However, no tree was found to be naturally infected by the two groups. To follow disease progression and to investigate if co-infection by the two groups could be detected, Robinvale stage 4 was sampled in spring 2006 and again in autumn and spring 2007. Several 1-2-year-old twigs were collected from each of the trees surrounding the infected trees and tested by both culturing and PCR.

5.2. Results

5.2.1. Phylogenetic analysis

The *gyrB* gene was successfully amplified from group A and group B isolates, sequences being identical within groups (100% homology) but different between groups (96% homology). The phylogenetic tree showed that *Xtp* clusters among *X. translucens* pathovars but also revealed that the two groups originated from two different lineages (Appendix 1).

5.2.2. Integron screening

Integron screening of a collection of *Xtp* isolates showed that the genome of the pistachio pathogen contains an integron, as do other *Xanthomonas*. However, the integron-associated genes in *Xtp* isolates are different from those in other *Xanthomonas* species. They are also different between the two groups of *Xtp* but they are similar within each group (Giblot-Ducray *et al*., in press).

Screening of integrons in 15 isolates of *X. translucens*-like bacteria isolated from grasses growing in pistachio orchards showed that they all contain a *Xanthomonas*-type integron. The grass isolates revealed five different patterns of integron-associated genes, with some isolates harbouring the same patterns, even when collected in different seasons. Although more closely related to that of the *Xtp*B isolates, none of the grass isolate patterns exactly matched *Xtp*.

5.2.3. Comparison of Xtp*A and* Xtp*B for their pathogenicity to pistachio*

a- Incidence of infection after artificial inoculation

In August 2005, *Xtp* was isolated from 8/10 trees inoculated with *Xtp*A, from 1/10 trees inoculated with *Xtp*B and from none of the negative control trees (Table 2). In March and September 2007, although there was a discrepancy between the results obtained by culturing and by PCR, *Xtp* was also detected in a higher proportion of trees inoculated with *Xtp*A (10/12) than with *Xtp*B (4/7). On no occasion were external symptoms observed. The final incidence of infection was 81 and 29% for *Xtp*A and *Xtp*B, respectively.

Table 2 – Comparison of infection rates between *Xtp*A and *Xtp*B after artificial inoculation

Treatment	August 05		March 07		September 07		Final % infected
	Culturing	PCR	Culturing	PCR	Culturing	PCR	
*Xtp*A	8/10[a]	N/A	4/12	4/12	6/12	10/12	81
*Xtp*B	1/10	N/A	0/7	1/7	1/7	4/7	29
SDW	0/6	N/A	0/6	0/6	NT	NT	0

[a] number of positive trees out of the total number of trees sampled

Note: some of the trees died in February 2005 because of a heat wave: 4 inoculated with Xtp*A, 3 inoculated with* Xtp*B and 3 non-inoculated controls.*

b- Spread in natural conditions

In 2005, Stage 4 of the Robinvale orchard was extensively sampled; all the trees contained in a designated area (limited by a black rectangle in Appendix 2) were tested. Other trees were sampled and tested based on symptom expression. Sampling over the duration of the project showed a slow progression of the disease within the orchard. Several trees were newly identified as being infected in spring 2006 and summer 2007 but none in autumn 2007. Altogether, three trees were found to be infected with *Xtp*A, all next to *Xtp*A-infected trees, and two with *Xtp*B, both next to *Xtp*B-infected trees. No tree was found to be infected by the two groups, even when trees infected by *Xtp*A and *Xtp*B were in close proximity.

6. Disease cycle and epidemiology

The location of *Xtp* in infected trees was studied previously, using the culturing technique (HAL NT99004 final report) and infection pathways and spread of disease were also investigated (HAL PS04015 final report). Here, we re-assessed the presence of *Xtp* in pollen, roots and buds, taking advantage of the more sensitive PCR and RT-PCR techniques developed in this project. We also looked again for *Xtp* in orchard floor vegetation and we completed and replicated the leaf inoculation experiment initiated during PS04015. This was designed to clarify the role of pollen, roots, buds and orchard floor vegetation in the disease cycle and determine if *Xtp* can enter pistachio trees through leaves.

6.1. Material and methods

6.1.1. Location of Xtp *in infected trees*

a- Pollen

In previous investigations (HAL NT99004 final report), *Xtp* was found in the pollen of one inflorescence out of 50 collected from infected trees. These results warranted

reassessment of pollen for *Xtp*, using the more sensitive RT-PCR technique. This necessitated development of a DNA extraction technique as the standard 'soaking' protocol was unlikely to release bacteria enclosed in pollen grains. The principle in this case is to extract whole DNA content of the plant material and to detect specifically the DNA of *Xtp*.

Early in November 2007, mature inflorescences were collected from male trees in the Waite orchard and left overnight at room temperature in sterile Petri dishes. The next day, pollen was recovered and used to compare the yields of three DNA extraction protocols, the 'PowerPlant' and 'Ultraclean' plant DNA isolation kits (MO BIO laboratories, USA) and the SARDI RDTS protocol (Ophel-Keller *et al*., 2008). In addition, as whole DNA extracts may still contain plant inhibitors, their potential to inhibit RT-PCR was assessed. For this purpose, extracted DNAs, pure or diluted 1:10 and 1:100, were artificially mixed with known amounts of *Xtp* DNA. Efficiency of detection of *Xtp* in these mixtures was compared to that of *Xtp* alone.

At the end of November 2007, 75 pollen samples were collected from three infected trees at the Robinvale orchard, as described above. All but 10 samples comprised 50 mg of pollen. One sample contained only 11 mg and nine others contained 30-44 mg each. DNA was extracted using the RDTS protocol and tested for *Xtp* by RT-PCR.

b- Roots

The presence of *Xtp* in roots was previously investigated using the culturing technique. Although isolation from roots did not yield *Xtp*, plates were overgrown by other micro-organisms, which may have inhibited or masked the growth of *Xtp*. The results were therefore inconclusive and this warranted further investigation using the more specific RT-PCR. Also, several observations over the years suggested that *Xtp* may need a 'trigger' to stimulate symptom expression. One of our hypotheses is that other micro-organisms could play such a role. Therefore, as well as *Xtp*, roots were also assessed for the presence of two soil fungi, for which RT-PCR diagnostics were available at SARDI. These were *Verticillium dahliae*, which causes Verticillium wilt in pistachio, and *Pythium* species belonging to the so-called 'clade F' containing important plant pathogens with a world-wide distribution (Lévesque and De Cock, 2004).

Preliminary experiments were conducted to determine the best processing technique. Superficial roots were collected from pistachio trees at the Waite orchard. The 'soaking' technique used to prepare wood samples for the PCR was rapidly abandoned because of the severe inhibition observed in subsequent RT-PCR. Roots were washed twice in water and dried. They were then cut into small pieces, which were subsequently placed in a plastic bag and hit with a mallet to soften tissues. The same three DNA extraction protocols tested with pollen were compared in terms of yield of DNA from roots and inhibition of PCR.

Roots from trees at the Robinvale orchard were sampled in autumn (mid May 2008) and again in winter (late July 2008) to take into account potential seasonal variation in the amount of *Xtp* and other fungi. Fifty-two root samples, collected from three *Xtp*A- and three *Xtp*B-infected trees, were tested in autumn. Twelve samples collected from two healthy trees and 34 samples collected from three *Xtp*A- and two *Xtp*B-infected trees were tested in winter. DNA was extracted from 1-2g of root tissues using the RDTS protocol and assessed for *Xtp*, *V. dahliae* and *Pythium* spp. by RT-PCR.

c- Buds

Pistachios are propagated either by grafting or budding onto rootstocks, which may represent a risk of spreading the disease. Previous investigations (HAL NT99004 final report) have shown that budding with a contaminated budding knife was unlikely to initiate infection

and suggested that buds collected from infected trees were free of bacteria. However, only the culturing technique was available at the time. Therefore, budwood was collected in January 2007 and 2009, at the same time as the industry collected budwood for propagation, to reassess the presence of *Xtp*.

In 2007, budwood was collected from two infected trees among the 'T5 daughter' trees in Kyalite orchard. Sample preparation was conducted in a laminar flow cabinet. Buds and their bud shield were individually removed from the twigs, flamed and cut into small pieces. All the pieces were collected and soaked in SDW, and the resulting suspensions were either spread on BSPA for isolation of *Xtp* or used in PCR after concentration by centrifugation. Altogether, 720 buds were assessed individually.

In 2009, budwood was collected from two healthy 'T5 daughter' trees, the two infected 'T5 daughter' trees assessed in 2007, two weakly positive trees from the New South Wales DPI orchard at Dareton and three severely diseased trees from stage 3 of the Kyalite orchard. As buds were small, they were processed in batches of 4-6. Buds and their bud shield were cut into small pieces. Meticulous precautions were taken to avoid cross contamination between samples. DNA was extracted using the RDTS protocol and *Xtp* detection was conducted using RT-PCR. Altogether, 165 samples were analysed, which represented a minimum of 660 buds.

6.1.2. Orchard floor vegetation survey

Orchard floor vegetation was surveyed in winter, spring and summer 2003 and in winter 2004 (HAL NT99004, PS04015 and NT02007 final reports). On all occasions, *X. translucens*-like bacteria were isolated but no *Xtp* has been found. Further sampling was conducted here to elucidate the role of grasses as potential sources of *Xtp* inoculum or as alternative hosts in infected orchards.

