The potential of a Novel Green Manure as a treatment for Replant Disease on *Sorbus aucuparia*

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Dedication

This thesis is dedicated to three special people in my life who have helped me throughout my journey

My soul mates David, Lesley and James

“The true meaning of life is to plant trees, under whose shade you do not expect to sit.”
—Nelson Henderson.

Declaration

This thesis describes original work by Julia Jayne Lock which was completed during the period of registration as a post-graduate/PhD student of the University of Northampton, except where reference is made to the work of others. No part of this work has been previously submitted for a higher degree at this or any other University.

Julia Jayne Lock. 27th September 2017

Abstract

Trees have been an integral part of our environment for millennia by providing many ecosystem services to the surrounding populous. Trees add to their environment through the provision of ecosystem services and are a positive contributor to public health which financially equates to £1.8 billion per year. The importance of trees is recognised at government level and their health and resilience is considered a priority.

Tree and shrub species in the Rosaceae are susceptible to replant disease (RD), which is expressed in the form of necrotic roots, stunting and reduced yield, and are thought to be the result of a build up of soil borne pathogens that are associated with root rotting, and can be exacerbated by abiotic factors such as poor soil condition, the presence of phytotoxins and a lack of available nutrients. Pathogens detected in replant soils cover a broad range of microorganisms often in the form of a complex consisting of organisms from bacteria, fungi, and stamenopiles to nematodes. As a result of this disease being incited by a complex of various pathogens it was treated with a broad spectrum pesticide called methyl bromide which is non-selective in its target species. This chemical treatment was revoked under the food and environment protection act 1985 and the control of pesticides regulations 1986 due to being a stratospheric pollutant. RD was identified as a specific problem on *Sorbus aucuparia* (rowan) which was previously associated with a complex of *Cylindrocarpon*, *Fusarium,* and *Pythium*. This species was found to be particularly sensitive to RD and was demonstrated as being a model species for soil susceptibility bioassays. *S. aucuparia* is an ornamental species which is used widely throughout Europe in urban forest plantings due to its tolerance to disturbance and poor conditions. Due to these factors this species was used to determine the effectiveness of green manure as a treatment for RD.

It was proposed that the use of novel green manures with specific antimicrobial properties as an organic soil addition had the potential to alter the condition of the soil to one that is conducive to tree growth. *Phytolacca americana* (American pokeweed) was selected for its potential as a biofumigant, with *Brassica juncea* (mustard) *Tagetes patula* (French marigold), *Triticum aestivum* (wheat), and *Allium sativum* (garlic) studied as comparative treatments. The research was split into three streams of work involving a three year bioassay running alongside microbial inhibition tests and DNA analysis. *In-vitro* studies (n=9) were conducted to determine the potential of green manure leaf matter to inhibit *Pythium ultimum* and *Rhizoctonia solani*, which were previously associated with RD. At the same time, bare root *S. aucuparia* saplings were arranged in a randomised block design in the research area of the Moulton College estate in Northamptonshire. After three months of establishment trees were categorised into 16 treatment groups of single tree replicates (n=25). Eight groups were potted in sterile soil, whilst eight were potted into diseased soil, obtained from Burbage Common in Leicestershire. *P. americana* was applied in three concentrations (1.67g/l, 8.33g/l and 15.00g/l), whilst comparative green manures were applied in one concentration (8.33g/l). During a period of three years trees were monitored initially after three months and then yearly, with growth variables including primary and secondary growth, leaf

