Angle Vale leaf tatter and defoliation

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Scholefield Robinson
HORTICULTURAL SERVICES

FINAL REPORT

Angle Vale Leaf Tatter and Defoliation disorder (LTD)

Prepared for : **Horticulture Australia Ltd**

HAL Project No. : **AL05003c**

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PURPOSE OF REPORT

This Final Report has been prepared at the conclusion of the second year of industry-supported field trials established to investigate the cause and control of an almond disorder. The disorder is widespread in the northern Adelaide Plains of South Australia and has resulted in economic losses over the past four seasons.

This report includes information (observations, survey responses, diagnostic test results etc) gained prior to trial establishment, in addition to the 2005/06 and 2006/07 trial details, results and conclusions.

ACKNOWLEDGMENTS

This research has been supported by Horticulture Australia Limited (HAL) and a Voluntary Contribution from the Almond Board Australia. Several crop protection companies have supported the research through the provision of technical guidance and product (Syngenta, Nufarm Australia Ltd, Bayer CropScience Pty Ltd, SACOA). Three grower co-operators and the Industry Development Officer, Mr Chris Bennett, gave generously of their time and resources. They are gratefully acknowledged.

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MEDIA SUMMARY

The Angle Vale Leaf Tatter and Defoliation disorder (LTD) causes economic losses in almonds on the northern Adelaide Plains (NAP). No other production district has reported similar losses or symptoms. The losses result from defoliation and loss of photosynthetic capacity, bud and twig dieback, yield losses as a direct result of bud death and indirectly as a result of affected trees having higher levels of sticktight nuts at harvest.

The failure of LTD to develop on feral trees, during any season to-date, suggests chemicals or a chemical-biological interaction may be involved in the LTD disorder. An investigation of this was the focus of initial field trials.

In 2005/06 non-pareils were confirmed as the variety earliest and most severely affected. Symptoms were widespread on most non-pareil trees by mid-October 2005. The first symptoms were yellow, translucent lesions randomly spread across the leaf blade. Some of these lesions were preceded by greasy, reflective spotting. Leaves in the outer canopy appear worse-affected, although it has been noted that symptoms are usually widespread by the time of first detection within any one season, in susceptible varieties. The onset of symptoms is often sudden. The leaves become tattered and shotholed as the lesions develop necrotic centres. Affected leaves fall while still green.

Although the cause of LTD remains unknown, progress was made in 2005/06, on its management. A fungicide with two active constituents of differing chemistry, successfully delayed the establishment of the disorder in the 2005/06 season. Trees treated with this product (BAS 51604F) on two occasions during the season did not defoliate, and remained of healthy appearance until six weeks before harvest. All other fungicidal treatment programs successfully controlled the known fungal diseases of almonds but were ineffective on LTD.

In the 2006/07 season, BAS 51604F and its components were trialled on a commercial scale and chemical combinations with similar efficacy range, were applied as superimposed treatments. Other blocks were maintained untreated. Some were ‘chemical free’ while others received no foliar nutrients. The trial orchard had an extended and consistent history of LTD, however LTD did not develop in 2006/07.

Only one orchard on the NAP was observed to have LTD in 2006/07. The extended dry period from winter through February, resulted in few chemical crop protectants being applied in any orchards, negligible development of common almond diseases and lower orchard humidity generally. It is considered likely that these conditions and the lack of applied chemicals had an effect on LTD development.

Laboratory and greenhouse work confirmed that neither of the fungi consistently isolated from LTD lesions are primary almond leaf pathogens, nor likely causes of LTD. The scanning electron microscopy (SEM) work revealed that chemically-induced LTD-like lesions have distinct, erumpent and vertically-severed margins. ‘Field’ LTD lesions have a similar appearance at the leaf surface. Under SEM, LTD lesions appear to have neither diffuse margins nor a halo, as may be expected around a pathogen-caused lesion.

Consideration of risk factors associated with LTD, revealed neither planting material nor harvested products to be likely mechanisms for LTD introduction or spread. The disorder has an economic impact associated with yield decline and potentially, input costs. With the cause of LTD remaining undefined, it has not been possible to assign risk ratings to the identified risk factors.

TECHNICAL SUMMARY

The Angle Vale Leaf Tatter and Defoliation disorder (LTD) has been an economic problem in almonds growing on the northern Adelaide Plains (NAP) for four years. The first investigation was peripheral and took place late in the 2003/04 season, as a result of enquiries about widespread 'bacterial spot' on the NAP. Bacterial spot, it was later concluded, was not present in these affected trees. In 2004/05 grower, orchard and feral tree surveys, diagnostic tests and scanning electron microscopy (SEM) were undertaken. In 2005/06 field trials were established to determine if treatment programs used by growers in the area were contributing to, or could manage, the disorder. 2006/07 trials aimed to define the conditions under which a promising protectant fungicide identified in 2005/06 trials, delayed LTD onset and severity.

The early surveys and observations revealed that feral trees did not develop LTD; non-pareils were the most severely-affected commercial variety; fungal hyphae and bacteria were not detected through SEM in initial lesions; that the fungi *Alternaria* sp. and *Cladosporium* sp. were frequently isolated from necrotic lesions; and that growers had recently changed some pest control practices and crop protection products in their orchards.

Symptoms of LTD develop on young and old leaf tissue of most almond varieties, with non-pareil the most susceptible. No symptoms have been observed on very young trees, suggesting this is not a problem attributable to nurseries. The typical symptoms of LTD are small, round-irregular translucent, chlorotic lesions randomly spread across leaf blades. These later develop necrotic centres which may fall out giving affected leaves a tattered and shotholed appearance. Symptom onset and the subsequent defoliation may be sudden. Twig dieback and bud death results in yield losses. During the 2004/05 and 2005/06 seasons, typical LTD symptoms developed. Fine, clear nut gumming also occurred in 2004/05.

In 2005/06 three field trials, each at sites with an LTD history, were established. Each treatment block included non-pareil rows that were used for weekly monitoring and diagnostic sampling. The treatment programs were designed to compare current practices and products with those proven for almonds elsewhere, whilst maintaining the full potential for control of known almond diseases in the area. These included Botrytis blossom blight, anthracnose, brown rot, shothole and rust. There was no confirmed history of bacterial spot at the trial sites. The treatment programs focused on fungal and mite control. Despite the practice being common on the NAP, no canola oil was applied in combination with any of the trialled treatments.

The intended variable between the trial sites was the water volume in which chemical applications were made, with the lowest being 1200 L/ha and the highest 2000 L/ha. Nutrient applications at each site included both foliar and fertigation applied products. These were applied as desired by each grower, across their particular trial site in 2005/06. In 2006/07 two blocks were maintained fungicide free, with one also being maintained free of foliar-applied nutrients. In 2006/07 only one orchard developed LTD and the trial site remained free of LTD.

Field trials have demonstrated the effectiveness of one product (BAS 51604F), a protectant fungicide. The trees treated with BAS 51604F remained free of LTD until 6 weeks before harvest. At no time did these trees defoliate. Symptoms identical to those of LTD have been induced by applications of above-label rates of canola oil, and by a tank mixed application of captan and copper. In neither case however did leaves that emerged after the cessation of the chemical applications, develop LTD-like symptoms.

Leaf samples and diagnostic isolations have demonstrated a consistent presence of *Alternaria* sp. and *Cladosporium* sp. in necrotic lesions. No organisms have been recovered from the translucent, yellow lesions. No insects of significance as potential vectors have been trapped or

identified from within rows of susceptible trees. No viruses have been detected and phytoplasma results have been inconsistent. Graft transmission testing is on-going.

Laboratory work has confirmed that neither of the fungi consistently isolated from LTD lesions are primary almond leaf pathogens or likely causes of LTD. SEM work revealed that chemically-induced LTD-like lesions have distinct, erumpent and vertically-severed margins and that the 'field' LTD lesions have a very similar appearance at both the macro and microscopic level. Neither field LTD lesions nor induced lesions initially have diffuse margins or a halo, as might be expected around a pathogen-caused lesion. There is evidence to suggest an association between LTD and applied chemicals, under certain environmental conditions.

Identification of the cause of LTD remains essential since symptoms alone do not define a disease/disorder. The potential for a complex cause has been recognised, i.e. interaction between applied chemicals and/or nutritional products, environmental conditions, and/or micro-organisms. Understanding the interactions and contributing environmental factors will assist in the management of LTD.

INTRODUCTION

The almond disorder now referred to as Angle Vale Leaf Tatter and Defoliation disorder (LTD) has been examined since 2004. Its cause remains undefined. While control and management strategies also remain ill-defined, an array of tests and observations has been made on affected leaf tissue over three seasons, and progress towards management has been achieved.

This report includes the early hypotheses, observations and tests, since they provide industry with documented history and intelligence about this disorder. The background intelligence was the basis of the 2005/06 and 2006/07 field trials. These trials are the major focus of this report.

Background

General

A disorder of almonds, not previously seen in Australia, was first reported in the Angle Vale area of the northern Adelaide Plains (NAP) in 2003. It was brought to the attention of Scholefield Robinson Horticultural Services Pty Ltd (Scholefield Robinson) in February 2004. At that time, many growers had assumed the symptoms to be those of 'bacterial spot' and accordingly, had applied copper on multiple occasions to affected trees and orchards (References 1- 4).

The symptoms observed by Scholefield Robinson in February 2004 differed from those typically caused by bacterial spot, and copper phytotoxicity appeared the most likely cause of the observed symptoms, at that time. The copper phytotoxicity hypothesis appeared to be supported by the greater incidence and severity of symptoms being in the outer canopy in a mid-height canopy 'band', the acknowledged widespread use of late copper, and the failure to isolate recognised pathogens from the leaf lesions (References 1- 4).

Symptoms

The early symptoms of LTD are translucent, yellow spots randomly distributed across leaf blades (Appendix 1 - Photo 1). The LTD lesions are not clustered along the leaf margins, at the tip or along the mid-vein. Greasy spots may form prior to the translucent lesions. These spots, which are somewhat reminiscent of honey dew spots, reflect light and 'glisten' (Appendix 1 - Photo 2). On occasions they have been the earliest sign of the developing disorder; however they are never as numerous as the yellow spots that develop soon after. Their significance in the development of lesions is unclear. The yellow spots are clearly seen when light is transmitted through the leaf. A good time to inspect trees early in the season is when the sun is out and high in the sky.

Affected leaves are found throughout the canopy but the intensity of lesions is generally greater in the outer canopy. As the weather warms, chlorotic lesions of LTD develop necrotic (dead) centres. The centres may fall out leaving a shotholed and tattered appearance (Appendix 1 - Photos 3-5). Affected leaves drop while still green and not wilted, thereby exposing extensive areas of new wood (Appendix 1 - Photo 6). Chlorotic and necrotic lesions continue to develop on new growth, until late in the season (late January), when new growth appears to stay symptom-free for longer periods. The early leaf loss is followed by bud and twig dieback, and severely-affected trees become bare in a mid-height canopy band. Severely-affected trees also have more sticktights at the end of the season. Yields of affected trees are also reduced the following year due to the loss of buds and budwood.

Varieties on Nemaguard and hybrid rootstocks develop symptoms more readily than those on almond rootstocks. Although all varieties have developed symptoms, the most susceptible appears to be non-pareil. Keane's Seedling and Price have a delayed onset of symptoms, even

when planted in rows of, or adjacent to, severely-affected non-pareils. Symptoms have not been observed in very young trees.

Surveys and Inspections 2004/05

Almond growers in the region include conventional and organic growers. Many were surveyed in 2004/05. The information they provided confirmed that many NAP orchards had suffered LTD, with non-pareils being reported as the most severely affected. The surveys also revealed that copper use, water source (mains, treated effluent or bore) and volume, were inconsistent factors across affected orchards. A summary of the chemical spray information provided by the growers is attached in Appendix 2.

Changes in management practices that coincided with the first appearances of LTD, were reported by the growers. These included increased applications of canola oil, either independently or as an adjuvant in tank mixes. Many growers had moved from using brand-name 'standard' active constituents to their generic equivalents. They had also increased their use of single site of action fungicides, and foliar nutrients. Zinc had increasingly been applied in the form of zinc nitrate (NZn). It was also revealed that a general move towards the use of urea for defoliation (rather than zinc), had occurred. Most growers in the region reported that they had ceased using oil for mite control, during the dormant season, but had increased overall the number of chemical applications for pest and disease management.

The survey of feral almond trees (those along roadsides or in abandoned orchards), revealed they were free of the specific LTD symptoms under investigation. Many however had severe 'shot hole'. It was assumed that the feral trees were predominantly seedlings and thus had a range of genetic variability. Such trees would not have received foliar fertilisation or pest control, and would have been watered irregularly, by street run-off and rain only.

Literature Review

A literature review revealed no reports of similar symptoms on almonds being associated with a biological organism. In California, a similar disorder with an unresolved cause has been described. It is referred to as 'corky spot' and results in leaf lesions visually similar to those caused by LTD. However corky spot lesions reportedly form more frequently along leaf margins.

Other suggested causes of LTD have included cool weather shothole; a toxicity – either chemically-induced (i.e. copper) or a mycotoxin (result of infection by a fungus - i.e. *Alternaria* sp.); oil applied too soon after particular fungicides (i.e. captan); peach silver mite; long distance chemical drift (i.e. paraquat); an unknown obligate pathogen (virus or phytoplasma).

Diagnostics

Symptomatic leaves were sampled for diagnostic analysis in 2004/05, 2005/06 and 2006/07. Such isolations have consistently recovered *Alternaria* sp. and *Cladosporium* sp. In 2004/05 and 2005/06 these were considered fungi secondary to the cause, since they were only recovered from necrotic lesions. Viral testing in 2004 did not detect Prunus Necrotic Ringspot Virus (PNRSV) nor Prune Dwarf Virus in symptomatic tissue.

Scanning electron microscopy (SEM) was undertaken to allow greater inspection of the surface of asymptomatic and affected leaves. Initially, those treated with copper were compared with those from untreated, feral trees. It was notable that both groups of leaves had surfaces free of bacteria, fungal spores and mycelium. Fungal spores were however clearly visible in the 'test sample' which was known to be rust-infected (Appendix 1 - Photos 7, 8). Leaves with lesions typical of LTD appeared to have disrupted epidermal and wax layers. Light microscopy work has shown that the LTD lesions are not vein-delimited (Appendix 1 - Photos 4, 9).

Conclusions 2004/05

At the conclusion of the 2004/05 almond season, the researchers drew the following conclusions from their preliminary and peripheral field inspections, observations and analyses:

- The disorder is widespread in NAP orchards, but not in the Riverland (SA) or Sunraysia (Vic, NSW) areas
- The disorder affects non-pareils more severely than other varieties
- The disorder is more severe on trees on Nemaguard rootstock, followed by those on hybrid stocks. Trees on almond rootstocks are less severely affected
- The first symptoms appear in mid-late November-December, often after a humid spell
- The first leaf symptoms are greasy spots or translucent yellow lesions, randomly distributed across leaf surfaces
- There are no consistent nut symptoms however clear, fine gum streams along the suture and sticktights appear more prevalent in LTD-affected trees
- The use of a generic product with the active ingredient chlorothalonil is widespread
- Late copper applications are frequently made on almonds on the NAP
- Tank mixing of chemicals with canola oil is widespread
- Canola oil has been applied on occasions, against manufacturer recommendations, within 10 days of other fungicide applications
- Unsprayed feral trees appear not to develop LTD symptoms
- Spray volumes (water) are highly variable across the region, but all are lower than used in the Riverland
- None of the three possible water sources is consistently associated with orchards with LTD
- Bud development is reduced in affected trees and twig dieback results in bud death
- The cause is more likely to be abiotic in nature, but remains undefined.

RESEARCH 2005/06

With the cause not being identified through the 2004/05 preliminary tests and observations, the almond industry resolved to support more in-depth research into the disorder during 2005/06 and 2006/07. This research was conducted in NAP orchards, and is hereafter, described and reported on (References 1, 2, 3).

Objectives

The research during 2005/06 aimed primarily to identify the cause of the LTD disorder, and secondly to develop potential management strategies for it. The agreed approach was to establish field trials in which:

- A range of varieties, including non-pareil, was available for evaluation
- Pre-determined, ‘standard’ and equivalent (generic) treatment programs for recognised pathogens of almonds in Australia, could be applied, monitored and compared
- Effectiveness of the programs could be determined from visual symptoms

The field evaluations were complemented by diagnostic analyses, fungicide resistance tests, and inspections of surrounding native vegetation and feral almonds.

MATERIALS AND METHODS

Trial Design 2005/06

Each trial was a commercial-scale block, with treatments applied to multiple rows. Replicates were single trees within each non-pareil sample row. Each treatment program was applied to (at least) three varieties including non-pareil and 60+ trees. Since evaluations were based on visual symptom development, an observational trial was both appropriate and practical for grower co-operators.

Trial Sites

The three chosen trial sites were located on the NAP. The details of each are given below:

- **Trial Site A** (Site A) – Trees 34 years-old, higher and more open canopy than at other sites. Row spacing was 7.5 m; tree spacing within row 3 m. Overall tree health - minor problem with the LTD disorder in 2004/05. This orchard is located close to the town of Virginia and is surrounded by other almond orchards.
- **Trial Site B** (Site B) – Trees within this trial were located at the western end of the orchard. Trees were 7 years-old and on row spacings of 7.5 m and tree spacings of 5.5 m. This orchard has suffered from LTD since 2003/04 with the trees on Nemaguard, and all non-pareils affected. The orchard is surrounded by pasture, almonds and some native plants including eucalypts. This trial site is located near Site C, in the Angle Vale area.
- **Trial Site C** (Site C) – Orchard with the majority of its trees being included in the trial. Trees (2-40 years) with row and tree spacing of 6.5 x 6.5 m. This orchard has suffered from LTD since 2003/04. An adjacent abandoned orchard has not shown symptoms of the LTD disorder in the past, however it routinely develops shothole and rust. This trial site is adjacent to the abandoned planting on one side and glasshouses and a small property that includes deciduous fruit trees on the other boundaries. This trial site is located near Site B, in the Angle Vale area.

At each of the trial sites, non-pareil sample rows were buffered by rows of either non-pareil or other varieties, within the treatment blocks. Sample rows did not receive drift from sprays applied to adjacent treatment blocks. Sample trees within each non-pareil row were identified and flagged while dormant. Coloured surveyors' tape was used to distinguish the planned treatment programs in the field. These are de-coded in Table 1.

Table 1: Planned treatment programs and colour codes

Trial site	Treatment program focus (colour code)						
(Spray volume L/ha)	BAS 51604F	Amistar®	Chlorothalonil (early)	Chlorothalonil (late)	Tilt®	Bio-Pest® mite control	(+ Oil)*
Site A (2000)		Yellow	Blue	Pink	Blue (tank mix)	Orange	
Site B (1200)	Yellow		Blue	Pink	Blue (tank mix)	Orange	✓
Site C (1700)	Yellow	Orange	Blue	Red	White		✓

* Limited application - Refined canola oil (Synertrol® Horti Oil) applied to marked branches only

Evaluations – In Field

Visual evaluations

Treatments were evaluated visually. A 'successful' protectant application in this research was one that curbed the onset of symptoms and/or the severity of symptoms. Sample rows and trees were inspected regularly (every 7-10 days) throughout the season from early green tip to 4-6 weeks pre-harvest. Leaves were inspected visually for the presence of lesions.

Leaves were collected from sample trees for diagnostic analysis, from early September. Samples were also collected from feral, unsprayed trees, and neighbouring native vegetation.

Evaluations - Laboratory

Diagnostics

After each collection, a sub-sample of representative leaves was analysed as fresh leaves, while another sub-sample was incubated in humid conditions, before laboratory analysis. The aim of leaf incubation was to activate quiescent infections, should they be present. Leaves from feral trees, and native vegetation (*Eucalyptus* sp.), were also sent for diagnostic testing. Leaf diagnostic work was performed at SARDI, by Barbara Hall. Fungal and bacterial isolations onto synthetic media, were made from leaves collected from the commercial, trial trees and also from feral trees.

At the conclusion of the formal trial (January 30, 2006) symptomatic leaves from spurs on first and second year wood were collected. They were examined for the presence of almond viruses and phytoplasmas by Dr Brendan Rodoni, CHS Knoxfield, Victoria.

Nutritional analysis

Samples of symptomatic, non-pareil leaves and asymptomatic Keane's Seedlings (from row adjacent to non-pareils) were collected in November 2005, from both the chlorothalonil and BAS 51604F treatment programs, at Site B. These were evaluated for their general nutritional status by CSBP Laboratory in Western Australia. The interpretation of results was made by Dr Ben Thomas, plant nutritionist, Scholefield Robinson.

Fungal Resistance

Some fungi isolated from blighted blossoms and areas of twig dieback in the orchards around the trial sites were also evaluated. Their resistance or susceptibility to two groups of fungicides (benzimidazoles and dicarboximides) was determined.

Treatments - Chemical

Dormant Season

The trees within the three trials were treated similarly during the dormant season: copper application for general fungal and bacterial management; summer oil, for mite control.

Growing Season

The treatment programs were aimed at protection against known, almond fungal diseases and LTD, rather than eradication. Each centred on fungicidal products and equivalent (generic) active constituents. Mite control was also included to ensure mites did not introduce an unwanted variable into the trials and tree performance assessments. The details of treatments applied at each site are given in Table 2. They are further summarised by product and site in Table 3.

The trialled chemicals and their time of application were chosen after review of several factors: necessary control of known and recognised diseases of almonds; reproduction of grower chemical regimes of previous seasons on the NAP; comparison of brand name and generic products with the same active constituents. The treatments were applied at label rates, unless otherwise stated.

Treatments - Other

Canola oil was not used independently or in conjunction with any other product in the main trials, since its label clearly states it cannot be used within 10 days of some fungicide applications. This practice however has occurred on the NAP in recent years, and to test its potential role in the LTD disorder, multiple oil applications were made to several labelled branches at Sites B and C. These have been referred to as "+ oil" treatments in Table 1. The canola oil was applied at label and above label rates, on two sample trees per treatment program.

The potential eradicant activity of one promising protectant was evaluated towards the end of the 2005/06 season. This product, BAS 51604F, has two active constituents and in January, 2006 each was applied independently, to symptomatic trees *outside* the main trial at Site B, i.e. the active constituents of BAS 51064F and the pre-mix product itself were trialled as eradicants.

At Site B, a range of nutrients (including potassium nitrate, urea, calcium nitrate and Hydro-Complex) was applied with each irrigation from September onwards. Other foliar nutrients were also added. The nutrient program at Site A was less complex with ammonium nitrate the main nutrient applied.

Exclusion Treatment

Several small branches with asymptomatic leaves were bagged early with cryovac plastic made into loose bags, in an attempt to create an 'exclusion' barrier. The objective was to evaluate the appearance of leaves on small branches that had been covered to exclude insects and chemicals. The enclosed leaves were evaluated after two weeks.

