

**Microbiological Aspects of Apple Replant Disease.**

**By**

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“Come now, a roundel and a fairy song.” An illustration by Arthur Rackham, to  
*A Midsummer Night's Dream*, written by William Shakespere.

Everything is connected to everything else.

Vladimir Ullich Illianov  
(Lenin)

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## Declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any degree or diploma in any university, and that, to the best of my knowledge and belief, the thesis contains no copy of any material previously published or written by another person except where due reference is made in the text.

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## Abstract

The apple industry represents a major part of Tasmanian, Australian and world wide fruit production. Continuing demand for new varieties in the changing global marketplace presents growers with an ongoing requirement for removal of uneconomic varieties and their replacement with the more currently favoured varieties, this particularly being the case for Tasmanian and Australian growers supplying competitive overseas markets. Planting designs using closely spaced early fruiting trees on dwarfing rootstocks, leading to quicker economic returns, also encourages faster turn-around of orchards. These factors have placed increasing pressure on land available for apple orchards.

Replanting of many plant species, including apple (*Malus domestica*), often presents growers with a well known management problem commonly referred to as “replant disease”. Trees planted in locations previously occupied by earlier plantings show reduced growth, and an atypical growth pattern. Severely affected plants can show significant stunting, often resulting in a characteristic “wave” pattern across affected fields, with weakest growth corresponding with tree positions in the previous orchard. Reductions in growth can adversely impact on orchard coverage and hence yield per unit area, with consequent economic impacts. Dwarfing rootstocks, popular under current management strategies, tend to be more susceptible to the effects of replant, making effective management of apple replant disease of significant importance to growers.

While the underlying cause of this condition has yet to be established it has been found to be associated with a range of biotic and abiotic factors. Fumigation with aggressive fumigants such as methyl bromide has been found to consistently prevent

occurrence of the problem. Other lower impact or cheaper alternatives are available but they have not gained wide acceptance due, in part, to inconsistent results and the economic need for young trees to achieve uniform growth to the appropriate size for the orchard design.

In 1990 parties to the Montréal Protocol 1987 agreed to a phase out of a range of ozone depleting substances including methyl bromide. Consequently the present thesis reports on components of research seeking an alternative control measure for the local (Tasmanian) industry. A range of possible measures were investigated on both potted and field conditions trees. Treatments included: sterilants methyl bromide, Telone C35<sup>®</sup> a mixture of Telone<sup>®</sup> 65% and chloropicrin 35%, Basamid<sup>®</sup> (dazomet), metham and Perlka<sup>®</sup> (calcium cyanamide), organic matter addition in the form of peat and biocontrol agents including two *Trichoderma* sp. Trichopel<sup>®</sup> and a locally isolated strain Td22 together with a commercially available *Bacillus subtilis* agent known by the trade name Companion<sup>®</sup>. In addition dominant microbiota were isolated from these treatments in order to find potential pathogens or biocontrol agents that may be involved in causing or countering apple replant disorder.

It was found that Telone C35<sup>®</sup> was the only alternate treatment that produced consistent growth results equivalent to methyl bromide. Given both fumigants utilize the same equipment and management strategies a change to Telone C35<sup>®</sup> provides growers with a suitable replacement for methyl bromide. Basamid<sup>®</sup>, in these trials, did not produce a consistent effect in countering apple replant. Other potential sterilants did not produce consistent effective results. Addition of organic matter produced some positive effects, but results did not suggest it as a consistent and suitable countermeasure for apple replant. Treatment of soil with commercial and locally isolated biocontrol agents did not produce conclusive results.

Application of mono-ammonium phosphate was effective under glasshouse conditions, confirming extensive earlier work, but was ineffective in field trials reported here, possibly due to enhanced weed competition.

Plant water stress is often said to be associated with replant disease, but there is little published evidence supporting the assertion. A trial comparing root hydraulic conductivity over the initial 6 weeks from planting into replant and non-replant soil demonstrated a reduction in conductivity in replant soil. The result is discussed in terms of a link between the replant status of the soil and a measurable physiological response in plants.

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## Abbreviations

ANOVA	Analysis of Variance
ARD	Apple replant disease
ATP	Adenosine - 5' - triphosphate
DGGE	Denaturing gradient gel electrophoresis
ITC	Isothiocyanate
LSC	Leaf specific conductance
LSD	Least significant difference
MAP	Mono - ammonium phosphate
ns	Not significant (at P = 0.05)
PCR	Polymerase chain reaction
PLFA	Phospholipid fatty acid analysis
RLSC	Root leaf specific conductivity
SPSS	Statistical package for social sciences
TCSA	Trunk cross sectional area
WFW	Wood fibre waste

## Chapter 1 Introduction

This study was carried out in response to interest by the Australian apple industry in alternatives to methyl bromide fumigation for control and management of specific apple replant disease. It follows on from previously published local work concentrating mainly on MAP and organic matter amendment for ARD management by Wilson, *et al.* (2004) and a series of more general studies by Ramona and co-workers on potential use of organism antagonism for soil pathogen biocontrol.

### 1.1 Historical Background

Apple replant disease also known as apple replant disorder is recognised world-wide, occurring wherever apple trees are replanted a short time after pre-existing trees in the same location. Although often referred to as specific apple replant disease, it is commonly referred to as apple replant disease, the term that shall be adopted here, although the acronym ARD has been retained. Replant disorders have been observed since Ancient Greek times, with a wide range of crop species including both annuals and perennials exhibiting poor growth, development and often increased incidence of disease when planted on sites where the same crop has previously been cultivated. The earliest recorded references to apple (*Malus domestica* Borkh.) replant problems date from 1610 in Holland (Reference cited by Mai and Abawi, 1981). During the twentieth century, commercial apple production has become established into traditional regions and there has been an associated rise in pressure on available land resulting in a need to replant after minimal fallow periods. Apple replant disease is now prevalent and well documented in all the major fruit growing regions of the world (Mazzola, *et al.* 2002).

## **1.2 Effects and Symptoms of Apple Replant Disease**

The symptoms of apple replant disease become evident relatively soon after planting (Granatstein and Mazzola, 2000), usually in the first growing season following winter planting. Typically, young affected orchards show substantially weaker growth than expected for the rootstock/scion combination. Growth is often uneven, sometimes visible as a “wave” along rows, with trees located at previous tree positions demonstrating the most pronounced reduction in size (Plate 1.1). The weaker and uneven growth persists for the life of the orchard, with most apple replant disease affected orchards failing to reach their full productive capacity (Peterson and Hinman, 1994). Symptoms include stunting, shortened internodes, rosetted leaves, small root systems, fewer lateral roots and root hairs, decayed or discoloured roots and reduced productivity (Granatstein and Mazzola, 2000).

## **1.3 Orcharding Systems and Specific Apple Replant Disease**

Older style apple orchards were planted with rows and tree spacing of around 6 m (Plate 1.2) with trees pruned heavily and allowed to establish a production framework or scaffold over several years. Economic returns were not expected for around 5 years. In most production areas, since the 1980's, there has been a gradual shift to closer planting (Plates 1.1 and 1.3) enabling increased yield per unit area and full production earlier in the life of the orchard (Robinson and Hoying, 2002). Plantings utilise dwarfing rootstocks that produce smaller trees, with early and more abundant crops of consistent quality fruit, which in today's market is becoming increasingly important. Reduced tree size also allows easier access to trees for orchard management ultimately reducing the unit production costs (Webster, 2002).

Profitability of such orchards is critically dependent on rapid establishment of trees to fill their “allotted space” in the planting design, requiring uniform growth to produce a planned tree size. The design is invariably based on a tree size managed by rootstock, and apple replant disease imposes an uncontrolled influence on growth, making choice of rootstock more difficult, as well as causing difficulties with lack of uniformity.

**Plate 1.1 Apple Replant Affected Orchard**



Plate 1.1 Recently replanted orchard in the Tamar valley, northern Tasmania, the site had been previously planted with apples, following removal of original apple trees the field had been pasture for 22 years consequently apple replant disorder had not been considered likely and the site had not been treated prior to planting. Stunted trees can be observed at 3 m intervals corresponding to original tree spacing showing the “wave” effect often observed in apple replant disorder situations.

**Plate 1.2 Older Style Orchard**

Plate 1.2 Older style orchard in the Tamar Valley in northern Tasmania, trees spaced at 6m intervals with 6m inter-row distance.

The commercial approach to apple replant disease in Australia has generally been to allow 5 - 10 years of pasture fallow between plantings or simply to select a fresh site for each new orchard planting. Recently, increased land values, pressure on orchard land and higher infrastructure costs (irrigation, centralized packhouses, etc) has encouraged growers to re-use existing orchard land at decreasing fallow intervals. This has necessitated control or management of apple replant disease under the wide range of soil and climate conditions across the Australian industry. Options include accepted methods such as fumigation of soil along the tree lines, addition of the fertiliser mono-ammonium phosphate (MAP), organic matter amendment and apple

replant tolerant rootstocks used as a primary stock or as an understock. Other methods have also been tried, usually with limited success. Given the high cost of orchard establishment, growers have tended to opt for the most reliable control measure, which is soil fumigation with methyl bromide or a similar material.

**Plate 1.3 Higher Density Modern Style Orchard**



Plate 1.3 Higher density planting at an orchard in Huon Valley, southern Tasmania demonstrating the more recent high density planting currently utilised by many growers.

#### **1.4 The Montréal Protocol**

World wide co-operation for protection of the stratospheric ozone layer began with negotiations leading to the Vienna Convention for the Protection of the Ozone Layer 1985. This agreement was further refined in the Montréal Protocol on Substances

that Deplete the Ozone Layer 1987, becoming effective in 1989. In London 1990 parties to the Protocol agreed to a phase out of controlled substances and this was accelerated in Copenhagen 1992. Substances included chloro-fluoro hydrocarbons, halons, carbon tetrachloride, methyl chloroform, hydrochlorofluorocarbons, hydrobromofluorocarbons and methyl bromide. (afeas.org web site). In developed countries, a total phase out of methyl bromide was due by 1<sup>st</sup> January 2005. While several developing countries have not yet agreed to the phase out those developing countries that are parties to the Montréal Protocol are expected to completely phase out the use of methyl bromide by 2015. As result of the Montréal Protocol, world wide use of methyl bromide has greatly reduced (Martin, 2003). In the short term, alternative sterilants including Telone C35<sup>®</sup> containing 35% chloropicrin, methyl-isothiocyanate based products including Basamid<sup>®</sup> and Metham<sup>®</sup> and a number of new products including methyl iodide and propargyl bromide are likely to replace methyl bromide for soil fumigation (Porter and Mercado, 2001).

There are provisions in the treaty of for the use of methyl bromide in specific applications such as quarantine purposes where the fumigant can be recovered so reducing escape into the atmosphere. Additionally there is provision for Critical Use Exemption under which affected industries may apply for an exemption under appropriate conditions where viable alternatives are unavailable (Martin, 2003).

## **1.5 Conclusion**

With the phase out of methyl bromide growers need an alternate management measure that provides the certainty of tree growth demanded by current orcharding systems. Alternative fumigants have been trialled, recommended and entered commercial use, but all present some difficulties as industry moves to reduce the use of high environmental impact chemicals. Thus, in the longer term, there is a need to move to a more environmentally sustainable management system based on an

improved understanding of the soil and plant response mechanisms involved in the condition.

Consequently initial trials examined effects of various commercial and semi-commercial antagonists to possible soil disease organisms in field and pot trials, with responses compared with conventional control measures including methyl bromide and mono-ammonium phosphate. Isolations from orchards and pot trials were also examined for antagonist activity. In this latter part of the study, aspects of the apple replant disease complex itself were also included in two small trials designed to investigate impacts on growth and development of young trees in the first growing season.

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## Chapter 2 Literature Review

### 2.1 Introduction

The term “replant disease” or “replant disorder” is a general one used for a range of usually vegetatively propagated cropping species. Its use often reflects the lack of detailed understanding of why a newly planted crop does not grow and yield according to expectation for the species, cultivar and site. Consequently it is not applied to situations where a known root pathogen builds during the life of a crop necessitating a period of fallow or crop rotation before replanting. Literature on replant diseases of various species is extensive but this review concentrates mainly on apple replant and particularly on what has been termed “specific apple replant” disease or disorder by Brown and Schimanski, (2000a) and others. Whilst two major classifications, specific apple replant and non-specific apple replant diseases, have been suggested (Ibid.) it is the “specific” type of greatest interest and economic importance to growers.

Specific apple replant disease only affects apples (*Malus domestica*) when planted in a location previously with apples (Brown and Schimanski, 2000b, Brown, *et al.* 2000), it has been attributed to a variety of biotic influences, mainly unspecified pathogens, and abiotic factors such as pH, phytotoxins, soil structure, and heavy metal contamination. The suspected cause has varied with region, even between different orchards in the same region. While the underlying cause has never been characterised it is probable that there are many factors involved (Brown, *et al.* 2000), however the growth response to soil sterilisation or fumigation confirms a biological component is involved in the disorder (Granatstein and Mazzola, 2000).

Non-specific apple replant disease affects apples replacing other fruit crops, such as stonefruit. The main biological component is thought to be plant pathogenic

nematodes which can be controlled with the application of a nematicide or aggressive fumigant prior to planting, but factors including degradation of soil structure, pH and agricultural chemicals and metal contamination may also be involved (Brown, *et al.* 2000).

Throughout this thesis the term apple replant disease, or the acronym ARD, is used for the disorder arising from a previous planting of the same species: specific apple replant disease or disorder according to these definitions.

## 2.2 Possible Causative Factors

No one causal agent has been found to produce apple replant disease, given that specific apple replant disease only occurs in locations previously planted with apple (Mazzola, 1998, McKenry, 1999). Exposure to apple roots (Mazzola, 1998) and likely interactions within the ecosystem and the micro-organisms associated with the previous planting (McKenry, 1999) are thought to be factors. This biological component is usually said to be confirmed by positive growth responses to soil sterilisation, pasteurisation or fumigation.

A range of potential fungal pathogens *Phytophthora*, *Pythium*, *Fusarium*, *Rhizoctonia* and *Cylindrocarpon* have often been found associated with apple replant. A complex of these has been suggested as a possible cause or contributor (Mazzola, 1998; Mazzola, 2002), but in some locations fungicides had little impact suggesting the causal agent may not be a fungus (Mazzola, 2002, Brown, *et al.* 2000). A number of bacterial groups have also been suggested as causing the disease including *Bacillus* sp. and *Pseudomonas* sp. and there is speculation that in some locations actinomycetes may play a role (Brown, *et al.* 2000; Locci, 1994; Otto, *et al.* 1993). Thus, while pathogens may represent an important and specific component of apple replant disease, there is likely to be a much more general component present (McKenry, 1999).