count, active bud count, and chlorophyll content. At the end of the three years the trees underwent destructive analysis which consisted of dry weight measurements of stems, roots, fruit and foliage. Roots were examined for nematode cysts. Soil samples were analysed by Scientia Terrae for a prescribed list of pathogens and beneficial microorganisms using a polymerase chain reaction and DNA Multiscan. The *in-vitro* laboratory study demonstrated that application method, concentration, and green manure species used can influence pathogen growth. All *P. americana* treatments significantly inhibited *R. solani* and *P.ultimum*. *P. americana* has properties which induce inhibition of pathogen growth, with these properties being liberated from the plant matter directly or as a water based extract. Of the two pathogens the former was found to be more sensitive to treatments than the latter. Application of comparative green manure species as plant matter in agar inhibited *R. solani* and *P.ultimum* growth. *T. patula* was the only plant species that did not inhibit *R. solani* and only temporarily inhibited *P. ultimum* growth. When the treatment was applied as a cold water extract *P. americana* successfully inhibited more than other treatments. The *in-vivo* field study determined that the influence of the sterile soil sub-set treatments on tree growth was no different to the control. Treatments did not have an influence on height, secondary growth, foliage and fruit production throughout the three years. There was also no significant difference in root and stem biomass. The use of green manures appears to produce variable results, which is especially true in terms of *P. americana* where improvement in growth parameters was not correlated with concentration. *T. patula* (8.33g/l) and *P. americana* (15.00g/l) were linked to increased height and vigorous roots with greater biomass, whilst *B. juncea* and *A. sativum* caused stunting in comparison to the diseased control. *T. aestivum* treated trees were associated with variable vigour. Molecular DNA results in relation to tree growth data indicated that the disease in this particular instance was exhibited in the presence of soil borne pathogens *Rhizoctonia solani*, *Pythium* spp., and *Pythium sylvaticum*. Results demonstrated that, regardless of concentration, *P. americana* does not have a consistent affect on soil microbes. However, *P. americana* is successful in eliminating *R. solani* when used at a rate of 15.00g/l.

Overall results suggest that *P. americana* and *T. patula* soil amendments improve root and shoot growth relative to the control. *P. americana* was consistently fungistatic *in vitro* whilst displaying variability in its activity in field conditions. *T. patula* demonstrated poor ability to suppress pathogens in the laboratory however was much more successful when added as an amendment. Surprisingly, two species known for their antimicrobial activity, *A. sativum* and *B. juncea*, were not found to be beneficial in this instance. *T. patula* may have potential as a green manure; however the other species are unreliable treatments. It is recommended that active ingredients of *P. americana* and *T. patula* are investigated further to deduce their activity against replant disease on *S. aucuparia*.

Keywords: Replant Disease, green manure, *Sorbus aucuparia*, methyl bromide, Rosaceae, microbial community, *Triticum aestivum*, *Allium sativum*, *Tagetes patula, Phytolacca americana, Brassica juncea*, antimicrobial.

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Chapter 1 – Introduction

1.1 Research scope: a move away from chemicals

Trees are an important facet of the political, socio-economic and environmental agenda in the United Kingdom. A healthy green infrastructure adds to the social, economic, and ecological fitness of an urban setting and the broader landscape. Trees mitigate climate change and provide ecosystem services, whilst contributing to public health and natural capital. The ecological and social value of woodlands in rural environs equates to £1.8 billion per year (Forestry Commission, 2014). The UK Government works with The Forestry Commission, The National Forest the Community Forest Programme, Food and Environment Research Agency (FERA) and the Department for Environment, Food and Rural Affairs (DEFRA) in order to promote tree health and resilience as a priority, the. On a national scale, various funding schemes are available, such as The Rural Development Programme for England (RDPE) and English Woodland Grant Scheme (EWGS) which successfully resulted in 12,480 hectares of woodland being planted by the end of 2013, whilst The Big Tree Plant campaign of 2010 culminated in 1,050,264 trees being planted (60% were placed in areas of deprivation or those lacking green space) by 2015 (Foresty Commission, 2016). Internationally, European Union funding was made available to farmers through the Common Agricultural Policy for tree planting (Lawson *et al.*, 2011).

In order to ensure an ecologically resilient treestock it is important to effectively manage the production of healthy nursery stock (Forestry Commission, 2014). The provision of healthy rosaceous stock as important top fruit and ornamental amenity trees is significantly impacted by disease, such as Replant Disease (RD). RD is a chronic global issue caused by a complex of pathogens, which elicit root destruction, limited canopy growth and insufficient fruit yields (Mai and Abawi, 1981; Willett *et al.*, 1994; Utkhede and Smith, 1994; Peruzzi *et al.*, 2017). O'Neill and Wiltshire (2007) reported that RD delays growth rates of marketable stock by 50% and that a 1% reduction in the output of UK field grown ornamental trees equates to a £264,000 loss per annum. Recently, a review by Mancini *et al.* (2017) concluded that RD causes a 30-40% decline in productivity. Historically, RD was controlled by the broad spectrum fumigant methyl bromide (MB), however this chemical was revoked under the Montreal Protocol in 1991 (Yates *et al.,* 2003; Appendix I). Global production of MB was due to end in 2005, however, growers in the US were still using the fumigant in 2016 under critical-use exemptions, due to no efficacious alternative being available (Guthman, 2017). RD is not easily treated due to the persistence of pathogens in the soil, as trees planted in a diseased soil can still exhibit symptoms after ten years in the absence of rosaceous species (O'Neill and Wiltshire, 2007). In line with the EU Policy for the sustainable use of pesticides and REACH, this research thesis investigated the use of an antimicrobial novel green manure, developed from the leaves of a hardy perennial herbaceous plant, *Phytolacca americana* (American Pokeweed) as a treatment for RD.