Table 2: Chemical and Nutrient Treatment* Regimes

Treatment Time	Date	Site A	Date	Site B **	Date	Site C
Dormant	30/05/05	Norshield 750 copper (All)	30/06/05 06/07/05	Summer Oil (All), Copper (All) Blue Shield copper (All)	28/06/05 19/07/05 31/07/05	Copper + Urea (All) Summer Oil (All) Copper + Agral (All)
Budswell/Green Tip	01/08/05	Norshield 750 copper (All)	24/07/05	Broadcast Hydro-Complex (NPK+) (All)		
Pink Bud-Early Bloom			03/08/05 11/08/05	Broadcast Ammonium Sulphate (All) Kocide (All)		
Mid Bloom					18/08/05	Rovral (All)
Full Bloom		No planned treatments		No planned treatments		No planned treatments
Late Bloom-Early Petal Fall	27/08/05	Barrack/Bravo (All)	26/08/05	BAS 51604F (Area 1)	27/08/05	BAS 51604F (Area 1) Barrack (All except Area 1)
Shuck Fall-Early Set	06/09/05	Amistar (Area 1)	06/09/05	Echo (Area 2) Barrack + Tilt (All except Area 2)	06/09/05 20/09/05	Echo + NZn + Potassium (Area 2) Bravo + NZn + Potassium (All except Area 2) Potassium + Agral (Area 3) Tilt + NZn + Potassium(All except Area 3)
2 Weeks Post Bloom, Early Leaves	14/09/05	Echo (Area 2) Bravo + Tilt (All except Area 2)	22/09/05	BioPest Oil + Penncozeb (Area 3) Lorsban™ + Barrack 720 (All except Area 3)	27/09/05	Amistar +NZn + Boron (Area 4) Tilt + NZn + Penncozeb + Boron (All except Area 4)
5 Weeks Post Bloom	28/09/05	Penncozeb + Tilt + NZn + Agral (All)	06/10/05	BAS 51604F + Potassium Nitrate (Area 1) Penncozeb + Potassium Nitrate + Tri-Base Blue (All except Area 1)	13/10/05 25/10/05	BAS 51604F (Area 1) Penncozeb (All except Area 1) Tri-Base Blue copper (All)
5-7 Weeks Post Bloom	15/10/05 16/10/05	Amistar (Area 1) Penncozeb + KNO_3 +Agral (All except Area 1) Ammonium Nitrate (All)				No miticide treatment
Late October/November	01/11/05 20/11/05 25/11/05	Ammonium Nitrate (All) BioPest Oil (Area 3) Penncozeb + Rogor + Agral (All except Area 3) Ammonium Nitrate (All)	17/10/05 27/10/05 09/11/05 19/11/05 29/11/05	Echo + Potassium Nitrate (All except Area 3) Penncozeb + Wetter +Urea (All except Area 1) Bravo 720 Weatherstik +Boron + Urea + Tri-Base Blue (All except Area 4) Potassium Nitrate + Urea + Zinc (All) Potassium Nitrate + Urea + Zinc + Dithane Rainstick (All)		No late chlorothalonil (Echo)

*All products have registered trademarks but Lorsban™ is not specifically registered for almond use. ** Site B – Regular fertigation with potassium nitrate, urea, calcium nitrate and Hydro-Complex

Table 3: Treatments during growing season 2005/06

APPLICATION	DATES OF PEST AND DISEASE CONTROL APPLICATIONS		
	Site A	Site B	Site C
ECHO®	14 Sept	6 Sept 17 October	6 Sept
BRAVO®/BARRACK®	27 August 14 September	6 September 22 September; 9 November	27 August 6 September
COPPER (in growing season)	---	6 October 9 November	25 October
BAS 51604F	---	26 August 6 October	27 August; 13 October
AMISTAR®	6 Sept; 15 October	---	27 Sept
PENNCOZEB®	28 September 15 October 20 November	22 September 6 October 27 October	27 September 13 October
TILT®	14 September 28 September	6 September	20 September 27 September
	DATES OF NUTRIENT APPLICATIONS		
Zinc – as NZn	28 September	19 November 29 November	6 September 20 September 27 September
Zinc			
Ammonium nitrate	16 October 1 November 25 November		
Potassium nitrate	15 October	6 October; 17 October 19 November; 29 November – at all irrigations	6 September 20 September
Ammonium sulphate		3 August	
Urea		27 October; 9 November; 19 November; 29 November – all irrigations	
Boron		9 November	27 September
FIELD SYMPTOMS	No symptoms developed during season 2005/06	*First* detection in 2005/06 - 21 Oct. (not in BAS 51604F) *Severe* – 15 Nov, 2005	*First* detection in 2005/06 - 3 Nov. (not in BAS 51604F) *Widespread* – 25 Nov, 2005

RESULTS

In-field Chemical Treatments

Despite the planned comparable treatments amongst and between sites, the variable weather conditions and disorder onset caused some intended applications to change or be delayed. However the intended variables in each treatment program were achieved at each of the sites. The most significant differences in applications were: Site A did not receive BAS 51604F and

Site B did not receive Amistar® applications. At Site A, copper was not applied after August 1. Tables 2 and 3 summarise the chemical pest control applications.

Although monitoring and evaluation of nutrient applications were not formal components of the trial, it is noted that the choice and application frequency of foliar and fertigation nutrients was variable across the sites. The significance of the various nutrient regimes in the onset of the disorder could not be determined from the 2005/06 trials.

LTD Development

The first appearance of LTD symptoms during the 2005/06 season was on non-pareils at Site B, on October 21, 2005. Inspections 10 days earlier had shown no sign of LTD. The translucent lesions were in non-pareil trees within the generic 'chlorothalonil' (blue) treatment block, and at their first sighting (for this season) were in fact already widespread within all trees within the non-pareil row. These trees had received applications in addition to chlorothalonil. The program was: Echo®, Lorsban™ + Barrack®, Penncozeb® + Potassium nitrate + copper, and Echo® + potassium nitrate. The disorder had a sudden, but extensive onset within this treatment.

At this time (October 21, 2005), there were no symptoms in the adjacent treatment program, being BAS 51604F (yellow) at Site B, nor in trees of other varieties. The mid-October onset of symptoms at Site B was earlier than expected. In previous seasons the first symptoms were found from mid-late November. In 2005/06 as in other years, the non-pareils were the most severely affected variety.

By early November, 2005, trees in all treatment programs at Sites B and C had visible LTD of variable severity. The chlorothalonil programs, across both Sites B and C, had the most extensive and severe symptoms. By mid-November however, LTD in all treatment blocks except the BAS 51604F program was extensive and similar in terms of symptom severity. The copper and Tilt® programs were not distinguishable from the chlorothalonil programs at either site. Each tree in these rows was symptomatic; however the Keane's Seedling and Price trees in adjacent rows were less severely affected and did not suffer significant defoliation.

Although detectable in mid-November, very few LTD lesions could be found in the BAS 51604F treatments at Sites B and C. This program was notably superior for several additional weeks in November at Site C and for a further 6 weeks at Site B. In these trees the lesions were sparse and trees did not defoliate. Not until early January 2006, two months after the last BAS 51604F application, did the non-pareil trees in this program at Site B display extensive symptoms. Unlike other affected trees, defoliation of the BAS 51604F-treated trees did not occur even when symptoms became extensive in early January.

No treatment program other than that of BAS 51604F curbed the development of LTD symptoms. This fungicide delayed the onset of symptoms and the extent and severity of symptoms. No symptoms of LTD were observed at Site A during 2005/06.

The trial of eradicant efficacy of BAS 51604F, boscalid and pyraclostrobin on severely-affected non-pareils outside the Site B trial area, took place in January, 2006. The eradicative efficacy of these products on LTD was not clearly demonstrated. It appeared however, three weeks later that there was more symptom-free new growth on the BAS 51604F-treated trees, than on others.

In general, the LTD development in 2005/06 was characterised by earlier onset, fewer greasy spots preceding chlorotic spots and very little nut gumming.

Other Almond Diseases – 2005/06

Despite variable LTD onset and severity across the trial sites, the treatment programs effectively controlled the other recognised almond fungal diseases in the area. The wet and mild spring

weather provided conditions that were conducive to a number of almond diseases, but each was well-controlled by the programs, despite some minor shothole and Botrytis appearing for a short period and some minor anthracnose detected as aborted nuts at Site B. The product choice and timing of applications resulted in good disease management and fewer chemical applications than were applied by the grower co-operators elsewhere on their properties. There is potential for growers in the region to effectively reduce their chemical inputs for these diseases.

Contrasted against the pest/disease control achieved in the trials was the severe shothole and rust that developed on the feral trees growing near Sites B and C.

Mite Treatments

Both chemical insecticide and Bio-Pest Oil® achieved mite control at Site B. Mite treatments were not required at Sites A or C.

A summary of field observation and inspection notes follows in Table 4.

Table 4: Field Inspection Reports

Stage of Development	Date	Leaf Symptoms			Brief Comments
		Site A	Site B	Site C	
Pink Bud – Shuck Fall	06/09/05	×	×	×	Canola oil applied at Site B and C.
Shuck Fall – Early Set	15/09/05	×	×	×	Early, minor shothole. Lesions on oil+ treated leaves.
	27/09/05	×	×	×	Lesions and chlorosis of leaves in oil+ treatments. Botrytis - Site C. No symptoms on feral or organic trees.
Nuts nearly full size	10/10/05	×	×	×	Minor shothole at all sites including feral trees.
	21/10/05	×	✓	×	Symptom development in all non-pareils *except* those treated with BAS 51604F.
Nuts Full Size	03/11/05	×	✓	✓	Increased severity and extent of LTD. Very sparse symptoms for the first time within BAS 51604F Site B and early symptoms at Site C.
Kernel Hardening	15/11/05	×	✓	✓	Significant leaf loss in chlorothalonil (blue) treatment Sites B and C. No symptoms Site A.
Kernel Hardening	25/11/05	×	✓	✓	More severe LTD within treatments; LTD in BAS 51604F at Site C now more widespread; very minor LTD in BAS 51604F at Site B.
Kernel Hardening	09/12/05	×	✓	✓	Defoliation stopped at Sites B and C. Most new growth is staying clean.
Kernel Hardening	28/12/05	×	✓	✓	BAS 51604F trees at Site B still significantly better than all others.
Kernel Hardening (~5 weeks until harvest)	12/01/06	×	✓	✓	No significant change at any of the 3 trial sites. BAS 51604F at Site B still better than other treatments.

✓ = Symptomatic Leaves
× = Asymptomatic Leaves

Other Treatments

+ Oil limited Trial

The application of above-label rates of canola oil induced symptoms visually identical to those of the LTD disorder. The sprayed leaves developed lesions evenly distributed across the blades. The lesions dried and leaves fell prematurely. However once the application of oil ceased, asymptomatic, new growth emerged.

Exclusion Treatment

The exclusion treatment could not be fully evaluated after two weeks since the cryovac plastic bags had not allowed sufficient air exchange and the leaves fell prematurely within the bags.

Nutritional Tests

Symptomatic and asymptomatic leaves from two varieties and two treatment programs were analysed, and their general nutritional status compared. It was found that the nutritional status of the two subsets (symptomatic and asymptomatic), was very similar. While it appears unlikely nutritional status can explain the LTD disorder, it cannot be concluded that individual nutrients applied in combination with other nutrients or fungicidal products (i.e. nutrients + trace element mixes and/or pest control products) are not LTD- contributing factors. Table 5 below summarises the comparative nutritional results.

Table 5: Nutritional analyses of leaves from two treatments at Site B

Nutrient		Chlorothalonil Treatment Program		BAS 51604F Treatment Program	
Date Sampled November 25, 2005		Keane's Seedling (Asymptomatic)	Non-pareil (Symptomatic)	Keane's Seedling (Asymptomatic)	Non-pareil (Symptomatic)
N	(%)	3.02	2.95	2.95	2.88
NO_3-N	(mg/kg)	193	122	157	117
P	(%)	0.18	0.19	0.18	0.18
K	(%)	2.17	2.24	2.13	1.94
Ca	(%)	3.6	3.12	3.49	3.04
Mg	(%)	0.66	0.63	0.67	0.6
S	(%)	0.2	0.24	0.2	0.19
Na	(%)	0.08	0.08	0.08	0.06
Cl	(%)	0.28	0.34	0.37	0.31
Zn	(mg/kg)	150	148	127	135
Mn	(mg/kg)	117	153	62	75
Cu	(mg/kg)	92	116	88	80
Fe	(mg/kg)	94	113	93	123
B	(mg/kg)	36	42	37	34

Laboratory Tests

Diagnostics

Neither LTD development, nor pathogen recovery from lesions on sampled leaves appeared to be enhanced by the process of incubation in humid conditions before attempting fungal and bacterial isolations.

No recognised pathogens were isolated from asymptomatic leaves at any time during the season. From symptomatic leaves, the most consistently recovered organisms were fungi in the genera, *Alternaria* and *Cladosporium*. These were predominantly recovered from necrotic leaf lesions. They were also recovered from some feral trees that did not display LTD symptoms. The recovery frequency from BAS 51604F-treated leaves was variable, and this might relate to the relative time before or after a BAS 51604F application that isolations were attempted.

The results of diagnostic analyses from both commercial (C), trial trees and feral (F) trees are provided in Table 6. The significance of the common fungi recovered in the development of LTD, was unclear in the 2005/06 season but pathogenicity tests conducted in 2006/07 suggest these are not primary almond pathogens.

Table 6: Schedule and results of diagnostic analyses

Sampled Material	Trial Site and Treatment program	Date Sampled	Incubated (+/-)	Organisms Detected, Recovered		Feral (F) or Commercial (C) Trees
		2005				
Flowers, Shoots	Independent	09/09/05		*Botrytis sp.* 3 of 8 isolates resistant to dicarboximides		C
Shoots	C	06/10/05		*Botrytis sp.* *Ps syringae*		C
	Independent			*Seimatosporium sp.*		C
Leaves Symptomatic - yellow mottle	B BAS 51604F program Oil Sprayed	18/10/05	+	Secondaries not associated with spots. Diagnostician queried oil damage		C
Leaves Symptomatic – *new* LTD*	B Chlorothalonil program	24/10/05	+	*Alternaria sp.* *Cladosporium sp.* *Stigmina sp.* (Shothole)		C
Leaves Symptomatic LTD	B All Treatments	03/11/05	+	*Alternaria sp.* *Cladosporium sp.*		C
Leaves Symptomatic LTD	B & C All Treatments	08/11/05	+	*Alternaria sp.*		C
	C BAS 51604F program		+	*Alternaria sp.*		C
Leaves Symptomatic LTD	B Chlorothalonil program - before Spray	14/11/05	+	*Alternaria sp.*		C
	B BAS 51604F program - before Spray		+	No organisms recovered		C
Leaves Symptomatic *not* LTD	Roadside	18/11/05		*Alternaria sp.* *Cladosporium sp.* *Stigmina sp.* (Shothole)		F
Leaves Symptomatic *new* LTD	C BAS 51604F program			*Alternaria sp.* *Cladosporium sp.* *Stemphyllium sp.*		C
		2006				
Shoots/twigs bud lesions	B	09/01/06		*Alternaria sp.*		C
Twig Dieback, diffuse margin	B			*Alternaria sp.* *Cladosporium sp.* Phoma –like fungus		C
Leaves/shoots Symptomatic LTD	B	30/01/06		Phytoplasma ? Virus ?	*Variable Results*	C
Leaves/shoots Asymptomatic	A			Phytoplasma ? Virus ?		C
Leaves Symptomatic – *not* LTD	C			*Stigmina sp.* *Alternaria sp.*		F
Twigs Canker	C			Sooty mould *Alternaria sp.*		C
Twigs Gumming lesion	C			No organisms recovered		C
Leaves *Eucalyptus sp.*	B			*Mycosphaerella sp.* (likely)		F

* LTD = Leaf tatter and defoliation disorder

Leaves and woody tissue collected in late January 2006 for viral and phytoplasma analyses revealed inconclusive results. There are indications however that a phytoplasma may be present in some of the sampled almond tissue. The testing, performed by Dr Brendan Rodoni, Crop

Health Services DPI-Vic Knoxfield, Victoria, is currently being repeated and reviewed in conjunction with work on grafting of LTD-symptomatic non-pareil budwood to 'clean' Nemaguard rootstocks. These results will be provided in an Addendum to this report, as soon as they are made available.

Fungicide Resistance

In the early part of 2005/06 season, *Botrytis* sp. was recovered from early flowering varieties with blossom blight. Three of the eight isolates of *Botrytis* sp. were confirmed to be resistant to the dicarboximides (i.e. Rovral®). There appear to be no isolates from this area currently resistant to the benzimidazoles used. It is important in an area like the NAP where grapes, vegetables and almonds are grown, and dicarboximide use is high, that product use be monitored across crops, as part of a resistance management strategy.

Summary - 2005/06

The preliminary findings from the 2005/06 field trials were reported in this project's Milestones 2 and 3 and in the Interim Report (Milestone 4).

In summary, one effective experimental treatment for LTD was revealed. It is a fungicide of dual chemistry, not registered for use on almonds in Australia, but registered for control of fungal diseases of almonds and other crops, in California. It successfully delayed the onset of LTD until January 2006 and in so doing reduced LTD's impact on the treated trees. It however remains unclear if the fungicide's efficacy was due to the direct control of a fungus, or was indirect, i.e. as a result of an induced host response, control of more than one biological agent (pathogen and/or vector), or as a result of its chemistry providing a level of protection or barrier at the leaf surface. The 2005/06 results do not discount the continued potential for an abiotic cause of LTD or abiotic contributing factors.

RESEARCH 2006/07

Objectives

Determining the cause of LTD remained the objective of the 2006/07 trials.

MATERIALS AND METHODS

Field Trial 2006/07

One field trial was established in July 2006 at Trial Site B (Angle Vale, SA) again. In the continuing effort to find the cause of LTD, BAS 51604F and its efficacy range became the foci of investigations during the 2006/07 season. This product and its two active constituents, and other chemicals/chemical combinations with similar efficacy ranges were applied on a 'commercial' scale, at the trial orchard.

Two treatment blocks within the trial were 'controls' and received no fungicides: one received neither pest control products nor foliar nutrients; the other received an insecticidal program but no foliar nutrients. The other treatment blocks received BAS 51604F, boscalid or pyraclostrobin. Super-imposed over each of the treatment programs were single tree applications of other products in combinations considered to have a similar efficacy range to that of BAS 51604F. A summary of the treatments, rates and the application dates is given below in Table 7.

Greenhouse-maintained trees that received the same treatments as applied in the field were used to support field work where appropriate.

Table7: Chemical treatments applied during growing season 2006/07

BLOCK TREATEMENTS			SUPER-IMPOSED, SINGLE TREE APPLICATIONS		
ACTIVE CONSITUENT	RATES	FOLIAR NUTRIENTS[X]	PRODUCTS APPLIED	RATES	DATE APPLIED
Control – Untreated	n/a	-	Cyprodinil + Amistar®	80g / 100L 65g / 100L	11/09/06
			Captan + Copper (BlueShield DF)	200g / 100L 200g / 100L	
Control – Insecticide	+	-	Cyprodinil + Amistar®	80g / 100L 65g / 100L	11/09/06
			Captan + Copper (BlueShield DF)	200g / 100L 200g / 100L	
			Confidor	25ml / 100 L	9/8/06
BAS 51604F	40g / 100L	+	Cyprodinil + Amistar®	80g / 100L 65g / 100L	11/09/06
			Captan + Copper (BlueShield DF)	200g / 100L 200g / 100L	
Pyraclostrobin	20.5ml / 100L	+	Cyprodinil + Amistar®	80g / 100L 65g / 100L	11/09/06
			Captan + Copper (BlueShield DF)	200g / 100L 200g / 100L	
Boscalid	20g / 100L	+	Cyprodinil + Amistar®	80g / 100L 65g / 100L	11/09/06
			Captan + Copper (BlueShield DF)	200g / 100L 200g / 100L	
Trifloxystrobin	10-15g / 100L	+	Cyprodinil + Amistar®	80g / 100L 65g / 100L	11/09/06
			Captan + Copper (BlueShield DF)	200g / 100L 200g / 100L	
Metiram + Pyraclostrobin	102.4g / 100L	+	Cyprodinil + Amistar®	80g / 100L 65g / 100L	11/09/06
			Captan + Copper (BlueShield DF)	200g / 100L 200g / 100L	

[x]Foliar nutrients and dates of application include: 28/08/07 Zinc, Urea; 29/09/07 Boron, Zinc, Urea; 22/10/06 Potassium Nitrate

Other Trials

Greenhouse - Pathogenicity

Greenhouse trials were established during 2006/07. Greenhouse-based research focussed on determining the pathogenicity of the *Alternaria alternata* and *Cladosporium* sp. isolated with

consistency from necrotic LTD lesions. This work was performed under controlled greenhouse conditions, utilising self-fertile almond trees. Leaves were inoculated with ten isolates of *Alternaria alternata* and one *Cladosporium* sp. recovered from necrotic LTD lesions in previous seasons.

The inoculation methods used were sprayed spore suspensions and mycelial plug attachments on both "injured" and intact leaves. Post-inoculation leaf management included both mist (and high humidity) and dry incubation. The experiment was repeated. The methods are detailed in the SARDI report attached in Appendix 3.

Greenhouse - Chemical Treatments

The same chemical solutions applied to the almond trees at the trial site were applied to almond trees growing under controlled conditions in the SARDI greenhouse (Appendix 1 - Photo 10). Leaves were regularly observed over a six week period at which time some of the trees were removed from the greenhouse and placed in the orchard.

Leaf surface Microscopy

Scanning and light microscopy were utilised to evaluate the nature of the LTD lesions. The methods used are detailed in the Adelaide Microscopy reports attached in Appendix 4.

Diagnostics

Samples from the one orchard that developed LTD in 2005/06 were sent to SARDI, Dr Chin Gouk (DPI-Vic – Tatura and Knoxfield, Victoria) and Christine Horlock (QDPI – Stanthorpe, Qld). The two pathologists have specific interest and experience in stone fruit. They had been made aware of the LTD problem, and expressed willingness to examine samples specifically for the presence of bacteria. The methodologies used by the Queensland team, are included in Appendix 5. SARDI attempted further isolations of fungi, using a technique referred to as the ONFIT system. This methodology is outlined in Appendix 3.

Sticky traps were placed in two rows within the trial to determine the presence of insects that may potentially be vectors of viruses, or of interest due to their feeding patterns. They were read and replaced on a 7-10 day schedule.

RESULTS

Field Trial 2006/07

LTD did not develop this season in the trial area, and therefore quantification of the LTD presence and severity, and evaluation of BAS 51604F, its constituents, and foliar nutrients, were not possible.

None of the commonly encountered almond diseases developed in the trial trees this season, regardless of the block treatment. Trees in the untreated blocks maintained a healthy appearance throughout the season although the canopies of these trees were less vigorous than elsewhere. This was expected since no foliar nutrients were applied. Anthracnose was observed but it is not new to this site. Minor incidences have been previously observed and reported.

Trees across the entire trial site suffered from high mite populations. The insecticide-treated block suffered less, despite the insecticide used early in the season not being specifically active on mites. Mite populations in general were higher across the production district this year.