Grasses and other weeds were collected in the Robinvale orchard. Sampling was difficult because of the scarcity of grasses due to the drought. In August 2007, 19 samples were collected in the proximity of trees infected with *Xtp*A (10 samples) or with *Xtp*B (eight samples) and along the track at the border of the orchard (one sample). Each sample contained 10-25 g of material, mainly barley grass and a few weeds such as capsella, clover, marshmallow, stinging nettle and capeweed. In September 2008, 34 samples were collected randomly along 10 rows, wherever grasses were growing. Each sample comprised 30-40 g of material, mainly rye grass that had been sown and left to grow by the grower for this study.

A protocol was established to isolate bacteria present on the surface of the plants, as reported previously (HAL NT99004 and PS04015 final reports), and from the internal leaf tissue: the material in each sample was cut into small pieces and soaked in 100 ml of SDW for 15 min with shaking. 1.5 ml of the resulting suspension, or 'surface extract' were transferred to a sterile Eppendorf tube. The rest was ground in a blender for 2 minutes and 1.5 ml of this 'leaf extract' were transferred to a sterile Eppendorf tube. 100 µl of both the 'surface' and 'leaf extracts' were plated either pure or diluted 1:100 on Antibiotic-BSPA (A-BSPA). Plates were incubated at 28ºC for up to 2 weeks. Yellow mucoid colonies were recorded and subcultured on BSPA. 1 µl aliquots of the leftover extracts, pure or concentrated by centrifugation, were tested by PCR using the T1 and T2 primer set, which allows detection of all *X. translucens* (Maes *et al*., 1996). Colonies, the morphology of which was consistent with *X. translucens*, were checked by PCR, using the T1 and T2 primers, to confirm identification.

6.1.3. Pathway of infection: leaf inoculation

In an experiment set up in November 2005, damaged and undamaged leaves of young pistachio trees were sprayed with a mixture of *Xtp*A and *Xtp*B. Analysis conducted in March 2006 on inoculated leaves showed that *Xtp* had penetrated the leaves and survived in the leaf blades for up to 4 months (HAL PS04015 final report). Here, we further analysed the trees inoculated in 2005 (1st experiment) and we repeated the experiment.

1st experiment: In May 2006, just after leaf senescence, stems that bore inoculated leaves were sampled. Two leaves that were still attached to one *Xtp*-inoculated tree were also collected. Leaf blades, petioles and stems were processed separately as described in PS04015 (HAL final report). In March 2007, two-three twig samples were taken from each of the 18 inoculated trees and tested by both plating on BSPA and PCR to check if infection had been initiated. Plates were incubated at 28ºC for up to 2 weeks and yellow mucoid colonies, if any, were confirmed by PCR.

2nd experiment: In December 2006, the leaf spray experiment was repeated. 18 young trees were selected; nine were sprayed with SDW and nine with a 10^6 *Xtp*A cells per ml. On each tree, all the leaves were sprayed. As there was no difference between damaged and undamaged leaves in the results of the first experiment, leaves were not damaged. Soil, trunk and stems were protected to avoid contamination. After inoculation, trees were covered with plastic bags until the next day and then maintained in the shadehouse. Assessments were conducted 2 and 4 months after inoculation. For each tree, two samples of four leaves each, and one stem sample were collected. Leaf blades, petioles and stems were processed separately as described in PS04015 (HAL final report), and assessed for *Xtp* using both plating on BSPA and PCR. In November 2007, twigs were collected from all trees and assessed both by plating on BSPA and PCR.

6.2. Results

6.2.1. Location of Xtp *in infected trees*

a- Pollen

Preliminary experiments showed that the RDTS extraction protocol was the more efficient. Also, DNA extracted from pollen, exhibited slight inhibition of detection of *Xtp* in RT-PCR, unless diluted 1:10. Therefore, for pollen assessment, DNA was extracted using the RDTS protocol and diluted 1:10 before being assessed for *Xtp* by RT-PCR.

Of 75 samples collected from 3 infected trees, one gave returned positive, but with a result suggesting that very few bacteria were present.

b- Roots

As with pollen the best yield of DNA was obtained with the RDTS protocol. Detection of *Xtp* was also slightly inhibited by DNA, unless extracts were diluted 1:10.

In autumn, *Xtp* was detected at very low levels in four root samples of 52, *Pythium* was found in 27 samples and *V. dahliae* in none (Table 3). The *Xtp*-positive samples came from four different trees, infected either with *Xtp*A or *Xtp*B. As results indicated very small amounts of bacteria (except for one sample), DNA was re-extracted from 11 samples, including three of the positive samples in the 1st test, using 2 g of root tissues instead of 1 g. *Xtp* was detected in all re-extracted samples (Table 3).

Table 3 - RT-PCR detection of *Xtp, Pythium* spp. and *V. dahliae* in pistachio roots collected in autumn from dieback-affected trees

Tree[a]	No. positive samples/Total No. per tree			
	Xtp		*Pythium* spp.	*V. dahliae*
	1 g	2 g		
R1T8 (*Xtp*A)	1/14[b]	1/1	3/14	0/14
R1T9 (*Xtp*A)	0/6	N/A	4/6	0/6
R1T10 (*Xtp*A)	1/9 [b]	5/5	2/9	0/9
R2T8 (*Xtp*B)	1/6 [b]	N/A	1/6	0/6
R2T9 (*Xtp*B)	1/17	5/5	17/17	0/17

[a] R: row number; T: position of the tree in the row
[b] Typical xylem staining was recorded for the positive sample

In winter, *Xtp* was detected in four of the 34 root samples collected from infected trees and in none of the samples collected from healthy trees. The positive samples came from four different trees infected either by *Xtp*A or *Xtp*B and again indicated low to very low levels of bacteria (Table 4). *Pythium* was detected in a large proportion of samples collected either from healthy (3/12) or infected (13/34) trees. No *V. dahliae* was detected.

Table 4 - RT-PCR detection of *Xtp, Pythium* spp. and *V. dahliae* in pistachio roots collected in winter from healthy and dieback-affected trees

Tree[a]	No. positive samples/Total No. per tree		
	Xtp	*Pythium* spp.	*V. dahliae*
R10T7 (healthy)	0/4	2/4	0/4
R20T33 (healthy)	0/8	1/8	0/8
R10T11 (*Xtp*A)	1/2	2/2	0/2
R10T12 (*Xtp*A)	0/5	2/5	0/5
R10T13 (*Xtp*A)	1/10	6/10	0/10
R3T19 (*Xtp*B)	1/4	3/4	0/4
R2T21 (*Xtp*B)	1/13	5/13	0/13

[a] R: row number; T: position of the tree in the row

c- Buds

In 2007, no *Xtp* was detected in any of the 720 buds assessed individually both by culturing and PCR.

In 2009, *Xtp* was not detected by RT-PCR in 165 samples prepared from a minimum of 660 buds.

6.2.2. Orchard floor vegetation survey

X. translucens was not detected, by direct PCR, in any of the 'surface' or 'leaf extracts' prepared from grass and weed samples collected in 2007 and 2008. At each sampling date, more than 30 yellow mucoid colonies were isolated from several samples, but they were shown not to be *X. translucens* in PCR using the T1-T2 primer set.

6.2.3. Pathway of infection: leaf inoculation

1st experiment: 4 months after inoculation, *Xtp* was isolated from the leaf blades of 14/18 inoculated trees, and from the corresponding petioles of three of these, but not from the stems

(PS04015 HAL final report). Just after leaf senescence, *Xtp* was not detected in stems, but could still be detected in the only two leaves collected from an *Xtp*-inoculated tree. More than a year after inoculation (March 2007), no *Xtp* was isolated nor detected by PCR in any of the trees, whether sprayed with *Xtp* or SDW.

2nd experiment: 2 months after inoculation, *Xtp* was isolated from leaf blades of five of the nine trees sprayed with *Xtp*, but not from petioles and stems. Identification of colonies was confirmed by PCR, but *Xtp* was not detected by direct PCR on any of the samples. *Xtp* was not isolated from, nor detected in any of the samples collected from the control trees sprayed with SDW. Four months after inoculation, similar results were obtained with *Xtp*A isolated from the same five trees of nine inoculated. One year after inoculation, no *Xtp* was isolated nor detected by PCR in wood collected from trees sprayed with *Xtp* or SDW.

7. Control, management and prevention

Control and management were a focus of HAL project PS04015. In that project, the role of pruning in spreading *Xtp* within infected orchards was demonstrated, as was the importance of cleaning pruning tools with a bactericide to reduce such spread. Also, based on the location of the bacteria in infected trees and on the assumption that severely affected trees might have received multiple re-infections via pruning, a long-term field trial was established to assess clean, drastic pruning as a strategy to manage infected trees. PS04015 also highlighted the importance of prevention and recommended the establishment of a clean source of propagation material to prevent spread of the disease through grafting or budding. Here, we further investigated the efficiency of a bactericide against *Xtp* and assessed the drastically pruned trees over a further 3 years to evaluate the benefit of the technique. We also assessed several orchards as a step forwards establishing a pistachio budwood repository.

7.1. Material and methods

7.1.1. Disinfection of pruning tool with a bactericide

Quaternary ammonium compounds prevented the spread of *Xtp* when used to disinfest pruning tools (HAL PS04015Final report). However, growers have reported concerns about achieving and maintaining complete coverage of the cutting surface with the disinfectant. One option was to use dormant oil to 'spread and stick' the quaternary ammonium to the surface of tools. An *in vitro* trial was conducted to determine the effect of mixing quaternary ammonium with dormant oil on efficacy.