1.2 Rosaceae: an overview of the family and the financial implications of RD

The Rosaceae (rose family) consists of over 100 genera and 2830 to 3100 species (Judd *et al.*, 1999), and is distributed globally in most areas, particularly in the Northern Hemisphere (with exception to deserts or tropical rainforests). The rosaceae consists of 104 genera and 28,293 species, of which 4,828 are accepted names (The Plant List, 2013). Genera *Cotoneaster*, *Crateagus*, *Potentilla*, *Prunus*, *Rosa*, and *Sorbus* contain between 244 and 380 species, with *Rubus* including the majority (1,494).

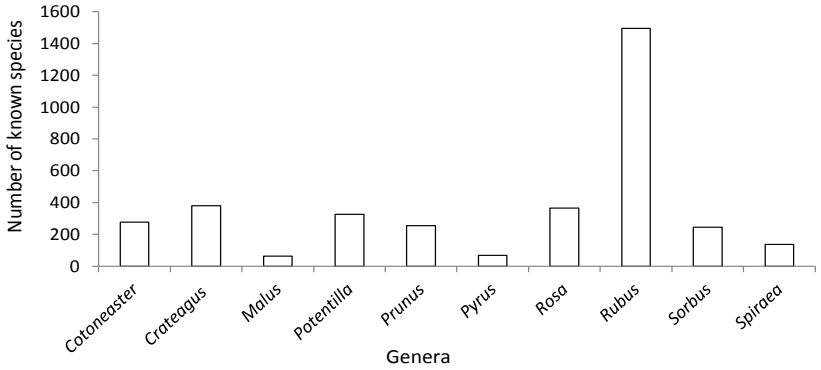


Figure 1.1: Number of accepted species for some major top-fruit and amenity rosaceous trees (data from The Plant List, 2013).

The Rosaceae includes herbs, shrubs, or trees that are utilised in various situations ranging from urban and ornamental garden landscaping, timber production, and soft fruit and tree fruit cultivation (Table 1.1). Species such as *Malus* spp., *Prunus* spp. (cherry), *Sorbus aucuparia* (rowan), and *Crateagus* spp. (hawthorn) are often used in hedgerow, woodland settings, and urban forests due to their tolerance to disturbance (Hummer and Janick, 2009).

Table 1.1: The uses of some rosaceous species (adapted from Hummer and Janick, 2009)

Amenity	***Fruit***
Amelanchier alnifolia (serviceberry)	*Cydonia oblonga* (quince)
Chaenomeles japonica (Japanese quince)	*Eriobotrya mespilus* (loquat)
Cotoneaster spp. (cotoneaster)	*Fragaria* x *ananassa* (strawberry)
Crateagus spp.	*M.* x *dometica* (apple)
Geum spp. (avens)	*Mespilus germanica* (medlar)
Kerria japonica (kerria)	*P. armeniaca* (apricot)
Malus spp. (crabapple)	*P. avium* (wild cherry)
Photinia spp. (photinia)	*P. cerasus* (sour cherry)
Potentilla spp. (cinquefoil)	*P. domestica* (common plum)
Pyracantha spp. (firethorn)	*P. dulcis* (almond)
Rosa spp. (rose)	*P. persica* (peach or nectarine)
S. aria (whitebeam)	*Pyrus communis* (European pear)
S. aucuparia	*P. serotina* (black cherry)
Spirea prunifolia (bridal wreath)	*Rubus* spp. (blackberry and raspberry)

Data on rosaceous produce indicate that fruits from the Rosaceae contribute to local and global economies. In the UK growers produce includes apples, strawberries, cherries, pears, plums and raspberries. Indeed in 2013 alone growers supplied 217,240 t of apples and 94,373 t of strawberries (FAOSTAT, 2016; Figure 1.2). In 2015 UK Home production marketed orchard fruit totalled £187.7 million (Anon, 2016). On a global scale, based on 2013 income (US dollars/t) figures, the USA, China, Iran, and Spain are some of the top producers of a variety of rosaceous fruit with the majority of income resulting from the sale of *P. dulcis*, *F.* x a*nanassa*, and *P. avium* cultivars. Other influential producers include Italy, Ukraine, Turkey, and Morocco (FAOSTAT, 2016; Appendix II).