Insect sticky traps did not reveal the presence of Western Flower Thrip as had been expected, nor aphids of potential concern. Other thrips and flies were commonly recovered from within the trial area.

Chemically-induced lesions similar in appearance to those of LTD were induced this season with a hand-applied mix. These 'chemical lesions' were chlorotic, not vein-delimited, round and similarly dispersed on the leaf surface to those of LTD. The induced chemical lesions although generally distributed across the sprayed leaf surfaces resulted in more coalesced lesions. No new lesions appeared on leaf tissue that emerged after the cessation of the fungicidal mix application.

The chemical LTD-like lesions were induced on each of the single tree replicates over-treated (two per treatment block) with captan/copper on 14th September, 2006. This treatment caused the same damage on each of the treated trees, regardless of the underlying block treatment. Trees not receiving this over-treatment did not develop any symptoms, even when adjacent to the symptomatic trees.

Environmental conditions are known to contribute to the impact of some applied chemicals and nutrients, i.e. the leaf burning attributed to the application of sulphur in hot conditions. Similarly, the presence of heavy dews as occurred frequently during spring at the trial site may have influenced not only the leaf wetness periods but also the degree of copper solubility and duration and frequency of release periods, at the leaf surface. Copper (and zinc) toxicities result from free ions in solution, with the solution concentration, time in contact with leaf and presumably osmotic potential contributing to the impact at the leaf surface. It has been shown in other crops that the extent of damage may also relate to leaf age (i.e. urea on grapes, citrus) and variety and it is likely that similar sensitivities may occur in almonds.

While it is not known what the pH of a captan/copper (or other nutrient/crop protectant) solution might be at the leaf surface, it is possible that acidity has an influence on the reaction of leaves to heavy metal solutions. There is some precedent in a range of crops for such hypotheses: leaf damage in strawberries from a foliar nutrient tank-mixed that included copper; in almonds, with both phosphorous acid/copper applications, and also mancozeb/copper applied together very late in the season; and in potatoes with a chlorothalonil-based tank mix applied at night.

The captan/copper induced chemical lesions in the 2006/07 trial were indistinguishable visually from those caused by high (off-label) rates of canola oil in the 2005/06 trials, and from 'field' LTD lesions seen in both 2005/06 and 2006/07. (Appendix 1 - Photos 11-13).

Although LTD was not observed in the trial orchard in 2006/07, it did develop on non-pareils (and some pollinators) in a nearby drip-irrigated orchard. The symptoms were first seen in mid-November, 2006. This orchard has suffered from LTD in previous years. The crop protectants applied in this orchard, prior to the onset of LTD, were: Champ Dry Prill (cupric hydroxide), Rovral® (iprodione), Bravo® Weather Stik® (chlorothalonil) and Tilt® (propiconazole). Trace elements applied as foliar nutrients included boron and chelated forms of zinc, manganese, copper and sulphur. LTD symptoms were observed two weeks after an application of chlorothalonil and trace elements. The trace element mix included copper and sulphur. As suggested above, the non-specific application of copper and other trace elements, in combination with some fungicides, may contribute to LTD. Crop protectant and foliar nutrient sprays are often applied late afternoon/early evening on the NAP, due to wind. The effect of spray timing (and therefore leaf wetness periods) on the efficacy of some tank mixes and the subsequent development of LTD, needs further investigation.

A chemical application to Keane's Seedlings outside the trial area but within the trial orchard caused leaf burn ('chemical burn'). The leaf symptoms however were not reminiscent of those resulting from LTD.

Other Trials

Greenhouse - Pathogenicity

In the greenhouse experiments, candidate pathogen inoculations by neither method resulted in the development of LTD symptoms. Some fungal isolates were capable of infecting almond leaves but the signs of infection included localised, concentric rings typical of other *Alternaria* - caused ringspots, but not LTD. In no case did a generalised infection occur with symptoms extending beyond the inoculated leaves. Most isolates did not establish any infection. We have concluded these fungal isolates are not primary pathogens of almonds, under the trial conditions. None of the isolates are now considered potential primary causes of LTD. The full SARDI report on pathogenicity is available in Appendix 3.

Since pathogenicity resulting in LTD, was not demonstrated, the proposed work on fungicidal resistance and fungal modes-of-action (including toxin production) was not undertaken.

Greenhouse – Chemical Treatments

The almond trees maintained in the greenhouse and treated with the same chemicals applied in the field, did not develop typical LTD. Some applications however resulted in severe leaf burn and defoliation.

Leaf Surface Microscopy

Leaves of healthy appearance (‘healthy’), with chemical burn (‘chemical burn’) or chemically-induced LTD-like lesions (‘chemical lesions’) and those with ‘field’ LTD from the nearby orchard, were examined under light (LM) and scanning microscopy (SEM). The SEM images of each at the leaf surface have provided a benchmark reference against which the LTD leaf surface images may be compared.

Healthy leaves appeared under SEM, to have uniform and intact leaf cuticles, on both upper and lower surfaces (Appendix 1 - Photos 14, 15). No microflora (fungi or bacteria) were observed on the scanned, healthy leaves. Rust fungal spores were readily detected by SEM on the collected rust-infected leaves (Appendix 1 - Photos 7, 8).

The ‘chemical lesions’ induced by the application of captan/copper (Appendix 1 - Photo 11), appeared as areas of broken cuticles corresponding on both the upper and lower leaf surfaces (Appendix 1 - Photos 16, 17). The circular pattern of the broken cuticles and the associated presence of precipitates, strongly suggested the lesions were associated with an applied chemical. The centre of some lesions fell out on processing which suggested the damage although visible predominantly on the upper leaf surfaces as translucent spots, in fact extended vertically through the leaf (Appendix 1 - Photo 18). Although the margins of some lesions were erumpent (eruption-like), all lesions had distinct margins with the area immediately outside the lesions appearing normal (Appendix 1 - Photos 16, 17). No ‘transition zone’ or halo was evident around the greasy spots or translucent lesions, as might have been expected had a pathogen been the cause of these initial lesions. Microflora were not observed on these leaves under SEM. However, it is known through the associated laboratory analyses that *Alternaria* sp. and *Cladosporium* sp. are frequently present in necrotic lesions and many of these have a visible halo.

The leaves that developed LTD in 2005/06 and 2006/07 (one orchard) appeared under SEM to have distinct similarities with those caused by chemicals in 2005/06 (canola oil) and 2006/07 (captan/copper). Greasy spots, translucent yellow lesions and chemical lesions were not vein-delimited; had distinct, vertically-severed margins; and had associated residues (Appendix 1 – Photos 19, 20, 21).

Diagnostics

From LTD lesions that had necrotic centres, *Alternaria* and *Cladosporium* sp. were again isolated by SARDI in 2006/07. No organisms were recovered from chlorotic lesions. The samples forwarded to Crop Health Services (from Dr. Chin Gouk) did not reveal any fungal or bacterial pathogens.

The methodology utilised by Christine Horlock resulted in the isolation of a bacterium, which initially appeared somewhat similar to *Xanthomonas arboricola* pv *pruni*, the cause of stone fruit 'bacterial spot.' Further attempts to identify this bacterium to species were carried out at the Australian Collection of Microorganisms (Brisbane, Qld) and utilised Biolog GN2 MicroPlates and API 20 NE test kits. One isolate has been identified as *Chryseomonas luteola* (formerly *Pseudomonas luteola*). The significance of this as a plant pathogen is not well-documented on any host and its potential as an almond pathogen while unknown, is considered unlikely.

Definitive identification of neither of the two isolates was achieved with the Biolog GN2 Microplates, beyond their being gram negative, oxidase negative bacilli, forming yellow colonies. The full report (and summary) on the bacterial identification by Jenny Spratley is included in Appendix 5.

In 2006/07, SARDI received an almond sample from SA's Riverland with symptoms they believed to be similar to the many they had observed from the LTD trial work and the NAP region. We did not view these samples but can report no pathogens were isolated from the lesions. The associated report and background notes indicated that all cultivars in the orchard were affected, and that copper, Rovral®, chlorothalonil and Tilt® had been applied at appropriate times during the season. It is unclear if this is the first detection of LTD from a region outside the NAP. It would be unusual, from our NAP experience, to find LTD affecting all cultivars equally.

Environmental Monitoring

Environmental monitoring in the trial site was not warranted during the 2006/07 season, given the record dry conditions in the area. Although early in the season dews frequently formed, none lingered long and the leaves dried out quickly. The humidity within the orchard in 2006/07 was generally low, given the quick drying time of irrigation applied through mini-sprinklers. There was so little ground moisture that even after irrigation, the humidity within the trial site fell quickly and trees were seen to wilt. The most difficult aspect of the orchard's management this season was the maintenance of sufficient moisture for the trees, given the weather and water restrictions.

Summary - 2006/07

LTD did not develop this season at the trial site, nor generally in the Angle Vale region. Crop protectant treatments have therefore not been fully assessed and the cause of LTD has not been confirmed. Only one orchard was observed to have developed LTD in 2006/07. The extended and severe dry weather from winter through February resulted in few chemical crop protectants being applied in any orchard, negligible development of common almond diseases and lower orchard humidity generally. Water restrictions affected watering in the trial orchard and indirectly, the humidity.

The pathogenicity work confirmed that neither of the fungi consistently isolated from LTD lesions are primary almond leaf pathogens or likely causes of LTD.

Light and SEM microscopy work revealed that the induced chemical LTD-like lesions have distinct, erumpent and vertically-severed margins similar to those observed under SEM, of greasy spots and chlorotic field LTD lesions. The field LTD lesions (from one orchard this season), were not distinguishable under SEM, from the chemically-induced lesions.

Detailed molecular bacterial work identified the bacterium isolated from LTD lesions (one orchard) in 2006/07 as *Chryseomonas luteola*. The consistency of its presence and its pathogenicity on almonds, are unknown.

There is reason to believe from the available evidence that the LTD cause could be directly or indirectly associated with an applied chemical, and environmental conditions during and after application. This is supported by the absence of LTD on feral trees but weakened by the knowledge that few, if any almond growers on the NAP apply off-label canola oil or captan/copper combinations. However there is evidence that many almond growers have applied oil and/or copper within 10 days of other fungicides, and that they do tank-mix foliar nutrients and a number of crop protectant products. It is recognised that some chemicals and foliar nutrients when mixed, cause leaf damage. In particular micro-climatic conditions, the mixes and the leaf wetness period, could increase both the solubility and release periods of the chemicals, and the time of exposure at the leaf surface, to these solutions. The choice of product (crop protectant and/or nutrient), time of spraying and orchard humidity may therefore directly influence the development of LTD.

RISK ANALYSIS - LTD

The incomplete knowledge of LTD and its cause/s precludes identification and detailed analysis of the critical control points associated with the disorder. Potential risk factors however have been listed below (Table 8) and discussed. It is relevant to consider some of these in management plans associated with various aspects of almond production, e.g. planting material, irrigation, harvest etc. and in risk reduction strategies. At this time, however there are no risk factors that we have confirmed as directly impacting on the introduction, establishment, spread or severity of LTD. What we have demonstrated and confirmed is non-pareil as the most susceptible variety and different crop protectant products that have either induced or delayed symptom onset. There is some evidence to suggest that dry conditions, either directly or indirectly, reduce LTD.

Table 8: Potential risk factors

Risk Category	Specific Risks	Comments
Financial:		
Revenue	*Variety choice*	Non-pareil most susceptible.
	Variety supply	Unlikely to be a nursery issue. No evidence of graft transmissibility. Supply, cost, distribution of ‘clean’ planting material should not be affected.
	Tree performance **NAP** 0-3 years	Young trees not affected.
	4-20 years	Leaf loss, dieback etc. reduce economic productivity.
	Tree performance **Riverland; Sunraysia** 4-20 years	Limited, unconfirmed evidence of LTD occurrence in these areas. Continued monitoring important.
	Tree longevity	Leaf loss, dieback effect on longevity.

Risk Category	Specific Risks	Comments
	Trade/distribution restrictions	No evidence to support curbed tree or product distribution. No evidence of LTD 'introduction' or 'spread' via nuts, hulls, shells, leaf debris.
	Marketing	No nut symptoms so no reasonable effect on product distribution or quality. Compliance – assumed to be complete. If chemical role in LTD demonstrated, appropriate disclaimers and warnings required for crop protection products. If new management product identified (ie. BAS 51604F) financial/research commitment to its registration needed.
Expenditure	*Input costs* **Crop protectants**	Increase if new product/s needed for LTD management. Potential for LTD cause to be directly related to choice of product - choice of crop protectants may change/be limited. Potential also for effective products to control multiple diseases/disorders, reducing chemical inputs.
	Water source/irrigation	Water source has no impact on LTD onset. Water frequency and volume may affect canopy humidity and potentially, but indirectly, LTD.
	Nutrients	Fungicide and nutrient combinations untested. Specific rates, products, foliar vs. fertigation, remain untested. Interactions may affect LTD. Potential cost impact unknown.
	Imposed changes to orchard practices	The specific nature of tank mixes and applied products might dictate suitable/safe application times. Potential need for additional or specific management/monitoring of non-pareil; weather conditions. Multiple harvest passes may result if sticktight numbers high. Pruning out dieback. No anticipated benefit of changes in hygiene, visitor/machinery entry or management.
	Monitoring	If demonstrated that environmental conditions influence LTD, on-site monitoring potentially beneficial. Industry input/cost: Review of LTD reports, spray diaries, weather conditions needed for several more years.

DISCUSSION

Two seasons of field trials were preceded by a series of orchard inspections, observations and grower surveys. Initial observations confirmed LTD was not 'bacterial spot' and suggested that LTD was in fact likely to be copper phytotoxicity. Growers reported extensive tank-mixing of crop protectant products and nutrients, widespread use of canola oil, late copper applications, and increased use of generic rather than brand name fungicides. There were few growers applying protective applications for mites and/or insects, and many growers had moved from zinc to urea, for defoliation.

In the LTD-affected NAP region there are three water sources routinely used but none have been consistently associated with LTD. By the end of the 2004/05 season, it was confirmed that feral trees were not affected, non-pareil was the most susceptible commercial variety and that an abiotic cause (i.e. chemical applications) of LTD was likely.

The first year of field trials included fungicide applications that allowed comparison between usual grower crop protection programs and proven almond disease control programs with brand-name fungicides. Judicious and early use of copper, and an experimental fungicide were included as treatments. The experimental (in Australia) fungicide registered in the United States for control of most fungal almond diseases, was BAS 51604F. During the 2005/06 season, it was demonstrated that two BAS 51604F applications effectively 'controlled' LTD and the other fungal diseases of almonds observed on the NAP. Other treatment programs revealed their lack of efficacy against LTD, and the existence of dicarboximide resistance (within *Botrytis* sp.) in the region. The trials also demonstrated that Keane's Seedling and Price have a useful degree of tolerance to LTD.

At the conclusion of the 2005/06 trials it was known that the onset of LTD could be significantly delayed by application of a fungicide; that feral trees remained LTD-free and that effective almond disease management in the region could be achieved with fewer, well-timed fungicide applications than usually applied by the region's growers. It was also demonstrated that LTD-like symptoms could be induced with above-label rates of canola oil. Although LTD in 2005/06 continued to closely resemble copper phytotoxicity, the trials were designed such that specific copper and canola oil applications (i.e. individually) could be eliminated as potential primary causes of LTD. However the non-specific application of copper for example, in trace element mixes with other products, cannot be ruled out as an LTD contributory factor.

The effectiveness of BAS 51604F in 2005/06 suggested that LTD may have a biological, rather than abiotic cause. BAS 51604F has two active constituents, each with a different chemistry and mode of action. Despite its demonstrated effectiveness across varieties in the severely LTD-affected orchard, it could not be determined if BAS 51604F provided direct (i.e. controlled a fungus) or indirect (i.e. induced host response) protection against LTD. It also remained possible that the lack of tank-mixed applications of other fungicides and nutrients to the BAS 51604F treated block, in fact resulted in LTD 'avoidance' rather than 'control'.

Despite fungi from the genera *Alternaria* and *Cladosporium* being consistently isolated from necrotic LTD lesions, and BAS 51604F providing effective LTD management in 2005/06, it was reasoned at the end of the 2005/06 season that LTD most likely resulted from interacting biological and abiotic factors. This was not sufficiently tested in the field but laboratory and greenhouse research showed that the *Alternaria* sp. and *Cladosporium* sp., although consistently associated with LTD necrotic lesions, were not primary pathogens of almonds, and therefore are unlikely causes of LTD.

In 2006/07, LTD did not develop at the trial site. We presumed the very dry season and the consequent reduction in humidity and necessary crop protectant applications, influenced LTD

appearance. Such conditions also affected the establishment of known bacterial and fungal diseases of almonds on the NAP, and therefore the failure of LTD to develop might again relate to both biological and abiotic factors. An applied chemical combination (copper with captan) however induced LTD-like lesions on every tree receiving the treatment. SEM work confirmed the similarity of field LTD, oil+ and induced chemical lesions, at the microscopic level. Our observations of sudden lesion appearance and uniform distribution both on leaf surfaces and across a variety, were consistent for both chemically-induced lesions and field LTD, and support the hypothesis of abiotic contributory factors or LTD cause.

The micro-climatic influences on the duration of leaf wetness after spraying, the resultant solution concentration and release periods for some active constituents and their pH at the leaf surface, require further consideration. Early evening and night spraying has been undertaken on the NAP in orchards with a history of LTD. Tank-mixing of products and nutrients occurs with some regularity.

At the conclusion of the 2006/07 season, it appeared that the cause of LTD was more likely to be abiotic, than biological, in nature. It however remains ill-defined and the planned work on BAS 51604F and its components, and nutrient and fungicide interactions, needs to be repeated and tested during 2007/08. A greater understanding of the normal epidemiology and host range of *Chryseomonas luteola* is needed before pathogenicity work with this organism is warranted. Graft transmissibility trials are continuing.

It is essential that the cause of the LTD disorder be found. Without this, it is possible progress towards registration of BAS 51604F in this country could be stalled. It is strongly recommended that the industry commence work on registration of BAS 51604F, regardless of the LTD situation, since its efficacy against a number of important almond diseases is widely acknowledged and its addition to the stable of registered almond products would be useful in terms of resistance management and overall chemical application reduction.

CONCLUSIONS

- BAS 51604F delayed the development of symptoms on susceptible almonds in a severely-affected orchard, in 2005/06.
- The basis of the effectiveness of fungicide BAS 51604F is unknown; i.e. direct (fungal control) or indirect (avoidance, induced host response).
- Onset of LTD is sudden and uniform within a variety.
- Three are no edge effects or apparent point sources of LTD within orchards.
- Feral trees have not developed LTD.
- Refined canola oil applied at above-label rates, and captan/copper in combination, induce lesions visually identical (to the naked eye and at the microscopic level) to field LTD lesions.
- Copper, canola oil and captan applied independently have not been primary causes of LTD on the NAP, when appropriately applied.
- Environmental conditions within orchards likely affect the development of LTD.
- Potential abiotic causes (applied chemicals +/- nutrients) have not been sufficiently tested under a range of spray and micro-climatic conditions (humidity, presence of dew).

- Non-pareils are the most susceptible variety. Keane's Seedling and Price demonstrate a useful degree of tolerance to LTD, even when planted adjacent to severely-affected non-pareils.
- The *Alternaria* and *Cladosporium* spp. consistently isolated from necrotic LTD lesions are not primary pathogens of almond.
- *Chryseomonas luteola* is of unknown significance to almonds and/or LTD.
- The cause of LTD is complex, and likely to include applied chemicals +/- nutrients and particular micro-climatic conditions.

NEXT STEPS

The trial established in 2006/07 will be re-established in 2007/08. An additional *water only* treatment will be included and leaf wetness periods relating to timing of sprays and micro-climatic conditions will be examined. Field inspections as planned will continue with some additional resources utilised to measure the area of translucent, yellow lesions prior to onset of necrosis. Determination of lesion size changes (or constancy) will provide further information on the likelihood of the lesions being biological or abiotic, in nature.

More work on foliar nutrient mixes (with and without copper, for example), tank mixed (or otherwise) with crop protectants, is needed. It is anticipated that these would be best tested by utilising intermittent spraying (on/off rig) within the same row. This will minimise the potential impact of humidity and leaf wetness variables, in these particular treatments.

The researchers are keen to assist with any industry pursuit of registration for promising crop protectant products. Incorporation of residue testing protocols into our trials, is possible.

TECHNOLOGY TRANSFER

This process has been on-going with articles, presentations and field days provided throughout the project.

RECOMMENDATIONS (SCIENCE AND INDUSTRY)

It is strongly recommended that the research identified above in Next Steps is undertaken and that industry commence work on registration of BAS 51604F, regardless of the LTD situation.

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HORTICULTURAL SERVICES PTY LTD

PRUE McMICHAEL
Plant Pathologist\Senior Consultant

May 2007

APPENDICES

Appendix 1 Photographs

Appendix 2 NAP grower survey - spray records 2004/05

Appendix 3 SARDI – pathogenicity trial report; ONFIT methodology

Appendix 4 Adelaide Microscopy reports

Appendix 5 Australian Collection of Microorganisms – Report on bacterial identification

REFERENCES

1. Prue McMichael and Lucy Pumpa. March 2006. Angle Vale Leaf Tatter and Defoliation. Australian Nutgrower 20 (1) pp16-19.

2. McMichael, PA and L Pumpa. 2005. Milestone Report 3 (AL05003c) Angle Vale Leaf Tatter and Defoliation (HAL, March 2006).

3. McMichael, PA and L Pumpa. 2005. Milestone Report 2 (AL05003c) Angle Vale Leaf Tatter and Defoliation (HAL, December 2005).