Five treatments were tested; each was combined 1:1 with a 10^7 cells/ml suspension of *Xtp*A and *Xtp*B and shaken for 1 minute. 0.1 ml of the mixture was then plated onto BSPA, 5 plates for each treatment. After incubation at 28°C for 7 days the plates were examined for colonies. The treatments were as listed:

1. 12.5% quaternary ammonium in undiluted dormant oil
2. 25% quaternary ammonium in undiluted dormant oil
3. 25% quaternary ammonium in sterile distilled water
4. 100% undiluted dormant oil
5. sterile distilled water

7.1.2. Drastic pruning

A long-term field trial was conducted to test the hypothesis that severely diseased trees may be restored to full production by drastically pruning the trees and ensuring that this and all subsequent pruning is performed with the simultaneous application of bactericide. In

September 2004, 24 severely diseased trees were cut back to secondary or tertiary branches, and a bactericide was sprayed on the cut surface at the time of pruning. The control comprised a matched set of 24 trees that were not pruned. Each cut branch was labelled and the excised wood assessed for the presence of bacteria.

a- Canopy assessments

The health of the canopy was assessed annually for each tree, after harvest. Canopy size was measured and percent of canopy with dieback and stunted growth was estimated. The number of new shoots arising from primary, secondary and tertiary scaffolds in the non-pruned controls was counted.

b- Presence of Xtp *in new growth*

New shoots were assessed for the presence of *Xtp* annually in August. One shoot was sampled from each cut branch each year. Each sampled shoot was divided into sections of different ages using bud-scale scars to indicate each year's growth (Schweingruber, 2007). Each section was assessed for the presence of *Xtp*.

c- Yield and nut quality

Yields of non-pruned trees were determined annually and those of drastically pruned trees when they resumed production of nuts in the fourth year after drastic pruning. Subsamples were collected, hulled, dried and graded. Nut size (United States Department of Agriculture 2003), percentage of nuts split, 'early-splits' and stained nuts were determined. Returns to growers were calculated taking into account nut size, percent split and staining.

7.1.3. Budwood repository

a- Choice and assessment of the repository

Discussion was conducted with the PGAI Research Committee to identify an orchard that would be suitable as a repository. It was also agreed that, prior to establishing a repository, the chosen orchard would not only be extensively assessed for *Xtp* but also for *V. dahliae*, the causal agent of verticillium wilt.

For the diagnosis to be as accurate as possible, it was decided that samples would be tested using RT-PCR on DNA extracts. The processing method had to be suitable for a large number of samples, give good yield of DNA extraction and allow good *Xtp* detection. Preliminary experiments were conducted to compare various processing methods in terms of DNA yield and *Xtp* detection. Samples were collected from known infected trees in Robinvale. Each twig sample was cut in half. One half was cut into small pieces which were placed in a plastic bag and softened with a mallet. The other half was drilled and the shavings were collected. DNA was extracted from the two types of materials using the RDTS method. DNA yields and detection of *Xtp* were compared.

In October 2008, trees in the orchard chosen to become a repository were sampled. For each of 53 trees, eight samples were taken by drilling directly into branches and collecting shavings. For each sample, DNA was extracted from 1 g of shavings. Then, leftover wood shavings taken from the same tree were pooled and DNA was extracted from 2 g of this material. All individual and 'pooled' samples were tested for *Xtp* and *V. dahliae* by RT-PCR and one individual sample per tree was tested by culturing.

b- Validation of results

After extensive assessment of the orchard identified as candidate for the repository, further investigation was deemed necessary to validate the results (see section 7.2.3). First,

asymptomatic trees from other orchards were tested for *Xtp* and/or *V. dahliae* as described for the budwood repository material. In this case, however, the drilling was done on twigs in the laboratory instead of directly on the trees. Samples were collected from Robinvale, Kyalite and Mallee (Pinaroo) orchards, and also from Sunraysia Nurseries (Gol-Gol), the Waite arboretum (Adelaide), and a planting at DPI (Irymple South). The Waite arboretum planting included various *Pistacia* species and cultivars. Altogether 207 trees were sampled. Second, PCR products obtained from some samples were purified, cloned and sequenced to verify the sequences were indeed *Xtp*.

7.1.4. Biological control

A review of the literature available for other plant pathogenic bacteria was conducted to identify biological control agents that might be useful and applicable to *Xtp*.

7.2. Results

7.2.1. Disinfection of pruning tools: bactericide

No colonies of *Xtp* were observed in any of the treatments containing quaternary ammonium regardless of whether it was mixed with dormant oil or water. Plates from the treatments without quaternary ammonium were covered in colonies of *Xtp* too numerous to count.

Dormant oil did not appear to affect the efficacy of quaternary ammonium when used at the above concentrations. These concentrations are higher than that recommended by the manufacturer.

7.2.2. Drastic pruning

a- Canopy assessments

The canopy size of the drastically pruned trees was initially smaller than the non-pruned controls ($p=0.002$). However, while the canopy size of the control trees did not change, the canopy of the pruned trees increased in size each year. By 2009, there was no difference in size between the pruned and control trees ($p=0.053$).

No difference was observed in severity of dieback in 2005, with approximately 9% of the canopy in both the drastically pruned trees and non-pruned controls having dieback. From 2006 on, the control trees had more canopy affected by dieback (20%) than the pruned trees (8%) ($p=0.004$).

In every year, pruned trees had less stunted growth in the canopy than the control trees ($p<0.001$). An average of 30% of the canopy of pruned trees exhibited stunted growth compared with 75-88% of the canopy in the control trees.

In 2005, each control tree had an average of 12.9 new shoots arising from major scaffold branches. In 2006, this dropped to 4.4 and decreased again in 2007 to 0.8, thereafter new shoot numbers remained below 1.5 per tree.

b- Presence of Xtp *in new growth*

The proportion of shoots from which *Xtp* was isolated changed little from 2006 to 2008 (Table 5). However, the age of the shoot section from which *Xtp* was most frequently isolated changed. There were significant differences in the isolation rates from shoot sections of different ages each year, with the oldest sections always having the highest isolation rate. The proportion of positive 2 and 3 year-old shoot sections decreased in each subsequent year

(Figure 1). *Xtp* was isolated from a small proportion of sections from current season shoot tissue.

Table 5 - Presence of *Xtp* in shoots sampled annually from drastically pruned branches

	2004*	2005	2006	2007	2008
Shoots from which *Xtp* isolated (%)	77	5	31	23	27
Number of shoots sampled	394	339	290	265	260

**Xtp* present in drastically pruned branch at time of pruning

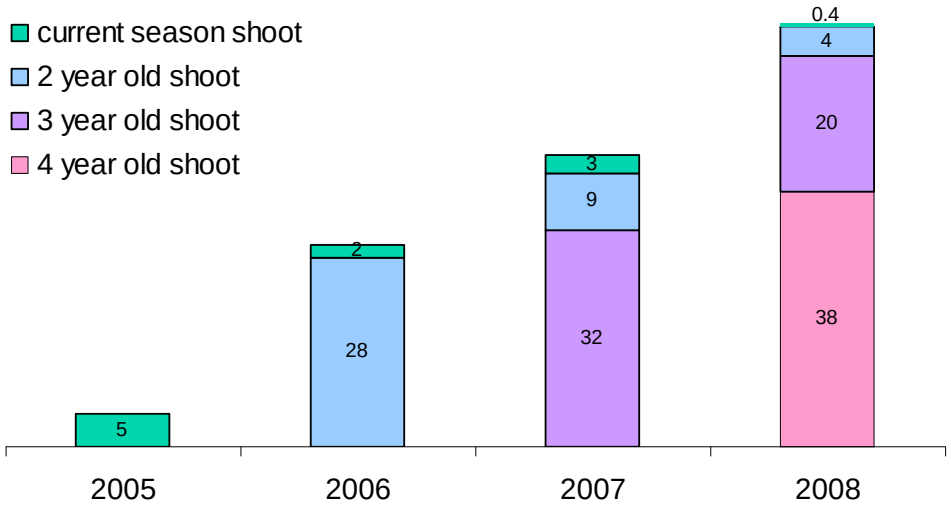


Figure 1 - Presence of *Xtp* in sections of different ages taken from shoots sampled annually from drastically pruned branches

c- Yield and nut quality

Drastically pruned trees yielded more crop than the non-pruned control trees ($p<0.0001$) in 2008 while in 2009, control trees yielded more than the pruned trees ($p<0.0001$) (Table 6). 2008 and 2009 were off-crop and on-crop years, respectively, for the industry as a whole, and the drastically pruned trees were in the opposite crop cycle compared with the non-pruned control trees. In the three years before the pruned trees returned to production, the control trees had an average yield per year of 11.8 kg, worth $62.85 per tree.

Table 6 - Average yield and returns per tree for drastically pruned and non-pruned control trees for 2008 and 2009

	2008		2009		Total	
	Yield (kg)	Return ($)	Yield (kg)	Return ($)	Yield (kg)	Return ($)
Pruned	18.8	116.98	13.0	80.98	31.8	197.96
Control	5.6	34.35	34.3	131.44	39.9	165.79

In 2009, there was a difference in nut size, with pruned trees producing medium-sized nuts compared with small nuts from the control trees ($p=0.002$). In 2008, pruned and control trees produced medium-sized nuts.