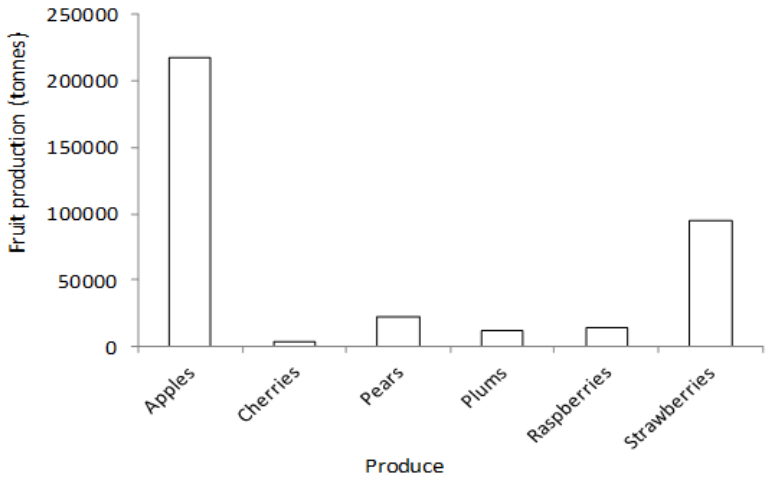


Figure 1.2: Quantity of rosaceous fruit produced (t) in the UK by growers in 2013 (adapted from data from FAOSTAT, 2016).

Top-fruit growers need to optimise production by maintaining high quality nursery stock to ensure a consistent contribution to the economy. However, plant species in the Rosaceae family are particularly prone to RD (Savory, 1967), with examples of susceptible rosaceous species spanning many genera; *Sorbus* spp. (rowan), *Rosa* spp. (rose), *Cydonia oblonga* (quince), *Mespilus* spp. (medlar), *Malus* spp., *Pyrus* spp. (pear), *Prunus* spp. (cherry, plum, peach, almond, apricots), *Rubus* spp. (raspberry) and *Fragaria* spp. (strawberry). RD is typified by a reduction in growth and a loss in yields (Section 1.4). A nursery can suffer productivity losses of 40-50% (Otto *et al.*, 1994) and a loss of faith when stock requires replacement due to poor growth.

Many studies on RD have been biased towards top-fruit trees, whilst very little research has been conducted on amenity species (Section 1.5). O'Neill and Wiltshire (2007) determined that ornamental cherry tree stock can take twice as long to grow to a marketable size and lose half of their value. The UK horticulture industry found that a 1% loss in rose production equated to £240,000/yr (AHDB, 2008). In terms of ornamental rosaceous trees, the loss of 1% of those that are marketable in the UK equates to £264,000 in a given year (AHDB, 2008). More recently a study by O'Neill (2011) determined that *S. aucuparia* is susceptible to RD and is suited for use in RD bioassays.

1.3 *Sorbus aucuparia*: autecology and association with RD

RD was identified as a specific problem on *S. aucuparia* (Sharp, 2010, pers. comm., 1 Nov; O'Neill, 2011). As stated (Section 1.5.2) where a complex of pathogens may be responsible for RD on rosaceous stock. In one of the few studies on RD on *S. aucuparia* O'Neill (2011) determined that this species is associated with *Cylindrocarpon-Fusarium-Pythium*. This research used *S. aucuparia* as a model tree species due to its sensitivity to RD (O'Neill, 2011).

S. aucuparia (rowan or mountain ash) are small to medium sized deciduous trees which have a comparatively low growing habit (15-20 m), are fast growing, with a life expectancy of 150 years in optimum conditions whilst remaining small and shrubby in limiting environments (Raspé *et al.*, 2000). The tree bears ripe red fruit in the autumn after a spring and summer bloom of white flowers, and has pinnately compound leaves (McAllister, 2005; Kingsbury, 2015) (Figure 1.3).


a


b


c

Figure 1.3: Phenology of *S. aucuparia* (a) specimen in a sheltered location at Moulton College, Northamptonshire The compound leaf form (b) red berries (c) (Photograph credit: Lock, 2015).