Given no underlying causal agent has been found, but with the same microbial species frequently found across different locations, it has been suggested that these could be secondary invaders and not the causal agent (Mazzola, 1998, Brown, 2000).

In a detailed review, McKenry (1999) suggested that apple replant disease consisted of at least four distinct but interlocking components. These are: the rejection component; the soil physical and chemical components; the soil pathogens and pests; and the initial nutritional needs component. Each component may not be present at every site so the presentation of apple replant may vary at different locations. The rejection component may be caused by an ecosystem of diverse microbes or metabolites of microbes that are inhospitable to new introductions into their ecosystem. The most successful means of treating this seems to be manipulating the soil system followed by the use of broad spectrum biocides. Fumigation destroys the rejection component of apple replant but it also destroys a diverse range of micro-organisms in the soil making it difficult to establish the causal agent involved. This component could be attributed to specific micro-organisms surviving on old roots and in the soil, but could also be due to interactions within the overall ecosystem accentuated by high populations of micro-organisms associated with the previous planting.

The second component involves the physical characteristics of the soil including structure, hardpans, plough pans or soil lenses or chemical characteristics including accumulation of salts, herbicide residues or other chemicals.

The third component is the presence of known pathogens and pests in the soil, and these are best treated prior to planting with methods such as fumigation. The negative aspect of broad spectrum biocides is that they also kill off beneficial microbiota which can have an adverse effect on long term pest or disease suppression. The use of resistant rootstocks can also give some long term protection. Rootstock resistance is often specific to a pest or disease and does not

guarantee protection against the wide range of potential pathogens that may be present. There is also potential for resistance to be countered by the development of new pathogen biotypes.

The fourth component relates to the nutritional requirements of the new trees which may be impacted upon by a change in the soil ecosystem. Newly planted bare rooted plants have been found to benefit from receiving trace amounts of a broad range of micro and macro nutrients and soil fumigation has been shown to produce an increase in nutrient levels.

Overall the cause of apple replant disease has remained elusive for decades. Whilst recent publications, such as those by McKenry and Mazzola and coworkers referred to above, have moved more towards the notion of a soil/plant complex rather than single causative factor there is little indication of the components or their mechanisms of interaction.

### **2.3 The Soil / Plant Complex**

The soil biological community is complex (Bever, 2003). While the cause of the apple replant disease has not been fully elucidated, regardless of whether it is a single or multi-factor syndrome, its aetiology is inevitably the result of interactions among soil micro-organisms (Catská, 1988). Consequently it will be widely influenced by soil microbial balance with potential to suppress soil pathogens (Manici, *et al.* 2003). A number of potentially pathogenic fungi have been proposed as playing a role in this complex and a consistency in the composition of the microbial population suspected of contributing to the disease has been observed (Mazzola, 1998).

Disease suppressive soils have also been observed to influence a number of soil borne plant diseases including apple replant. Short term wheat cropping has been found to suppress some aspects of the fungal complex (potentially similar to

apple replant) and result in increased growth (Mazzola and Gu, 2000a; Mazzola and Gu, 2000b) implying that apple replant is associated with the overall soil community.

### **Bacteria**

Many bacterial species have been found in association with apple trees affected with replant and could be implicated as playing a role in the disease (Mazzola, 1998). Reported species have included unspecified *Pseudomonas* spp., *Pseudomonas pudita* and *Pseudomonas fluorescens* with changes in the populations of fluorescent *Pseudomonas* sp. having been found associated with apple orchards. Additionally some *Flavobacterium*, *Citrobacter*, *Enterobacter*, *Klebsiella*, *Achromobacter*, *Arthrobacter* and *Chromobacterium* spp. are commonly encountered and these can be associated with the deleterious rhizobacteria phenomenon that causes crop yield losses without an obvious pathology, though deformed root hairs and increased susceptibility to root diseases are associated with this condition.

### **Fungi**

Studies conducted in Washington State USA (Mazzola, 1997; Mazzola, 1998) found a fungal complex including: *Cylindrocarpon destructans*, *Phytophthora cactorum*, *Pythium* spp. and *Rhizoctonia solani* were consistently found to be associated with trees showing symptoms of replant in all orchards examined and it was concluded that this fungal complex was the primary cause in the disease development.

Similar studies conducted in Poland (Kowalik, 1999), found a similar range of pathogens or potential pathogens including: *Alteraria alternaria*, *Cylindrocarpon destructans*, *Fusarium culmorum*, *Fusarium heterosporum*, *Fusarium oxysporum*, *Rhizoctonia solani*, *Verticillium albo-atrum* and *Verticillium nigrescens*. The

abundance and composition of soil fungi gradually increased during three successive seasons and it was suggested that apple replant disease may be due to the effect of the accumulation of soil pathogens colonising the rhizosphere of the replanted tree.

### **Actinomycetes**

Actinomycetes have attracted particular attention since around 1990, (eg Szabó, 1999). Apple trees are said to have a generally low tolerance for actinomycetes, with some species known to be pathogenic and high infection with actinomycetes has been observed in trees showing replant symptoms. It has been proposed that these are the cause of specific apple replant disease (Catská, 1988; Catská, 1994; Szabó, *et al.* 1998; Szabó, 1999), that a combination of phytotoxic bacteria and actinomycetes (Stern, *et al.* 1991) or that soil borne pathogens: fungi, actinomycetes and saprophytic phytotoxic micro-organisms are responsible for the condition (Biró, *et al.* 1998).

### **Mycorrhiza**

More than 90% of terrestrial plants form mycorrhizal associations with root colonising fungi (Strack, *et al.* 2003). The more common of the two major groups of mycorrhizae are the arbuscular mycorrhizae, found in association with 80% of plant species, mostly angiosperms, along with some gymnosperms, pteridophytes, lycopods and mosses (Smith and Read, 1997). Fossil records and molecular data suggest arbuscular mycorrhizal fungi originated at least as early as the Ordovician period some 450 - 500 million years ago (Remy, *et al.* 1994; and Redecker, *et al.* 2000). The symbiotic relationship may have assisted plants in colonisation of the terrestrial environment facilitating water and nutrient, particularly phosphorous, uptake (Simon, *et al.* 1993). Influx of phosphorous into roots colonised by mycorrhizal fungi can be three to five times higher than in

non-mycorrhizal roots (rates of  $10^{-11}$  mol m<sup>-1</sup>s<sup>-1</sup>) (Smith and Read 1997 cited by Schachtman, *et al.* 1998)

The other main group is the ectomycorrhizae, which evolved more recently with a suggested origin between 353 - 462 million years ago, coinciding with the late Ordovician and Devonian periods (Simon, *et al.* 1993). These comprise the Zygomycetes, Ascomycetes and Basidiomycetes and are adapted to environments rich in organic material (Strack, *et al.* 2003).

Arbuscular mycorrhizal fungi are common soil micro-organisms, which form symbiotic relationships with roots of many plant species, colonising both within and around the root system and forming structures known as arbuscules in the roots. These structures act as a bridge between the roots and surrounding soil (Toro, *et al.* 1997). The fungi are also known to reduce pathogen associated root problems and plant disease (Giananazzi-Pearson and Giananazzi, 1983; Smith and Read, 1997; Strack, *et al.* 2003) and reportedly increase tolerance to water stress (Kaushik and Dabas, 2003).

Under natural conditions mycorrhizae form a permanent network, which often do not persist under arable cropping, and mycorrhizal associations have to become re-established with replanting. This could leave newly transplanted plants susceptible to unfavourable conditions or pathogens.

Interactions between vesicular-arbuscular mycorrhizal fungi and apples growing in apple replant disease soils have been speculated upon, but not widely studied (Taube-Haab and Baltruschat, 1993). Apple rootstocks are likely to establish mycorrhizal relationships while in stoolbeds (Brown, 1998), but the introduction of young trees into a new environment carrying the biota of a previous orchard may disrupt the association. While the soil acts as a “biological buffer”, such that changes in the microbial population are temporary (Bashan, 1999) changes in the

rhizosphere could be conducive for the development of specific and or non-specific apple replant disease or other conditions.

One year old apple rootstocks (cv. M26) inoculated with *Glomus macrocarpon* had significantly increased growth at the lowest rate of phosphorous application (20mg elemental P per kg soil) (Wang, *et al.* 2001). In this study, uptake of Cu and Zn was also increased and it was concluded that apple stocks utilise vesicular-arbuscular mycorrhizae to assist in P-uptake, at least in low-P soils. In high-P soils the plants may profit from improved uptake of Zn and Cu.

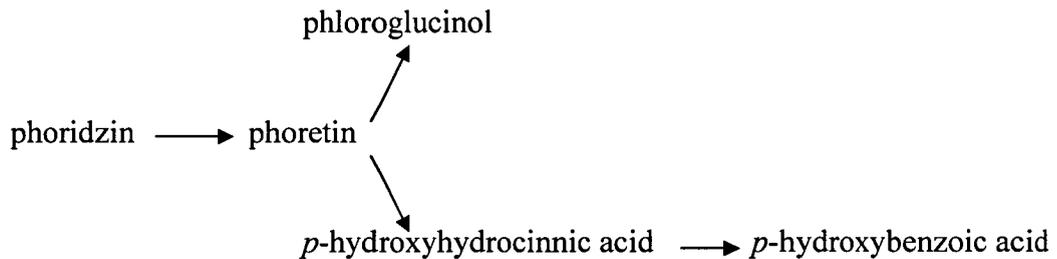
Colonisation by vesicular-arbuscular mycorrhizal fungi have been shown to increase root survival in roots exposed to known pathogens. Mechanisms may include improved phosphorous nutrition, which reduces root exudation, thus making the roots less attractive to root diseases (Graham, 1988; Stroble and Sinclair, 1992) as well as direct initiation of plant defence compounds (Benhamou, *et al.* 1994).

### **Toxins**

While not conclusively demonstrated that substances released from apple residues into the soil are the cause of specific apple replant disease it has been found that such residues can produce reduction in the growth of apple seedlings, (Börner, 1959). Five phenolic compounds were found present in water extracts of soil, of these phloridzin could be identified as a natural constituent of bark and wood of apple roots, the remaining compounds were found to be breakdown products produced by micro-organisms in the soil and water.

With the exception of phloroglucinol all inhibited root growth while mainly phloridzin and phloretin had an inhibitory effect on stem length (Börner, 1959; Börner 1960). Phloridzin however, under field condition concentrations has been found not to produce inhibition to apple roots suggesting that if specific apple

replant disease is produced as a result of toxins then these would be microbially produced rather than coming from apple roots (Hudska, 1988).



(Börner, 1959; Börner, 1960).

## 2.4 Prevention and Treatments – fumigants and sterilants

Currently the only consistently effective means of preventing the development of symptoms of apple replant using soil treatment is by sterilisation, pasteurisation or the use of aggressive sterilants including methyl bromide, trichloronitromethane (chloropicrin) (Martin, 2003), 1,3-Dichloropropene (tradename Telone<sup>®</sup>) (Dow, AgroSciences), metham sodium (Anon, 1998) and Basamid<sup>®</sup> (Brown and Schimanski, 2002a and b). Telone has been applied alone or in formulations with chloropicrin as Telone C17<sup>®</sup> containing 17% chloropicrin and the recently approved in Australia for orchard use Telone C35<sup>®</sup> containing 35% chloropicrin.

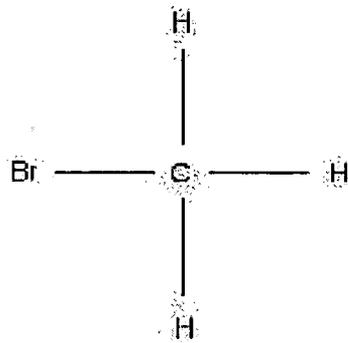
Other possible methods proposed to control apple replant include: introduction of fresh soil (Wilson, *et al.* 2004) mono-ammonium phosphate fertilisation (Nielsen and Yorston, 1991), correction of potassium deficiency (Merwin and Stiles, 1989), planting hole amendments including combinations with fungicides and peat (Nielsen, *et al.* 1994), formaldehyde (Daemen, 1994), Mancozeb<sup>®</sup> (Magarey and Bull, 1994), planting of antagonistic plants on apple ground (Edwards, *et al.*

1994), planting cover crops for orchards (Halbrendt, 1995) and utilising potential biological control agents (Catská and Taube-Haab, 1994). All of these have however given inconsistent results with positive and negative results reported in the published literature. Consequently any effective alternate soil treatment as effective and reliable as the aggressive fumigants will likely need to be integrated into a combined approach within the context of overall orchard management (Granastein and Mazzola, 2000).

Fumigation with agents such as methyl bromide and chloropicrin while reducing pathogens loads has also been found to produce improvements in root health, growth and fruit yields but the mechanism involved has not been fully established. This growth response may be connected to a temporary inhibition of nitrification and increased levels of nitrogen in soil, though other changes in soil microbiology may be of significance. Fumigation with aggressive fumigants does not sterilise soil, studies on strawberries found significant populations of bacteria survive and roots were rapidly recolonised with a range of *Pseudomonas* spp. (Duniway, 2002). Consequently root colonisation with beneficial bacteria could be a factor in the positive response associated with fumigation.

### **Methyl Bromide**

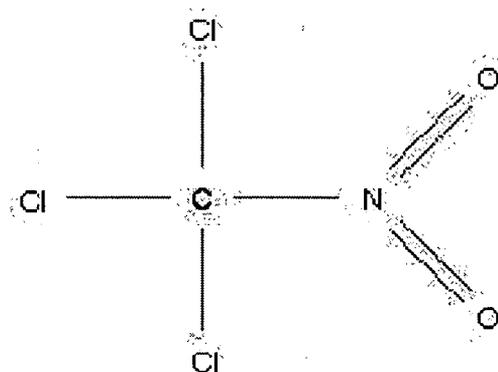
Because of its broad spectrum of activity towards bacteria, fungi, nematodes and a broad range of weeds, methyl bromide (structural formula below) has been used as a pre-plant fumigant for over 40 years. High volatility, allowing effective penetration through the soil, cost effectiveness, relatively short with-holding periods and consistent results have lead to it becoming the predominantly used fumigant within the apple, other industries and quarantine applications (Duniway, 2002; Martin, 2003).



Application is by injection into the soil using a modified spring tined implement like a harrow or cultivator and the soil is immediately covered /sealed with plastic sheeting for 5-6 days. The plastic is then removed a few days before planting to let any residual methyl bromide dissipate.

### **Chloropicrin**

Chloropicrin, trichloronitromethane (structural formula below) has been widely used as a pre-plant fumigant. It has a significant anti fungal action but is less effective against nematodes and weeds than methyl bromide (Duniway, 2002).