4. Stuart Pettigrew and Prue McMichael. June 2004. Bacterial Spot on Almonds. Australian Nutgrower 18 (2) pp 24-25.

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Appendix 1

Photographs

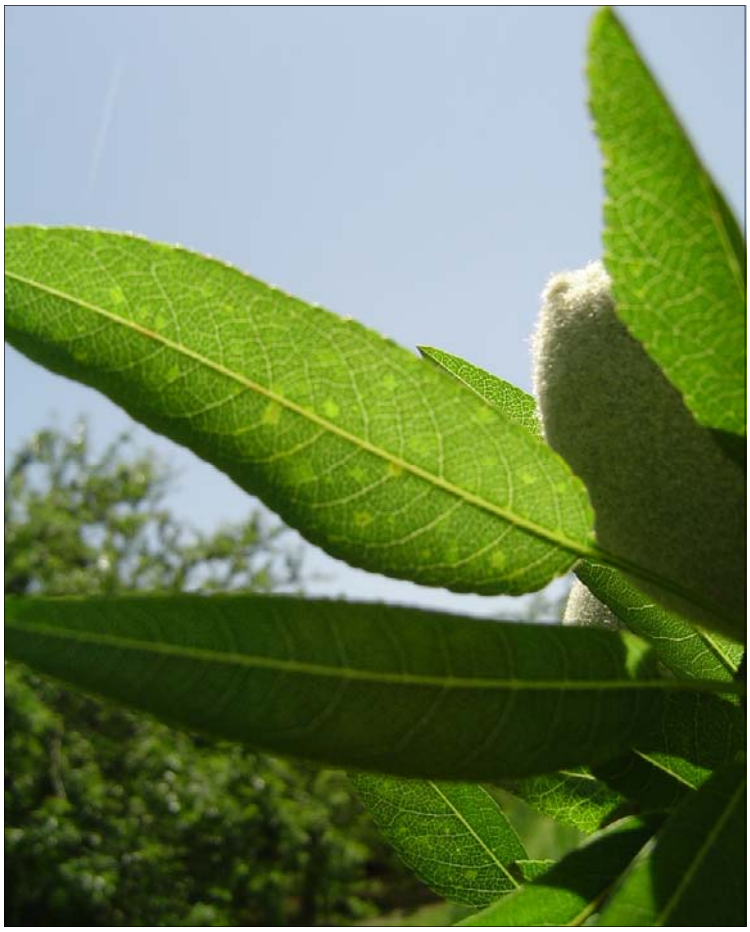

Photo 1: Translucent, yellow spots across leaf blade

Photo 2: Greasy spots

Photo 3: Chlorotic and necrotic lesions, some resulting in shotholing

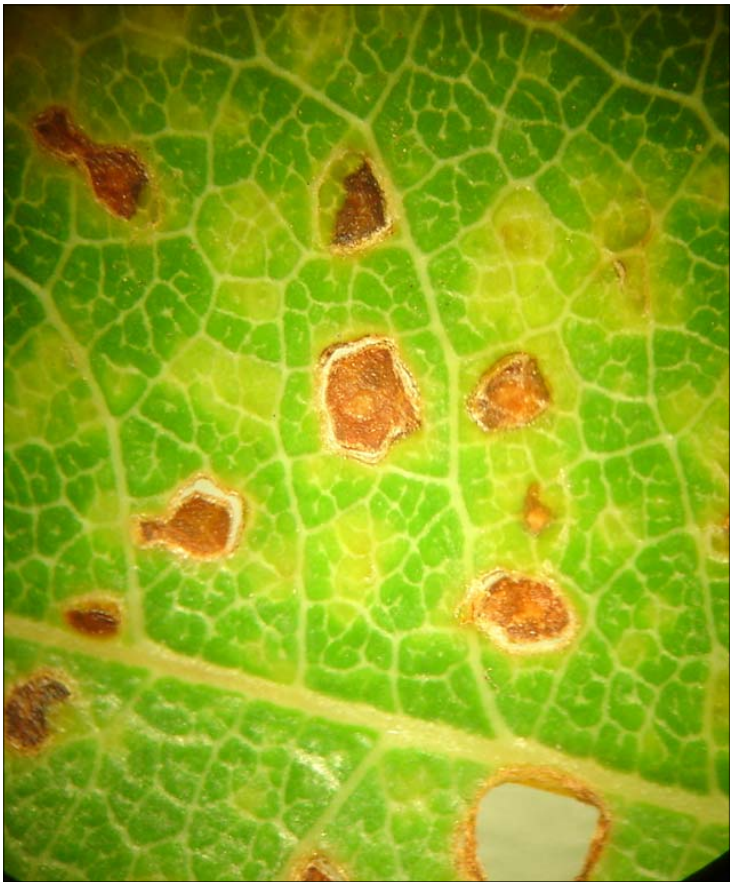

Photo 4: Chlorotic and necrotic lesions, some resulting in shotholing.

Photo 5: Chlorotic and necrotic lesions, some resulting in shotholing.

Photo 6: Bare wood and branches in canopy.

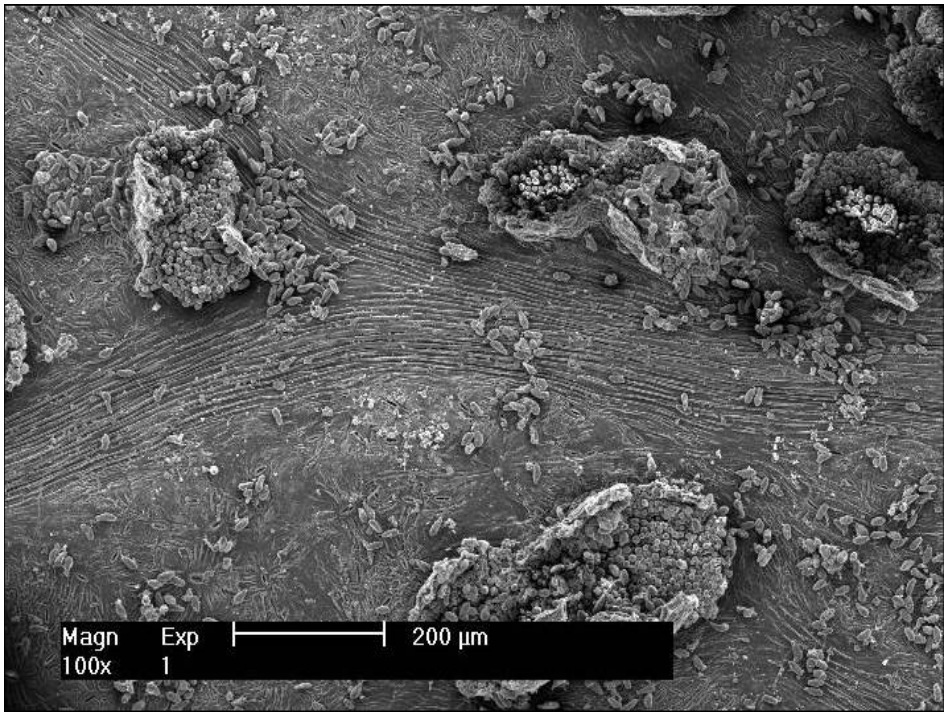

Photo 7: Feral trees with rust pustules.

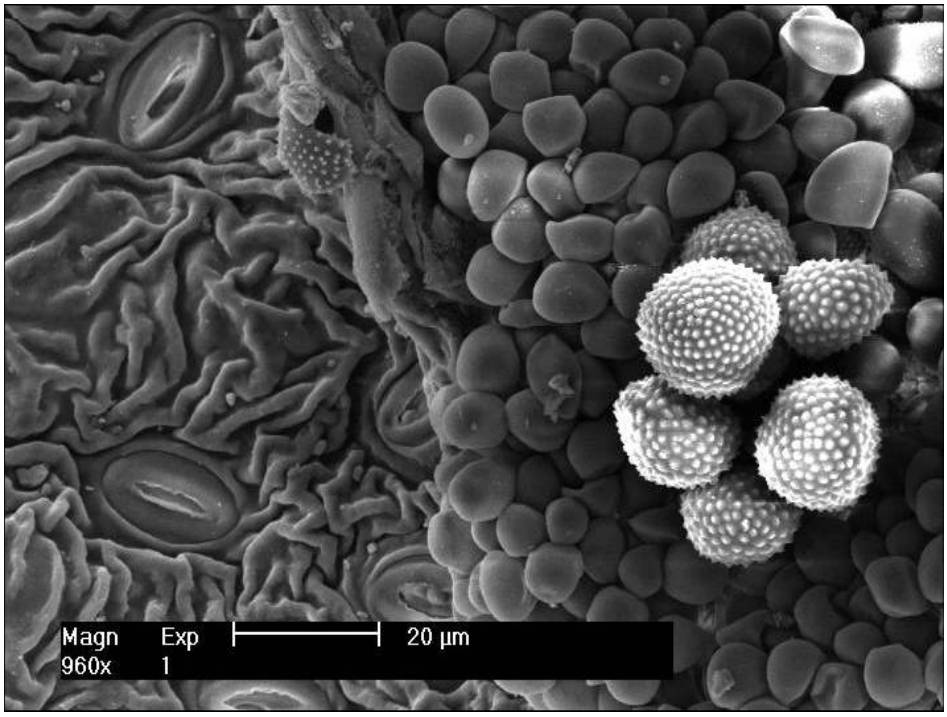

Photo 8: Feral tree leaf surface showing rust spores.

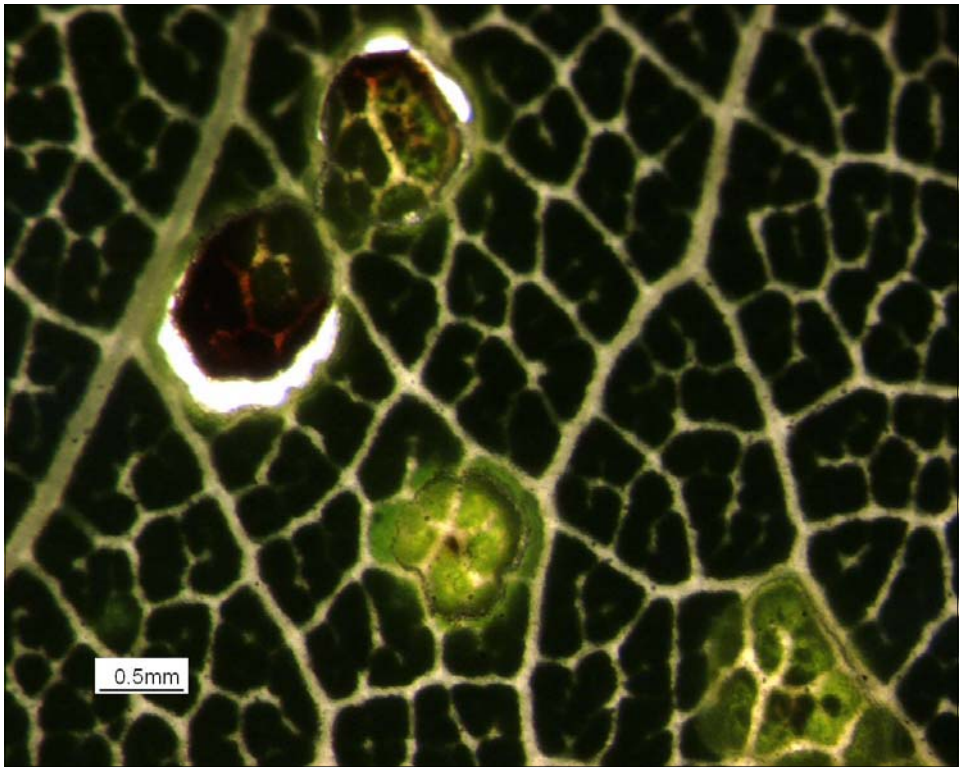

Photo 9: LTD lesions—light microscopy, no vein delimitation.

Photo 10: Trees in glasshouse treated with chemicals applied in the field.

Photo 11: Chemical lesions - copper and captan treatment

Photo 12: LTD lesions on leaf.

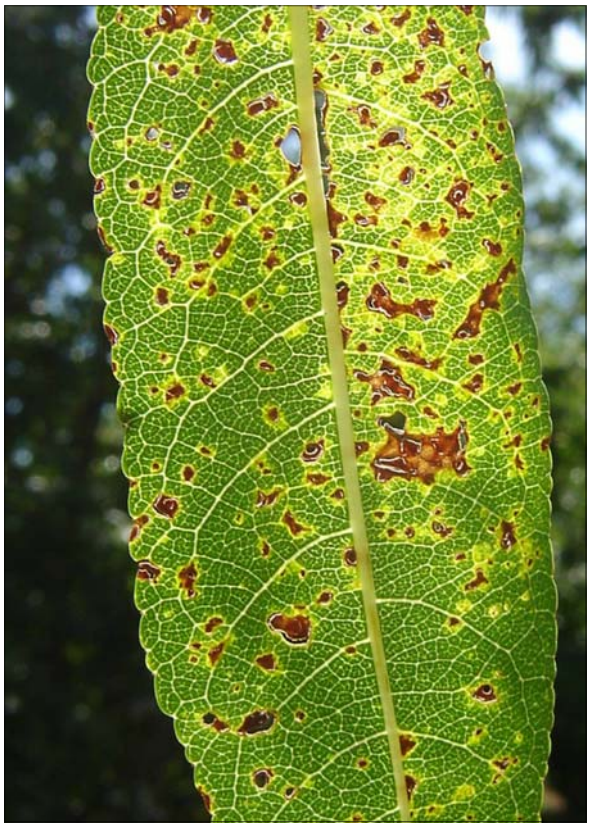

Photo 13: LTD lesions on leaf.

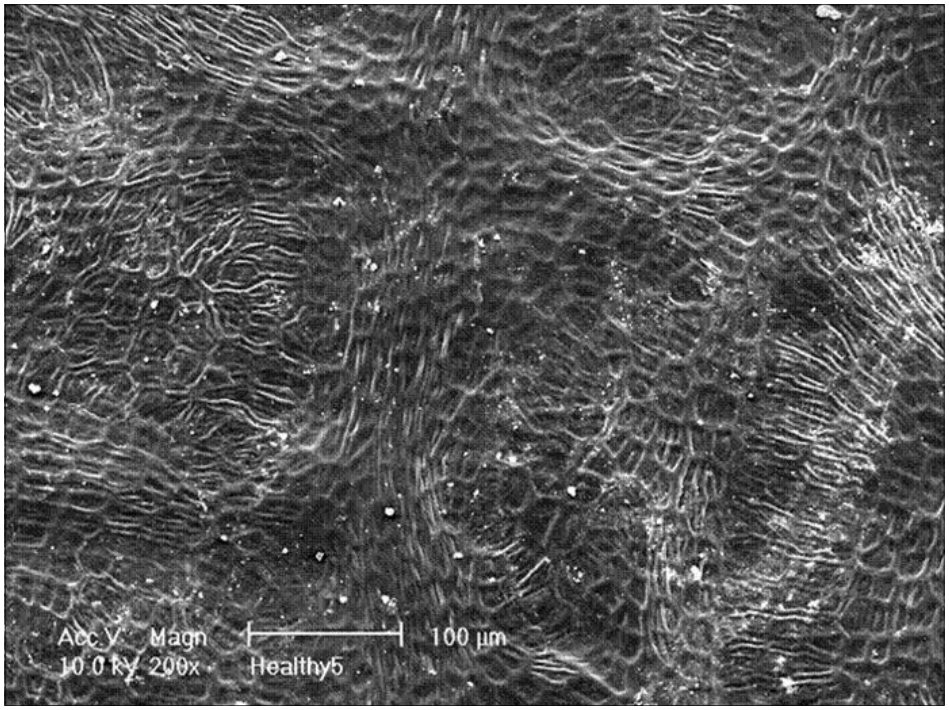

Photo 14: Healthy leaves, upper surface.

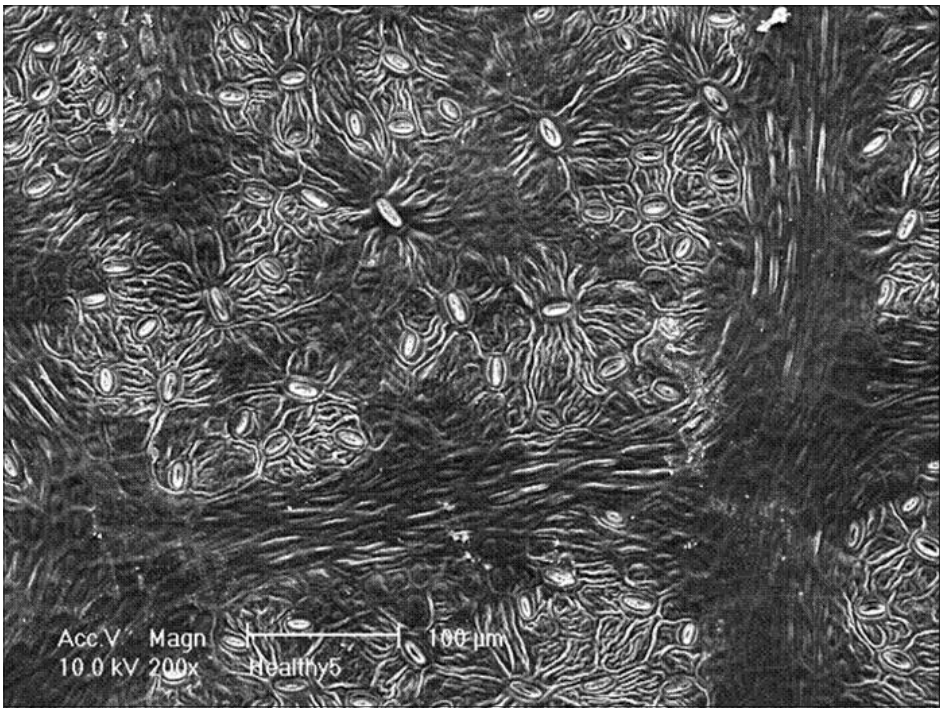

Photo 15: Healthy leaves, lower surface.

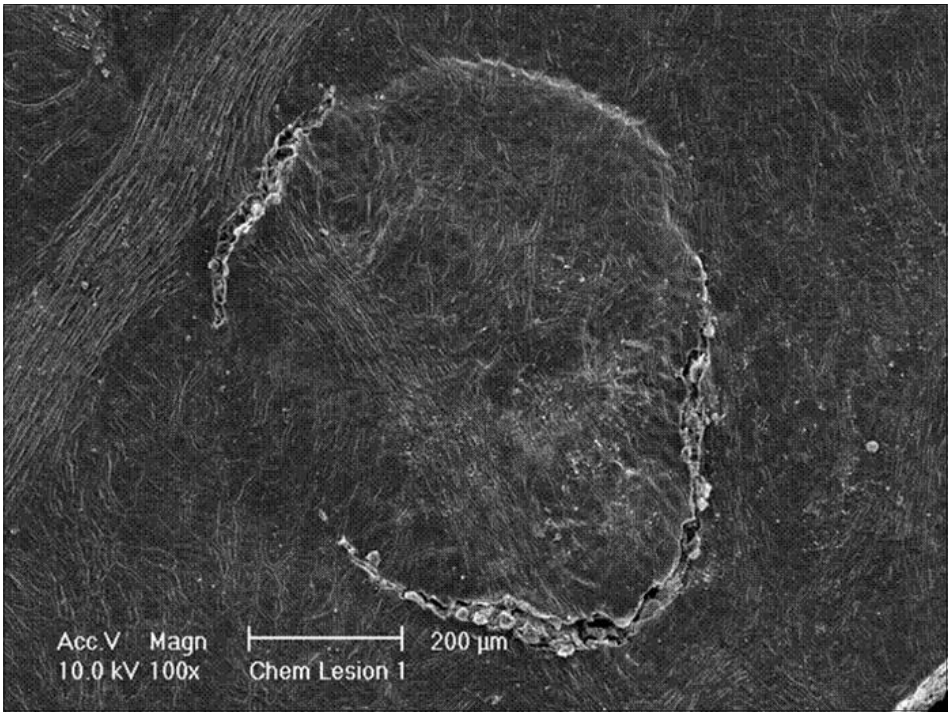

Photo 16: Chemical lesions, upper surface.

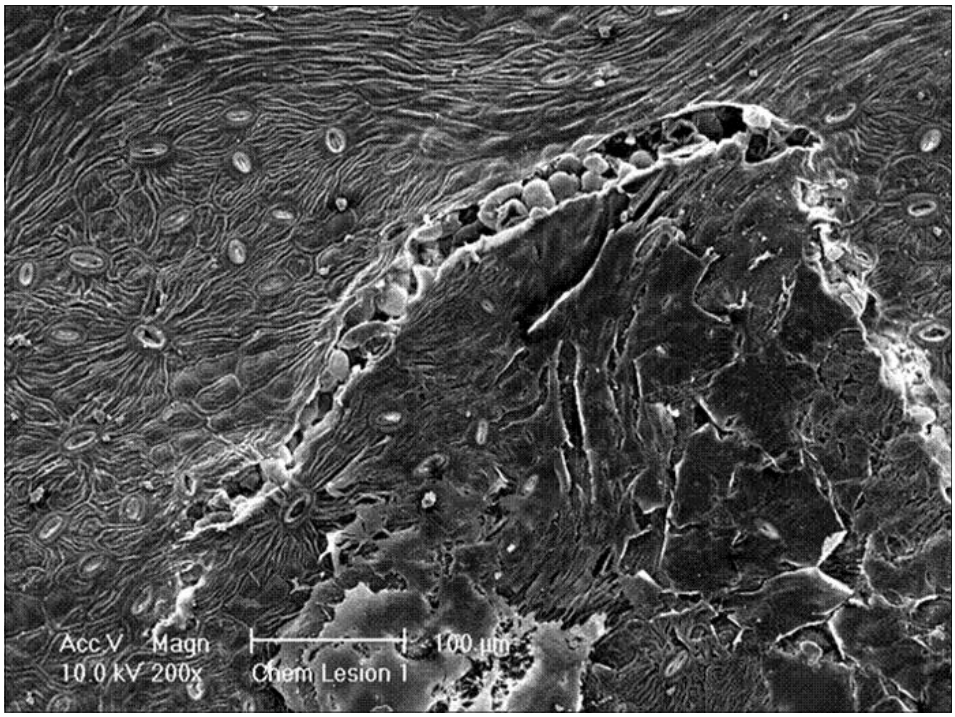

Photo 17: Chemical lesions, lower surface.

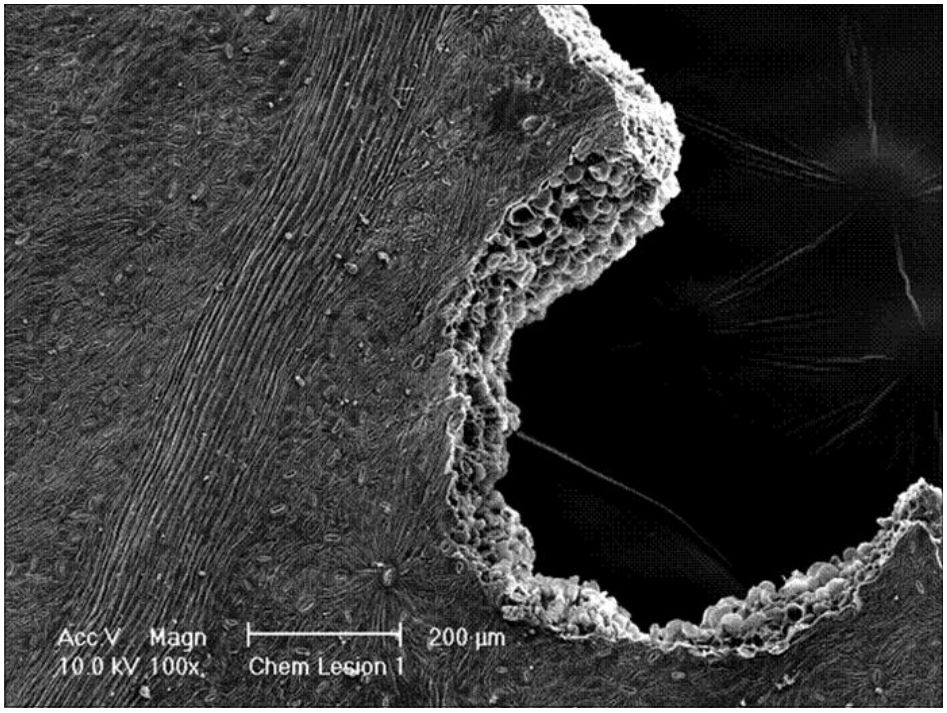

Photo 18: Damage, vertical at margin of fallen chemical lesion.