In 2009, control trees produced more than twice as many early-split nuts than the pruned trees ($p<0.01$), but there was no difference in percent of early-split nuts in 2008.

Staining in nuts from control trees was twice that observed in nuts from pruned trees in both 2008 and 2009.

No difference in percentage of split nuts (a desirable trait) was observed in either year. Split rates were acceptable, ranging from 90-94% in 2008 and 96-97% in 2009.

7.2.3. Budwood repository

a- Choice and assessment of the repository

It was deemed necessary that the orchard chosen as a repository would provide for the future expansion of the industry. The experimental orchard managed by New South Wales DPI at Dareton, located away from infected commercial orchards, satisfied the required criteria, such as the cultivars and the number and age of trees. It contained 53 trees comprising three males and 50 females on different rootstocks (PG-1, UCB-1 and Terebinthus) that were planted in August 1991.

Extensive assessment of each tree revealed low levels of *Xtp* in 6/53 trees (Appendix 3). To rule out any false positive due to contamination during the processing, the results were confirmed on a second DNA extraction. However, *Xtp* was not isolated from any tree.

V. dahliae was detected by PCR in nine of the trees (Appendix 3), but attempts to isolate it from one of these trees failed.

b- Validation of results

Despite the high specificity of the RT-PCR, the inability to isolate *Xtp* from trees identified to have low levels of bacteria raised questions about the veracity of the results. However, low levels of *Xtp* were also detected by RT-PCR in asymptomatic trees sampled from several other orchards (Table 7). Most of the time, results could be confirmed on a separated DNA extraction, but again, no *Xtp* could be isolated from the samples identified to have low levels of bacteria in RT-PCR. Overall, 13% of the trees tested revealed low levels of *Xtp*.

Apart from Dareton, low levels of *V. dahliae* were also detected in one tree from the Mallee Orchard (Table 7).

Table 7 - Assessment for *Xtp* and *V. dahliae* of asymptomatic trees from various orchards by RT-PCR

Orchard	No. trees positive/Total No. tested	
	Xtp	*V. dahliae*
Dareton	**5/54**	**9/54**
DPI Victoria mid area	13/23	0/23
Arboretum	11/19	NT
Kyalite Pistachios	0/42	0/42
Robinvale	1/40	0/40
Mallee Orchard	2/60	1/60
Sunraysia Nurseries	3/23	0/23

The sequences of the PCR products obtained from selected samples from Dareton, DPI Victoria mid area and the arboretum, confirmed that the amplified products had the expected *Xtp* sequences.

7.2.4. Biological control

The literature review showed that biological control may have some potential for management of *Xtp*. There are numerous reports of antagonistic activity by non-plant pathogenic bacteria and fungi against microorganisms that are pathogenic to plants. Amongst the microorganisms reported to have antagonistic ability, some of the best studied are *Trichoderma*, *Streptomyces* and *Bacillus* spp. *Trichoderma* spp. have been used mainly for the biological control of fungal diseases, such as Eutypa dieback in vineyards (John *et al*., 2005), and non-pathogenic *Streptomyces* spp. are particularly efficient at controlling plant pathogenic *Streptomyces* spp. (Emmert and Handelsman, 1999). In contrast, *Bacillus* spp. have been used successfully to control both fungal and bacterial diseases. One example particularly relevant to *Xtp* is the use of commercial formulations of *B. subtilis* to control *Erwinia amylovora*, the bacterium responsible of fire blight in pome fruits (Broggini *et al*., 2005).

In recent years, beside antagonistic microorganisms, the use of antimicrobial proteins and peptides as an alternative strategy in plant disease control has received a lot of attention (Marcos *et al*., 2008). Plants can produce these antimicrobial compounds as part of their specific defence mechanisms, but some antimicrobial peptides can now be synthesised and their sequence and length modified to optimise their activity. For example, Badosa *et al.* (2007) reported that one synthetic peptide, BP100, was very effective against three plant pathogenic bacteria, including *E. amylovora* and one *Xanthomonas* species. BP100 also reduced colonisation of plant tissues by *E. amylovora*

Experiments were conducted by Masters research student Ms Asmah Salowi (with funding from the Government of Sarawak and the University of Adelaide), to assess the ability of various bacterial isolates, including one of *B. subtilis*, to inhibit the growth of *Xtp*. The peptide BP100 (provided by Professor E. Montesinos, University of Girona, Spain), was also tested. Of eight bacterial isolates tested, one strongly inhibited the growth of *Xtp in vitro*, as did the peptide. These promising results indicate that further experiments should be set up to assess efficacy *in planta*.

8. Golden Stain

Staining of pistachio nuts has been associated with various causes. For example, insect feeding has been shown to cause shell staining in California (Daane *et al.*, 2005). Also, early-split nuts (which occur when the shell splits before the hull detaches from the shell, resulting in the hull splitting and exposing the shell and kernel) can develop staining along the split line and are also prone to fungal infection (Doster and Michailides, 1995). Pistachios can flower over an extended period resulting in nuts ripening unevenly during the harvest period, and this may also affect GS development. Temperatures in field bins and the time between harvest and processing can also influence the development of shell staining. Thompson *et al*. (1997) found that (1) if the temperature of unhulled nuts is greater than 25ºC for more than 48 hours staining increases and (2) early harvested nuts have less staining than late harvested nuts. To investigate the possible cause of GS in Australia, we surveyed several orchards in search of factors that might affect development of GS. We assessed the effect of temperature, possible pathogen infection, damage to nuts, nut maturity, position of nuts in the bunch and position of bunch in the canopy, on final incidence of GS.

8.1. Material and methods

8.1.1. Orchard survey

To determine if uniformity of budbreak, insect damage or some phenological changes during the growing season may influence the development of GS, the following surveys were conducted.

Two orchards in the Riverland region of South Australia and one in north west Victoria were visited every 2-3 weeks, from shell hardening until harvest, during the 2006-07 growing season. Several bunches were monitored at each orchard and any new damage to the hull of nuts was recorded at each visit. Nuts were picked when ripe, sorted according to hull symptom and the presence or absence of GS was recorded.

In 2008, one week prior to harvest, six orchards were visited and surveyed for hull damage, early-split nuts and hull condition. The results were then compared with the grading reports, prepared by the processing plant, for each orchard.

In spring 2008, five orchards from across the growing region were visited during flowering and the uniformity of break of flowering buds was determined. This was then compared to the grading reports for each orchard.

8.1.2. Temperature effect

a- Bin temperature

Temperature in harvest bins was monitored to determine if the temperature of nuts increased during transportation to the processing plant and if this was associated with an increase in the incidence of GS.

A 500 g sample of ripe, hand-harvested nuts enclosed in a plastic mesh bag with a temperature logger was placed in the centre of each of 10 harvest bins prior to the bins being filled and before transport to the processing plant. Samples were then retrieved from the harvest bins immediately prior to processing at the plant, placed in a cool store and transported to the laboratory. Another ten 500 g samples were placed into the cool store at the orchard and transported to the laboratory. All samples were assessed for the presence of GS. This was conducted at two orchards, 100 km (Site 1) and 330 km (Site 2) from the processing plant. Temperatures were recorded in harvest bins from a further two orchards, 260 km (Site

3) and 140 km from the processing plant (Site 4). Air temperatures were obtained from the nearest Bureau of Meteorology weather station. In 2008, bin temperatures were monitored from Site 4 and from another orchard (Site 5), 250 km from the processing plant.

b- Postharvest storage trial

A trial was conducted to determine if storage temperature and duration affected the incidence of GS in early- and late-harvested nuts.

In 2008, ripe nuts were harvested from selected trees early in the harvest period, prior to the first shake (Harvest 1). Nuts were transported to the laboratory and approximately 2 kg of nuts were placed into sealed 5 l plastic buckets. The nuts were then stored at either 20ºC or 31ºC for 0, 2, 4 and 7 days. At the end of each storage period nuts were hulled and assessed for incidence of GS. The trial was repeated 2 weeks later with freshly harvested nuts from the same trees (Harvest 2).

8.1.3. Pathogen

Previously, samples of hulled and dried golden stained nuts had been analysed for the presence of pathogens. Several potential pathogens, including *Alternaria, Penicillium, Cladosporium* and *Aspergillus,* were found, but no analysis had been undertaken on fresh, unhulled nuts.

In 2007, ripe nuts were collected from three orchards and moist-incubated to isolate fungi or bacteria from the hull. Nuts selected for incubation had a range of hull conditions: intact hulls, ruptured hulls, early-splits and over-ripe. Before being moist-incubated, nuts were either surface sterilised using 10% $NaOCl_2$, placed in sealed bags with an SO_2 pad for 24 hours or had no pre-treatment. Nuts were incubated at 21ºC for up to 4 weeks and observed for formation of sporulating lesions. Spores or other fungal growth were subcultured onto potato dextrose agar and nutrient agar growth media for further identification on the basis of colony morphology.

8.1.4. Damage trial

Earlier work (HAL PS03003 final report) showed that damaging hulls in the month before harvest increased the incidence of GS. This work was expanded to include damage applied from shell hardening to pre-harvest.

In 2006-07, bunches of nuts were damaged using various methods to determine if hull damage led to an increase in the incidence of GS. Nut hulls were damaged by piercing, cutting or bruising; a non-damaged control treatment was also included. All the nuts in a bunch received the same damage treatment, once. Treatments were applied to new bunches four times, approximately 4 weeks apart beginning at shell hardening in late November and finishing 2 weeks before harvest. The nuts were collected as they ripened and assessed for the presence of GS.