S. aucuparia is an important pioneer species and part of mountain Sorbeto-Piceetum forests (Homolka and Heroldová, 2003), recovering well from herbivory laying down compensatory growth. It has been demonstrated that the adventitious roots of *S. aucuparia* act to stabilise erosion by reinforcing the structure of the soil, and have been found to enhance forest regeneration and ongoing stability (Raspé *et al.*, 2000; Räty *et al.*, 2016); historically, Tandy (1972) recommended that this tree should be used in street planting and the landscaping of roof gardens due to its tolerance to these limiting factors. Indeed, the main value of *S. aucuparia* is in its use as an ornamental tree cultivated for garden planting schemes and street settings (Räty *et al.*, 2016; BSBIDB, 2016; Kingsbury, 2015). The saplings are a prominent

feature of urban forests in Finland and mature specimens are typically used as street trees in Scandinavia (Sæbø and Johnsen, 2000; Hamburg *et al.*, 2009). Harvested wood is used for woodworking and turnery due to the low economic value of the timber, whilst their fruit are a vital food resource for migratory and overwintering birds (Raspé *et al.*, 2000; Homolka and Heroldová, 2003; Hester *et al.*, 2004). In its natural habitat, *S. aucuparia* is found in a range of altitudes from sea level to over 900m in Scotland and Wales in the British Isles and at altitudes of over 1500m in France and Norway (Raspé *et al.*, 2000). *S. aucuparia* has been found in many UK settings (Appendix III) The distribution of this species in Europe stretches from Iceland and Scandanavia to Russia in the East, Sicily in the South, and Spain to the West (Raspé *et al.*, 2000). *S. aucuparia* has also been introduced to North America where it has naturalised at higher latitudes (Räty *et al.*, 2016).

According to adjusted Ellenberg's indicator values *S. aucuparia* is noted for its stress tolerance and ability to compete with other species. This species is well adapted to acidic clay soils with average nitrogen availability and no presence of salt (Hill *et al.*, 1999; Hill *et al.*, 2000); and is rarely found on naturally alkaline soils with a pH above 7.0 (Räty *et al.*, 2016; Raspé *et al.*, 2000). This species is tolerant of a lack of nutrients, temporary water logging, soil erosion, wind exposure, atmospheric pollution, and coppicing (Raspé *et al*, 2000; Räty *et al.*, 2016). *S. aucuparia* is well suited to a semi-shaded position, although *S. aucuparia* is tolerant of unsuitable conditions its distribution is limited by temperature and water availability (Räty *et al.*, 2016). Cold temperatures are required to break seed dormancy (stratification) and to stimulate bud burst (McAllister, 2005; Raspé *et al.*, 2000), whilst a lack of water in high summer temperatures leads to tree stress. *S. aucuparia* in a forest situation prefers annual precipitation levels of 500mm to 1500mm at a temperature between 5^{o}C and 10^{o}C. This species has the ability to tolerate cold temperatures and less photosynthetic active radiation (Räty *et al.*, 2016). It is adapted to survive continental and oceanic climates in both sunny and partially shaded positions and survives short growing seasons by being independent of photoperiod signalling (Sæbø and Johnsen, 2000; Räty *et al.*, 2016).

The relative value of *S. aucuparia* was assessed by O'Sullivan *et al*. (2017) and has been noted for its ability to enhance air quality through the reduction of net volatile organic compounds and add to local biodiversity (where data includes mycorrhizal interactions, richness and biomass of soil invertebrates, leaf litter communities, pollinators, and fruit set). Indeed, Suhonen and Jokimäki (2015) acknowledged that *S. aucuparia* berries are associated with the abundance of some bird species, namely *Turdus pilaris* (Fieldfare), *Bombycilla garrulous* (Bohemian Waxwing), and *Pyrrhula pyrrhula* (Bullfinch), from autumn through winter in the Northern Hemisphere due to food resource provision. This species is also known for it's a medium degree of winter hardiness and suitable performance in carbon sequestration (determined by wood density and growth rate) (O'Sullivan *et al*., 2017).