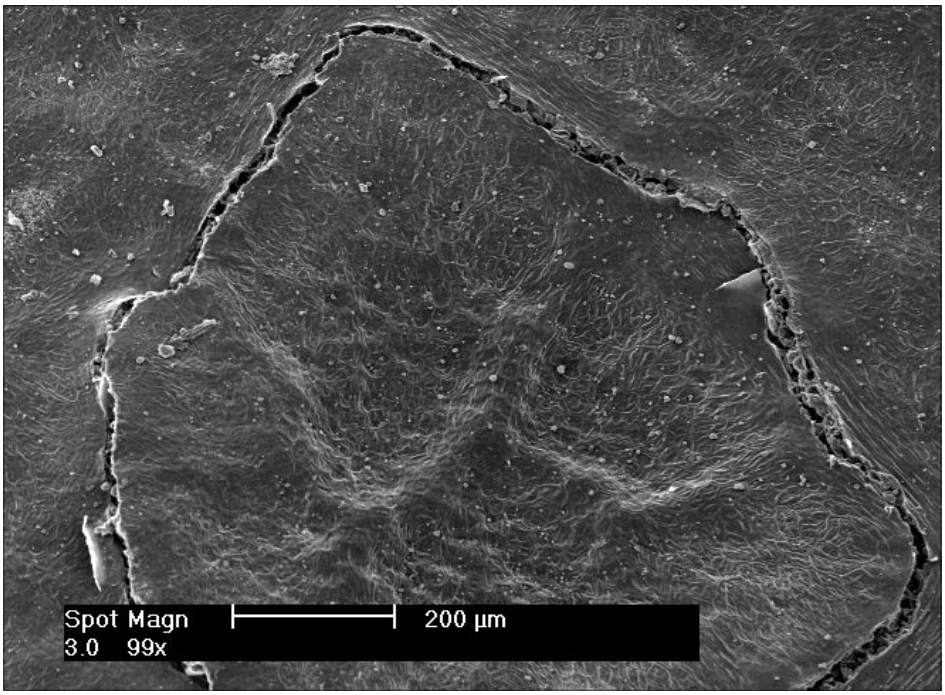

Photo 19: SEM of greasy spots of field LTD lesion 2006/07.

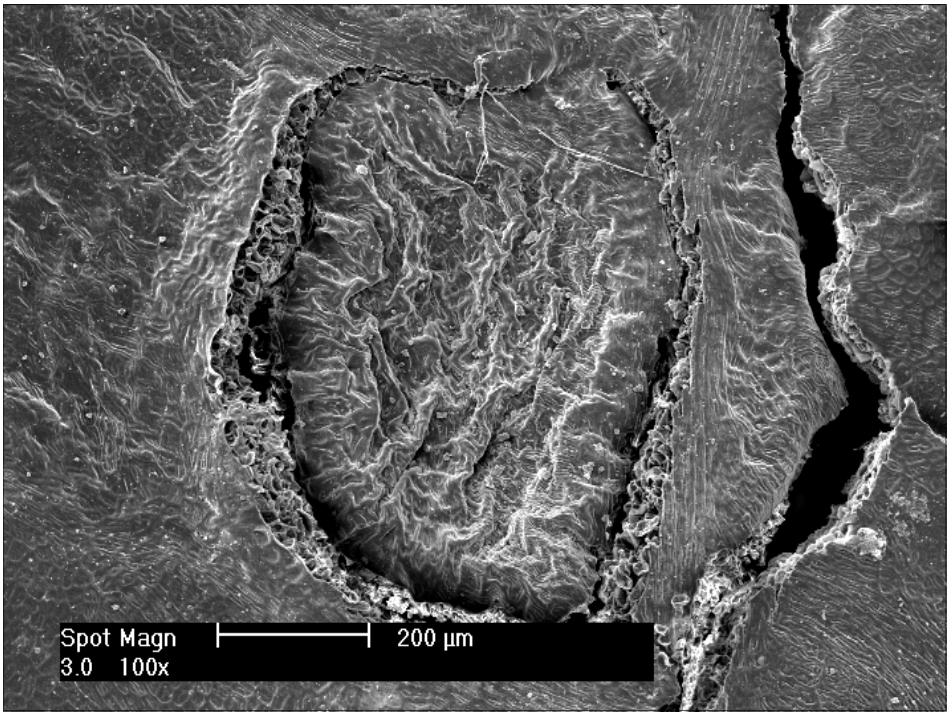

Photo 20: SEM of yellow field LTD lesions 2006/07.

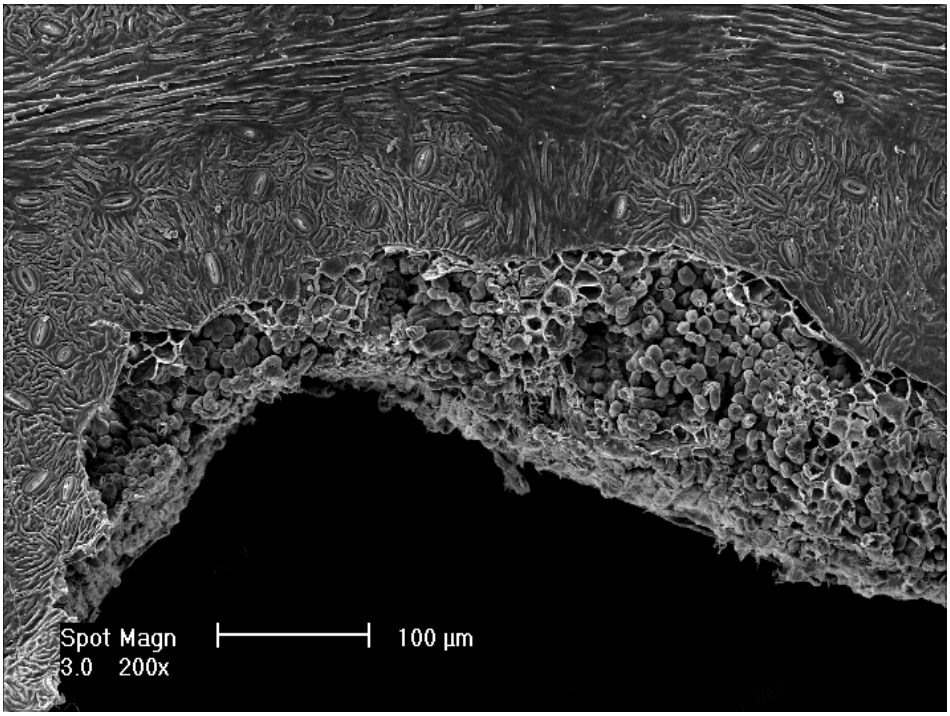

Photo 21: SEM of yellow field LTD lesions 2006/07.

Appendix 2

Northern Adelaide Plain Grower Survey 04/05

Survey of NAP almond growers –Pest control and nutrient application records 2004/05

Grower	Application Date Range	Total No	Tank Mixes	LTD Symptom Severity/extent – Grower Rating
PROPICONAZOLE				
1	0	0		Medium
2	11/8 - 10/12	5	✓ Tilt*, copper, iron chelate, boron, calcium	Med-severe
3	1/9 - 15/1	4	✓ Aurora, copper	Med-severe
4	7/8 -17/1	4	✓ Tilt, boron, NZn	Med-severe
5	14/9, 11/10	2	✓ copper	?
6	5/8 -11/12	6	✓ Tilt (shuck fall), copper, boron, K nitrate	Minor- Medium – Non-pareil, older trees most severe
7	2/9, 14/1	2	✓ Tilt	?
8	11/8 - 30/9	3	✓ Tilt	Minor – most severe on alternate rows that were also sprayed with copper
9	17/8 - 13/1	7	✓ Tilt, copper, calcium, zinc, K nitrate	Minor
CHLOROTHALONIL				
1	15/12, 15/1	2	✓ Dithane, Barrack, K nitrate	Medium
2	27/8, 3/9	2	✓ Echo, canola oil, K nitrate	Med-severe
3	-	0		Med-severe
4	2/9, 16/9	2	✓ Echo, K nitrate	Med-severe
5	13/12	1	✓ Elect	?
6	25/8 - 13/12	3	✓ Echo, canola oil	Minor – Medium
7	1/11, 14/1	2	✓ Echo, copper	?
8	11/8 - 1/2	4	✓ Echo, Agral	Minor
9	28/8, 5/9	2	✓ Echo, canola oil	Minor
1	Dormant -29/7	3	✓ Agral	Medium
2	21/7 - 10/12	4	✓ Echo, canola oil, zinc, urea	Med-severe
3	10/8 - 26/10	3	✓ Echo, canola oil	Med-severe
4	15/7 - 2/10	3	✓ Rogor, canola oil	Med-severe
5	14/4 - 11/10	4	✓ Echo, canola oil	?
6	14/4 - 11/12	4	✓ Echo, boron	Minor-med
7	5/7 – 1/11	3	✓ Tilt, Thiovit, Agral, K nitrate	?
8	7/7 - 19/10	3	✓ zinc sulphate	Minor
9	21/4 - 12/12	6	✓ Echo, canola oil	Minor
FERTILIZERS (FOLIAR, FERTIGATION)				
1	?	3	Potassium nitrate	Medium
2	10/9 – 9/11	3	Zinc, Urea	Med-severe
	27/8 – 7/1	6	Potassium nitrate	
	14/9, 28/10	2	Boron, calcium	
3	13/9 – 9/11	9	NZn, Potassium nitrate. K chloride (trial)	Med-severe
4	16/9 -17/1	5	NZn, Potassium nitrate	Med-severe
	25/10	1	Boron, manzate	
5	April	1	Urea (defoliation)	?
6	8/9, 13/10	2	Zinc chelate, calcium nitrate	Minor-med
	16/9 - 8/11	3	Potassium nitrate	
	16/9, 8/10	2	Boron	
7, 8				
9	8/9, 18/9	2	Zinc chelate, calcium nitrate	Minor
	15/10	1	Potassium nitrate	

- Products listed by name are trademarked or registered.

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Appendix 3

SARDI Reports and ONFIT Protocol

ALMOND PATHOGENICITY TESTING.

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December 2006

Almond trees from the Angle Vale area of South Australia were suffering from a condition known as Almond tatters, where transparent spots appear on the leaves and develop into necrotic lesions. Several tests undertaken by the Horticulture Pathology unit of SARDI recovered *Cladosporium* sp. and *Alternaria alternata.* Further tests were undertaken to determine if these fungi were pathogenic and caused leaf tatter symptoms.

MATERIALS AND METHODS.

Isolates.

1 isolate of *Cladosporium* and 10 of *Alternaria alternata* recovered from almond leaves in 2005/2006 were used (Table 1). Inoculum was prepared by growing the fungi on potato dextrose agar (PDA) at 22^0C for 14 days under a 12hr/12hr light/dark cycle. Inoculation was undertaken either by placing on the pant surface a 2mm by 2mm mycelial plug cut from the edge of the growing fungal colony, or by applying a spore suspension to the plant with a hand held atomiser. To make the spore suspension, the fungal growth on the agar surface was scraped off with a sterile scalpel and strained through muslin. The spore mass was mixed with sterile distilled water to make a suspension of $1x10^6$ spores per ml.

Test plants.

Nine 1-2 year old potted almond seedlings were purchased from Bunning's nursery (2 cv Nonpariel, 5 cv "all in one" self pollinating variety, 2 cv "Zaione" self pollinating variety). Another four 2 year old potted almond trees were obtained from the Adelaide University (lbT32). Trees were maintained in the greenhouse at 22-25^0C, watered daily and fertilised as necessary with osmocote.

Pathogenicity tests 2006.

Test 1. 9th March.

Leaves and stems of the almond plants were wounded by pricking the surface with a sterile needle then inoculated by placing a mycelial plug of either 298 A *Alternaria* or 306 A2 *Cladosporium* on the wound. All inoculation sites were placed in a moistened plastic bag for 6 days to provide near 100% humidity and encourage infection. Leaves and stems were examined for symptoms at 12 days after inoculation.

This test was aborted as there was significant leaf drop on some of the trees at 12 days and many of the inoculation sites were missing. All trees were pruned back, placed in the cold room at 4^0C for 4 weeks, then replaced into the greenhouse at 25^0C to encourage new growth.

Test 2. 12th July.

Leaves and stems of the almond plants were wounded by pricking the surface with a sterile needle then inoculated by placing a mycelial plug of each of the 11 fungal cultures on the wound, several cultures per tree. All inoculation sites were placed in a moistened plastic bag for

6 days to provide near 100% humidity and encourage infection. Leaves and stems were examined for symptoms at 13 days after inoculation.

Any suspect lesions were surface sterilised in 1% sodium hypochlorite for 2 mins and pieces of infected tissue placed on PDA. The cultures were examined after 7-10 days for the presence of fungi.

Test 3. 11th August.
Leaves and stems with and without wounding were reinoculated by spraying with suspensions of one of the two cultures that showed some reaction in the 2nd test (306 A1 *Alternaria*, 306 A2 *Cladosporium*). The inoculation sites were covered with the plastic bags as previously described. After the bags were removed 3 of the trees were placed in a misting chamber, which consisted of a "Defensor" atomiser humidifier (capacity 3L/hr) placed in a clear plastic tent located within the greenhouse. After 1 month the plants were removed from the humidifier. Leaves and stems of all plants were examined for symptoms approx 6 weeks after inoculation.

RESULTS.

Test 1. 9th March.
No symptoms were observed on the leaves or stems.

Test 2. 12th July.
Necrotic lesions, some with concentric rings, were observed on leaves inoculated with four of the *Alternaria* cultures (306 A1, 306 B1, 4-1a) and the *Cladosporium* 306 A2 (Fig 1 & 2, Table 2). However these were localised around the inoculation site and did not induce the typical leaf tatter symptoms.

Alternaria was only recovered from the lesions inoculated by *Alternaria* 306 A1 isolate.

Test 3. 11th August.
Localised necrotic lesions were observed at the inoculation site on some leaves (Fig 3), particularly on plants placed in the humidifier. However the lesions were not typical of those seen with leaf tatter.

Chlorotic lesions were visible on the new growth on these plants (Fig 4), however again they were not typical of those seen with leaf tatter.

CONCLUSION.

Both *Alternaria* and *Cladosporium* induced localised lesions when inoculated onto almond leaves, however these differed to the leaf tatter symptoms which developed in the field. It is likely that these fungi are secondary infections and not the casual agent.

Some chlorotic spotting was observed on leaves inoculated with the *Alternaria* and this seemed to be induced with the humidity. While these symptoms were not typical of those seen with the leaf tatter, they were unusual and may indicate more work is needed to determine the cause.

Table 1. Fungal cultures recovered from leaf tatter lesions and used in pathogenicity tests.

Culture number	Species	Source
306 A2	*Cladosporium* sp.	Baker – yellow, 15/11/2005
306 A1	*Alternaria alternata*	Baker – yellow, 15/11/2005
306 B4	*A. alternata*	Feral trees, 15/11/2005
298 A	*A. alternata*	14/11/2005
298 A1	*A. alternata*	14/11/2005
298 B	*A. alternata*	14/11/2005
28/06 A	*A. alternata*	Baker – yellow 30/1/2006 old lesions
28/06 B	*A. alternata*	Baker – yellow 30/1/2006 old lesions
28/06 C	*A. alternata*	Baker – yellow 30/1/2006 old lesions
4-1a	*A. alternata*	Pezzaniti 28/12/2005 lesions
4-2A	*A. alternata*	Pezaniti 28/12/2005 tip die back

Table 2. Symptoms on almond leaves 13 days after inoculation with *A. alternata* or *Cladosporium* sp.

Tree	Variety	Culture used for inoculation	Symptoms observed at 13 days
1	Nonpariel	*Cladosporium* 306 A2	No symptoms
		Alternaria 28/06 A	
		Alternaria 298 B	
		Alternaria 4-1 a	
		Alternaria 306 A1	
2	Nonpariel	*Alternaria* 306 A1	No symptoms
		Alternaria 298 B	
3	All in one self pollinating	*Cladosporium* 306 A2	No symptoms
		Alternaria 306 B1	Concentric rings at inoculation site (Fig 2)
		Alternaria 4-1 a	
		Alternaria 306 B4	No symptoms
		Alternaria 28/06 B	
4	All in one self pollinating	*Cladosporium* 306 A2	No symptoms
		Alternaria 306 A1	Concentric rings at inoculation site
		Alternaria 306 A1	
		Alternaria 306 B1	
		Alternaria 306 B1	
		Alternaria 4-1 a	No symptoms
5	All in one self pollinating	*Alternaria* 28/06 B	No symptoms
		Alternaria 4-1 a	
		Alternaria 306 A1	
		Alternaria 298 A1	Concentric rings at inoculation site
6	All in one self pollinating	*Alternaria* 306 A1	No symptoms
7	All in one self pollinating	*Cladosporium* 306 A2	No symptoms
		Alternaria 28/06 A	
		Alternaria 306 B4	
		Alternaria 306 A1	
		Alternaria 298 B	
		Alternaria 298 B	Local lesion (Fig 1)
		Alternaria 4-1 a	Concentric rings at inoculation site
		Alternaria 298 A1	
		Alternaria 306 A1	
		Alternaria 306 B4	

8	Zaione self pollinating	*Alternaria* 28/06 A	No symptoms
		Alternaria 298 B	
9	Zaione self pollinating	*Cladosporium* 306 A2	No symptoms
		Alternaria 306 A1	
		Alternaria 306 B1	
		Alternaria 28/06 B	
10	1bT32	*Cladosporium* 306 A2	Dry patches, chlorotic spots at inoculation site
		Alternaria 306 A1	No symptoms
11	1bT32	*Cladosporium* 306 A2	Chlorotic spots at inoculation site
		Alternaria 306A1	No symptoms
12	1bT32	*Alternaria* 306 B1	No symptoms
		Alternaria 306 A1	
13	1bT32	*Alternaria* 4-1 a	No symptoms
		Alternaria 298 A1	

Table 3. Leaf symptoms developing on almond trees inoculated with *Cladosporium* sp. or *Alternaria alternata*.

Plant location	Inoculation	Symptoms after 6 weeks
Greenhouse	*Alternaria* 306 A1 Not wounded	No symptoms
	Cladosporium 306 A2 Not wounded	No symptoms
Greenhouse	*Alternaria* 306 A1 Wounded	No symptoms
Greenhouse	*Cladosporium* 306 A2 Not wounded	No symptoms
Greenhouse	*Alternaria* 306 A1 Wounded	No symptoms
Greenhouse	*Cladosporium* 306 A2 Wounded	No symptoms
Greenhouse	*Alternaria* 306 A1 Wounded	Necrotic lesions at wound sites (Fig 3)
Humidity tent	*Alternaria* 306 A1 Not wounded	No symptoms
	Cladosporium 306 A2 Wounded	Lesion at wound site, lesions forming on new leaves (Fig 4)
Humidity tent	*Alternaria* 306 A1 Not wounded	Lesion at wound site, lesions forming on new leaves
	Cladosporium 306 A2 Wounded	No symptoms
Humidity tent	*Cladosporium* 306 A2 Wounded	No symptoms
	Alternaria 306 A1 Not wounded	No symptoms

Fig 1. Localised lesions at wound sites inoculated with *Alternaria alternata*. (Test 2)

Fig 2. Concentric ring lesions at wound site inoculated with *Alternaria alternata*. (Test 2)

Fig 3. Localised lesions at wound site inoculated with *Alternaria alternata*. (Test 3)

Fig 4. Chlorotic lesions developing on new leaves developing above the leaves inoculated with *Alternaria alternata*. (Test 3)

ONFIT Protocol provided by
Dr. Themis Michailides (University of California, Davis)

PROTOCOL No. 1

Protocol for freezing stone fruit (plums and prune) to reveal latent infections by brown rot and gray mold

(prepared by Themis J. Michailides**)**

1. Mix 160 ml chlorine household bleach with 160 ml ETOH 95%, and 0.5 ml surfactant Tween-20 in 10 liters water (actually, 9.680 liters).
2. Surface sterilize fruit for 4 minutes (by stirring fruit gently in the above solution)
3. Using gloves, place fruit in sterile containers in rows and in an orderly fashion and add about 200 ml water.
4. Freeze fruit from 5:00 p.m. to 8:00 am the next day at -16°C*.
5. Remove containers with fruit from freezer and let fruit thaw on a laboratory bench (about 20-25°C); do not open covers until you are ready to record the brown rot disease developed on the green fruit.

Use 50 fruit per container so that calculation of the disease incidence (% infected fruit) is easy and meaningful.

* For grapes freeze for 1.0 hour at -10°C.

Appendix 4

Adelaide Microscopy Reports

Dec 2006

SEM and LM observations of almond leaves

(2)

Samples

1. Yellow spots
2. Greasy Spots
3 'Something a bit odd'

Method

3-5 mm samples of leaf from the 3 groups received were cut and placed into fixative solution and left in the fridge for 24hrs. They were then taken through a graded series of ethanol to 100% ethanol over a 2 day period.
Samples were then critical point dried, coated with gold/palladium and viewed in the Philips XL20 scanning electron microscope (SEM).
SEM images were taken of up to 3 areas in each of the leaf samples with images collected of both lower and upper surfaces.
Images were taken at approx. 100x and 200x magnification.

Images were coded as follows:

S1	Sample1
S2	Sample2
S3	Sample3
U	Upper leaf surface
L	Lower leaf surface
A,B,C	Sample region of leaf

Comments.

Sample 1.