In 2007-08, damaged and undamaged nuts were inoculated with either *Colletotrichum* or *Alternaria* to determine if the presence of pathogens increased the incidence of GS. Treatments were applied to whole bunches and damaged nuts had a 5-mm cut made to the hull. Inoculum was applied as a spore suspension sprayed to run-off. Bunches were moist-incubated for 18 hours by enclosing in a plastic bag moistened with SDW. A set of non-inoculated control treatments, damaged or undamaged, was also included; these nuts were not moist-incubated. Treatments were applied in late January. The nuts were harvested when ripe and assessed for the presence of GS.

8.1.5. Maturity and position

a- Maturity

The results from the storage trial suggested that nut age or maturity influences the incidence of GS. As a pistachio nut ripens the hull changes colour and detaches from the shell, the shell usually splits after the hull has detached. Once ripe, the hull begins to break down, the outer surface splits (tatter) and, if left too long, will dry and shrink back onto the shell.

To investigate the relationship between flowering time and maturity, early, mid and late flowering buds were marked during the flowering period. When nuts started to ripen, bunches for each flowering time were sampled and this was repeated every 9 days. There were four harvest times, covering the duration of nut ripening. Hull condition of each nut was noted and the presence of GS assessed. For each flowering time, the number of days taken for 90% of nuts to ripen was determined.

b- Position

Sampled trees were located throughout the orchard. Early, mid and late flowering buds were selected from either inner or outer positions in the tree canopy. The position of each nut within the bunch, terminal or lateral, was recorded. The date when nuts within each bunch ripened was recorded. Ripe nuts were harvested immediately before the first harvest and again before the second harvest. Nuts from lateral and terminal positions were separated at harvest and assessed for the presence of GS.

8.1.6. Pack out analysis

The grading reports from 2004-2009 for all grower deliveries to the Australian Pioneer Pistachio Company (APPC) processing plant were analysed for any patterns that might indicate a possible cause of GS. Parameters that were considered included time of harvest, geographic location, location in orchard, and first or second shake.

8.2. Results

8.2.1. Orchard survey

A number of different types of damage were observed on the nut hulls. The most common types of damage (11% of nuts) were stings, small wounds with resin typically at the apical end of nuts. Most stings appeared before the end of January and 20-30% of nuts with stings had GS. Early-split nuts occurred in the month before harvest, 6% of all nuts were early-splits and 47% of early-split nuts had GS. GS was also observed in 3% of nuts with no hull symptoms.

Immediately before harvest 2008, three of the orchards had very few visible defects. Two of these produced no or very low levels of GS, whereas the other orchard, harvested 18 days into the harvest period, had above average levels of GS. The remaining three orchards had either a large number of early-split nuts or ripening was uneven with some nuts already having tattered hulls. These orchards had levels of GS one and a half to twice the industry average.

In orchards visited in spring 2008, uniformity of flowering bud-break ranged from very uniform or compact through to extended bud-break where some clusters had 10-20 mm nuts while others were just emerging on the same tree. Poor winter chill can exacerbate uneven flowering, and applications of dormant oil in late winter have been shown to promote even bud break (Beede and Ferguson, 2002). Identical oil applications at adjacent orchards resulted in one orchard with very even bud-break while the other had extremely variable bud-break with 10 mm nutlets and emerging buds observed on the same tree. All five orchards produced nuts with GS levels above the industry average. No relationship was found between uniformity of bud-break and the incidence of GS.

8.2.2. Temperature effect

a- Bin temperature

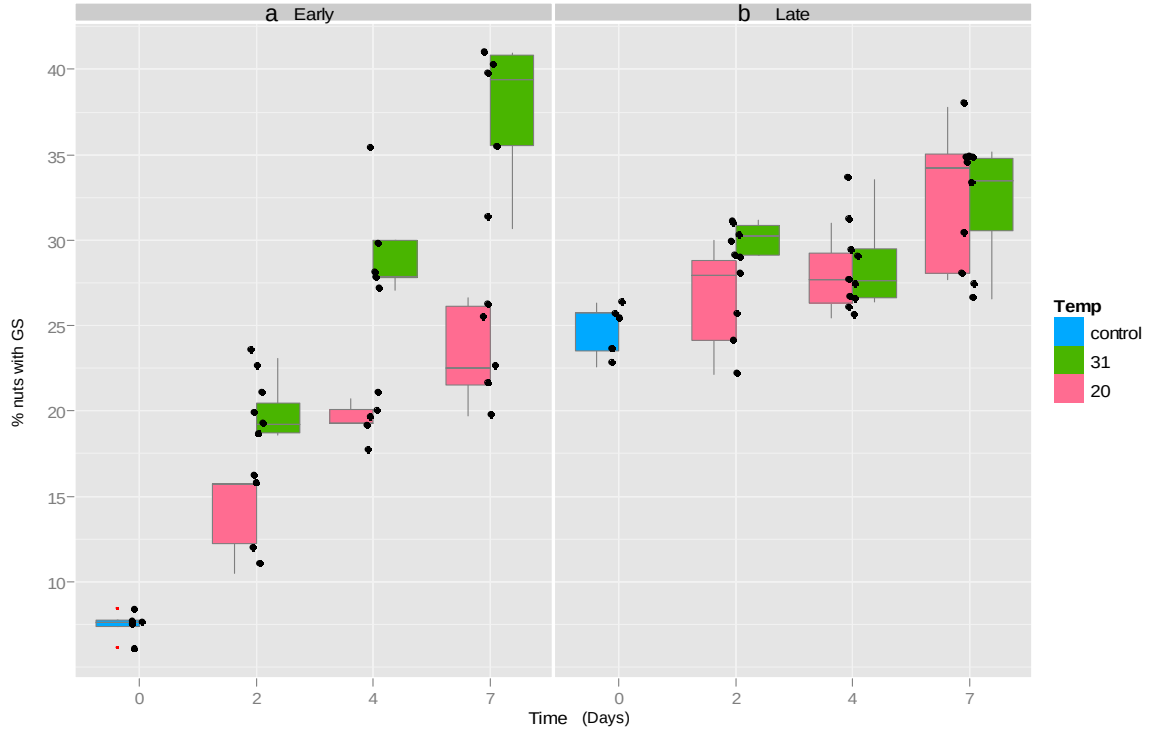
There was no significant difference in the incidence of GS in relation to transportation of the nuts from the four orchard sites to the processor ($p>0.27$). All orchards were harvested in the afternoon except Site 1 which was harvested between 8:00-10:00 am following a cool night (12^{o}C). Nuts from Site 1 were processed 14 hours after harvest; the other sites were processed 20-38 hours after harvest. Bin temperatures decreased 2-10^{o}C in transit except for those from Site 1 in which transporting had no effect on bin temperature. In all but Site 1, bin temperatures rose from harvest until around midnight, at times up to 13^{o}C above air temperature, and then slowly decreased until mid-morning when they again increased. Temperatures in bins from Site 1 increased steadily but remained 7-12^{o}C below air temperature.

b- Postharvest storage trial

For nuts from Harvest 1, the level of GS at harvest (control) was significantly less than that for all stored nuts regardless of storage temperature (Fig 2a). Nuts stored at 20^{o}C for 2 days had less GS than those stored for 4 and 7 days while GS increased with each increase in storage time in nuts stored at 31^{o}C ($p<0.001$) (Figure 3a).

In Harvest 2 (Figure 2b), all nuts stored at 31^{o}C and those stored at 20^{o}C for 7 days had higher incidence of GS than the harvest (control) sample; nuts stored at 20^{o}C for 7 days had more GS than those stored for 2 or 4 days and when nuts were stored at 31^{o}C, length of storage made no difference to the incidence of GS. GS on samples examined immediately after harvest (controls) was less for Harvest 1 than for Harvest 2 ($p<0.001$) (Figure 2).

Figure 2 – Effect of storage time and temperature on incidence of GS in (a) early- and (b) late-harvested nuts



Dots represent individual samples. Boxes contain 25-75th percentile. Central horizontal lines represent the median value. Vertical lines indicate the smallest and largest non-outlier observation

8.2.3. Pathogens

There did not appear to be any association between any of the hull conditions assessed and the fungi isolated. *Alternaria* and *Cladosporium* were the most commonly isolated fungi and were obtained from nuts with all hull conditions, including intact hulls. Other fungi isolated included *Colletotrichum, Stemphylium, Fusarium, Rhizopus* and various *Penicillium* species. No bacteria were isolated.

8.2.4. Damage trial

2006-2007 trial: Cutting the hull, regardless of the timing of the damage, and piercing the hulls of nuts at the start of shell hardening resulted in greater incidence of GS than other treatments and the control ($p=0.002$). Piercing the hull with a needle at the start of shell hardening also resulted in fewer nuts being present at harvest compared to other treatment times ($p<0.001$).

2007-2008 trial: Damaged nuts had a greater incidence of GS than undamaged nuts ($p<0.001$). The presence of either *Colletotrichum* or *Alternaria* did not affect the incidence of GS.

8.2.5. Maturity and position

a- Maturity

Incidence of GS increased linearly when harvest was delayed. Nuts harvested late in the harvest period (1 April) had almost twice the incidence of GS as those harvested in the first sampling (5 March) ($p<0.001$). When nut hulls were ripe or tattered, delaying harvest increased the incidence of GS ($p<0.001$). Once nut hulls had become shrivelled or dry, at least 70% of nuts had GS regardless of time of harvest.