In order to sustain these key features of *S. aucuparia* it is important to optimise its survival rates when it is transplanted into an urban environment. Most trees have a mortality rate of 30% during their first year *in-situ* (Hirons and Percival, 2012). Transplant stress can be a limiting factor resulting in slow growth and tree decline once a tree has been placed into a landscape. It is therefore important that root structure and aerial growth is healthy, which is not always feasible in replant conditions (Section 1.4). RD poses a problem in terms of establishing an economically viable and resilient stock and is a globally distributed problem, and has been reported as occurring throughout the world in the major fruit growing regions (Utkhede and Smith, 1993; Mazzola, 1998; Van Schoor *et al.*, 2009; Kandula *et al.*, 2010; Zhang *et al.*, 2012) and as stated above, it has been established that this disease affects S. aucuparia (O'Neill, 2011).

1.4 Symptoms: Replant Disease (RD) and the phenotypic responses of hosts

RD was first recorded in the writings of noted agriculturalist John Worlidge in Systema Agriculturae 1698 (Suranyi, 1998). The disease affects edible crops and ornamental nursery species, such as *Asparagus*, *Citrus*, grapevine, and tree, soft fruit and shrub species within the Rosaceae (O'Neill and Wiltshire, 2007). Replant disease accumulates in the soil and more predominantly the rhizosphere throughout the duration of plant growth (Traquair, 1984) and it is when a tree is removed and the infected area replaced with the same species, that the symptoms are first displayed. Intensively cultivated land (25,000 trees per hectare) is affected in nursery rows rather than in patches (AHDB, 2008). Indeed, Hoestra (1994) argued that RD is a product of monospecific stands of crops with minimal rotation, which is a common in tree nurseries.

RD has the effect of increasing tree deformity and reducing growth and vigour which in some cases can lead to increased mortality rates in *Prunus* species such as cherry, almond, apricot and peach (Browne *et al.*, 2006). The disease can result in severe stunting of 30-73% (Brown, 2009), shortened internodes, reduced productivity, and small rosette leaves with a light appearance. RD may result in the inhibition of the enzyme that integrates magnesium into the porphyrin ring of chlorophyll (Wang *et al.*, 2014) and thus decreases photosynthetic activity.

The reduction in the growth and general vitality of above-ground physiology is symptomatic of small compact root systems, short feeder roots and decay. The premature destruction of epidermal cells and cortical tissue results in visually discoloured roots and a reduction in root hairs. This diminished root surface limits the uptake and translocation of water and minerals that are used in the molecular pathways that support plant growth and development (Caruso *et al.*, 1989; Mazzola, 1998; Messenger and Braun, 2000; O'Neill and Wiltshire, 2007; Mazzola and Mancini, 2012). Most of these symptoms are seen in the first two years of tree growth, after which the tree partially recovers, although not to its full expected vigour. The same recovery or a reversal of symptoms can be seen in RD affected cherry and apple trees when planted in new soil (O'Neill and Wiltshire, 2007).

The level of disease has been shown to vary across different sites and is often linked to site age, cropping history, soil textural class and nutrient status, and relative degree of damage to mature tree roots (Mai and Abawi, 1981; AHDB Horticulture, 2009). In 1961 Oostenbrink and Hoestra defined two types of RD in apples: specific and non-specific replant disease, both of which caused the plant to exhibit the same symptoms (also acknowledged by Bent *et al.*, 2009). The former only affects fruit tree species *Malus* (apple) and occurs more consistently across an orchard, whilst the latter affects more than one genus of fruit tree.

1.5 Causes of RD: the general consensus of what causes the disease

The causal agent of RD has been a matter for much debate and still remains unknown for some plant species, although the current consensus is that a build up of pathogens (viruses, nematodes, fungi and bacteria) in combination with abiotic factors detailed in section 1.5.1 are the main causal agents (Mai and Abawi, 1981; Willett, 1994; Utkhede and Smith, 1994; Westphal, 2002; O'Neill and Wiltshire, 2007; Tewoldemedhin *et al.*, 2011b). Abiotic factors can be ruled out as the exclusive causal agent due to the improvement in growth demonstrated subsequent to fumigation or pasteurisation of 'sick' soil. However, these soil sterilisation methods treated only 50% of a replanted apple orchard suggesting this multifactorial cause involving both abiotic and biotic factors (Yao *et al.*, 2006). The causes differ between sites and geographic regions which are explained in section 1.5.2.