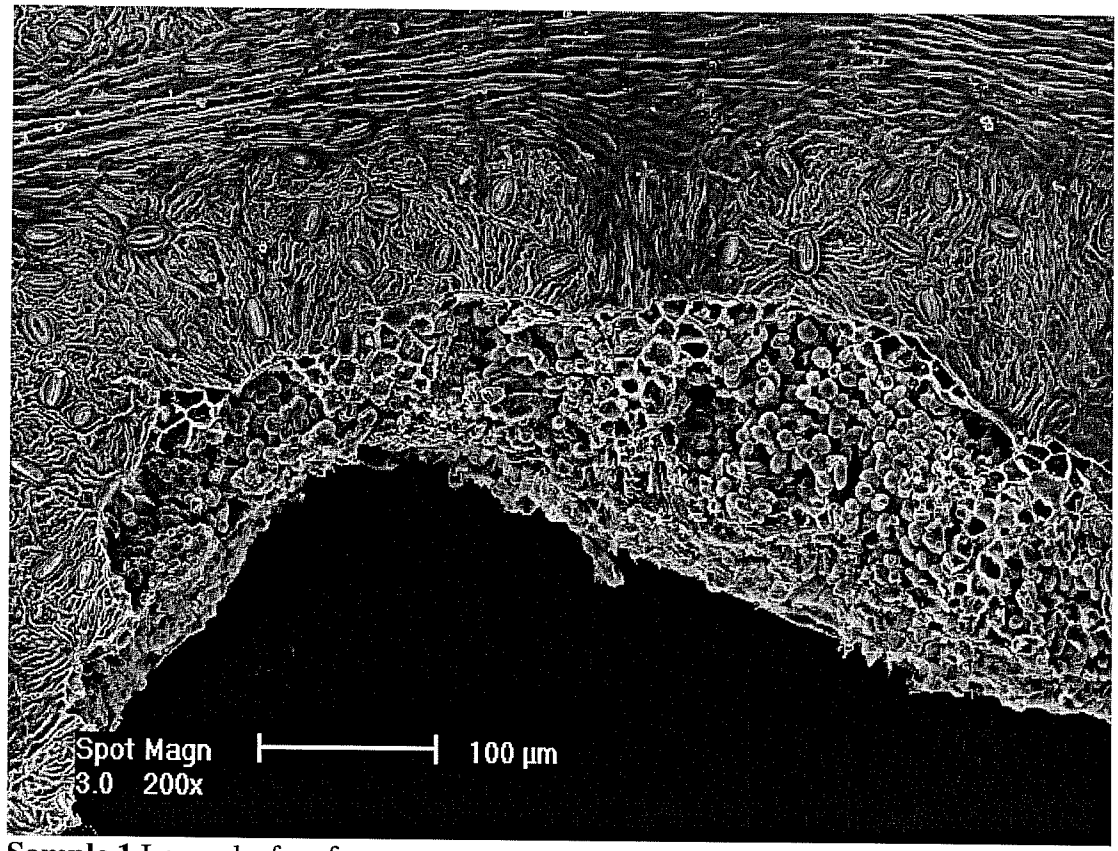
The light microscopy (LM) images show chlorotic regions or 'spots.'
The upper surface of the leaf reveals damage to the waxy surface.

Sample 2.

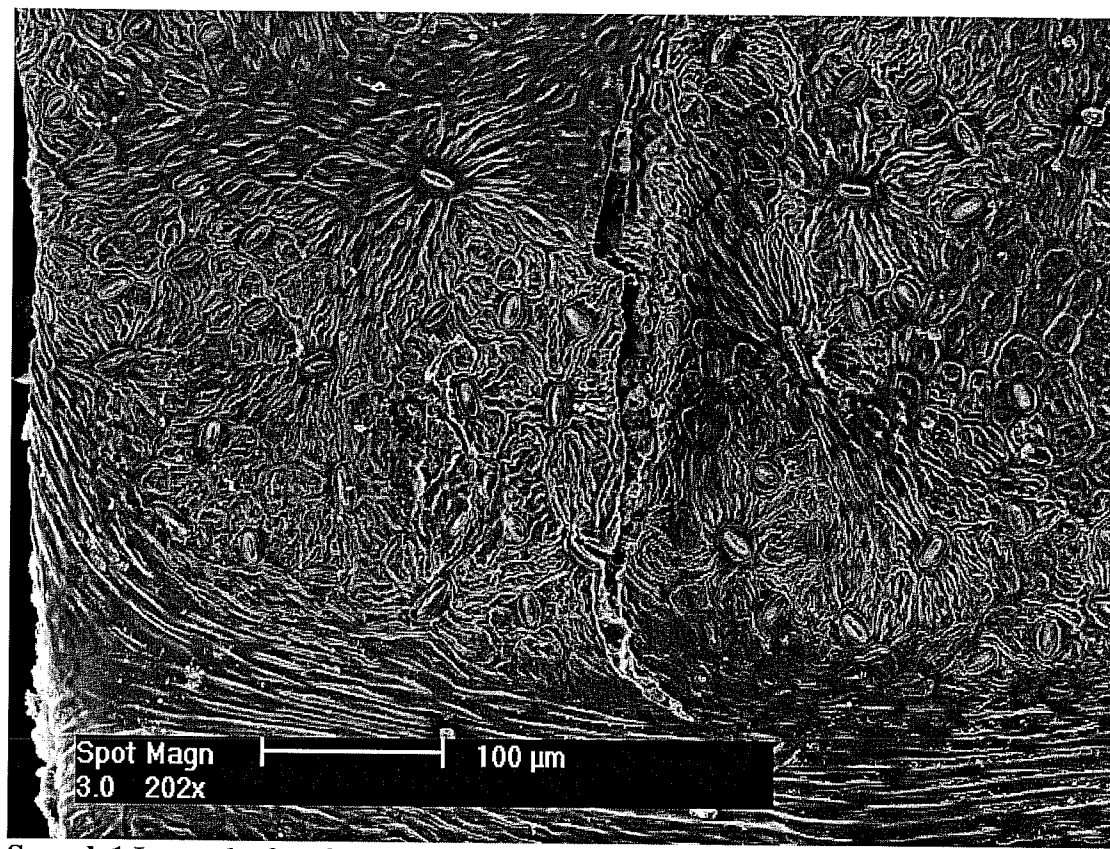
Images taken from sample 2 show residue along the junctions on both lower and upper surfaces. Areas of damage are not always defined by the veins. Dirt particles appear on both lower and upper surfaces.

Sample 3.

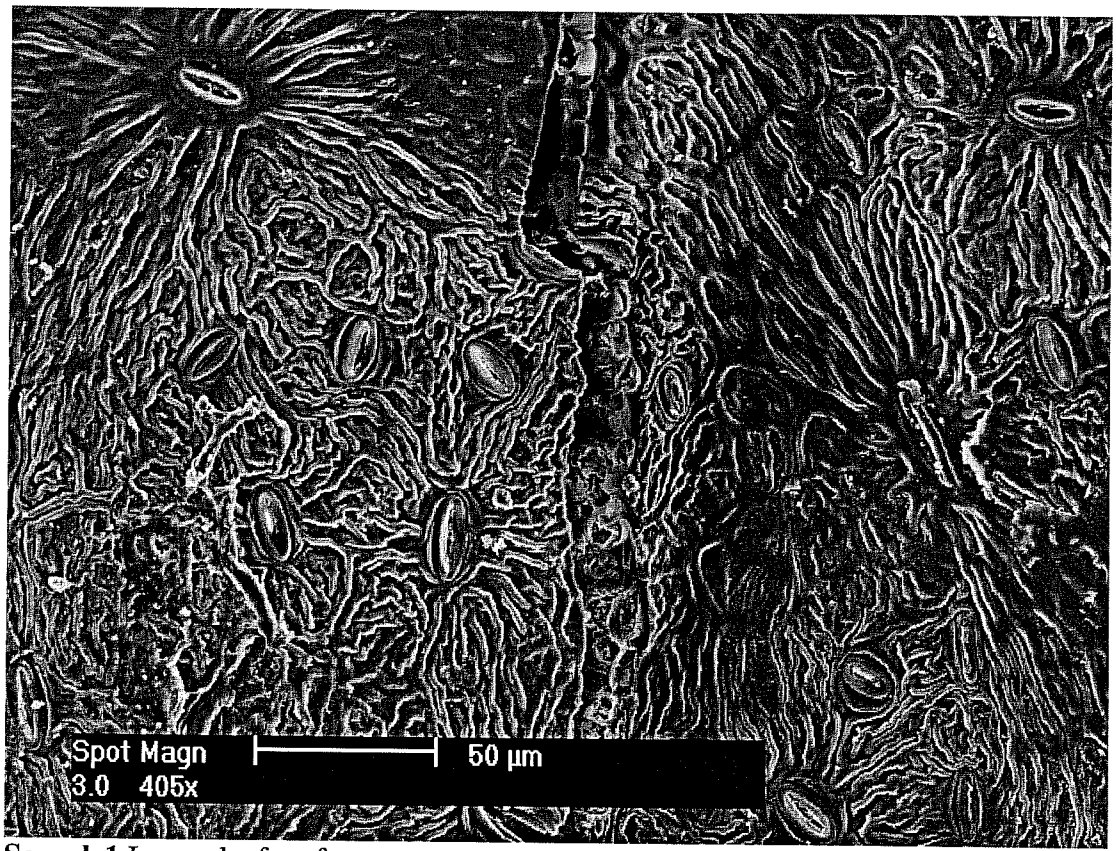
The LM images of the 'odd sample' show chlorotic regions which do not coincide with damaged areas (circular spots). Chlorosis is fairly advanced.
SEM images of lower leaf surface identify ridges near area of damage.