There were 5-6 days between each flowering time. It took 163 days for early and mid flowering nuts to mature compared with 167 days for late flowering nuts. Flowering time made no difference in incidence of GS regardless of time of harvest. Once nut hulls had become tattered or dry, late flowering nuts had less GS than early and mid flowering nuts ($p=0.003$).

b- Position

Canopy position influenced the incidence of GS, with nuts from bunches in the outer part of the canopy having greater incidence of GS than those from bunches in the inner canopy ($p=0.003$). No difference in incidence of GS was found due to position of nuts within a bunch or position of trees within the orchard. Flowering time had no influence on the incidence of GS. The position of the nut in the bunch or the bunch in the tree had no influence on the ripening time. Mid flowering buds produced more nuts per bunch than early or late flowering buds ($p=0.007$).

8.2.6. Pack out analysis

The industry average of GS since 2004 has been between 1.2-2.3% except 2006 when only 0.3% of the crop was affected (Table 8). Incidence did not appear to be related to crop load. Almost all growers delivered some GS-affected nuts each year, the only exception being a small-scale grower (2100 trees) in Junee, NSW, who did not deliver any GS nuts in 2004, 2007 or 2009. This grower did deliver some GS-affected nuts in 2005 and 2006 but only in the first load (about 25% of total deliveries). Other small-scale producers have occasionally delivered GS-free nuts.

Typically GS was rare (<1%) in early harvest deliveries, however, incidence increased as the harvest season progressed. In off-crop years a distinct pattern was observed, with GS being more common in nuts delivered in the last third of the harvest period.

Analysis of the grading reports from on-crop years did not reveal any obvious pattern. In 2005, GS was most common at the beginning of harvest and again near the end of the harvest. In 2007 there was an increase in GS that coincided with the start of the second shake. In 2009, GS was most common in nuts delivered in the middle of the harvest period but this rise did not coincide with the start of the second shake.

There was no obvious relationship between incidence of GS and temperature or rainfall during the growing season.

Table 8 - Industry average GS levels for the 2004-2009 period.

Year	Average GS%	Crop
2004	2.3	Off
2005	1.2	On
2006	0.3	Off
2007	1.5	On
2008	1.6	Off
2009	2.1	On

9. Discussion

9.1. Detection

Development of reliable detection tools is an important step in disease management and control. The three methods developed to detect *Xtp* from a range of pistachio materials are compared in Table 9. Culturing is a reliable, efficient and low cost method to detect *Xtp*. It is however slow and not adapted to high throughput testing. PCR is quicker, allows *Xtp*A and *Xtp*B to be distinguished and has sensitivity similar to culturing. Although more expensive, RT-PCR is the most sensitive and specific of the three methods and it is amenable to high throughput testing. More importantly, it has shown ability to detect *Xtp* in asymptomatic trees, when applied to DNA extracts. This is of particular interest given the long latent period (*i.e.* the time between infection and external symptom expression) in pistachio dieback.

Table 9 - Comparison of the three methods available to detect *Xtp*

	Culturing	PCR	RT-PCR
Time required	4-15 days	2 days	1 day
High throughput	No	No	Yes
A/B distinction	No	Yes	Yes
Specificity	+	++	+++
Sensitivity	++	++	+++
Cost	+	++	+++

A PCR machine and ancillary equipment have been installed at DPI Mildura to allow rapid assessment of samples after collection, as this has been shown to increase the sensitivity of PCR detection. Thus, culturing and PCR facilities are available at the University of Adelaide and DPI Mildura. The two techniques are considered complementary; used in conjunction they will provide consistent and reliable diagnosis. RT-PCR facilities are available through SARDI, Adelaide. Although more reliable results are obtained with DNA extracts, the technique can be used with wood extracts obtained by the 'soaking' method.

The availability of the three techniques will assist the industry to manage dieback in affected orchards and control its spread.

9.2. Origin and pathogenicity

Both phylogenetic analyses based on the *gyrB* gene, and integron screening confirmed the close proximity of *Xtp* to *X. translucens* and strongly support the hypothesis that *Xtp* originated from a grass-infecting *Xanthomonas*. Screening of integrons in 15 isolates of *X. translucens*-like bacteria isolated from grasses growing in pistachio orchards has revealed a high diversity, suggesting that these bacteria may be part of a larger pool from which *Xtp* has emerged.

The *gyrB* phylogeny also shows that *Xtp*A and *Xtp*B originated from two different lineages, which suggests that the introduction of *Xtp* into pistachio has happened as two different events. This is also supported by the consistent distinction of the two groups based on integron screening as well as previous results (HAL NT02007 final report, Marefat *et al*., 2006b).

Altogether, these results have allowed the description of *Xtp* as a new pathovar of *X. translucens*, for which the name *Xanthomonas translucens* pv. *pistaciae* has been proposed (Giblot-Ducray *et al*., in press). The recognition of the pistachio pathogen as a taxonomic entity distinct from other *Xanthomonas* is an important step that will facilitate scientific communication.

In terms of pathogenicity, *Xtp*A seems more efficient than *Xtp*B at initiating infections after artificial inoculation. This corroborates field and growers' observations and is in agreement with the wider observed distribution of *Xtp*A in affected orchards. This also suggests that *Xtp*B may be lacking pathogenicity attributes necessary for colonisation of the plant tissues in the early stages of the infection.

The two groups seem to spread in a similar manner as shown by the results obtained over 3 years in Robinvale stage 4, but any comparison is made difficult by the small number of trees that became infected. However, these data support the hypothesis that during the two major outbreaks, some unknown factor(s) were at play to trigger disease spread and symptom expression.

9.3. Disease cycle/Epidemiology

Further assessment of pollen, roots and buds as sources of *Xtp* was warranted by the development of the more sensitive PCR and RT-PCR detections.

With only one sample of 75 collected from severely diseased trees showing a small amount of bacteria at the limit of detection, pollen is unlikely to play an important role in the disease cycle. Likewise, no other plant pathogenic bacterium has been reported to be transmitted by pollen (Card *et al*., 2007).

Although we found *Xtp* more frequently in roots from infected trees than in pollen, levels were also low, whatever the season. No *V. dahliae* was detected, but *Pythium* was found in roots of both healthy and infected trees. It was shown previously that inoculation through the roots did not initiate infection in Sirora on PG-1 rootstock and rarely in Sirora on Terebinthus, but only when roots were damaged (HAL NT99004 final report). So, in Australian orchards, where PG-1 is widely used, roots are unlikely to play a key role in spread of the disease. In contrast, further investigation is needed to determine if the presence of *Pythium* in roots affects disease expression.

The inability to detect *Xtp* in buds collected from infected trees, including by use of the very sensitive RT-PCR, strongly suggests that *Xtp* does not colonise buds, or, if at all, only below detection thresholds. Based on these results, and as previously suggested (HAL PS04015 final report), transmission of *Xtp* through budding is considered unlikely if buds are taken from non-symptomatic trees and standard hygiene for propagation is practised.

In agreement with previous research (HAL NT99004, PS04015 and NT02007 final reports), *Xtp* was not found in orchard floor vegetation. However, in contrast to previous findings, *X. translucens*-like bacteria were not isolated either, which could be linked to the particularly dry conditions. As such, this aspect of the research was inconclusive.

Both leaf spray experiments confirmed that *Xtp* can penetrate and survive in leaf blades until leaf senescence but, in our experimental conditions, it did not spread further in the tree. This strongly suggests that leaves do not play an important role as infection pathways.

In summary, pollen, roots and buds are unlikely to play a role in the cycle of pistachio dieback; grasses do not represent obvious alternative or reservoir hosts for *Xtp*, although they may have been the source of the bacteria from which *Xtp* has evolved; leaves are unlikely entry sites through which the bacterium can become established in the tree.

9.4. Prevention/Control/Management

9.4.1. Drastic pruning

Canopy size measurements showed that it took approximately 5 years for drastically pruned trees to reach the size of the control trees. To date the pruned trees have not yet reached full size and increases in yield can be expected as canopy size continues to increase.

After an initial burst of activity, new shoot initiation in control trees declined, for reasons unknown. However, production of new, adventitious shoots can be an indication of stress (Lockhart *et al.*, 2006). It appears that, although non-pruned trees can produce new shoots, drastic pruning stimulates more new growth in targeted parts of the tree, allowing development of a well structured canopy.

Facelli *et al.* (2009) reported that *Xtp* was isolated from most current season shoots from moderately symptomatic trees. The low rate of isolation of *Xtp* in current season growth in drastically pruned trees may suggest that they have a lower bacterial titre. Isolation rates on 3 and 4 year-old shoot sections were also lower in our study.

From 2006 to 2008, the proportion of positive shoots in each age category decreased slightly. *Xtp* appeared to remain 'deeper' in the twig as the twig aged, with the oldest section of shoots having higher isolation rates, and isolation rates decreasing as the shoot sections decreased in age. If this is the case, then all pruning after drastic pruning should be done using disinfested tools to minimise the risk of infecting younger shoots.

Sampling of the drastically pruned branches in 2004 revealed that 75% of branches contained *Xtp*, and it can be assumed that incidence of infection would have been similar in the control trees. This figure is similar to the percent of canopy with stunted growth observed in the control trees. This result was reflected in the pruned trees, in which severity of stunted growth and frequency of isolation from shoots were approximately 30%.