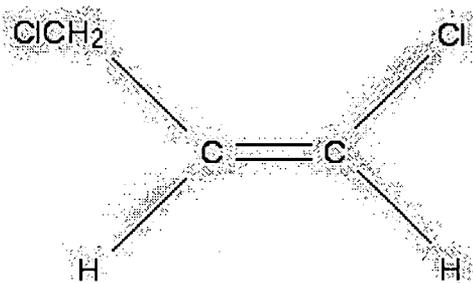
Chloropicrin is commonly applied in combination with other fumigants including methyl bromide, Telone<sup>®</sup> and methyl isothiocyanate generators in order to broaden its effectiveness (Martin, 2003). In field applications chloropicrin is subject to microbial degradation (Gan, *et al.* 2000) and has been observed to have a relatively short half life of one day (Ajwa, *et al.* 2002). Chloropicrin additionally has the practical disadvantage of being a powerful mammalian irritant, irritating the eyes, lungs and mucus membranes and presents a risk for users. Application either injection in gaseous form as with methyl bromide or a more recently developed emulsifiable form (TriColor, Shaddow Mountain Products) applied via drip irrigation.

### **Telone<sup>®</sup>**

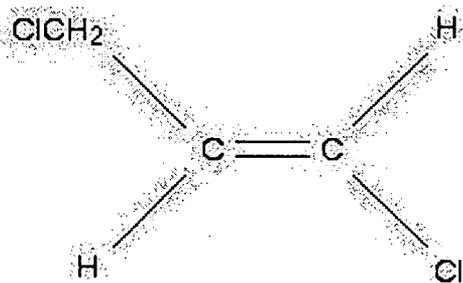
The agent 1,3-Dichloropropene is present as two isomers, (structural formulae below) the *cis* - isomer is an effective nematicide and is registered as a fumigant under the tradename Telone<sup>®</sup> (Dow, AgroSciences).

### Isomers

*CIS*



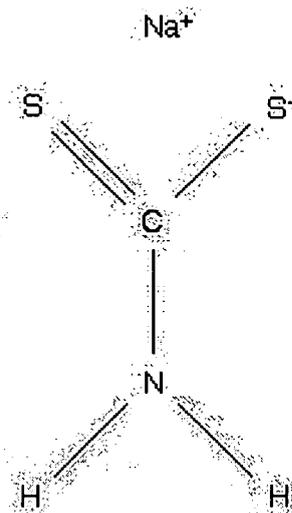
*TRANS*



Additionally it has some fungicidal properties and can be applied alone or mixed with chloropicrin. Although normally injected into soil in gaseous form similar to both methyl bromide and chloropicrin, recent product releases include combinations with chloropicrin in an emulsified form containing 33% chloropicrin that can be applied via drip irrigation lines (Martin, 2002). Telone C35<sup>®</sup> is a mixture of Telone<sup>®</sup> and 35% chloropicrin that has recently been approved for orchard use in Australia

### Methyl Isothiocyanate

Methyl isothiocyanate (structural formula below) is a liquid reaction product commonly used as a fumigant. The most common parent compound is metham sodium which reacts when added to soil to produce methyl isothiocyanate. The chemical acts in the liquid phase does not move through the soil as effectively as methyl bromide and the other gaseous fumigants and consequently inconsistent results are common (Anon, 1998; Martin, 2003).



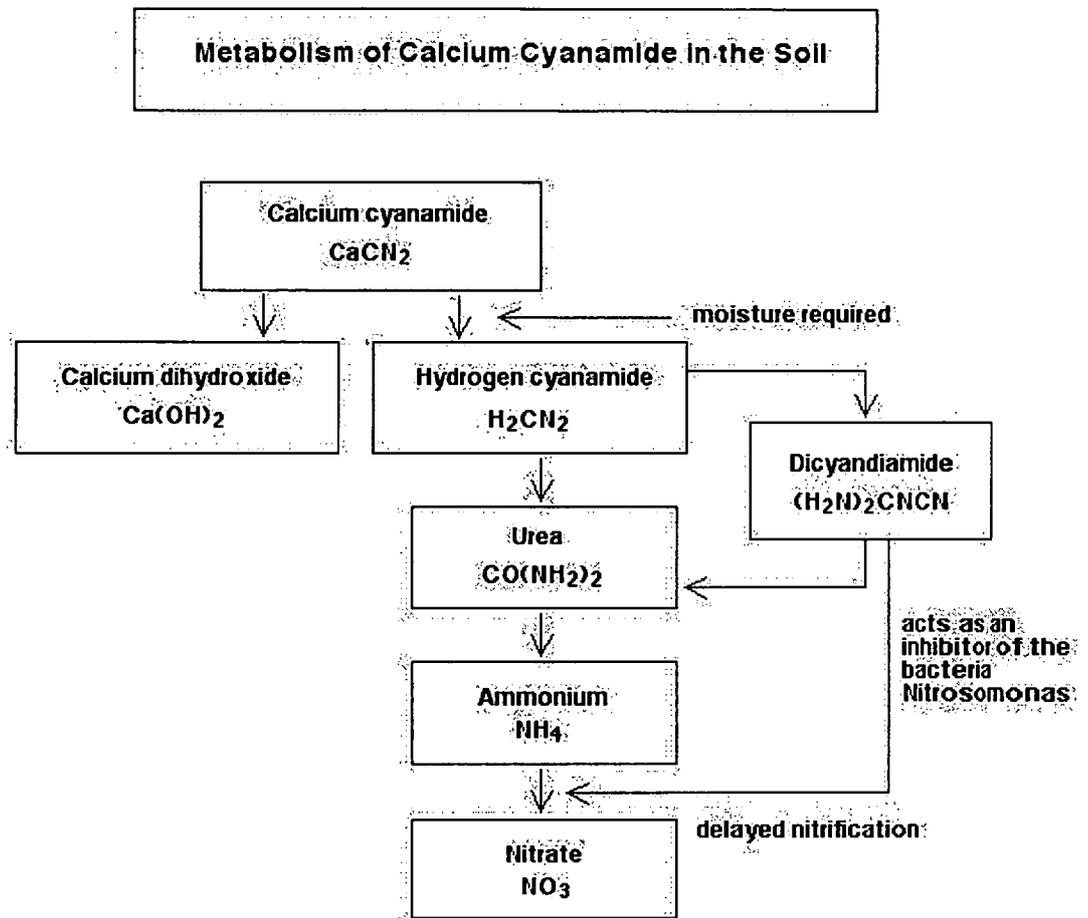
Methyl isothiocyanate can also be generated using the granular product Dazomet, known by the tradename Basamid<sup>®</sup>. The active ingredient is tetrahydro-3,5,-dimethyl-2H-1,3,5-thiadiazine-2-thione and the manufacturer, BASF Corporation, recommends its use for control of a range of fungi, weeds, bacteria and nematodes (BASF Corporation. 1998). A problem of distribution within the soil can be encountered with effective application requiring thorough mixing to ensure even distribution (Martin, 2003). The effectiveness of Basamid<sup>®</sup> for treating apple replant soil has not always been reliable in Tasmania (Brown, and Schimanski, 2002a), though field trials conducted recently in southern Tasmania (Brown, and Schimanski, 2002 a and b) has demonstrated that it can provide suitable control of apple replant when used in the appropriate manner. Basamid<sup>®</sup> can be phytotoxic (BASF Corporation. 1998) and its use in managing apple replant may require changes in management strategies with a suitable withholding period before replanting.

## Calcium Cyanamide

Calcium cyanamide known by the tradename PERLKA<sup>®</sup> has been used in Europe as a nitrogenous calcium fertiliser that can counter weed and pest build up over time. In soil, a reaction sequence resulting in a transient release of hydrogen cyanamide, produces some sterilant activity.

As shown below in the diagram taken from Klasse (2007), when moisture is added calcium cyanamide undergoes hydrolysis forming hydrogen cyanamide and calcium oxide (lime). Hydrogen cyanamide is present for a short period before undergoing further hydrolysis to form urea, ammonium and finally nitrate. While present, hydrogen cyanamide is toxic to germinating seeds, fungi, nematodes and insects in the soil. Additionally a dimerised isomer, dicyandiamide, is also formed and this acts as an inhibitor of nitrifying bacteria, which can delay nitrification for several months (Cathcart, 2003).

Microbial sensitivity to hydrogen cyanamide differs between species present. Some *Phytophthora* spp. have been found to be very sensitive and *Fusarium* spp. although more tolerant, have also been controlled under greenhouse conditions. Some non pathogenic fungi are able to tolerate hydrogen cyanamide and some, in particular *Aspergillus* spp. and *Penicillium* spp., can utilise it as a nitrogen source for cellulose degradation. It is perhaps notable that isolates from both these groups have been utilised as biocontrol agents (Klasse, 2007). A combination of calcium cyanamide, promoting suitable conditions with the incorporation of straw followed by solarisation has been found to provide a successful alternative to methyl bromide for soil fumigation under glasshouse conditions (Bourbos, *et al.* 1997).



## Nematicides

There are three fumigants registered in various countries for use as pre-plant nematicides in apple orchards. These are sodium methylthiocarbamate, (1,3-dichloropropene), a 1,3-dichloropropene and chloropicrin formulation and methyl isothiocyanate. In addition two post-planting nematicide sprays are available for use where high populations of plant parasitic nematodes are found in a young orchard, these being 3 ethyl 3-methyl-4-(methylthio)phenyl (1 methylethyl) phosphoramidate (sold as Nematicure<sup>®</sup>) and L methyl N'N'-dimethyl-

N-[(methyl carbamoyl)oxy]-1-thiooxamimidate (sold as Vydate®) (Koehler, 2000).

### **Biofumigants**

Biofumigation was first recorded by Theophrastus in 300 BC involves the use of naturally occurring volatile chemicals, allelochemicals, from plant tissues mainly from the order *Capparales*. The group includes the families *Tovariaceae*, *Resedaceae*, *Capparaceae*, *Moringaceae* and in particular *Brassicaceae* (Fenwick, *et al.* 1983; Brown and Morra, 1997; Rosa, *et al.* 1997). Traditionally these have often been part of a green manure rotation system (Bianco, *et al.* 2001; Mattner, 2001) for control or suppression of soil-borne pathogens and pests (Bianco, *et al.* 2001). These allelochemicals are the hydrolysis products of a group of sulphur containing chemicals produced as secondary metabolites known as glucosinolates. They vary between species and the type of tissue but are consistent within species and consistently occur in conjunction with a hydrolytic enzyme thioglucosidase hydrolase, otherwise known as myrosinase (Rosa, *et al.* 1997).

Released following tissue damage, myrosinase enzymes hydrolyse the glucosinolates to form isothiocyanates (ITCs), nitriles and oxazolidinethiones. The isothiocyanates, regarded as the most biologically active (Brown and Morra, 1997; Rosa and Rodrigues, 1999), are of similar chemical structure to the active ingredient produced by the commercial fumigants metham sodium and methyl isothiocyanate, acting in a manner similar to soil fumigants. They can be toxic to fungal pathogens including: *Phytophthora*, *Pythium*, *Fusarium*, *Rhizoctonia*, *Colletotrichum*, *Gaeumannomyces* (Kirkegaard, *et al.* 1996), *Aphanomyces euteiches* (Smolinska, *et al.* 1997), *Pythium ultimum* and *Sclerotium rolfsii* (Gamliel and Stapleton 1993), can produce varying effects on fungal and bacterial populations, and have nematicidal, insecticidal and phytotoxic effects (Brown and Mora, 1997). Their natural function is thought to be as part of a

defence against insects and pathogens (Bianco, *et al.* 2001; Brown and Morra, 1997; Matthiessen and Kirkegaard, 2006). Their effects could be due to microbial suppression and, from their variable effects, changes in the rhizosphere microbial community (Smith and Kirkegaard, 2002). Field experiments with wheat grown after canola (*Brassica napus*) and Indian mustard (*Brassica juncea*) produced greater growth than those grown after other intermediate crops including oats (*Avena sativa*) or linseed (*Linum usitatissimum*) (Angus, *et al.* 1991; Kirkegaard, *et al.* 1994). The level of pest or disease suppression has been linked to varying concentrations of glucosinolates present, which varies with the species of *Brassica* grown, and the age and part of the plant (Mojtahedi, *et al.* 1991). Other compounds including carbon-disulphide, dimethyl-disulphide, dimethyl-sulphide and methanethiol formed during decomposition of brassica tissues may also have allelopathic effects (Bending and Lincon, 1999).

Compounds released from undamaged *Brassicac*s during growth may also be suppressive of pests or diseases as demonstrated by Schreiner and Koide (1993) who showed that isothiocyanates released from roots of *Brassica kaber* (white mustard) and *Brassica nigra* (black mustard) were inhibitory to mycorrhizal fungi (see also Kirkegaard, *et al.* 1996).

Brassicacs can also have suppressive effects not associated with glucosinolates. It has been suggested that non-glucosinolate compounds are toxic to nematodes or that the incorporation of decaying Brassica tissue in soil results in increased levels of antagonistic organisms (Matthiessen and Kirkegaard, 2006). Brassicacs can also act as trap crops. Nematodes enter and develop within their roots but their sexual differentiation is disrupted producing very low numbers of females and consequently resulting in a population decline in subsequent generations. This has been used as a means of controlling sugar beet nematodes (*Heterodera schactii*) in northern Europe (Matthiessen and Kirkegaard, 2006) and similar results have also been observed with nematodes associated with non-specific apple replant disease (Mazzola, *et al.* 2001). Brassica sourced material

containing isothiocyanates such as seed meal or oil can also be used as soil amendments and these have been found to control *Rhizoctonia solani* and *Pratylenchus penetrans* in apple replant soil (Mazzola, *et al.* 2001 and references therein).

Any crops acting as green manure provide organic matter that can also influence microbial populations. Such changes may lead to disease suppression as well as altering soil structure, reducing subsoil compaction, lessening erosion, improving water penetration all of which could improve future crop health and possibly reduce requirements for artificial fertilisers. Brassicas, in addition to their biofumigation activity, have a combination of large tap root and fine root structure that can be advantageous in this regard (Pung, *et al.* 2004) and consequently contribute to overall crop management.

Practically, biofumigants may not replace aggressive fumigants such as methyl bromide, but could have potential as part of an integrated management strategy (Stephens, 1999). They can be phytotoxic to following crops with a report by Granatstein and Mazzola (2000), quoting unpublished data showing that canola (*Brassica napus*) seed meal caused damage and death to Gala on M.26 rootstocks.

## **2.5 Prevention and Treatments – soil amendments**

### **Mono-ammonium Phosphate**

Freshly transplanted trees have impaired roots systems, and addition of highly available phosphate such as mono-ammonium phosphate can be beneficial for new plantings in both non-replant (Schupp and Moran, 2002) and replant soils (Neilsen, 1994). Phosphorous is an important plant macronutrient, coming second after nitrogen as the common limiting macronutrient for plant growth (Schachtman, *et al.* 1998) and is a key component in compounds such as nucleic

acids, phospholipids and ATP. Consequently it is an essential nutrient for growth, involved with regulation of metabolic pathways and key enzyme reactions (Theodorou and Plaxton, 1993).