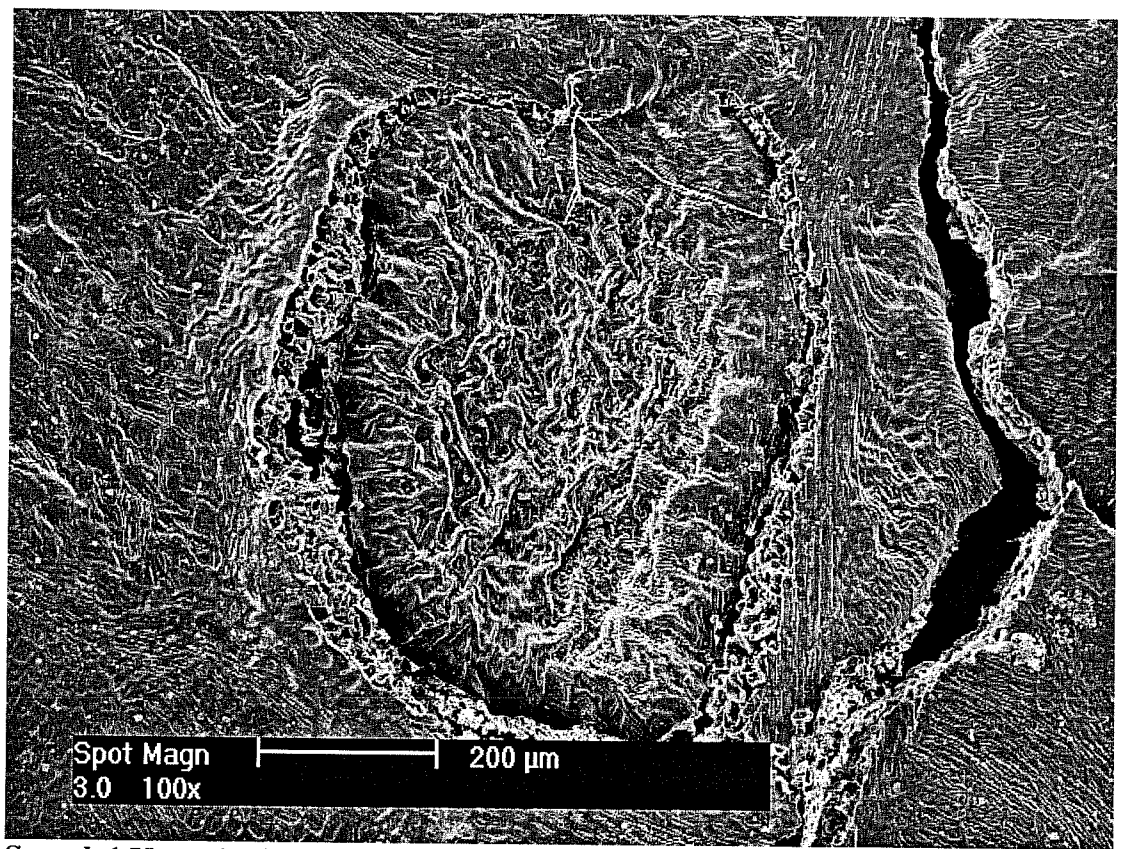
Sample 1 Lower leaf surface



Sample1 Lower leaf surface

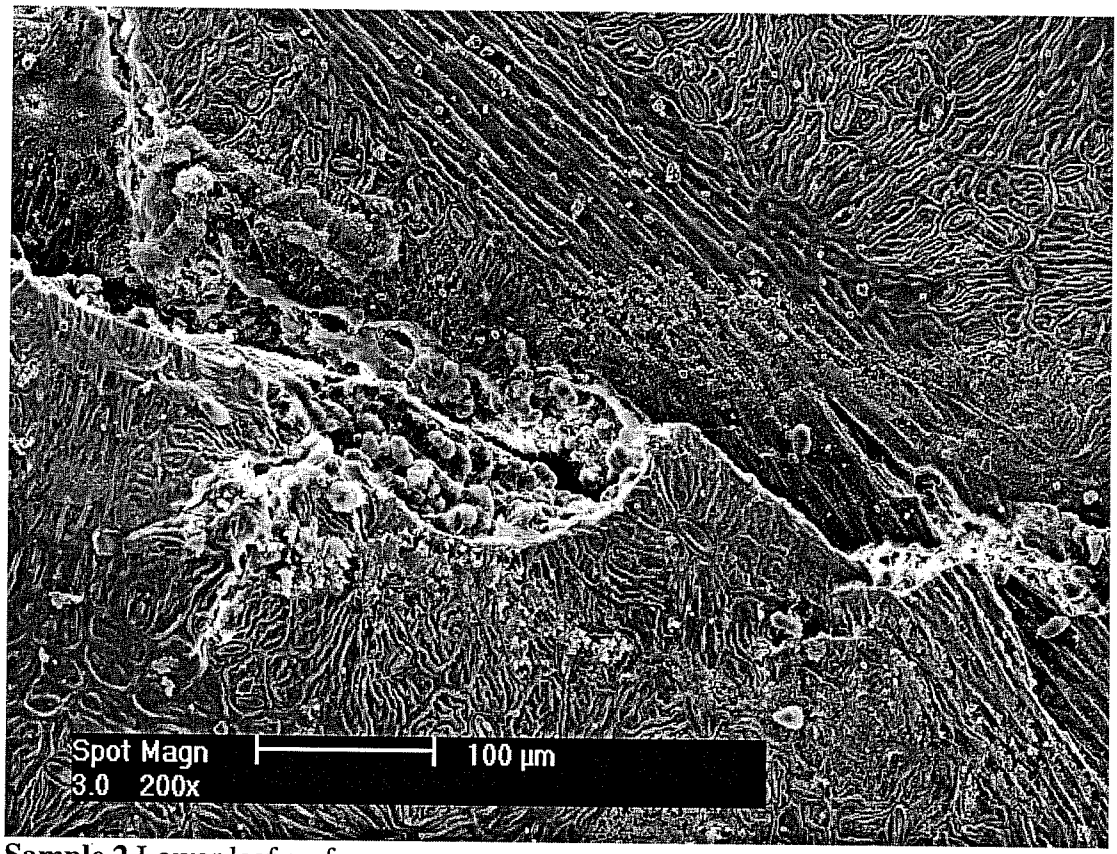


Sample1 Lower leaf surface

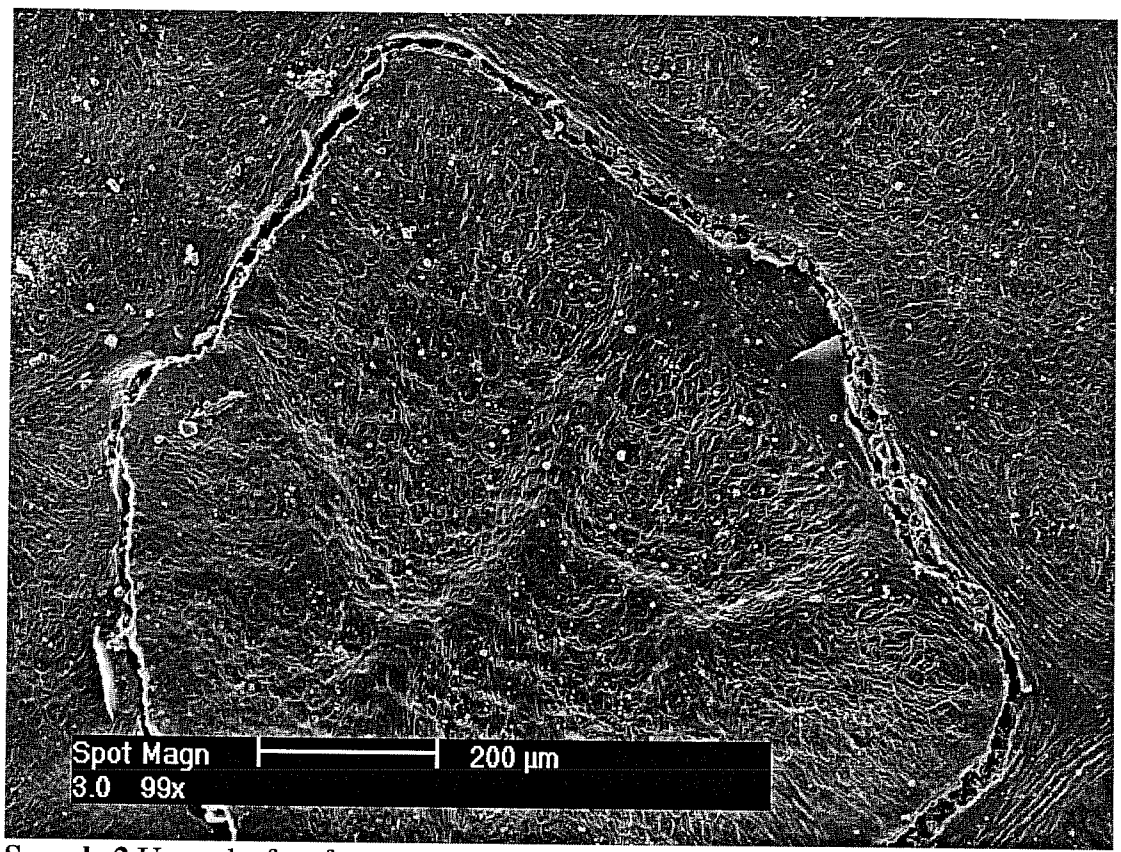


Sample1 Upper leaf surface

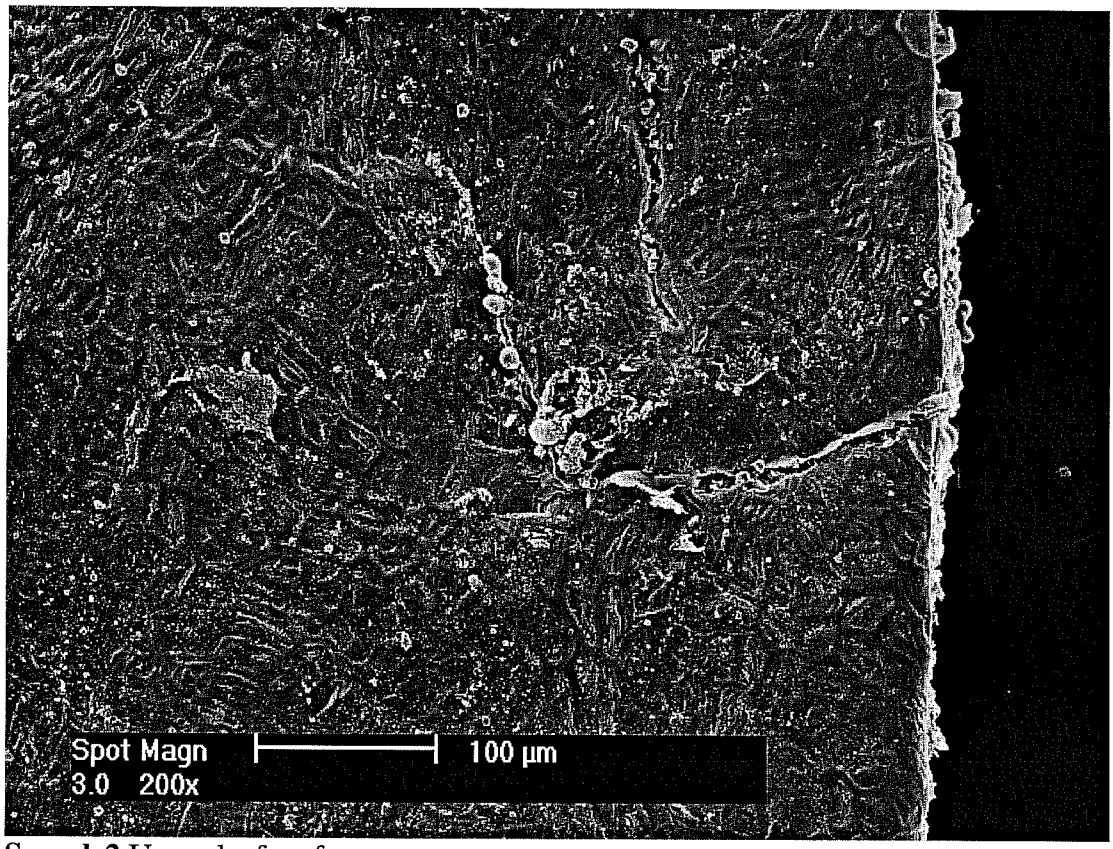
21



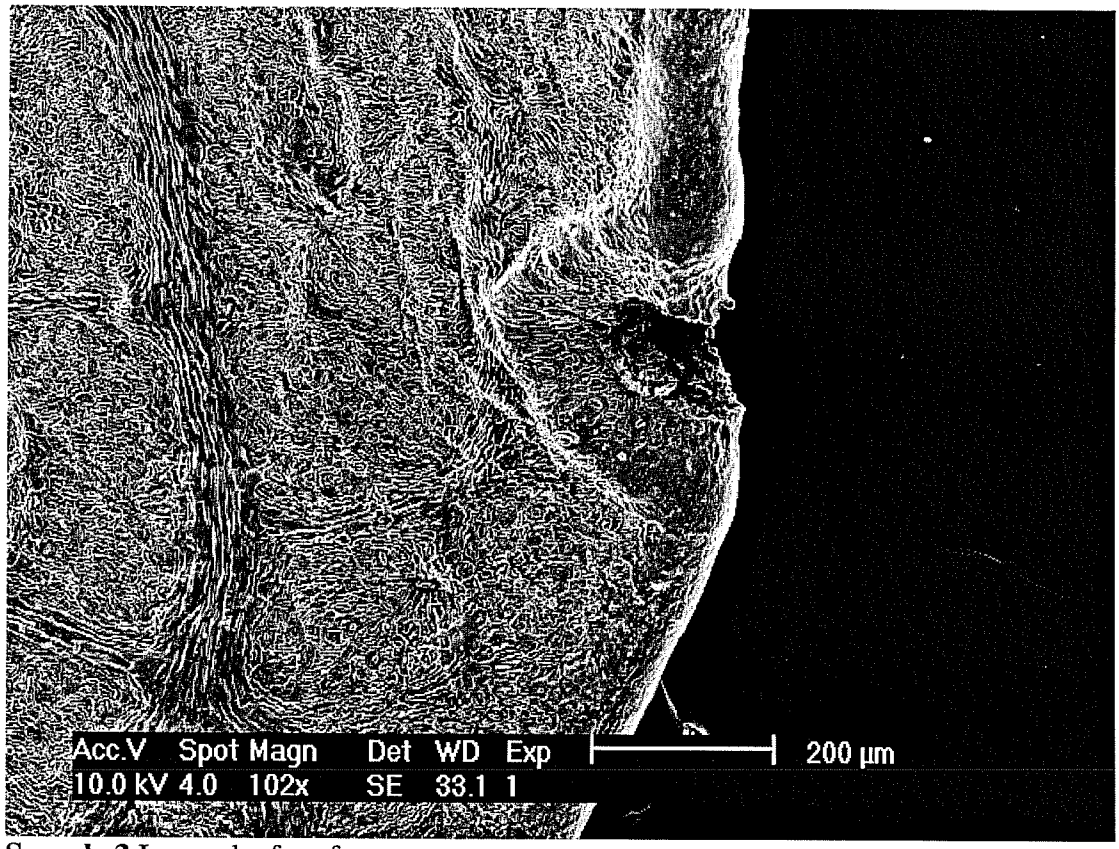
Sample 2 Lower leaf surface



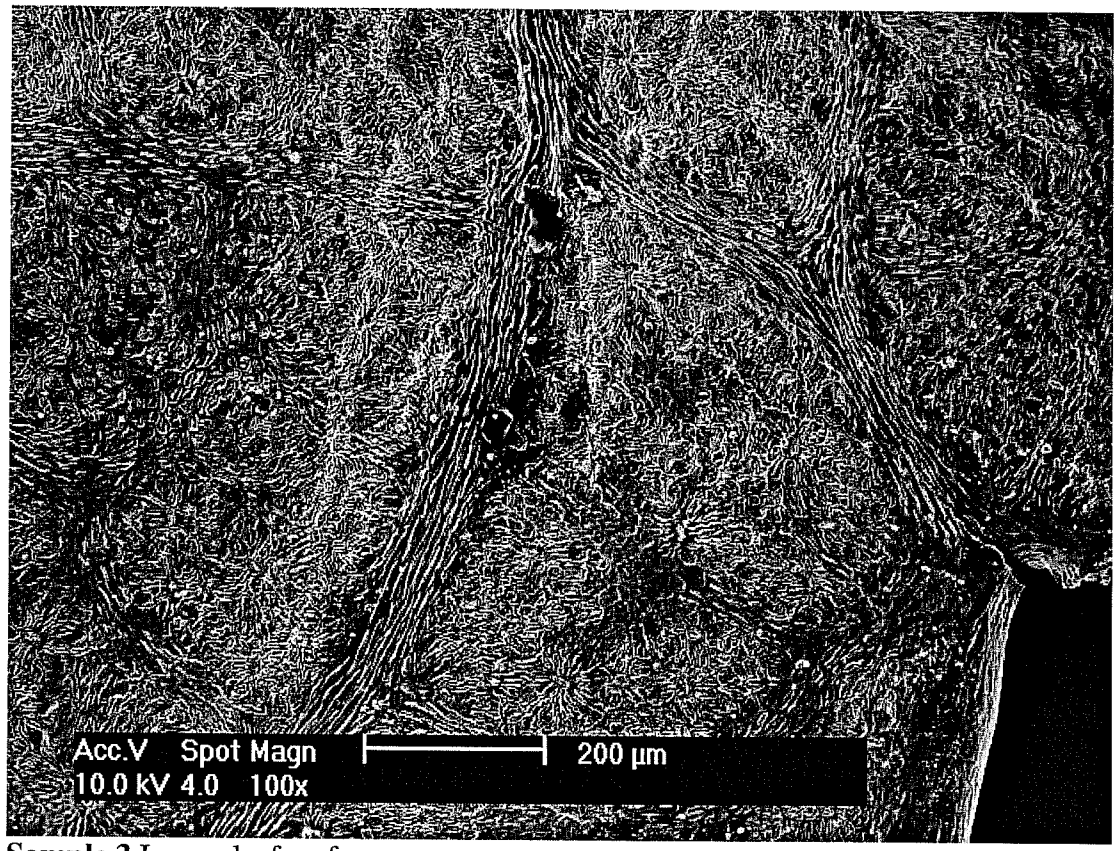
Sample 2 Upper leaf surface



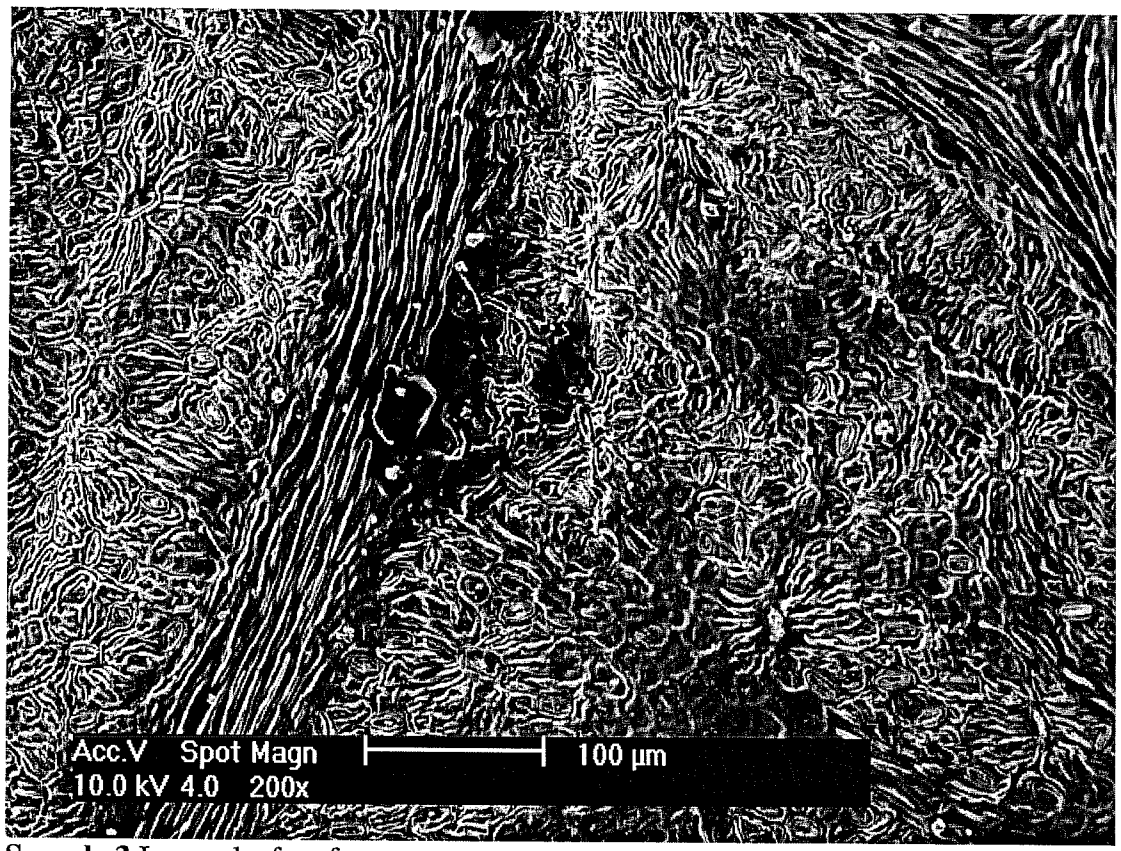
Sample2 Upper leaf surface



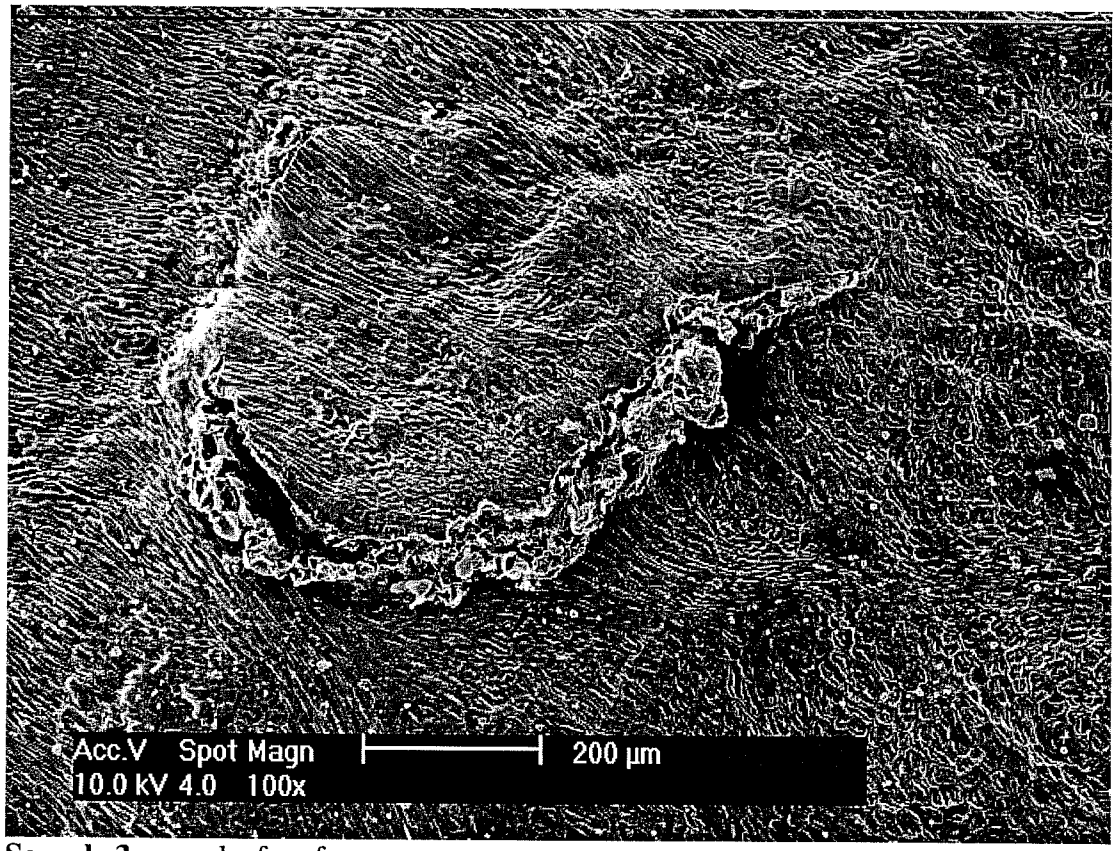
Sample 3 Lower leaf surface



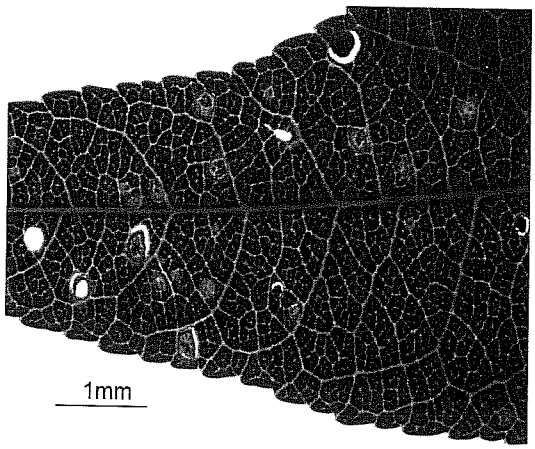
Sample 3 Lower leaf surface

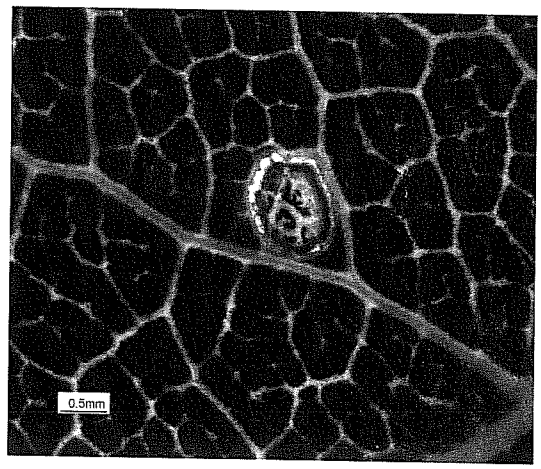


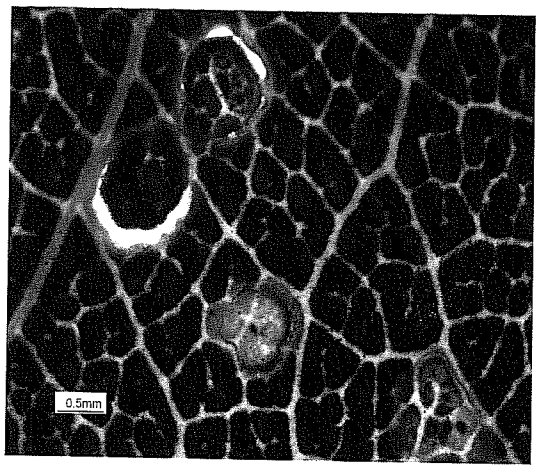
Sample 3 Lower leaf surface



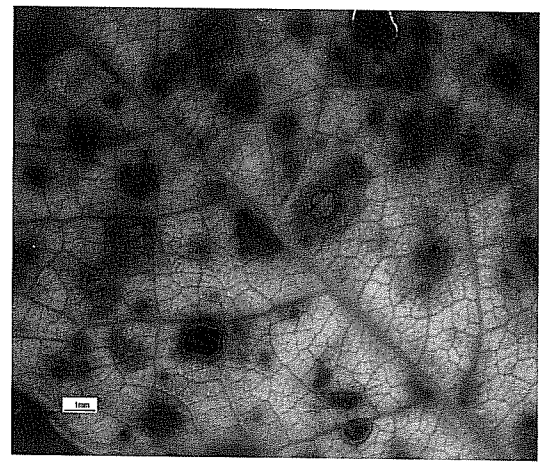
Sample 3 upper leaf surface







Sample 1



Sample 3 'odd sample'

Report on SEM observations of Almond Leaves

Monday 6 November 2006

Samples

1. Healthy leaves
2. Chemical Burn
3. Chemical Lesions

Samples of material (approx 2mm square) were cut from several leaves
Samples were cut into fixative solution, and after 24 hours taken through an alcohol dehydration series to 100% ethanol over 2 day period.

Samples were critical point dried, coated with gold/palladium and imaged in Philips XL20 scanning electron microscope.

(Let me know if you need details of this process to be written out again in full)

Scanning electron micrographs were taken of 3 areas in each of the leaf samples (1-3), with images collected of both upper and lower leaf surfaces.
Images were taken at 100x and 200x magnification.

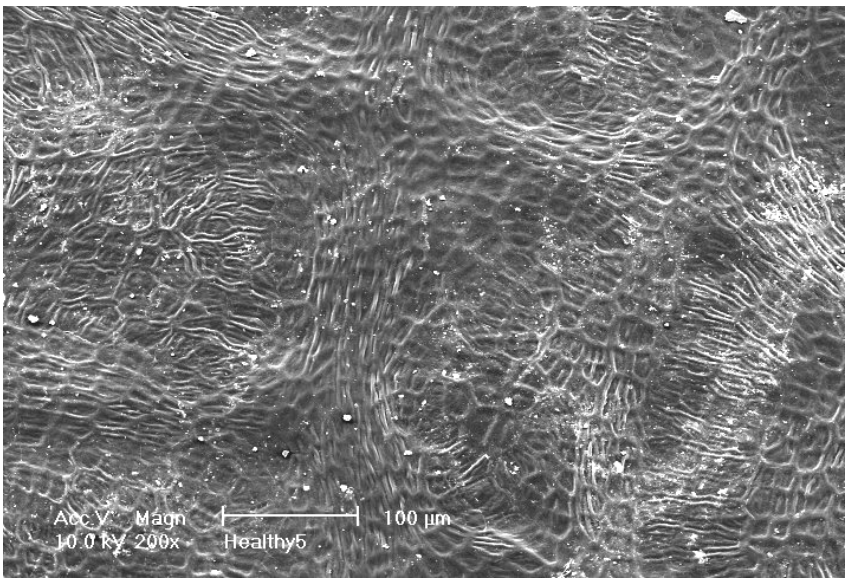
Images were coded as follows:

H	Healthy
CB	Chemical Burn
CL	Chemical Lesion
U	upper leaf
L	lower leaf
A,B,C	sample region of leaf
1	100x
2	200x
3	additional image

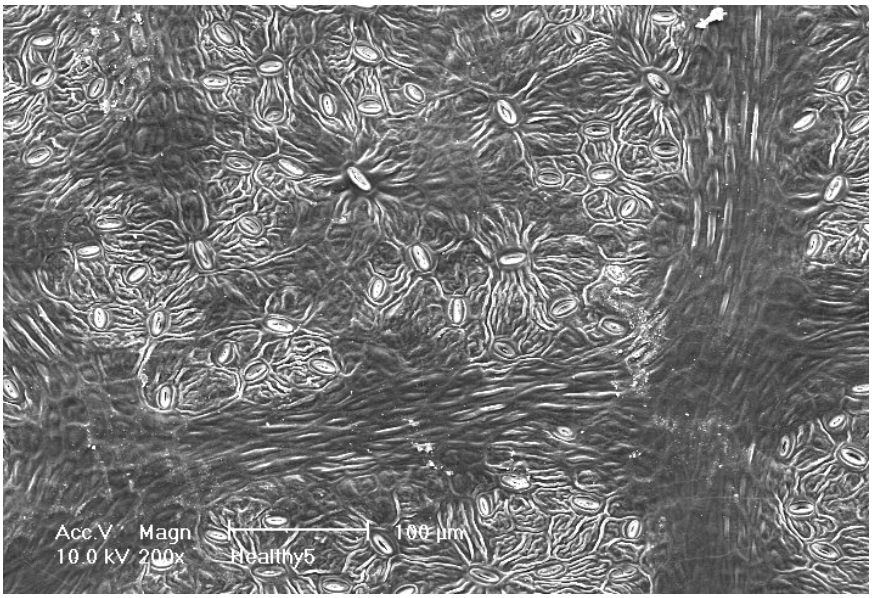
Comments:

1. Healthy leaf samples

Images taken of healthy leaves showed characteristic pattern of leaf cuticle with predominance of stomata located on lower side of leaf. Occasional dirt particles appeared on the upper surface, and both upper and lower leaf surfaces were intact and uniform in appearance.