Detection of *Xtp* in a maximum of 31% of the new canopy in drastically pruned trees indicates that, 4 years post-pruning, the pruned trees are healthier and the infection rate has remained static for the last 3 years. It would be of interest to sample the control trees to determine the proportion of positive branches.

Pistachio trees exhibit a strong biennial bearing cycle as can be seen from the large differences in yield for each treatment in the 2 years. The drastic pruning has resulted in the two treatments being in opposite crop cycles. This makes direct comparisons of yield per year difficult. It is apparent that although the pruned trees have not yet returned to full production, the nut quality and size are better than the unpruned controls and returns per tree are greater. It is expected that the yield lost from pruned trees during the regrowth stage will be recovered by the sixth year post-pruning. The extremely large 2009 on-crop from the control trees will almost certainly result in a correspondingly small off-crop in 2010, while the pruned trees

would be expected to produce an on-crop equal to or larger than they produced in 2008 (on-crop). Our results indicate that although *Xtp* is still present in the trees, drastic pruning results in larger, better quality nuts.

If the reduction in percentage of early-splits in pruned trees in 2009 is maintained in future years, this would be of benefit, as early-split nuts almost always result in shell staining and a subsequent reduction in returns. Early-split nuts are also more prone to infection by *Aspergillus* fungi, some of which produce mycotoxins (Doster and Michailides, 1995).

9.4.2. Budwood repository

The assessment of the Dareton orchard, selected to become the repository, and of other plantings suggested that *Xtp* is present at low levels in pistachio trees as an endophyte (*i.e.* living there without causing damage). However, the presence of *Xtp* does not mean dieback: in this scenario, as yet unknown external factors trigger multiplication of *Xtp* from a very small initial population until it reaches a threshold and starts causing symptoms. Such a hypothesis is supported by the unexplained hiatus in spread and expression of dieback after the two main outbreaks. It may also explain why *Xtp* could be isolated from apparently healthy trees (Facelli *et al*., 2009). Following these results, no orchard could be presumed free of *Xtp* and the establishment of the repository was suspended, the cost/liability *vs* benefit ratio being considered too large to proceed with this option.

9.5. Golden Stain

Our research identified factors that contribute most to GS to be late harvesting, over-ripe nuts and full thickness damage to hulls after shell hardening, including early-split nuts. No association was found between incidence of GS and potential pathogens or crop load.

Timing of harvest will always be problematic, as the availability of contract harvesters governs the harvest schedule for many growers. The economics of double shaking in an off-crop year also mean that harvest is often delayed to maximize the ripe nuts in a single shake. Our research has shown that flowering time had very little influence on the incidence of GS; however there were only 2 weeks between early and late flowering buds in the relevant years. If the period of flowering increased, perhaps through poor chilling, then nuts may ripen over a longer period and develop GS.

Damage to the hull can be caused by insect feeding, early-split nuts and mechanical damage such as wind or hail. Feeding by Hemipteran insects or bugs usually occurs before shell hardening although some larger bugs are present in orchards after shell hardening, but usually few in number (Daane *et al.*, 2005). Feeding by light brown apple moth larvae may also cause full thickness damage to nut hulls. Early-split nuts increase substantially when insufficient irrigation is applied during shell formation in late spring and increase slightly with excessive irrigation in late February (Michailides, 2000). So, irrigation management may help minimize the incidence of early-split nuts. Mechanical damage to nuts may be minimized through canopy management.

Storage temperature and time have been identified as leading to increased staining in California (Thompson *et al.*, 1997). Likewise, the results of our trial show that increased storage temperatures and time increased the incidence of GS. The increase was greater in early harvested nuts than in late harvested nuts where GS seems to have developed while the nuts were on the tree. Long storage times would not normally be a factor for the Australian industry as most production areas are within 5 hours of the processing plant and nuts are usually processed within 24 hours of arrival. Also, the Chep® vent-sided horticulture bins used by the industry appear to be well ventilated and small enough to prevent the temperature rises observed in the bulk bins used in California. However, some smaller orchards, where

nuts are hand harvested, do store nuts for a few days to accumulate enough nuts to transport to the processing plant, although there was no evidence that this contributed directly to GS.

More research is needed to identify all the factors involved in the development of GS. It would be particularly interesting to look at nutritional factors.

10. Technology transfer

10.1. Meetings with growers and field day

Research findings were regularly communicated to the Pistachio Growers' Association Research Committee. Meetings were held in Mildura on the following dates: 17 November 2006; 17 May, 7 August and 4 December 2007; 10 April, 26 June and 17 December 2008; 24 June and 3 September 2009.

Grower information field days were held on 8 August 2007 and 25 June 2009 at DPI (Mildura) and at CMV Farms (Robinvale), respectively. Both were well attended. Research updates were generally well received and raised interesting discussions and suggestions from the growers.

10.2. Conferences

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Giblot-Ducray D. and Taylor C., 2009. Pistachio dieback and Golden Stain – What do we know? Australian Nutgrower Vol. 23 (4), in press.

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Taylor C (2007). Golden Stain in pistachios. Australian Nutgrower 21 (4), 27-28.
Giblot-Ducray D, Taylor C, Ophel-Keller K, Emmett R and Scott E (2006). Challenges to the Australian pistachio industry – Bacterial dieback and nut quality. Australian Nutgrower 20 (4), 35-37.

10.4. Website

In early 2007, the 'Pistachio dieback' information webpage was made available to the growers through the PGAI website (http://www.pgai.com.au/), including all the results from the previous projects (HAL NT99004, PS04015 and NT02007). A new version, updated with the results of this project, will be released early in November 2009.

11. Recommendations

Pistachio dieback

We recommend using diagnostic techniques to confirm suspected infections to help manage orchards, particularly during the pruning season. We also recommend testing trees to be used for propagation and using only those trees that yield negative results.

Culturing and PCR are recommended for routine testing. The two techniques should be used in a complementary way as they can be performed on a single extract. The cost is estimated at $5-10 per sample.

RT-PCR is recommended for high value-samples such as trees to be used for propagation and/or when large numbers of samples have to be tested. The test will be available at SARDI RDTS at a cost of $50-$60 per sample, although cost per test will decrease with large sample numbers.

Given the widespread occurrence of *Xtp* at close to the limit of detection, the establishment of the budwood repository cannot be recommended. Grafting has not been tested, but we can recommend budding as a low risk propagation method, if good hygiene is practised for propagation.

Drastic pruning with simultaneous application of bactericide is recommended as a method for rejuvenating moderate to severely symptomatic trees. Care should be taken to conduct all subsequent pruning with disinfested tools. A further year of yield and quality data should be collected from this trial to determine the time taken for drastically pruned trees to be restored to full production

Golden Stain

To reduce the incidence of GS, every effort should be made to ensure that harvest is carried out promptly. Also, irrigation and canopy management should be manipulated to reduce the incidence of early-split nuts and mechanical damage, respectively, as both factors were associated with increased GS. Given the importance of storage temperature and time, if an extended period of storage is expected between harvest and processing, then half-filling bins and placing them in cool stores would minimize increases in temperature. Harvesting nuts early in the day before they warm up with ambient temperature will also minimise the temperature in the bins.

Further research

We consider that the following issues need further attention:

- Identify potential *Xtp* ancestors and establish a precise phylogeny of *Xtp* among other

Xanthomonas in order to determine from where *Xtp* has originated;
- Identify pathogenicity-related genes in the genome of *Xtp* to elucidate the means by which *X. translucens*, a species typically pathogenic to grasses, became able to infect and cause disease in pistachio;
- Determine the conditions that are conducive to pistachio dieback and identify environmental and/or biological factors that trigger disease expression.
- Determine if nutritional factors affect the incidence of GS.