Phosphorous is generally very immobile in soil and uptake can present problems for plants. The preferred form for assimilation is orthophosphate, which is not readily accessible to most plants due to adsorption to clay minerals, conversion to organically bound forms or formation of insoluble precipitates with iron, aluminium and calcium (Holford, 1997). The movement of orthophosphate through the soil is mainly due to diffusion, which, being relatively slow, can together with its low availability, result in a zone of depletion around plant roots and so limit uptake (Rausch and Bucher, 2002).

The form in which orthophosphate exists in solution varies with pH, and the pKs for the dissociation of  $\text{H}_3\text{PO}_4$  into  $\text{H}_2\text{PO}_4^-$  and  $\text{HPO}_4^{2-}$  are 2.1 and 7.2 respectively. Consequently at less than pH 6.0 most orthophosphate is present as  $\text{H}_2\text{PO}_4^-$  while  $\text{H}_3\text{PO}_4$  and  $\text{HPO}_4^{2-}$  will be present in smaller proportions. The pH range in which higher plants take up orthophosphate is between 5.0 and 6.0 where  $\text{H}_2\text{PO}_4^-$  is the dominant form suggesting that orthophosphate is taken generally up in this form (Ullrich-Eberius, *et al.* 1984; Furihata, *et al.* 1992).

Mono-ammonium phosphate  $\text{NH}_3\text{H}_2\text{PO}_4$  (MAP 11-55-0) addition has been found to produce increased growth, flower production and fruit set in the initial years following planting for apple (Schupp and Moran, 2002). Results have been consistently positive in most situations (eg Sewell, *et al.* 1988; Slykhuis and Li, 1985), but there have been a few reports, including Peryea (1990), Olszewski (2001) and Utkhede (1998), finding no positive effect. These results have suggested that factors including existing phosphorous levels, application rate, pH, moisture and even weather conditions can influence the efficacy of mono-ammonium phosphate application (Nielsen, 1994; Peryea, 1997).

Wojcik and Wojcik, (2007) suggested that vegetative and reproductive responses in apple trees to phosphorous fertilization is related to phosphorous availability rather than phosphorous levels. Mono-ammonium phosphate also acidifies the soil (Wojcik and Klamkowski, 2005) and can cause osmotic stress, which can result in injury or death of trees (Peryea, 1997; Wilson, *et al.* 2004). Additionally mono - ammonium phosphate provides nitrogen which may also influence tree growth, and it has been further suggested that the uptake or utilization of phosphorous may be more efficient in the presence of ammonium present as mono-ammonium phosphate (Schupp and Moran, 2002).

The use of mono-ammonium phosphate in combination with a fumigant or fungicide is common practice for replant situations (Nielsen, *et al.* 1990; Nielsen and Yorston, 1991). Under these circumstances, application of a readily available form of phosphate may counter the loss of potentially beneficial mycorrhiza through fumigation and provide a readily accessible source of phosphate.

It was suggested, by Sewell, *et al.* (1988), that there is a relationship between soil phosphorous levels, the growth response of transplanted apple nursery stock to soil fumigation and mycorrhizal associations. It has been suggested that phosphorous requirements for apple trees may be greater during the first three years than for mature trees and that this may be due to development of plant mycorrhizal associations (Wojcik and Klamkowski, 2005). Given the nature of plant microbe and in particular mycorrhizal associations and the role these play in phosphate acquisition, the effectiveness of mono-ammonium phosphate may be due to changes in soil biology rather than a direct effect on nitrogen and phosphate nutrition of trees (Wilson, *et al.* 2004).

## Organic matter and soil replacement

It has long been known that soil organic amendments such as animal manures, green manures, can provide some control of diseases caused by soil borne pathogens. Prior to the introduction of commercial fertilisers and the broad spectrum fumigant methyl bromide, these, in conjunction with crop rotation and in some cases steam pasteurisation, were the principle means of controlling soil borne diseases. Such amendments, together with composts, biocontrol agent fortified composts or fresh soil (Moran and Schupp, 2005) could provide potential control of diseases, insects and weeds when combined with herbicides and appropriate cultural practices (De Ceuster and Hoitink, 1999). Soil microbial populations have been found to increase following compost amendment. Mature composts can contain a high population and complex community, while immature composts contain lower populations and may serve as a substrate for existing soil microbiota. Knowledge of the mechanism by which such amendments provide disease suppression is limited, though there is evidence for microbial consortia rather than a single organism being involved. Many of these species exhibit biocontrol capabilities and could have potential as biocontrol agents or inoculants for microbially amended composts (Nelson and Boehm, 2002b).

The ability to control potential pathogens varies with the type and maturity of compost used as well as the target pathogen (Blok, *et al.* 2002). Most weeds and insect pests are not controlled with composts (De Ceuster and Hoitink, 1999) and additionally there have been few documented reports on the successful use of compost in the management of apple replant disease (Yao, *et al.* 2005). The effects of organic matter on apple replant disease may be due to a physical isolation of initial root growth from surrounding replant soil or simply a dilution of the causal agent(s), rather than any changes in soil microbiota (Wilson, *et al.* 2004).

Removal of replant soil and replacement with several litres of fresh soil has been used as an effective control measure (Wilson, *et al.* 2004), but there is no clear indication whether this is a simple dilution effect, or whether there is a more complex mechanism. As the method clearly protects the freshly planted tree only until roots extend beyond the replacement soil, its effectiveness raises questions about the role of tree physiology in the disease syndrome. In particular, why does a tree that is able to commence growth without exposure to replant soil able to tolerate any negative impacts later in the growth cycle.

### **Biological “Control”**

Biocontrol may be seen as a means of controlling pests and diseases and is generally perceived as being safer than pesticide use. There have, however, been few cases of commercially effective biocontrol and in most cases there is no patentable or marketable product, consequently limiting commercial interest (Campbell, 1994). As noted above, several groups of plants, most notably the *Brassicaceae* have been shown to control pests, diseases and nematodes (Mazzola, 2006). Populations of *Pratylenchus penetrans* within the root zone of apple rootstocks were also reduced after inoculation with *Glomus* sp (Forge, *et al.* 2001). A similar inoculation was found to reduce infection of roots by pathogenic fungi (Azcon-Aguilar and Barea, 1996).

A number of bacteria including *Bacillus* sp. and *Agrobacterium radiobacter* have shown some promise for biological control. *Agrobacterium radiobacter* suppressed some harmful groups of rhizosphere microorganisms including *Penicillium claviforme*, *Penicillium expansum*, *Penicillium griseofulvum* and *Alternaria alternaria* (Taube-Haab and Baltruschat, 1993). The authors also noted that efficient vesicular-arbuscular mycorrhizal strains alleviated “soil sickness”, suggesting possible use in the biological control of specific apple replant disease. Catská (1988) also observed improved growth of apple seedlings in replant soil treated with *Agrobacterium radiobacter* and *Bacillus subtilis*.

A number of commercially available biocontrol agents such as *Trichoderma harzianum*, (Trichopel<sup>®</sup>, Trichoflow<sup>®</sup>) *Trichoderma* spp. (Ramona, 2003), and a *Bacillus subtilis* strain (Companion<sup>®</sup>) are now available for treatment or management of a range of plant diseases. While these products are yet to prove a successful countermeasure for apple replant, they may provide part of an overall treatment strategy once the nature of specific apple replant disease has been more fully established.

Crop rotation, arguably a form of biological control, has been subjected to little detailed study for apple replant although it has been widely investigated for short term cropping. In greenhouse trials, planting orchard replant soils to certain wheat cultivars enhanced subsequent growth of apple trees (Mazzola and Gu, 2000). The authors attributed the effect to induced activity of resident soil microbial antagonists.

## **2.6 Prevention and Treatments – Rootstocks**

For many centuries the most common means of propagating apple and pear scion cultivars has involved budding or grafting onto suitable rootstocks, originally wild type seedling and later using seed collected from selected cultivars such as "Sturmer Pippin" for apple and "Bartlett" ("Williams") for pears. Seedlings are still used in some regions, but since the 1950's (or earlier), vegetatively propagated clonal rootstock have been preferred as they offer a means of controlling or managing scion growth or performance (Webster, 2003).

Super dwarfing, dwarfing and semi - dwarfing rootstock varieties have an additional advantage in that the scion varieties generally fruit earlier than on stronger stocks, often producing commercial crops by the second year. Additionally dwarfing varieties produce precocious, abundant and seasonally consistent cropping, and some, such as Malling 9, result in a larger average fruit size and slightly earlier ripening (Webster, 2003).

Resistance to soil borne pests and diseases is an important attribute of some rootstocks, but tolerance of apple replant disease is generally associated with the more vigorous rootstocks, such as Malling 793 and seedling, while the dwarfing varieties (such as Malling Merton 106 and Malling 26) preferred in modern orchards, are susceptible. One option to control tree size on a vigorous stock like Malling 793, is to use a size controlling interstock variety such as Malling 9.

Rootstock variety has been found to influence soil microbial populations. Specificity in the interactions between plant cultivar and arbuscular mycorrhizal fungi have been reported (da Mota, *et al.* 2002; Mazzola and Gu, 2000; Mazzola and Gu, 2002). In wheat, a genotype specific influence on the fluorescent pseudomonas population resulted in suppression of *Rhizoctonia* root rot. A similar relationship was observed between apple and the resident fluorescent pseudomonas population (Fazio and Mazzola, 2004). To date however there is no clear evidence to show whether rootstock based tolerance to replant disease is simply a function of induced scion vigour, or a more complex interaction with the soil conditions.

## 2.7 Conclusion

Almost all of the published literature on apple replant disease concentrates on possible disease organisms and their control. However, in spite of the wealth of information there is little conclusive evidence to implicate any one organism or complex of organisms. There are also certain issues around tree response that appear to invite investigation, but which have attracted little attention. One example is the apparent effect of replacement soil, resulting in successful control even though roots are exposed to the “disease” early in their growth as suggested above.

Another apparent anomaly is the effect on tree water status. There seems to be general acceptance (Willet, *et al.* 1994 and more generally Joseph, *et al.* 1998)

that one of the mechanisms of apple replant growth suppression is water stress. There are however few studies demonstrating such an effect and Nair (2003) found no evidence of water stress at the end of the first growing season.

## Chapter 3      Testing Commercial Antagonists and Other Biocontrol Agents

### 3.1 Introduction

A field trial, planted in freshly grubbed orchard soil, and a glasshouse trial, using plants potted into soil mixtures containing specific apple replant disease, were carried out. The objective was to evaluate effects on initial (first season) growth of materials marketed as biocontrol agents or pathogen antagonists against untreated soil and the standard control measures of mono-ammonium phosphate and fumigation with methyl bromide. Whilst commercial effectiveness needs to be demonstrated under field conditions where there is less control of the overall environment, field trials may present difficulties with confounding effects, preventing an objective evaluation of treatments. As soil may have marked effects on factors influencing growth other than apple replant disease, including weed germination and competition, soil structure and chemistry and other non-target organisms, the inclusion of pot trials was considered essential in spite of the difficulties associated with field soils used in potting mixes.

Field trials comparing fumigation and other treatments under commercial replant conditions have been reported from many apple growing areas. Locally, Wilson, *et al.* (2004) reported positive responses to mono-ammonium phosphate on a site replanted immediately after removal of an established orchard. Removal of an experimental (rootstock by pruning system) orchard from a similar soil type presented an opportunity to repeat this earlier work using a range of additional treatments including fumigation options and a range of potential antagonists and materials marketed as having some “biological” activity. Commercial fumigants Basamid<sup>®</sup> and Telone C35<sup>®</sup>, previously trialed locally by Brown and Schimanski (2002), were included along with methyl bromide. Biocontrol agents included a commercially available *Trichoderma* spp. isolate marketed as Trichopel<sup>®</sup> and a

local non-commercial isolate with known (Ramona, 2003) antagonist effects against soil borne pathogens. A local *Bacillus* sp. with similar antagonist potential was also obtained from the same laboratory. These treatments were combined with various organic matter amendments, mono-ammonium phosphate alone and in combination, and calcium cyanamide. The latter, marketed under the trade name Perlka<sup>®</sup>, had not been included in previously reported local trials on apple replant disease.

Following difficulties with weed control and the effect of weed competition in this field trial a greenhouse trial was designed with similar set of treatments. Two commercially available *Trichoderma* spp. preparations (Trichopel<sup>®</sup> and Trichoflow<sup>®</sup>) were included, along with a commercial *Bacillus subtilis* preparation marketed as Companion<sup>®</sup>. These were compared with the range of conventional treatments including the three fumigants trailed previously, mono-ammonium phosphate and organic matter amendment. Calcium cyanamide was tested again, this time with an “incubation” period to allow for any potential changes in soil microflora that are claimed from promotional literature. Soil was available from the same site as the field trial and this was collected at the end of the first season after the original orchard was removed.

### 3.2 Field trial

#### Materials and Methods

The trial was conducted on a low fertility duplex soil originally classified as Huon Loam and currently classified as a brown sodosol (Wilson, *et al.* 2004) at the Department of Primary Industry Research Station at Grove in southern Tasmania. The area had been planted with a mixed variety orchard set out as a variety by rootstock by pruning system trial with trees spaced at 5 m by 3m. The

previous orchard had been in production for 15 years, with management following normal commercial practice for the region, including herbicide strips in the tree line. The present trial was planted immediately after grubbing with the new planting following the previous tree lines to maximise both level and uniformity of exposure to apple replant disease.

After the previous trees had been lifted and removed the area was cultivated and any remaining root pieces or other woody material collected. Prior to application of preplanting treatments the area was rotary hoed and graded to form raised beds. The following treatments were then applied before trees were planted.

- (1) Telone C35<sup>®</sup> at 50g/m<sup>3</sup>.
- (2) Methyl bromide at 500g/m<sup>3</sup>.
- (3) Untreated control.
- (4) Mono-ammonium phosphate at 2g/L of soil.
- (5) Trichopel<sup>®</sup> incorporated at 5g/L of soil.
- (6) Perlka<sup>®</sup> incorporated in the soil at 0.4 g/L of soil (equivalent to the commercial application rate of 300 – 400 kg/ha ).
- (7) Basamid<sup>®</sup> (Dazomet) incorporated in the soil at 40 g/m<sup>2</sup>, rotary hoed into the soil and covered with plastic sheeting for 7 days after which the sheeting was removed, soil rotary hoed and left fallow for 32 days.
- (8) Perlka as per treatment (6) and mono-ammonium phosphate at 2g/L of soil.
- (9) *Bacillus* sp. (L20) grown in tryptone soya broth for 72 hours with added to the soil at 1.3ml/L.
- (10) A locally-isolated *Trichoderma* spp. (Td22) cultured in wood fibre waste (80%):barley (20%) spent grain provided by Cascade Brewery, Hobart, Tasmania added to the soil at 10% by volume.
- (11) A locally-isolated *Trichoderma* spp. (Td22) cultured as per treatment (10) and added to the soil at 20% by volume.