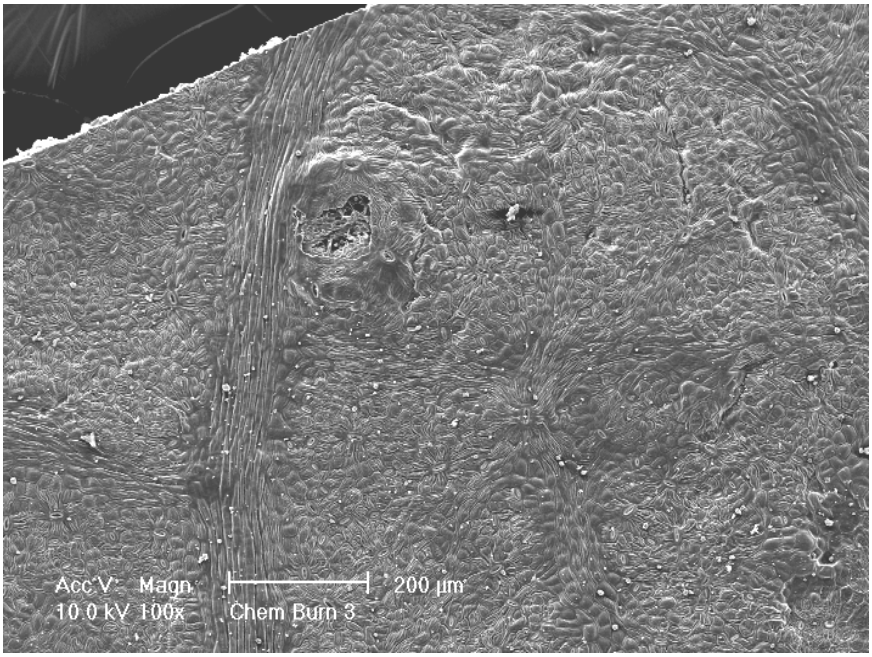
Upper leaf surface – healthy



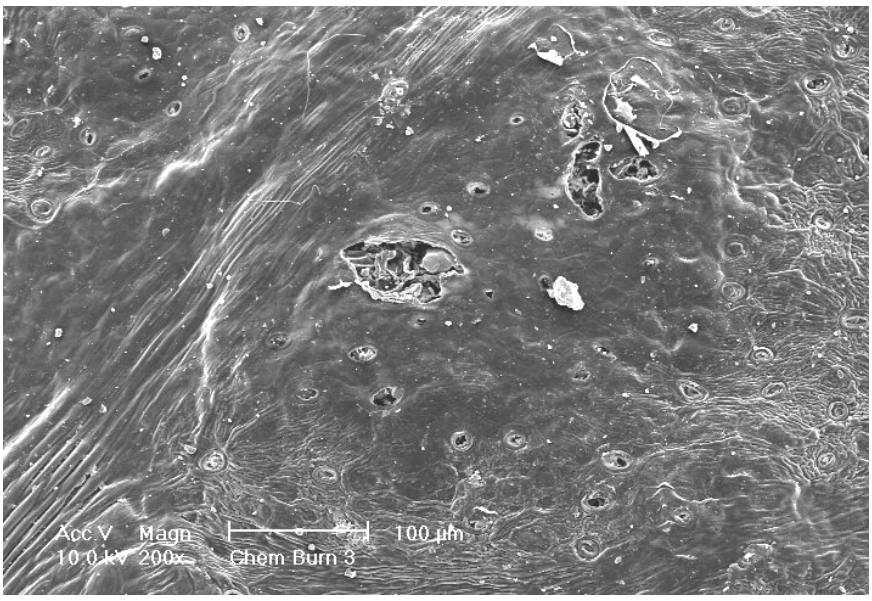
Lower leaf surface – healthy

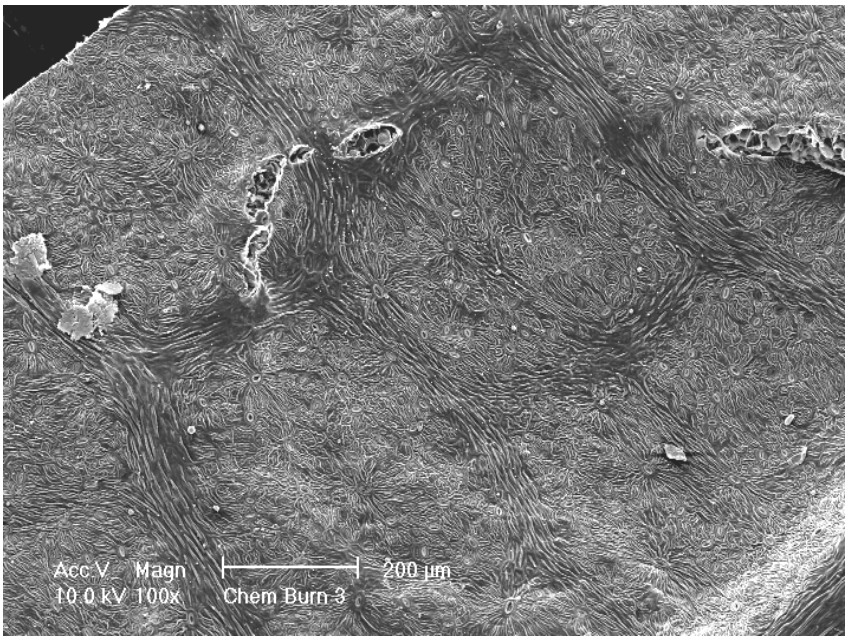
2. Chemical Burn samples

In the samples viewed, leaves showed damage on both upper and lower surfaces

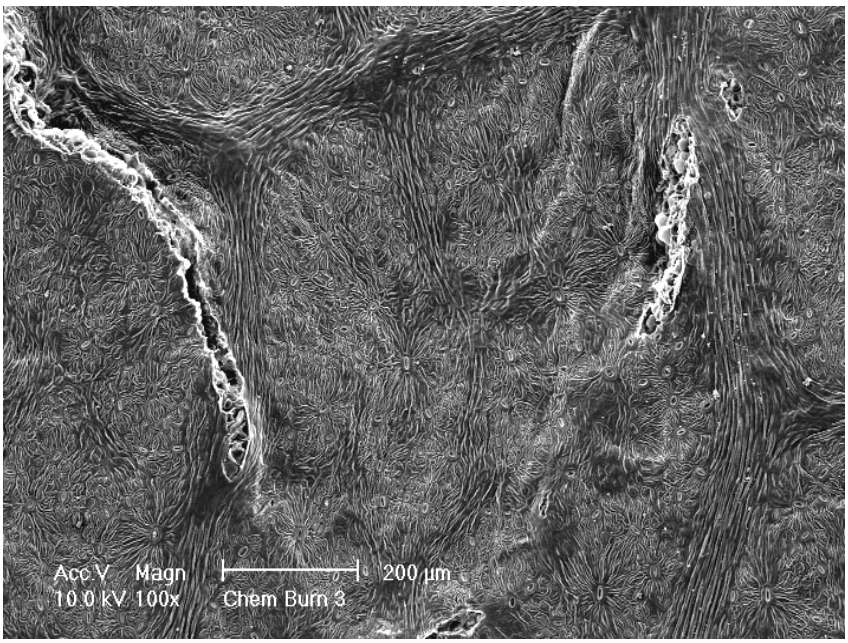


Examples of upper leaf surface





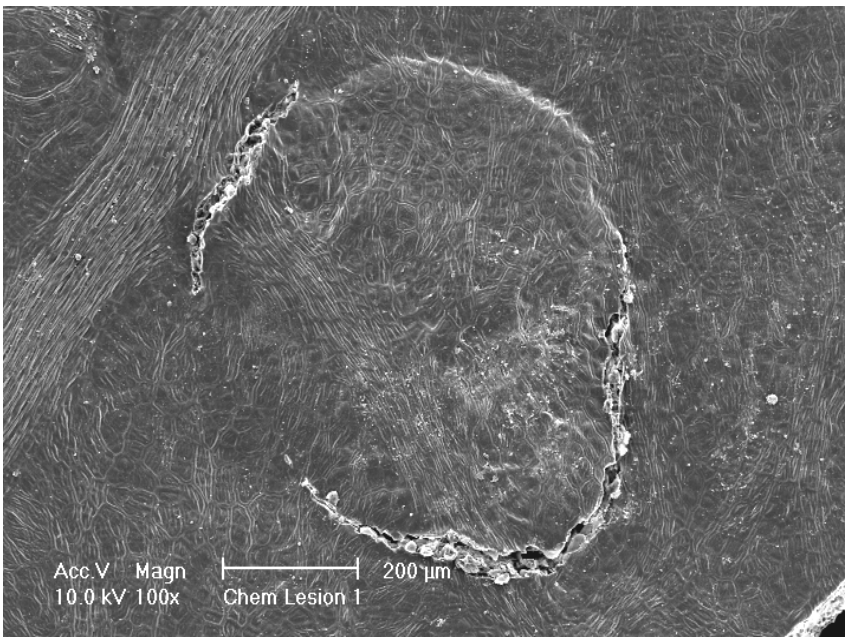
Examples of lower leaf surface



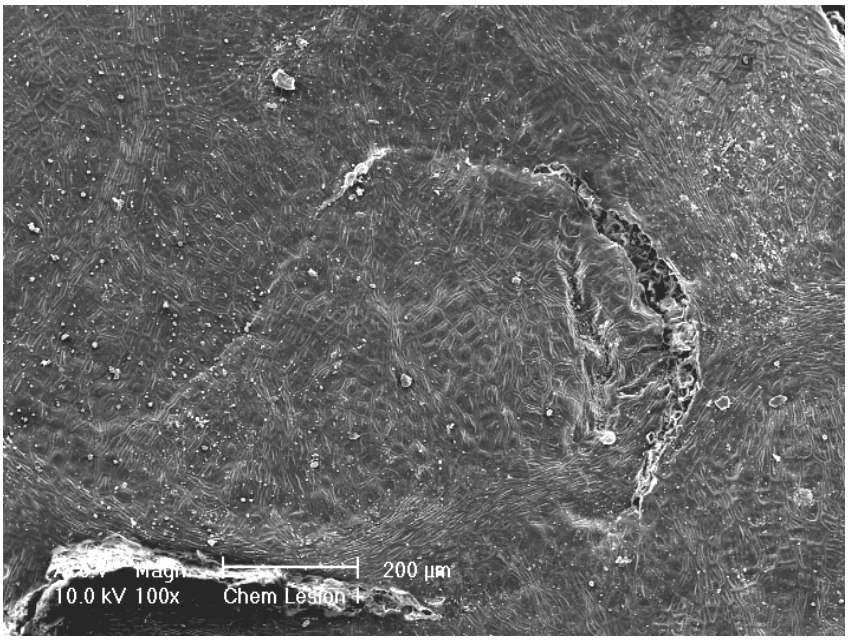
3. Chemical Lesion samples

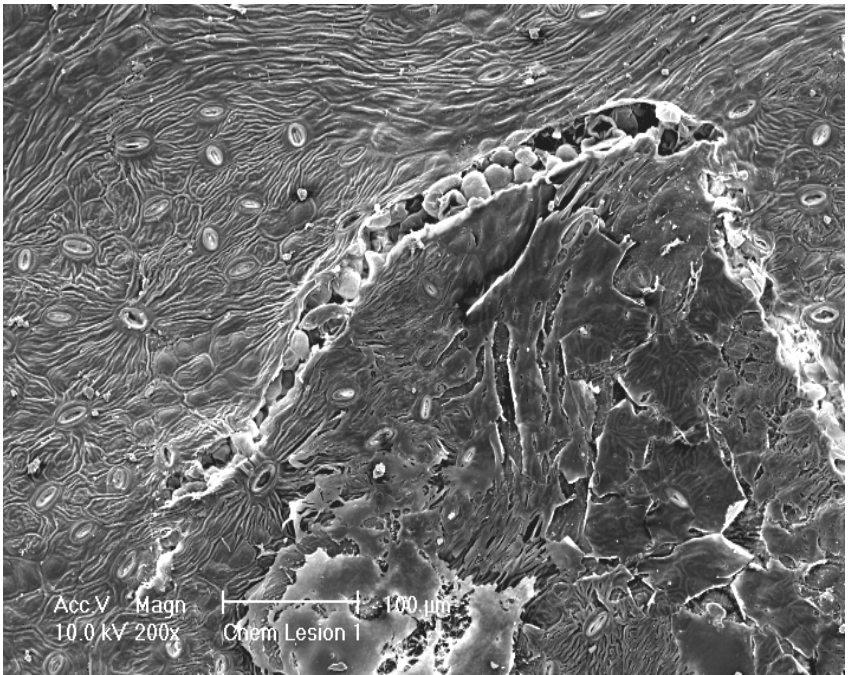
Some areas looked physically damaged, others with the some sort of precipitate, regions of leaf surface show damage (post-infection?)

Damage looks equally significant on both leaf surfaces – upper and lower. Interesting to note the circular appearance in some instances…

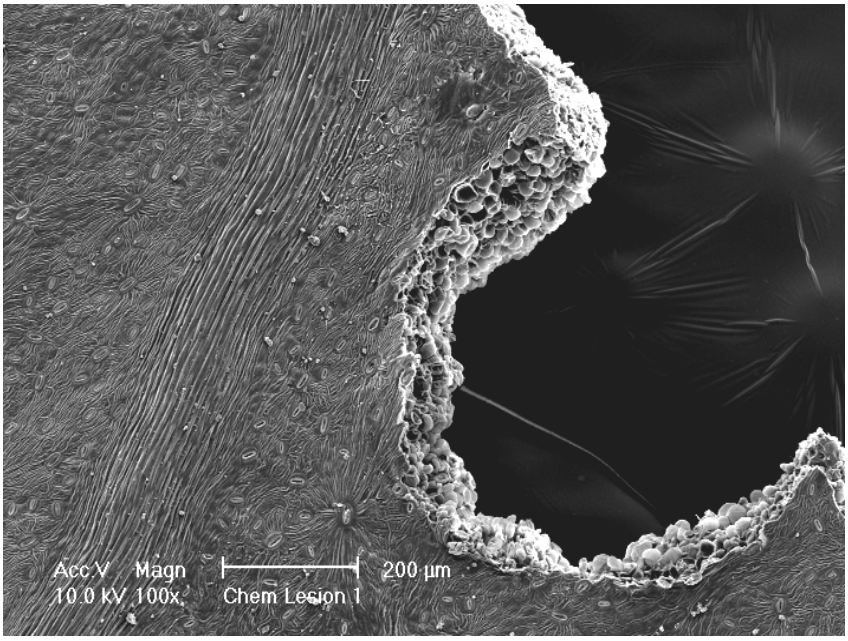


Upper leaf surface





Lower leaf surface



Note precipitate in some instances?
Severe damage resulted in portion of leaf being lost – this happened during processing of this tissue, suggesting that damage was quite extensive throughout the tissue.
The eruption-like appearance of damage emerging through leaf surface at the extremities of affected region.

Appendix 5

Australian Collection of Microorganisms – Bacterial Identification

BACTERIAL ID – METHOLOGY OPTIONS

Australian Collection of Microorganisms
University of Queensland
Jenny Sprately

Identification methodology options

The options for identifying the bacterial isolate from almonds (suspect the strain of Xanthomonas) are provided as follows:

Biochemical test kits: Biolog GN2 MicroPlates* and *API 20NE (BioMerieux) test kits

The Biolog GN2 (for aerobic, Gram -ve organisms) MicroPlate performs 95 discrete metabolic tests simultaneously, producing a metabolic "fingerprint" by which the organism may be identified by reference to a database. Please note that the Biolog database includes over 500 Gram negative aerobic organisms but only a limited number of *Xanthomonas* spp. (*X. albilineans*, *X. oryzae* pv. *oryzicola* and 18 pathovars of *X. campestris*). If the candidate isolate is not one that is included in the database, the test kit will identify which strain/species is closest metabolically to your isolate but may not provide a definitive identification.

The cost for attempted identification using the! Biolog GN2 MicroPlate is $110 per isolate (GST inclusive) and results may be expected within a few days of commencing the testing.

Partial 16S ribosomal RNA gene sequencing

Identification using partial 16S ribosomal RNA gene sequencing we charge $385 per isolate (GST inclusive). The procedure is more labour intensive and time consuming but is more likely (but not certain) to produce a definitive identification. Results are likely to take a week or so, depending on how things go with the procedure. (*This test was not undertaken*).

BACTERIAL IDENTIFICATION RESULTS – Summary April/May 2007

Initial testing of two isolates (isolate 1 and 2) recovered from symptomatic almond leaves from NAP, utilised the Biolog GN2 MicroPlates test kits (for suspected *Xanthomonas* spp.). Follow-up testing used API 20NE (BioMerieux) test kits. These proved more definitive tests and isolate #1 was confirmed as ***Pseudomonas luteola*** (re-classified as ***Chryseomonas luteola***). A summary of the results follows and a full report is also included in this Appendix.

Biolog GN2 MicroPlates test kits

Growth from the two plates submitted were tested separately because colonial morphologies were slightly different and sub-cultured growth from each also produced slightly differing colonial morphologies. A **definitive identification was not achieved with this test method, for either of the cultures submitted**.

Isolate #1 (sub-cultured from your isolate labeled "1")

Gram negative, oxidase negative bacilli, producing very intense-yellow, shiny, slightly convex, entire colonies (non-mucoid). Cells/bacilli are medium-sized, plump rods with rounded ends, generally uniform cellular morphology/size/shape, slightly more slender than isolate #2.

Biolog GN2 MicroPlates inoculated on 9 May and incubated at 28C/air before being read after approx. 17 hours and 45 hours incubation):

"*No identification*" (after both 17 and 45 hours). The results include a list of species to which the isolate was most similar biochemically but please note that *none of these* were even a close enough match to rate a statistically poor identification. The species from the Biolog database that is given as being most similar metabolically is ***Vibrio metschnikovii*** (at 17 hours: PROB -, SIM 0.08, DIST 18.92, at 45 hours: PROB -, SIM 0.12, DIST 18.30), though the colonial morphology and source of the strain doesn't support this (this species doesn't normally produce yellow colonies and is usually isolated from marine sources).

Isolate #2 (sub-cultured from isolate labeled "2"):
Gram negative, oxidase negative bacilli, producing lemon-yellow (slightly paler yellow), very mucoid/wet, shiny colonies. Cells are medium-sized, plump rods with rounded ends, generally uniform cellular morphology/size/shape, slightly more plump than isolate #1.

Biolog GN2 MicroPlates inoculated on 9 May and incubated at 28C/air before being read after approx. 17 hours and 45 hours incubation): :

"No identification" (after both 17 and 45 hours). The results included a list of species to which the isolate was most similar biochemically but please note that none of these were even a close enough match to rate a statistically poor identification and the species listed most similar metabolically is given as ***Vibrio metschnikovii*** (at 17 hours: PROB -, SIM 0.15, DIST 16.92, at 45 hours: PROB -, SIM 0.29, DIST 12.54).

For isolates 1 and 2, other species listed from the biolog database were even further statistically from a definitive identification than the *Vibrio* species. These included ***Chryseomonas luteola***, ***Pseudomonas syringae*** (various pathovars), ***Sphingomonas*** (several species) and ***Xanthomonas campestris*** (several pathovars). Most of these are more likely than the *Vibrio* given the colonial morphology (yellow, yellow and mucoid) and source of the isolates.

API 20NE (BioMerieux) testing
The test kits were inoculated/incubated for 48 hours @ 28C/air and read after 24 and 48 hours. The test strips may be re-read after 48 hours if reliable results are not produced after 24 hours. Although both strains were identified as ***Pseudomonas luteola*** after 24 and 48 hours incubation, only isolate #1 had a strong confirmation. This identification is consistent with the colonial morphology (yellow colonies) for each and with the source of isolation.

Pseudomonas luteola has been re-classified as ***Chryseomonas luteola*** and is generally isolated from soil, plants, water. It is a Gram negative, oxidase negative, catalase +ve bacilli that produces yellow colonies.

Isolate #1 (non-mucoid, bright yellow colonies): Test strip read after 24 hours: "Very Good Identification: significant taxa: *Pseudomonas luteola* 99.8% (T = 0.62)". **T**his is considered to be a very reliable identification. Test strip read after 48 hours: "Low Discrimination: Significant taxa: *Pseudomonas luteola* 92.5% (T = 0.56)"**.** This is considered to be a marginally acceptable identification but the results after 24 hours are accepted/very reliable.

Isolate #2 (mucoid, lemon-yellow colonies): Test strip read after 24 hours**:** "Doubtful profile: significant taxa: *Pseudomonas luteola* 86.4% (T = 0.29)"**.** This is NOT considered to be a reliable identification. Test strip read after 48 hours: "Doubtful profile: significant taxa: *Pseudomonas luteola* 86.4% (T = 0.29)". This is NOT considered to be a reliable identification (same result as at 24 hours).

These results suggest that Isolate #2 is likely to be a species that is closely related biochemically to ***Pseudomonas*** (***Chryseomonas***) ***luteola*** but which is not in the API database**.**

Please note that the above results are consistent with the Biolog results obtained previously, as follows:

For Isolate #1, even though Biolog did not produce an ID, the closest "fit" metabolically after 17 and 45 hours incubation apart from the *Vibrio* species (which is not consistent with colonial morphology or source of isolation) was *Chryseomonas luteola* (which is consistent with colonial morphology and source of the isolate).

For Isolate #2, (slightly different morphology and slightly different results), even though Biolog did not produce an ID, the closest "fit" metabolically after 17 and 45 hours incubation apart from the *Vibrio* species (which again is not consistent with colonial morphology or source of isolation) were a number of *Pseudomonas syringae* pathovars (which is consistent with colonial morphology and source of the isolate). Note: *Pseudomonas syringae* produces yellow colonies, is an oxidase negative *Pseudomonas* species and is NOT in the API database.

THE UNIVERSITY OF QUEENSLAND

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AUSTRALIAN COLLECTION OF MICROORGANISMS

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Curator: Dr Lindsay I Sly
Assistant Curator: Jenny Spratley (E-mail: j.spratley@uq.edu.au)

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31 May 2007

TO: Dr Prue McMichael
Senior Consultant/Plant Pathologist
Scholefield Robinson Horticultural Services Pty Ltd
PO Box 650
Fullarton, South Australia 5063

FAX: 08 – 8373 2442

RE: Identification of Isolates submitted on your behalf by Christine Horlock (Isolates #1 and #2, isolated from almond tree leaves)

Dear Prue,

Identification of the two isolates submitted on your behalf by Christine Horlock using commercially available biochemical test kits was completed on Wednesday 23 May 2007. This report provides details of the results which were previously summarised in my e-mails to you dated 17 May 2007 and 24 May 2007.

Growth from each of the two agar plate cultures that were submitted was treated separately because colonial morphology of the two cultures differed slightly (one being mucoid and the other non-mucoid on Trypticase soy agar/TSA). The isolates were subcultured to fresh TSA plates and incubated at 37°C and 28°C for 48 hours (as expected, given the source of the isolates, growth proved to be better for each isolate at the lower temperature).

Gram stains and oxidase tests were undertaken initially for each isolate. Based on the results, Biolog GN2 MicroPlates (used for identification of Gram negative, aerobic bacteria) were inoculated in an attempt to identify the organisms. The Biolog GN2 MicroPlate performs 95 discrete metabolic tests simultaneously, producing a metabolic profile by which many organisms may be identified by reference to a database. A definitive identification was not achieved for either isolate using the Biolog GN2 MicroPlates. However, I have attached copies of the results for your information and included some comments.

BioMerieux's API 20NE test kits (for identification of non-enteric, non-fastidious, Gram negative rods/bacilli) were subsequently inoculated, producing a reliable identification for Isolate #1 but not for Isolate #2. The API 20NE test kit utilises 8 conventional biochemical tests and 12 assimilation tests to identify a wide range of Gram negative organisms by reference to a database.

Test results for each isolate are as follows:

Isolate #1 (non-mucoid, bright yellow colonies on TSA):

(i) Gram negative, oxidase negative, catalase positive bacilli (medium-sized, plump bacilli/rods with rounded ends, generally uniform cellular morphology/size/shape, slightly more slender than those of Isolate #2), producing very intense-yellow, shiny, slightly convex, entire, non-mucoid colonies on TSA,

(ii) Identified as *Pseudomonas (Chryseomonas) luteola* ("Very Good Identification", probability 99.8%, T = 0.62) using the API 20 NE (BioMerieux Inc.) biochemical test kit incubated for 24 hours @ 28C/air **(this represents a very reliable identification)**. After 48 hours' incubation, the isolate also identified as *Pseudomonas (Chryseomonas) luteola* ("Low Discrimination", probability 92.5%, T = 0.56) – this is considered to be an acceptable (though slightly less discriminating/reliable) result. **Test results and a copy of the reactions/enzymes incorporated into the API 20 NE test kit are attached.**

Please note: *Pseudomonas luteola* has been reclassified as *Chryseomonas luteola* (the BioMerieux/API nomenclature is out of date).

Comment: the above result is consistent with the colonial morphology and source of the isolate (***Chryseomonas luteola*** is ubiquitous in the environment and is commonly isolated from soil, water and plants; it produces yellow colonies on nutrient medium and is oxidase negative and catalase positive).

This isolate did not identify using the Biolog GN2 MicroPlate test kit, though I have attached a copy of the test results and copy of the assimilation tests included in the GN2 MicroPlate test kit for your information.

Comment: The Biolog results include a list of species to which the isolate is most similar metabolically but please note that none of these were a close enough match to rate even a statistically poor identification. The species from the Biolog database that is given as being most similar when the plates/kits were read after 17 and 45 hours incubation is *Vibrio metschnikovii*, though the colonial morphology and source of the

isolate does NOT support this (*V. metschnikovii* doesn't normally produce yellow colonies and is usually isolated from marine sources). You'll note that the second match from the Biolog database is given as *Chryseomonas luteola* in most cases (consistent with the API results), though the profile is similarly matched to a number of other closely related species including *Pseudomonas syringae*, *Sphingomonas* species and *Xanthomonas campestris*. The Biolog test kit is apparently unable to distinguish between your isolate and a number of different species in the database.

Isolate #2 (mucoid, lemon-yellow colonies on TSA):

(i) **Gram negative, oxidase negative, catalase positive bacilli** (medium-sized, plump bacilli/rods with rounded ends, generally uniform cellular morphology/size/shape, slightly plumper/thicker than those of Isolate #1), producing lemon-yellow, shiny, slightly convex, entire, mucoid colonies on TSA,

(ii) **Identified as *Pseudomonas (Chryseomonas) luteola* ("Doubtful Profile", probability 86.4%, T = 0.29)** using the API 20 NE (BioMerieux Inc.) biochemical test kit incubated for 24 & 48 hours @ 28C/air **(this does NOT represent a reliable identification). Test results and a copy of the reactions/enzymes incorporated into the API 20 NE test kit are attached.**

The results suggest that isolate #2 is likely to be a species that is closely related biochemically to *Pseudomonas* (*Chryseomonas*) *luteola* but which is not in the API database.

This isolate did not identify using the Biolog GN2 MicroPlate test kit, though I have attached copies of the test results and a copy of the assimilation tests included in the GN2 MicroPlate test kit for your information.

Comment: The Biolog results include a list of species to which the isolate is most similar metabolically but please note that none of these were a close enough match to rate even a statistically poor identification. Again (as for Isolate #1), the species from the Biolog database that is given as being most similar when the plates/kits were read after 17 and 45 hours incubation is *Vibrio metschnikovii*, though the colonial morphology and source of the isolate does NOT support this. Other species listed as being most similar metabolically from the Biolog database are various pathovars of *Pseudomonas syringae* and *Chryseomonas luteola*. Note also that *Pseudomonas syringae* is NOT included in the API database.

For your information: *Chryseomonas luteola* is NOT part of the normal human flora but has been isolated from skin, respiratory tract etc. as an environmental contaminant. This species is not considered pathogenic to humans but can be an opportunistic infection in compromised patients, particularly catheter-related infections, septicaemia and peritonitis usually associated with continuous ambulatory peritoneal dialysis etc. The organism is usually associated with other infectious/pathogenic organisms when isolated clinically and is not considered to be the primary pathogen in such cases but merely an environmental opportunist. I don't know whether it is a

plant pathogen but none of my reference texts mention this (not just clinical references) so I suspect that it is not a known plant pathogen.

Please accept my apologies for the delay in supplying you with this report. If you require further information or clarification, please do not hesitate to contact me by phone or email.

Yours sincerely,

Jenny Spratley

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