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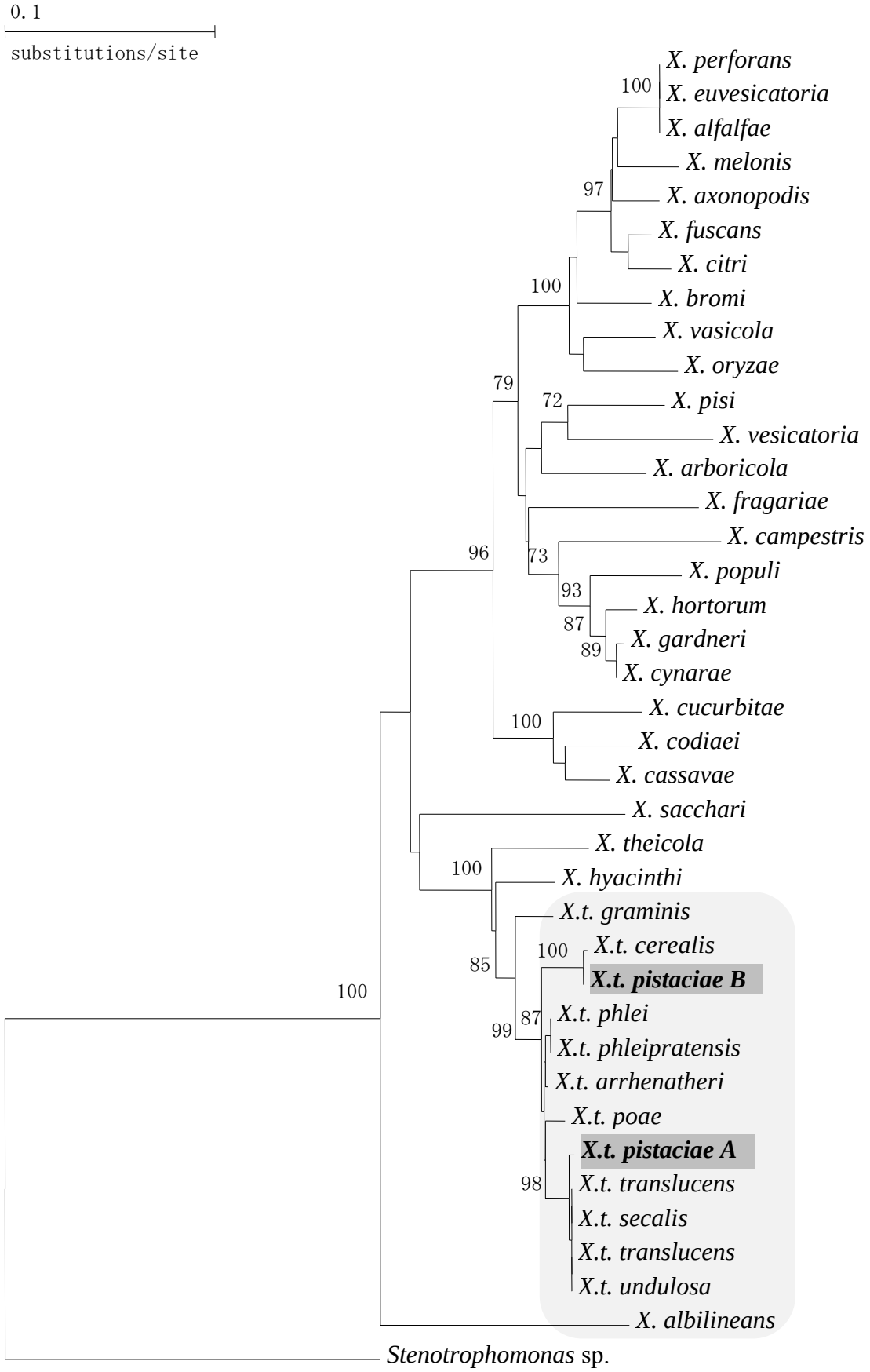
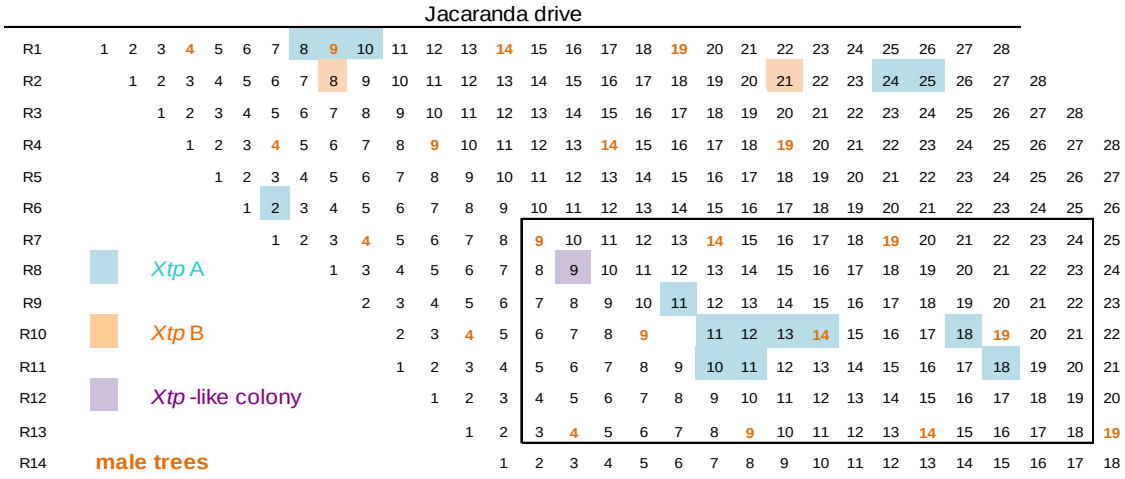
13. Appendices

Appendix 1 - Phylogeny of the genus *Xanthomonas* highlighting the position of the two groups of *Xtp* (*X.t. pistaciae* A and B) among other *X. translucens* (*X.t.*) pathovars

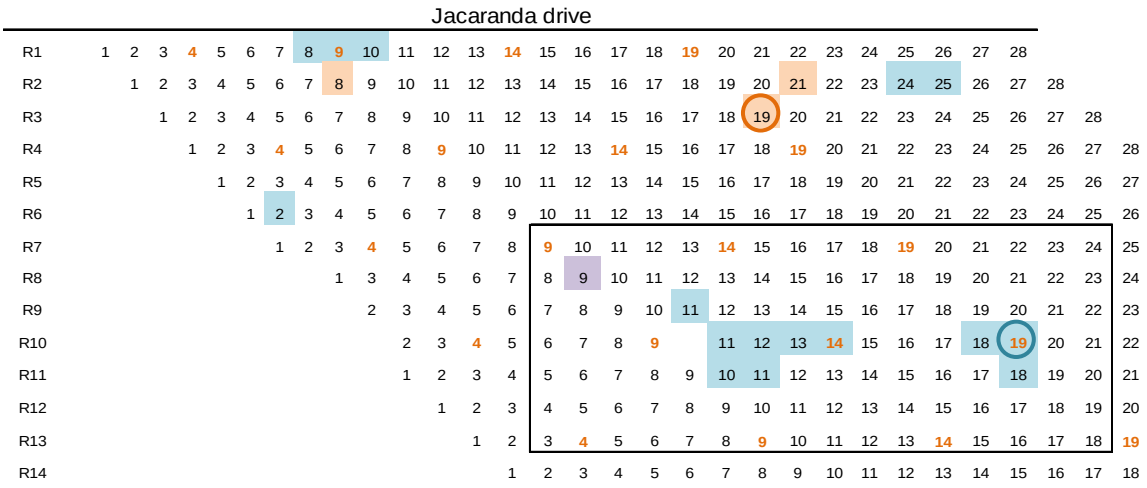


Appendix 2 – Spread of *Xtp*A and *Xtp*B in stage 4 of Robinvale orchard over 3 years

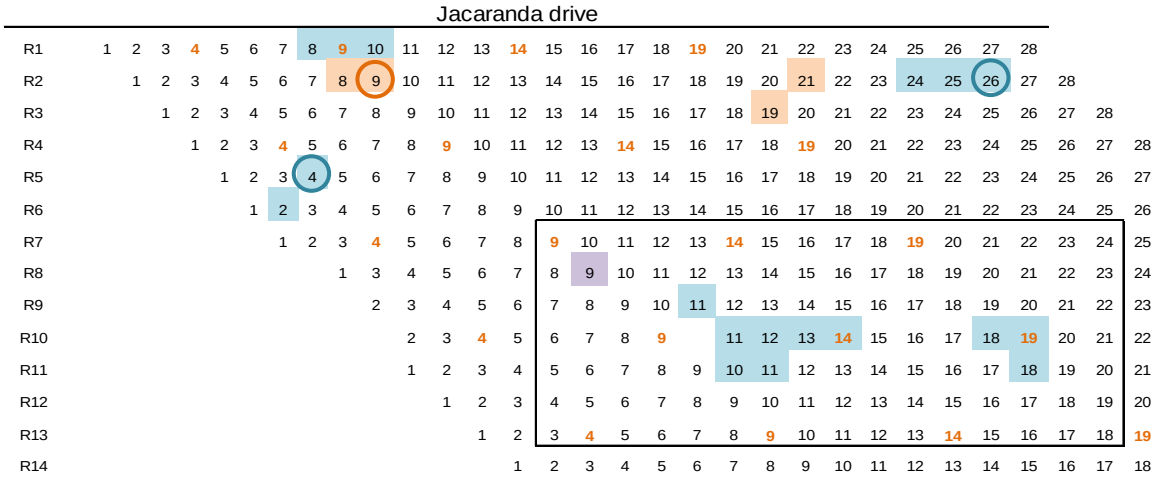
Notes: All the trees in the area marked by a black rectangle were tested in 2005 - Trees diagnosed as positive in subsequent years are marked by circles



2005



Spring 2006



Summer 2007

Appendix 3 – Summary of assessment (culture and RT-PCR) of the Dareton orchard for *Xtp* and *Verticillium dahliae* (*Vd*)

	Row 1			Row 2			Row 3		
		Xtp	*Vd*		*Xtp*	*Vd*		*Xtp*	*Vd*
Guard row	PG-1			PG-1			PG-1		
18				UCB-1			Terebinthus		
17	PG-1						PG-1		+
16	PG-1	+		UCB-1			UCB-1		
15	Terebinthus		+	PG-1			UCB-1		+
14	UCB-1			Male PT134	+	+	Terebinthus		
13	Terebinthus			PG-1		+	UCB-1		+
12				UCB-1			PG-1		
11	PG-1	+					Terebinthus		
10	PG-1			Terebinthus			UCB-1		+
9	UCB-1			PG-1			UCB-1		+
8	Terebinthus	+	+	Male PT134	+		Terebinthus	+	
7	UCB-1			Terebinthus			PG-1		
6	PG-1			UCB-1			Terebinthus		
5	Terebinthus			Male PT198			PG-1		
4	PG-1			UCB-1			PG-1		
3	UCB-1			Terebinthus			UCB-1		
2	PG-1						Terebinthus		
1	UCB-1			Terebinthus			PG-1		

Each tree is indicated by its rootstock *i.e.* PG-1, Terebinthus or UCB-1