- (12) Wood fibre waste:barley spent grain as per treatment (10) with no *Trichoderma* spp. amendment and added to the soil at 20% by volume.

Telone C35<sup>®</sup> and methyl bromide plots were 8 m in length to enable lead-in and lead-out distances for tractor mounted fumigation equipment. All other plots were 6 m in length.

Treatments were applied either on a per unit area basis or 4 - 5 litres of soil was removed from the planting position and mixed appropriately, for the per unit volume rates shown, and returned to position before planting. Fumigation with methyl bromide and Telone C35<sup>®</sup> was conducted by a local contractor in mid November (late spring), using a 2 m wide sheet of plastic sheeting sealed with soil around the edges to contain fumigants for the following 7 days. Application of Basamid<sup>®</sup> was carried out two days later, with granules incorporated into the soil using a rotary hoe before plots were heavily watered, after which they were also covered with 2 m wide plastic sheeting as above. Plastic sheeting was removed from all treated plots on the same day and the soil rotary hoed to increase aeration and dissipation of remaining fumigants. Other treatments were applied at planting, three weeks later.

The trial was planted with commercially graded MM106 rootstocks, which had been cool stored from lifting the previous winter. Spacing was a nominal 80 cm to give eight plants per plot. No fertiliser was used (except for the treatments with mono-ammonium phosphate and calcium cyanamide) and the area received a heavy application of overhead irrigation the day after planting. Dripper lines were then installed and all subsequent irrigation used dripper application with scheduling based on Class A pan evaporation measured at the site. Pest and disease management followed normal commercial practice and weed control was based on spot sprays using a broad spectrum desiccant type spray.

Six trees were measured in each plot with trees at each end used as buffers. Initial girth measurements were taken at marked positions within a week of planting and girth and extension growth was then measured at the end of the growing season.

### **Design and Analysis**

The trial was laid out as a randomised complete block with five replicates. Blocking was according to position only as trees were of uniform size and there were no obvious soil conditions to be taken into account. Treatment effects on new shoot growth, shoot growth per initial trunk cross section, final trunk cross section and percent increase in trunk cross sectional area were compared using analysis of variance calculated with the general linear models package in SPSS. Treatments were then compared using Fisher's LSD (Steele and Torrie, 1988). Trunk cross sectional area data was subjected to arc sine square root transformation before analysis. A regression between mean extension growth and mean percent increase in trunk cross section across all treatments was plotted using SPSS.

### **Results**

Although trees established well, weed seed germinated strongly in the non-fumigated treatments and weed control remained a serious problem throughout the growing season. Few weeds appeared in the fumigated plots and weed competition seemed strongest with the treatments containing mono-ammonium phosphate and calcium cyanamide.

There was a significant ( $P < 0.001$ ) treatment effect on shoot growth (Table 3.1) with both Telone C35<sup>®</sup> and methyl bromide treatments giving significantly

greater extension and radial growth increases than all other treatments. There were other minor treatment differences. The two containing calcium cyanamide, and the *Bacillus* sp. antagonist all produced weaker growth than compost but were not significantly different from any other treatments including the control. These extension figures were not reflected in the radial growth figures.

A plot of extension versus the proportional increase in cross section (Fig 1) produced a significant ( $P < 0.05$ ) linear regression with an  $R^2$  of 0.7.

#	Treatment	Mean extension growth (mm)	Extension Growth per Initial tesa	Final cross sectional area	Increase in tesa (%)
1	Telone C35 <sup>®</sup>	1660	21.4	152.5	96 <sup>b</sup>
2	Methyl bromide	1597	20.4	147.1	87 <sup>b</sup>
3	Untreated control	908	11.1	103.7	26 <sup>a</sup>
4	MAP	818	10.9	100.6	32 <sup>a</sup>
5	Trichopel <sup>®</sup>	843	11.0	99.8	31 <sup>a</sup>
6	Perlka <sup>®</sup>	715	10.2	109.2	43 <sup>ab</sup>
7	Basamid <sup>®</sup>	982	12.4	107.3	36 <sup>a</sup>
8	Perlka <sup>®</sup> /MAP	780	11.5	98.7	46 <sup>ab</sup>
9	Bacillus (L20)	598	7.5	90.9	15 <sup>a</sup>
10	Cultured Td22	878	11.7	102.1	36 <sup>a</sup>
11	Cultured Trichopel <sup>®</sup>	919	12.4	95.0	29 <sup>a</sup>
12	Compost	1176	14.1	111.6	32 <sup>a</sup>
	LSD ( $P < 0.05$ )	393	5.5	27.8	

**Table 3.1** Mean first season extension and radial growth for trees growing in apple replant soil subject to the treatments shown. Different superscripts in the percentage increase in trunk cross section column show significant differences based on the analysis of arcsine square root transformed data.

### 3.3 Greenhouse Trial

#### Materials and Methods:

Soil, as described in the field trial above, was collected after one season of fallow following grubbing of the original orchard. Surface soil, approximately 15 cm depth was collected from the original tree lines and placed in plastic bins for transport to the glasshouse complex. To improve air – filled porosity and drainage, soil was mixed 30/70 v/v with perlite to give an air filled porosity of approximately 13% (measured according to Australian Standard AS 3743, 1996). Treatments requiring time for fumigants to dissipate, or amendments to incubate, were carried out first and the remaining soil stored in covered bins outside at typical Tasmanian winter temperatures with maxima around 14 °C and minima above 0 °C. The following treatments were used and, unless noted otherwise, applied immediately before planting.

- (1) Trichopel<sup>®</sup> at 5g/pot and Trichoflow<sup>®</sup> at 5g/pot as a drench applied pre-planting.
- (2) Companion<sup>®</sup> a commercial strain of *Bacillus subtilis* applied as a pre-plant drench at 13ml/10L water equivalent to the commercial rate.
- (3) Mono-ammonium phosphate pre-mixed with the soil at 2g/L soil.
- (4) Basamid<sup>®</sup> mixed into the soil at 7g/30L soil sealed in a plastic bag for 5 days, then aerated for 36 days prior to planting.
- (5) Methyl bromide applied at 7ml/30L soil equivalent to the commercial application rate, sealed in a plastic bag for 1 day then aerated for 35 days.
- (6) Perlka<sup>®</sup> applied at 12g/30L soil equivalent to the commercial application rate of 300 – 400 kg/ha, sealed in a plastic bag for 1 day then aerated for 37 days.
- (7) Untreated soil (Control: soil with known ARD).
- (8) Commercial (eucalypt bark) potting compost applied at 20% by volume.

- (9) Trichopel<sup>®</sup> pre-cultured in a wood fibre waste (80%):barley (20%) spent grain provided by Cascade Brewery, Hobart, Tasmania added to the soil at 20% by volume.
- (10) Locally-isolated biocontrol *Trichoderma* spp. cultured as per treatment (9) and applied at 20% by volume.
- (11) Wood fibre:barley spent grain mix applied alone at 20% by volume.

Treated soil was placed in 3.5 L plastic pots (20 cm diameter) and commercial grade MM106 rootstocks were planted one per pot. The rootstocks were pruned to 40 cm high after potting. Potted plants were placed on a glasshouse bench and irrigated by hand as required throughout the growing season, with two applications of commercial complete nutrients watered in during the season. Weed seedlings were removed by hand at weekly intervals. After leaf fall at the end of the growing season, trees were carefully removed from the pots, washed free of soil and roots and shoots separated. Extension growth was measured as the total length of new shoot growth and root volume determined using the displacement method of Burdett (1979). (Root volume was not measured before planting). Roots and shoots were then dried to constant weight at 65 °C, weights recorded and shoot to root dry weight ratios calculated.

Trial design was a randomised complete block, with six replicates, blocked according to bench position in the glasshouse. Plots contained single trees. Results were subjected to analysis of variance of untransformed data using the General Linear Models package in SPSS and means compared using Fisher's least significant difference (Steele and Torrie 1988).

## Results

There were significant ( $P < 0.05$ ) treatment effects for each of the parameters measured (Table 3.2). *Trichoderma* spp. applied with 20% wood fibre waste and

barley spent grain produced the strongest extension growth with significant increases compared with all other treatments except the equivalent organic matter treatment without the antagonist. Across the four measures of total growth (shoot length and weight and root volume and weight) the two fumigants, Basamid<sup>®</sup> and methyl bromide both resulted in significant increases compared with the control. All treatments containing high levels of organic matter and the two inorganic amendments, mono-ammonium phosphate and calcium cyanamide, also produced significantly stronger growth responses but were not as consistent across the four measures. For example mono-ammonium phosphate failed to produce a significant response in shoot length but shoot growth measured as shoot weight showed a significant increase compared with the control. The two antagonist root drenches applied without other amendment failed to produce any significant growth responses compared with the control.

All shoot to root ratios except in the calcium cyanamide treatment were significantly higher than the control. The *Bacillus subtilis* (Companion<sup>®</sup>) treatment and mono-ammonium phosphate both produced shoot to root ratios higher than several other treatments (Table 3.3).

Table 3.2 Root and shoot growth responses for potted MM106 rootstocks grown in potted ARD soil treated as shown.

Treatment	Extension growth (mm)	Root volume (ml)	Root Dry Weight (g)	Shoot Dry Weight (g)	Shoot:Root ratio
Trichopel <sup>®</sup> + Trichflow <sup>®</sup>	1,050	101	22	50	2.27
Companion <sup>®</sup>	815	82	18	51	2.83
MAP	1,658	155	32	91	2.84
Basamid <sup>®</sup>	1,838	200	43	86	2.00
Methyl Bromide	1,798	208	44	70	2.05
Perlka <sup>®</sup>	1,680	163	39	59	1.51
Untreated Control	1,135	120	29	51	1.75
Compost – 20%	1,909	167	39	84	2.15
Trichopel <sup>®</sup> in WFW/barley – 20%	1,727	190	40	82	2.05
<i>Trichoderma</i> spp. in WFW/barley – 20%	2,415	202	43	99	2.30
WFW/barley – 20%	1,975	187	40	90	2.25
LSD (P = 0.05)	498	54	13	18	0.77

### 3.4 Discussion

In the field trial, the two gaseous fumigants clearly resulted in improved vegetative growth suggesting a confirmation of their effectiveness in controlling ARD. The results should however, be treated with caution. Both of these treatments suffered little weed competition compared with all others, except Basamid<sup>®</sup>, and results may be more indicative of weed competition effects on growth than any suppression by ARD. Further, treatments previously shown to be effective on this soil type (Wilson, *et al.* 2004) failed to produce any response compared with the control but the organic matter amendment (compost alone) did result in stronger growth than the *Bacillus* sp. (Companion<sup>®</sup>) and the

two calcium cyanamide treatments. Notably, Basamid<sup>®</sup> although a fumigant and providing some weed control, did not produce growth significantly greater than the control. This may have been due to residual phytotoxicity (BASF Corporation, 1998), suggesting that, in view of the earlier excellent field results for this product reported by Brown and Schimanski (2002), growers may need to modify application or planting strategies to minimise this negative effect.

With potted plants in the greenhouse, the results for the conventional treatments were much more in line with expectation. The fumigants, mono-ammonium phosphate and organic matter amendments all produced similar increases in growth when compared with the control treatment, confirming earlier greenhouse trials on this soil type reported by Wilson, *et al.* (2004) and Nair (2003). The failure of Basamid<sup>®</sup> to enhance growth in the field, in spite of reduced weed competition, was not repeated in potted plants providing further evidence that poor growth in the field may have been due to some residual phytotoxicity.

For the amendments to soil biota included in the two trials, results were inconclusive and in both the field and greenhouse trials there was no clear separation between the effects of added organic matter and any positive effects of any of the *Trichoderma* spp. isolates. There was poor growth in response to the *Bacillus* sp. in both trials. The significant reduction in growth compared with all other effective treatments remains unexplained at this stage.

The regression between trunk growth increment and extension growth suggests that although growth was suppressed in some treatments under field conditions, there was no overall change in tree form. While radial growth does provide some indication of the capacity to supply water to the shoot (Nair, 2003) it is not necessarily an indicator of the physiological balance between root and shoot. In contrast to the trees subject to field trial conditions, in the greenhouse there were significant treatment effects on shoot to root

ratio. This result suggests that apple replant disease may influence tree growth pattern as well as having an overall dwarfing effect. The significant differences between treatments in shoot to root ratio, also suggest that different treatments may target different aspects of the response of trees to apple replant disease.

## Chapter 4 Isolation and Testing of Potential General Root Disease Antagonists

### 4.1 Introduction

In the previous chapter, results for commercial “biocontrol” agents were inconclusive, and possible beneficial effects of *Trichoderma* spp. isolates could not be separated from added organic matter. Additions of *Bacillus* sp. in the root zone appeared to give no benefits and there was some evidence of a deleterious effect. It was also notable that pre-incubation of soil with calcium cyanamide (a source of urea nitrogen) in Trial 3.2 produced a growth increase equivalent to mono-ammonium phosphate, but a significant change in growth pattern. Whilst this could simply reflect a tree response to different nitrogen sources it may also reflect changes in soil biota during the incubation period.

In addition to the commercial products evaluated in Chapter 3, various biocontrol agents have been developed to counter a range of plant diseases (Ramona, 2003) and a biocontrol agent to counter apple and other replant disorders has led to a United States Patent being granted for a strain of *Pseudomonas putida* NNRL B-30041 (Mazzola, 1999).

Given the claims for commercial and patented biocontrol agents, and the uncertainty about indirect (ie non-nutritional) effects of chemical ameliorants arising from Chapter 3, the existence of other naturally occurring antagonists was investigated in soil used in apple replant disease trials and from local orchards. Antagonists were isolated from apple replant disease soil used in a greenhouse (pot trial) study reported previously by Nair (2003). Screening was aimed at determining whether there was any apparent change in rhizosphere or within root biota at the end of a

growing season in response to selected treatments including soil pasteurisation, mono-ammonium phosphate, fumigation or a *Trichoderma* spp. drench, (For full details of treatments used in the trial see Nair, 2003). Isolations were also prepared from commercial orchard samples and included in second stage screening.

Further evaluation of isolates as potential biocontrol agents were then carried out using lettuces and strawberry plants inoculated with *Sclerotinia minor* and grown under non-sterile conditions. Final evaluation of isolates showing antagonistic activity was carried out using potted apple seedlings grown in an apple replant disease soil mix.

## 4.2 Isolations and Initial Screening

### Materials and Methods

#### Isolations

For in-vitro screening, root samples were collected at the conclusion of a study comparing various soil treatments for apple replant disease reported by Nair (2003) and included in the later paper by Wilson, *et al.* (2004). For the second stage of screening using a seedling bioassay method, additional isolates from root samples collected from commercial apple orchards in north west, northern and southern Tasmania were also included. These latter samples were taken from trees exhibiting replant responses, non-replant trees, trees of varying age, variety and rootstock.

To obtain isolates from the rhizosphere, root samples were washed under running water for 2 minutes, and 1 g wet weight of the finest roots was placed in a stomacher bag with 100 ml sterile isotonic saline and stomached (Colworth 400 Stomacher)

for 30 seconds. The liquid was then discarded and stomaching (repeated with 100 ml fresh sterile saline for 2 minutes). Serial dilutions to  $10^{-5}$  were plated on to tryptone soya agar plates to obtain bacterial isolates and potato dextrose agar plates for fungal isolates. A selection of dominant bacterial and fungal isolates were then isolated onto tryptone soya or potato dextrose agar plates respectively to obtain pure cultures.

To obtain isolates from within root tissue, samples were first washed under running water for 2 minutes, before 1 g wet weight of the finest roots were selected and surface sterilised in 1.5% chlorine bleach  $\text{NaClO}_3$  for 2 minutes. The sample was then washed in sterile water, and ground in a sterile mortar and pestle with 1 ml sterile saline. Serial dilutions to  $10^{-5}$  were then prepared in the same manner as with the rhizosphere isolations. In both series of isolations note was taken of any isolate that demonstrated antagonistic or other competitive capabilities against adjacent colonies, thus indicating possible biocontrol potential (Ramona, 2003).

### Initial Screening

Initial antagonist testing was conducted using two *Fusarium* sp. isolated from an apple tree at Grove Research Station Southern Tasmania, a *Bacillus cerius* isolated from the orchard used in trial 3.2 in the present study and a *Sclerotinia minor* isolate from a culture collection held at the School of Agricultural Science, University of Tasmania.

To test for antagonistic potential a dual culture *in vitro* assay was conducted (Ramona, 2003). For evaluation against the bacterial isolate, *Bacillus cerius* was spot inoculated in the centre of a tryptone soya agar plate. For evaluation using the fungal isolates, round plugs were cut from a 48 hour culture grown on potato dextrose agar (Ramona, 2003) with the large end of a sterile glass pipette and placed

in the centre of the agar plate. Isolates that demonstrated antagonism toward the target pathogen in the form of a clear zone around the antagonistic colony were selected for further study. Results were qualitative only and not subjected to statistical analysis.

## Results

A total of fifty nine isolates were selected from the initial *in vitro* screening. Overall, effectiveness against the fungi was more common than against *Bacillus cereus*, with 42 showing anti fungal activity and 28 some inhibition of bacterial growth. Several isolates showed activity against both fungi and the bacterium, but only one was active against all fungi and the bacterium. Of the isolates showing antagonistic activity, 49% came from rhizosphere extracts with the balance from within the roots.

There was no particular pattern in response to treatments used in the source material with several isolates showing both fungal and bacterial antagonism obtained from "control" plots. Isolates with multiple antagonistic effects were also obtained from mono-ammonium phosphate treated plots. *Trichoderma* spp. treatments produced isolates with anti bacterial effects and pasteurized or fumigated treatments tended to produce isolates with anti fungal activity.

### 4.3 Second stage screening – selected pathogens on plants in sterile culture

#### Materials and Methods

To test for possible biocontrol activity, isolates were evaluated for their ability to protect radish seedlings inoculated with the *Fusarium* sp. and *Sclerotinia minor* used in the *in vitro* screening. Potting mix using Grove Research Station soil as described

for Trial 3.2, was prepared and 20 ml placed in 100 ml polycarbonate screw top containers (pottles) and autoclaved at 121 °C for 20 minutes. Four surface disinfested radish seeds (Long Scarlet) were placed in each pottle and allowed to germinate, then a plug of *Fusarium* sp. or *Sclerotinia minor* was introduced on to the soil surface. Containers were examined daily to record seedling survival. The trial was duplicated using 4 barley seeds instead of the radish.

The initial screening for antagonism was based on visual estimates of the single plate responses and results were recorded as yes/no for evidence of an antagonistic effect. Seedling survival ( /4) was recorded for each container of each of the two seedling species. Results were not subjected to statistical analysis.

## Results

Most isolates showed some apparent antagonist activity but results varied markedly between the two species tested, with barley seedlings apparently much more tolerant of the pathogen. Inoculation with one particular isolate (coded L36) resulted in all seed germinating to produce healthy seedlings of both species and with another (coded F41) only one radish plant had died and all barley seeds had germinated to produce healthy seedlings. In all others, seedling survival was less than 3/4 for one or both of the test species.

#### 4.4 Third stage screening - selected pathogen on plants in non sterile culture

##### Materials and Methods

Antagonists selected for strongest activity from the previous trials were cultured for 48 hours in 0.3 % tryptone soya broth. Lettuce seedlings, previously grown in sterilised standard potting mix for 2 weeks, were removed from pots and roots washed in tap water before being suspended in the antagonist in tryptone soya broth medium for 5 minutes. Plants were then potted into a standard but unsterilised potting mix at four per 15 cm diameter pot.

After one week acclimatisation in a shade house the surface of each pot was inoculated with *Sclerotinia minor* grown on millet seed at the rate of 2 g infected seed per pot. Plants were then maintained in a shadehouse for 8 weeks after which survival was recorded. The formally designed trial included two control treatments, using plants treated with the tryptone soya without antagonist, planted into potting mix with or without the *Sclerotinia minor* inoculation, twenty two isolates were compared with these two controls.

There were seven replicates in a completely random design, with results calculated as mean number of seedlings ( /4) surviving at the end of the trial. Statistical analysis was as described earlier, with results subject to analysis of variance and treatment (isolate) effects compared using Tukey's Honest Significant Difference.

##### Results

There was a significant ( $P < 0.001$ ) treatment effect, but mean survival was high in the diseased control treatment resulting in no significant difference ( $P > 0.05$ ) between the disease inoculated and non-inoculated controls. Further, no antagonist isolate treatments resulted in improved survival compared with the diseased control treatment. Results are shown in table 4.1.

Isolate	Mean survival	Group
L44	0.83	a
L7	1.00	ab
L26	2.14	abc
L49	2.14	abc
L55	2.14	abc
L3	2.28	abc
GA3	2.43	abc
Diseased control	2.57	abc
L5	2.57	abc
L51	2.57	abc
L32	2.71	bc
L2	3.00	c
L20	3.00	c
F41	3.00	c
L47	3.00	c
L8	3.14	c
L12	3.29	c
L4	3.29	c
L38	3.29	c

L8	3.43	c
L14	3.43	c
No- disease	3.57	c
L36	3.71	c
L54	3.86	c

Table 4.1 Mean survival of lettuce seedlings ( /4) exposed to *Sclerotinia minor*. Numbers with the same letter are not significantly different at P=0.05 based on Tukey's HSD

inoculated control. Isolates L44 and L7 both resulted in significantly poorer survival than both the inoculated and non-inoculated controls.

#### 4.5 Evaluation of selected isolates on apple seedlings in ARD soil

##### Materials and methods

Eight bacterial isolates demonstrating antagonistic ability selected in the previous screening trials were grown in 2 litres 0.3 % tryptone soya broth under sterile conditions and aerated with air filtered to 0.2 µm for 72 hours. Orchard soil was collected as a pooled sample from the trial site at the Grove Research Station described earlier. Half of the soil was autoclaved (as a bulk sample) shortly after collection at 121 °C for 1 hour. Just prior to planting, the soils were mixed with 30 % perlite (v/v) to improve air filled porosity as described earlier.

Roots of dormant, ungrafted commercial grade MM106 rootstocks were soaked in 2 litres of the antagonist/tryptone soya broth preparations for 5 minutes prior to planting in apple replant disease or sterilised soil mix in 10 cm round plastic pots. Pots were arranged on a galvanised steel mesh bench in a glasshouse operating at a set temperature of 20 °C and watering was provided by overhead sprinkler twice

daily. Nutrients were supplied using Hoaglands solution watered-in at four-week intervals and pest and disease control was carried out as required.

The treatment design was a factorial (two soils by nine isolates) in a randomised complete block design, blocked on bench position, with five single tree replicates. Total extension growth was measured at the end of the growing season and statistical analysis was by analysis of variance with means compared using Fisher's least significant difference as described for earlier experiments.

### Results.

The antagonists tested on apple seedlings in the ARD soil mix failed to produce any significant protection against the effects of ARD as shown in Table 4.5.

Bacterial Isolates	Non-ARD Soil	Mean ARD Soil
Control	516.7	310.6
L20	435.0	300.0
L36	454.0	335.0
GA3	395.0	308.3
F41	455.0	306.7
L54	390.0	315.0
AS17	472.0	285.0
AS14	390.0	266.7
AS8	357.5	276.7
LSD (P < 0.05)	ns	ns

Table 4.2 Extension growth (mm) of MM106 rootstocks in ARD and non-ARD soil treated with the antagonistic bacterial agents shown. Numbers are laboratory codes for the isolates and the control is with no added isolate.

There was no significant ( $P>0.05$ ) interaction between treatments and soil, or treatment effect, but there was a significant difference ( $P=0.005$ ) in overall growth between the two soils. Isolates AS17, AS14, AS8 were isolated in another study (Ramona 2003) and were included because of their demonstrated biocontrol potential.

#### 4.6 Discussion

Overall, the results suggest that soil conditions developing as newly planted trees establish or as an orchard matures, do not result in establishment of any single organism providing potentially useful antagonism to developing apple replant disease. The screening procedures produced several isolates with strong antagonistic activity against multiple pathogens effective in both sterile culture and in plants grown in open non-sterile conditions, but none produced a significant improvement in apple tree growth in replant soil. The result therefore extends the negative results for general-purpose commercial biocontrol agents in the previous chapter to include isolates from apple replant field soils and potting mix grown trees subject to various apple replant disease control pre-treatments.

In each case however it is important to note that organisms with known antagonism were introduced only on at one time in each trial and there was no evaluation of different methods of introduction.

It therefore remains possible that biocontrol agents could be used as part of an overall strategy to counter apple replant, but further study is clearly required. Given the complex soil community associated with plant roots, addition of a large quantity

of a selected organism may not necessarily alter the balance of the community sufficiently to counter apple replant disease. Nevertheless, use of a complex of active isolates and/or use of biocontrol agents in combination with other treatments remains worthy of investigation. While the cause or causes of apple replant disease remain unknown, the testing of biocontrol agents continues to depend on chance associations. With identification of a cause, use of selected and targeted biocontrol methods may yet contribute to consistent results.

Although not the focus of the present investigation, other aspects of the results were notable. Culturable bacterial isolates showing antagonistic activity to the pathogens tested were equally common in the rhizosphere and root tissue, in spite of the suggestion by Ramona (2003) that the rhizosphere has been traditionally preferred as a source of antagonists towards pathogens. Many researchers (eg Renwick, *et al.* 1991; Ramona, 2003) have found relatively few isolates ultimately are able to provide protection under natural conditions. However, the results from the lettuce trial with *Sclerotinia minor* in non-sterile potting mix suggest that, against this pathogen, several isolates from the various apple tree sources may be worthy of further investigation.

## Chapter 5      Tree Growth and Development under Apple Replant Disease

### 5.1 Introduction

As noted in the first experimental chapter (Chapter 3), there are inconsistencies in the pattern of tree growth and development in response to treatments providing some tolerance or protection against apple replant disease. Under field conditions, with weed competition there were no obvious treatment differences. Although the trial was not designed to show such differences and data on (for example) shoot:root ratio was not collected, a strong positive linear regression between radial and extension growth across all treatments suggests no change in tree form.

In contrast, the pot trial in Chapter 3 in which more data on tree form was collected showed marked differences in tree form with similar extension growth. For example, the two nitrogen fertiliser treatments (calcium cyanamide and mono-ammonium phosphate) produced similar shoot growth, but the shoot:root ratio for mono-ammonium phosphate was almost double that of calcium cyanamide. Conversely, although the biocontrol agent "Companion<sup>®</sup>" failed to produce any increase in shoot growth compared with the control, it produced a marked increase in shoot:root ratio. The latter confirming that treatment resulted in a marked suppression of root growth in addition to that imposed by ARD. Consequently, this final series of trials focused on tree form and growth patterns in response to ARD and or ARD treatments, with the first trial measurements being taken at the end of the growing season and the remaining three evaluating changes during early growth.

The first trial was a further investigation of the marked difference in tree form in mono-ammonium phosphate treated trees compared with an alternative nitrogen source. In this trial responses of two different rootstocks to mono-ammonium phosphate and equivalent non mono-ammonium phosphate nitrogen and phosphorous sources were compared.

Plant root diseases generally impede water uptake (Joseph, *et al.* 1998), and although several popular journal articles have commented that ARD causes water stress, few studies on any aspects of water relations and ARD have been published. Recently, Nair (2003) was unable to find any evidence of change in hydraulic conductance at the end of the first growing season in response to ARD and various management treatments including mono-ammonium phosphate, fumigation and pasteurisation. Differences in shoot:root ratio in Table 3.2, however suggest that water stress due to reduced leaf specific conductivity (Koide, *et al.* 1999) will be inevitable. Consequently, the second trial in this chapter involved a detailed examination of early season growth of trees exposed to ARD, concentrating on factors likely to influence water relations.

The third in the series of experiments further investigated the effect on total extension growth of a nutrient rich, sterile, broth added in the root zone at planting. The experiment also completes the screening trials in the previous chapter, evaluating the *Bacillus* sp. isolate L20 as a possible biocontrol agent. The apparent conflict in results obtained by Nair (2003) and the assertion by Joseph, *et al.* (1998) that invasive root disease causes water stress, raises the question of whether ARD involves any invasive disease organism. This then was the basis of the final trial, examining for a possible water soluble toxin. Although this possibility had been considered previously, early trials using water extracts did not include treatments subject to fine filtration and did not use extracts returned to a similar soil matrix with

no ARD history. In both of these two trials, growth was measured shortly after planting to determine whether an initial growth difference had occurred and been missed in previous studies because of compensatory growth later in the season.

## **5.2 MAP as an ARD Treatment or Fertiliser**

### **Materials and Methods**

An orchard soil/perlite mix was prepared as described earlier, with the mix pasteurised to provide for a non-ARD treatment. The following three nutrient treatments were applied:

- (1) pasteurised soil with added nitrogen and phosphate,
- (2) non-pasteurised soil with added mono-ammonium phosphate,
- (3) non-pasteurised soil with added nitrogen and phosphate.

For the nitrogen plus phosphorous treatment, nitrogen was added as ammonium nitrate phosphate as single-superphosphate, at rates equivalent to mono-ammonium phosphate. The nutrient treatments were incorporated into the soil/perlite mix at a rate of 2g/L for mono-ammonium phosphate and equivalent for the separate mix. Incorporation was immediately prior to planting.

Commercially graded MM106 or M9 rootstocks were planted into 3.5 L pots, which were placed on porous matting for irrigation from below by capillary to avoid leaching. Additional nitrogen and phosphorous and other nutrients were applied using a surface application of 50% Hoaglands solution every second week throughout the growing season. Pest and disease control was as required. The trial was carried out in a temperature controlled glasshouse operating at a set temperature

of 22 °C. Trunk circumference was measured approximately 10 cm above soil level at planting and at the end of the growing season, total extension growth was measured and number of new shoots counted and recorded.

Treatment design was a factorial with the two rootstocks by the three soil treatments listed above. There were seven replicates in a randomised complete block design and results were subject to analysis of variance with means compared using Fisher's LSD as described earlier.

## Results

In trial 5.1 (Table 5.1) there was a significant ( $P < 0.05$ ) interaction between rootstock and soil treatments for total extension growth, number of branches and number of branches per trunk cross section. There was no interaction for mean shoot length or total growth per unit cross section, but there was a significant ( $P < 0.01$ ) rootstock effect on mean shoot length and total extension growth per unit cross section. There was also a significant ( $P = 0.005$ ) effect of soil treatment on the number of new shoots per unit cross section. Overall there was a marked difference in shoot growth between the two rootstocks with M9 producing significantly ( $P < 0.01$ ) weaker growth. Total growth per unit cross sectional area was significantly reduced by the separate nitrogen and phosphorous treatments compared with both mono-ammonium phosphate and steam pasteurized treatments.

For MM106, there was a significant ( $P < 0.05$ ) increase in mean shoot growth in response to mono-ammonium phosphate compared with both other soil treatments, but no significant ( $P > 0.05$ ) difference between pasteurized and the separate nitrogen and phosphorous treatments. For M9, there was no significant ( $P > 0.05$ ) difference between mono-ammonium phosphate and pasteurized, with both producing

significantly stronger growth than nitrogen and phosphorous separately. There were no significant ( $P>0.05$ ) treatment effects on shoot growth per unit trunk cross sectional area, but there was a significant ( $P=0.01$ ) difference between rootstocks. Shoot number per unit trunk cross section was greater for M9 in pasteurized than nitrogen and phosphorous and for MM106 in all soil treatments.

Treatment	Total growth (mm)	Shoot number	Shoot Length (mm)	Growth/ tcsa ( $\text{mm}^{-1}$ )	Shoots/ tcsa ( $\text{mm}^{-1}$ )
MM 106 - pasteurised	1,205	2.14	703	13.8	.023
MM106 – MAP	1,847	4.00	516	17.6	.037
MM106 – N+P	1,254	3.64	437	13.5	.038
M9 - pasteurised	631	4.47	124	7.7	.058
M9 - MAP	718	3.57	205	9.3	.048
M9 – N+P	288	3.01	124	4.5	.040
LSD for the interaction ( $P = 0.05$ )	255	1.55	ns	ns	.018

Table 5.1 Shoot growth for MM106 and M9 rootstocks in ARD soil subject to the treatments shown.

### 5.3 Growth and development during first six weeks from planting

#### Materials and Methods

Replant soil was collected as pooled samples from a freshly grubbed orchard at the Grove Research Station in southern Tasmania, mixed with perlite and prepared as described above. MM106 rootstocks were taken from cool storage, planted in

standard potting mix (Australian Standard AS 3743, 1996) and placed in a shadehouse for 3 weeks. Plants were then removed and their roots gently washed before transplanting them into replant or non-replant (pasteurized ARD) soil. After intervals of 1, 8, 15, 22 and 43 days, plants were removed from pots and their roots gently washed, before taking growth and hydraulic conductivity measurements.

Stem circumference was measured immediately after planting. At each time, stem circumference, leaf area (measured using an electronic planimeter), and root volume (using the displacement method of Burdett, 1979) were recorded. Root leaf specific hydraulic conductivity (RLSC) was then measured using the pressure flow method described by Carlson and Miller (1990) and Wilson and Clark (1998). Rootstocks were cut 150 to 200 mm, above the roots and the 3 cm of outer bark removed, from the stem below the cut surface. This section of stem was then passed through a seal in the lid of the pressure chamber leaving approximately 2 cm of stem outside the chamber. The chamber was filled completely with water and connected to the domestic water supply at 1,100 KPa. A pressure regulator was then used to adjust water pressure in the chamber and the roots were allowed to equilibrate for 10 minutes before the cut end of the rootstock was wiped, and a calibrated fine tube attached. Flow rate was determined at two pressures, 150 and 220 KPa and the hydraulic conductance was determined as the difference in pressure (0.07 MPa) divided into the difference in volumetric flow rate between the two pressures (Maherali, *et al.* 2002). Leaf specific resistance was then determined by dividing the calculated conductance by the total leaf area.

Results were subjected to repeated measures analysis of variance using the general linear models package of SPSS and means compared using Fisher's least significant difference as described above. The data were not normally distributed and an arcsine square root transformation was applied prior to statistical analysis.

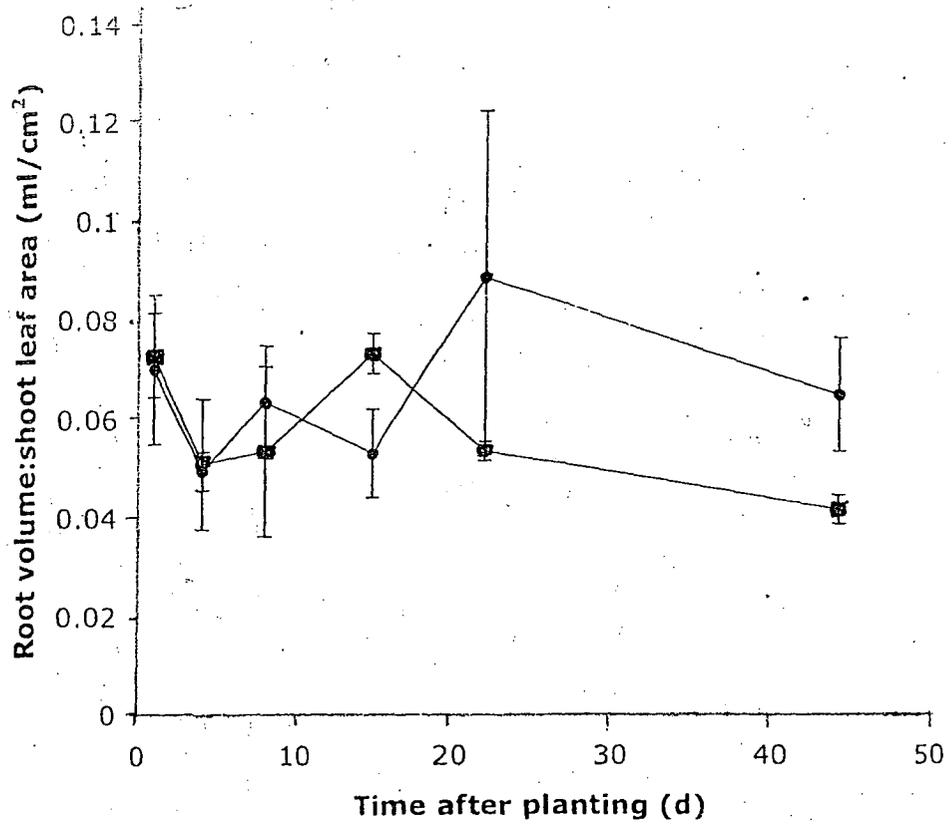


Figure 5.1 Root volume ; shoot leaf area (ml/cm<sup>2</sup>) over time (days) after planting. ● non-ARD soil ■ ARD soil

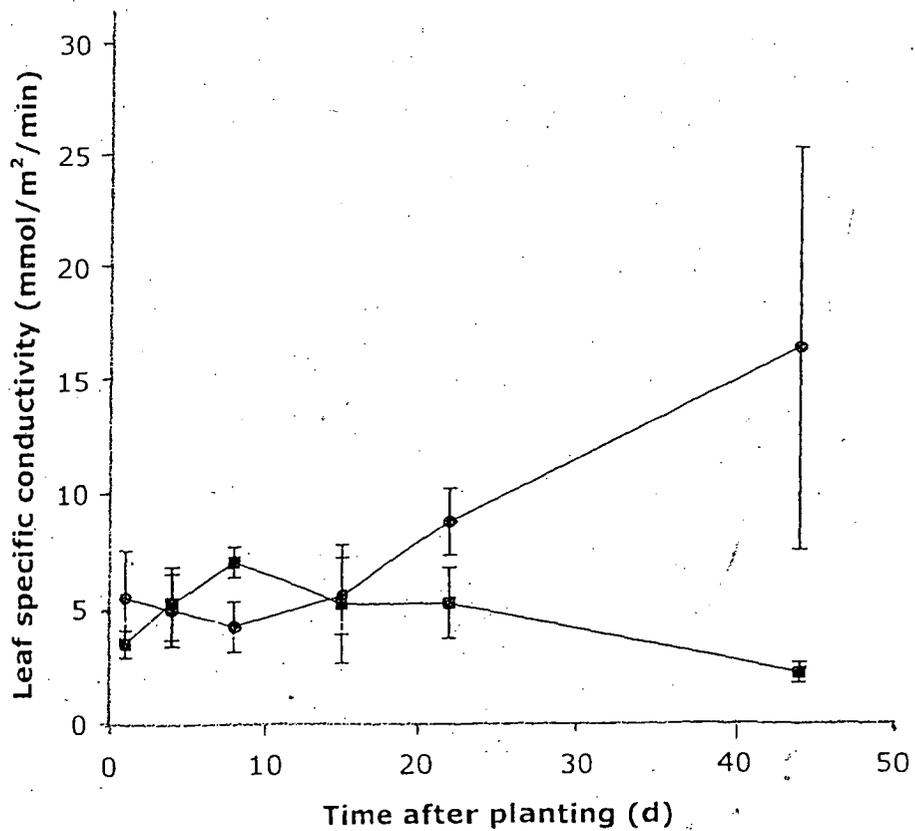


Figure 5.2 Leaf specific conductivity (mmol/m<sup>2</sup>/min) over time (days) after planting. ● non-ARD soil ■ ARD soil

## Results

For root conductivity there was a significant ( $P=0.035$ ) interaction between the two soil treatments and time, with mean LSC for the non-ARD treatment significantly higher at the final time of measurement (Fig 5.2) and having a marked increase in conductivity between days 22 and 43. In the ARD soil there was no significant change in conductivity over the measurement period. There was no similar interaction for any of the growth measurements, with extension increasing significantly over time, but no difference between treatments ( $P>0.05$ ). There were similar time effects on leaf area and root volume (Fig 5.1), and significantly ( $P<0.03$ ) reduced leaf area and root volume in the ARD soil. There were no significant treatment or time effects ( $P>0.05$ ) on shoot to root ratio.

### 5.4 Bacterial culture effects on tree growth

#### Materials and Methods

An orchard soil/perlite mix was prepared as described earlier, with the mix pasteurised to provide for a non-ARD treatment. Seed (cv. Pink Lady) was collected from a commercial apple processor, washed and stratified at 4 °C for 13 weeks before being surface sterilised and germinated in autoclaved orchard soil. Seedlings were allowed to grow on for 3 weeks before treating and transplanting into ARD or non-ARD soil.

A selected bacterial isolate *Bacillus* sp. (L20) demonstrating antagonistic ability in the Chapter 4 screening trials was grown in 850 ml 0.3 % tryptone soya broth under sterile conditions and aerated with air filtered to 0.2 µm for 72 hours. For treatments without the culture medium, 425 ml of the tryptone soya broth containing *Bacillus*

sp. (L20) was centrifuged under sterile conditions and supernatant replaced with an equal volume of autoclaved distilled water.

For treatments, seedlings of similar size were selected, removed and roots rinsed in tap water before being soaked in one of the following: (1) sterile distilled water, (2) 0.3 % tryptone soya broth, (3) antagonist *Bacillus* sp. (L20) in 0.3 % tryptone soya broth and (4) antagonist *Bacillus* sp. (L20) in sterile water. After soaking, seedlings were planted into either ARD or non-ARD soil mixes in 95 mm long x 85 mm wide x 85 mm high rectangular plastic pots with approximately 300 ml of soil mix per pot. They were then placed on a steel mesh stand in a shadehouse. Watering was provided by an automatic overhead sprinkler system, but no additional nutrient was supplied either before planting or during growth. Extension growth was measured after 58 and 71 days from planting.

Treatment design was a factorial with four soaking treatments and the two (ARD and non-ARD) soils. There were eight replicates in a randomised complete block layout blocked according to position on the bench. The trial was analysed as a repeated measures design using the ANOVA package in SPSS. Means were compared using Fisher's least significant difference as described above.

## Results

There was no significant interaction between soil, treatment and time, or between soil and treatment ( $P > 0.05$ ). There was however, a statistically significant ( $P < 0.05$ ) interaction between soil and time with stronger growth in the non-ARD soil becoming more marked with time as shown in Table 5.3. There were no significant treatment effects ( $P > 0.05$ ) and detailed results are not shown.

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	ARD soil (mm)	Non-ARD soil (mm)
Time 1	51	61
Time 2	96	151

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Table 5.2 Growth of seedling apple trees exposed to ARD. Table shows the interaction between period of growth and soil. Measurements are in mm of extension growth and the LSD ( $P=0.05$ ) for comparisons within the table is 24.5 mm

## 5.5 Evaluation of possible toxin effects

### Materials and Methods

Orchard soil was collected and combined with perlite to give an ARD soil mix as described previously. The trial also included a standard potting mix treatment prepared as described above. Apple seedlings (cv. Pink Lady) were prepared as described in 5.4 above.

A non-ARD soil extract was prepared by placing 250 g of a soil, collected from the margin of the Grove ARD site, in 1 litre distilled water. It was shaken in a mechanical shaker for 30 minutes then left to stand for 48 hours before filtering through gauze to remove large particulate matter, (treatment 1). An ARD extract (treatment 2) was similarly prepared using the Grove ARD site soil. For treatment (3) the coarse filtered ARD extract was filtered to 0.2  $\mu\text{m}$  to remove bacterial cells,

fungal hyphae and other particulate matter. Treatment (4) used the coarse filtered ARD extract sterilised by autoclaving at 121° C for 20 minutes.

Selected seedlings of similar size were removed as described above and planted in 95 (l) mm x 85 (w) mm x 85 (h) mm rectangular plastic pots with 300 ml of an ARD soil mix as described above or standard pasteurised potting mix. For each planting medium, after – planting treatments were: (1) watered with non-ARD soil extract filtered with gauze (2) watered with ARD soil extract filtered with gauze (3) watered with ARD extract filtered to 0.2 µm (4) watered with autoclaved ARD extract. Potted plants were placed on a steel mesh bench in a shadehouse as for 5.2 above. During the first week after planting, watering was provided by overhead sprinkler twice daily after which overhead watering was replaced with daily hand watering with 30 ml soil extract corresponding with treatments, per plant. No additional nutrients were supplied. Initial extension growth and trunk girth was measured immediately after planting and extension growth again after the trial was concluded at 15 weeks.

Treatment design was a factorial with two soil treatments (standard potting mix and the non-ARD soil) by the four extracts. Statistical design was a randomised block with 8 replicates, blocked according to bench position. Analysis and comparison of means was based on ANOVA as described for similar trials above.

## Results

There was a significant ( $P=0.044$ ) interaction between soil and treatment for total growth per unit trunk cross sectional area, as shown in Table 5.3, with the non-ARD extract watered onto sterile potting soil resulting in a significant increase in final growth per unit trunk cross sectional area compared with all other treatments. There

were no other treatment differences. For the growth increment per cross sectional area, there was a similar interaction ( $P=0.044$ ) between the two growing media and irrigation with ARD soil extracts (Table 5.3), with the non-ARD extract on potting mix producing stronger growth than all except ARD soil with fine filtered or autoclaved extract. There were no other treatment differences and there was no significant ( $P>0.05$ ) treatment effect on total extension growth.

There were no significant treatment or potting medium effects, or interaction between the two, on total growth or growth increment and results are not shown.

Table 5.3 Extension growth of seedling apple trees watered with extracts of apple replant diseased soils as shown. LSD ( $P=0.05$ ) figures shown are for the soil by treatment interaction

	Total extension per TCSA (mm/mm <sup>2</sup> )	Extension growth increase per TCSA (mm/mm <sup>2</sup> )
<b>Soil mix – apple replant disease</b>		
Non-ARD soil coarse filtered extract	25.0	14.0
ARD coarse filtered extract	26.3	13.1
ARD extract filtered to 0.2 micron	28.2	16.2
ARD extract coarse filtered, autoclaved	26.6	15.7
<b>Potting mix – no apple replant disease</b>		
Non-ARD soil coarse filtered extract	38.4	20.7
ARD coarse filtered extract	25.1	12.6
ARD extract filtered to 0.2 micron	27.1	14.4
ARD extract coarse filtered, autoclaved	23.1	10.6
LSD ( $P=0.05$ )	8.8	5.9

## 5.6 Discussion

In all trials except 5.2, there was a marked suppression of extension growth in response to ARD, or where ARD treatment was ineffective when compared with an effective "control" treatment. Using shoot growth as the primary (or conventional) measure of ARD impact on growth, the effect was evident in all except 5.4. With growth expressed on a per unit trunk cross sectional this trial also showed significant shoot growth effects in response to ARD. In Trial 5.2, which did not show any response in measured extension growth, there was however a significant reduction in leaf area averaged across the time series. In trials concentrating on initial growth following transplanting (Trials 2, 3 and 4) these growth differences were apparent within weeks of transplanting. Origin of the trial trees did not appear to influence the onset of ARD symptoms, with glasshouse raised seedlings used in Trials 3 and 4, showing similar timing of the response to Trial 2, which used rootstocks sourced from a commercial nursery.

Over the time frame of the short term experiments there were apparent changes in growth pattern in response to ARD or to ARD treatment. In Trial 5.2, both leaf area and root volume were significantly reduced by ARD exposure. In Trial 5.4, although there was no significant effect of treatment on extension growth, when measured as extension per unit cross section, there was a marked increase in the only treatment presenting no exposure to ARD. Means for all other treatments grouped within a narrow range, suggesting that, in the presence of ARD, trees allocated a smaller proportion of resources available for growth, to the developing shoot apex. Over the full growing season in Trial 5.1, the two high nitrogen treatments both increased shoot (branch) number compared with pasteurised soil in the more vigorous of the two rootstocks.

Over the full growing season in Trial 5.1, there were notable differences in response to the two nitrogen and phosphorous supply treatments between the two rootstocks. The more vigorous of the two (MM106) responded strongly to mono-ammonium phosphate, compared with both the nitrogen and phosphate (N+P) and pasteurised soil (non-ARD) treatments, with significantly greater total growth than both and greater shoot number than the non-ARD control. This result is consistent with observations by Schupp and Moran (2002), showing a stronger growth response to mono-ammonium phosphate, compared with other nitrogen and phosphate sources, across a range of soil conditions.

The marked increase in branch number in the present trial is probably of commercial value if the effect is also expressed in weak branching scion varieties. Although growth was strongest with mono-ammonium phosphate, the equivalent separated nitrogen and phosphorous application also resulted in similar extension growth to the non-ARD soil. In contrast with strongly dwarfing M9, mono-ammonium phosphate resulted in growth equivalent to non-ARD soil, but the nitrogen plus phosphorous treatment failed to overcome the growth suppression by ARD.

In the final trial (Trial 5.4), the only treatment to result in a significant increase in shoot growth per unit trunk cross section was the potting mix watered regularly with non-ARD soil extract. Thus in all treatments where trees were exposed to ARD either via the soil medium or applied leachate, there was a significant growth reduction. The result appears to present a strong argument for ARD mediation via a toxin or soluble plant growth substance. This suggestion has previously been dismissed by Savory (1969), but later work by Zhang, *et al.* (2006) has shown that apple seed germination, radicle extension and subsequent shoot growth are inhibited by root exudates of apple seedlings.

Water relations data in trial 5.2 indicated that, for nursery trees, ARD influenced root growth and/or function within 45 days of planting under the conditions of the trial. The result suggests that there is an initial direct impact on root function, which later influences other aspects of growth. Combined with the observation by Nair (2003) that there is no evidence of water stress associated with ARD measured at the end of the growing season, this suggests that the tree has adapted to the ARD environment by the end of the first growing season. At the conclusion of this trial, there was an overall reduction in leaf area, suggesting commencement of a water stress effect on leaf expansion, possibly associated with the reduction in root volume. However, after 45 days there was no measurable overall change in shoot to root ratio, in spite of the change in LSC, suggesting that the initial response to ARD was related more to suppression of root function than root growth.

## Chapter 6      General Discussion and Conclusion

### 6.1 Discussion

Considering that planting an orchard invariably uses grafted nursery stock sourced originally from (often) long established stool beds, new trees presumably have a history of exposure to whatever causes ARD. It appears then that the almost inevitable growth suppression following planting into old orchard soil is unlikely to be due to infection by a root pathogen. An assumption that it must therefore be caused by a toxin was questioned by Savory (1969) who dismissed tree derived "toxins" as a causal agent. Subsequently, most recent research has concentrated on the author's suggestion that;

*The Causal agent is a micro-organism which is a normal component of the (apple) rhizosphere population but which, after removal of the first crop, is present in such numbers as to cause temporary damage to roots of newly replanted apples.*

If this is the case, biocontrol agents with potential to influence the rhizosphere population could play a role in countering the disease. However published results have been inconsistent, with some reports of a positive effect (Mazzola's patent) and others and the present results (Chapter 4) reporting no measurable growth response in soils with a clear ARD presence.

The consistent reports of positive responses to mono-ammonium phosphate and to added organic matter are difficult to explain in this model for the disease. As indicated in the discussion by Wilson, *et al.* (2003), the possibility that added organic matter has a dilution effect has been considered as a possible explanation for the latter. In the present trials, failure of the organic matter drench (with and without

antagonist) to elicit any growth response tends to support the organic amendment diluent theory. The mono-ammonium phosphate response is, however, much more difficult to explain as there are consistent reports, confirmed here in Chapter 5, that equivalent application of nitrogen and phosphorous in different forms are ineffective.

Whilst Savory (1969) concluded that “toxins” were not responsible for ARD, it was conceded that they may have a role in non-specific replant situations. The present results (Chapter 5) provide strong evidence for an active “toxin” but the trial did not establish any specificity and did not distinguish between tree or micro-organism derived substances or substance.

A notable omission from almost all publications on ARD is detailed information on the pattern of tree growth. Most authors have recorded end of season extension and/or radial growth as a measure of an overall growth response with little attention paid to any preferential allocation of photosynthate in response to ARD or ARD treatments. Few papers report differences in root growth, root to shoot ratios, radial to extension growth ratios or branch development. Although the present trials were planned with conventional growth measurements in mind, Trial 5.3 was designed to specifically target key initial growth responses of plants exposed to ARD. These results, showing a substantial change in plant water relations before other growth changes became evident, resulted in a more detailed examination of measurements made in the other trials and re-examination of some trial results reported by Nair (2003).

While reduced ability for water uptake in ARD exposed rootstocks, compared with controls at 43 days, demonstrated a physiological response, it does not necessarily indicate a role for water stress in expression of the disease. It is notable that the marked difference in LSC evident at the end of this trial was not reflected in

measurements taken at the end of the first growing season in trials reported by Nair (2003). Further, there is no consistent difference in the present trials (or those reported by Nair) in root to shoot ratio as the usual measure of balance between water uptake capacity and transpiration demand.

These results suggest an initial impact of ARD on root physiology rather than root extension growth. Differences in leaf specific conductivity found in Trial 5.3 are consistent with results reported by Kyllö, *et al.* (2003) and the effect may be similar to that observed in peach rootstocks where water relations (Basile, *et al.* 2003a), in particular, hydraulic conductance (Basile, *et al.* 2003b) appears to play a central role in the dwarfing mechanism produced by size controlling rootstocks. A reduced water supply could reduce photosynthetic carbon uptake because of the effects of water stress on stomatal opening, and hence growth and potentially yield (Sperry, 2000). This has been suggested as a possible mechanism for dwarfing by rootstocks in apple (Higgs and Jones, 1990).

It has been previously established that apple replant disorder has a biological component, although the implicated biota are diverse and often site specific (Mai, *et al.* 1994; Mazzola, *et al.* 2002). It has also been shown that mycorrhizal colonisation can play a role in apple replant disorder (Gamiet, 1989), and their role in nutrient acquisition, in particular phosphate, is well known (Schachtman, *et al.* 1998; Strack, *et al.* 2003). Additionally mycorrhizal fungi can be associated with water uptake (Mushin and Zwiazek, 2002), with a role in modifying plant water relations. An increase in hydraulic conductivity and a positive effect on plant water potential, particularly under drought stress, has been suggested by Augé (2001).

A reduced ability to take up water could result in trees responding by adjusting growth and reducing water demand with lesser leaf area. Consequently, established

trees may not demonstrate water stress when examined later in life because tree size has been balanced against water availability. This was recognised in the suggestion by Nair (2003) that water stress could not be dismissed as part of the apple replant disorder mechanism, as measurements were conducted at the end of the growing season and may not adequately reflect conditions shortly after planting.

Hydraulic conductivity data included in Fig 5.2 shows no statistically significant change in root LSC, during the initial 22 days after planting. Whilst this could indicate that there is no initial effect on root growth it is more likely an indication of the start of root growth after transplanting as discussed for forestry species transplants by Wilson and Clark (1998).

## 6.2 Conclusion

These results suggest ARD is not a condition that can be readily rectified with the use of a biocontrol agent alone. Additionally it appears that suggestions that it is due to the actions of a chemical agent or nutrient status of affected trees cannot be dismissed without further study.

The efficacy of certain antagonistic bacterial isolates obtained from apple roots in protecting lettuce seedlings from the fungal pathogen *Sclerotinia minor* suggests that while bacterial antagonism is specific in some respects could have an application in countering plant disorders not necessarily associated with their initial origin.

The overall conclusion from these trials is that apple replant is likely to be a more complex interaction and any biologically oriented solution to countering apple replant will require a better understanding of the underlying plant and microbial relationships involved. In particular, the detail of the changes in tree growth

response to ARD (or its control), and further questions on a role for toxins, suggest that the role of the tree in the “disease” syndrome deserves greater attention in future work. This could include examination of interactions and relationships between tree (including variety), and soil microbiota (including mycorrhizal associations) and whether these then change over time as stoolbed produced rootstocks are introduced into an ARD environment. Tracking changes in soil microbiota can utilise molecular techniques such as polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE) as used by Rumberger (2004), Yao, *et al.* 2006 and phospholipid fatty acid analysis (PLFA) Ebekwe, *et al.* (2001). A more detailed examination of root growth and development also appears warranted, with particular reference to root function (water relations and nutrient uptake) rather than the conventional measure of root growth. The preliminary results on temporal changes in hydraulic conductance obtained here can be verified and extended using the same techniques.

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## **Appendix A    *In vitro* Growth Media**

### **A.    Standard Potting Mix**

50 L potting mix contains 35 L composted pine bark, 10 L sand, 5 L Sphagnum peat moss, 90 g dolomite, 90 g limil, 25 g FeSO<sub>4</sub> and 300 g Osmocote slow release fertiliser. All ingredients being combined in a cement mixer, pH approximately 6.0.

### **B.    Wood Fibre Waste : Barley Growth Media**

The wood fibre waste : barley growth media consisted of wood fibre waste provided by Norske Skoog, New Norfolk, Tasmania that had been air dried under cover for three weeks mixed with spent grain (remains of barley after the beer making process) provided by Cascade Breweries, Hobart, Tasmania at the ratio of 80 : 20 (WFW : barley) respectively. This mixture was brought to approximate field capacity with the addition of 1.5 L / kg basal mineral salts (BMS), mixture was prepared immediately before use.

### **C.    Trypticase Soya Agar (TSA)**

Trypticase soya agar (TSA) contains per litre, 3 g tryptocase soya broth (Oxoid), 1 g yeast extract (Oxoid) and 15 g agar (Davis). All components were dispersed in distilled water and autoclaved for 20 minutes at 121 °C for 20 minutes, upon cooling dispensed into sterile petri plates.

**D. Potato Dextrose Agar (PDA)**

Potato dextrose agar contained 4.0 g instant dried mashed potato, 20.0 g dextrose and 15.0 agar dispersed in 1 L distilled water. The medium was autoclaved at 121 °C for 20 minutes and upon cooling dispensed into sterile petri plates. (Lacy and Bridgmon, 1962).

**E. Basal Mineral Salts (BMS)**

Basal mineral salts contain per litre: 5.0 g  $\text{NH}_4\text{NO}_3$ , 2.0 g  $\text{K}_2\text{HPO}_4$ , 0.2 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.01 g  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  and 0.01 g  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ . All components were dissolved, dispensed into 500 ml bottles and autoclaved at 121°C for 20 minutes.