1.5.1 Abiotic factors that contribute to RD

Poor tree growth in replant conditions can be ascribed to pathogens exacerbated by abiotic conditions such as the compaction of soil which results in a lack of aeration; poor structure and the presence of pans; insufficient nutrient supply or an imbalance in those available; stress caused by a lack of water; waterlogging and gleying; contamination of soil by inorganic and organic chemicals and phytotoxins; cold injury, and the affects of pH on nutrient availability (Traquair, 1984; Mazzola, 1998).

Soil type was correlated to specific nutrient deficiencies in leaves of *Malus* spp. Rootstocks. CG.401, B.9, and B.396 were more sensitive to replant conditions and linked to low leaf phosphate concentrations when grown in sandy soil. Clay soils on the other hand were found to be limiting to G.16, CG.6143, and CG.6001 and linked to lower leaf zinc concentrations (Fazio *et al.*, 2012). Mai and Parker (1972) demonstrated that an application of potassium, magnesium, calcium, and phosphorus fertiliser increased yield of *P. cerasus* by 54%. Conversely, Proebsting and Gilmore (1941) did not demonstrate a growth response from *P. persica* after application of nutrients. More recently Fazio *et al.* (2012) determined that 'replant' soil was linked to low levels of leaf iron and molybdenum concentrations in 38 different *Malus* spp. rootstocks. Zinc, sodium, potassium, manganese, calcium, magnesium and phosphorus levels were not significantly different irrespective of whether the trees were planted in diseased or pasteurised soil. Low pH and acidification of the soil (<4.5) was correlated with a decrease in the occurrence of the disease (Donoho *et al.*, 1967; Savory, 1967; Hoestra, 1968), whereas Li and Utkhede (1991) demonstrated that macronutrients (N, P, K) were positively correlated with plant height of *Malus* spp. at a pH ≥8, whilst phosphorus was exclusively linked to increased growth at a pH <5.4. These studies combined would appear to indicate that there is no prescribed set of factors that contribute towards the occurrence of RD.

1.5.2 Biotic factors: organisms ascribed to RD and host-pathogen interactions

Hoestra (1977) described RD as a site-bound self-induced disease that is the result of the 'presence or activities of a plant species'. This categorisation was associated with *Malus* spp., *Prunus* spp., and the non-rosaceous *Vitis* spp. (grape). At this stage there was no solid evidence to rationalise this self-induced state. However, in 1994, Hoestra stated that the RD phenomena existed in nature as a factor that contributes to succession whereby plants alter the environment to a point that it suits other species. *Sorbus* and other rosaceous species are considered as facilitator species, allowing for the establishment of other species and the discouragement of monospecific stands which are devoid of diversity. In addition to affecting the establishment of same species saplings it was observed that *Sorbus* spp. create conditions that are not conducive to the growth of related fruit trees (Otto *et al.*, 1994), possibly due to a change in microbial composition and soil chemistry. Mazzola *et al.* (2002) found that transformations in the soil microbial complex occurred within the first three years of an apple orchard being established. These changes included the reduction of *Burkholderia cepacia*, an increase in *Rhizoctonia solani* AG-5 (root rot), and reduction of *Pseudomonas putida* with an increase in *P. fluorenscens* and *P. syringae*.

The presence of phytoxic plant residues such as tannins, hydrocyanic acid and benzaldehyde liberated from necrotic roots can have the effect of influencing plant development indirectly through interactions with soil biology (Benizri *et al.*, 2005; Nicola *et al.*, 2017). The effect can be strongly associated with the replanting of a site with the same species. Gur and Cohen (1989) demonstrated that the deleterious effect of the prunasin by-products, hydrocyanic acid (hydrogen cyanide in solution) and benzaldehyde on tree growth and survival rates was greatest in *P. persica*, whilst being less severe in *M. domestica* and *P. amygdalus* (almond). The authors explained that this was due to the specific heat resistant *bacilli* in the rhizosphere of *P. persica* roots which effectively hydrolysed the prunasin. Likewise, Sotomayor *et al.* (2006) determined that RD on *P. persica* x *P. davidiana* could be due to an "amygdalin-associated autoalleopathic factor", in which amygdalin also degrades to form cyanide. Conversely, Rumberger *et al.* (2007) found that rhizosphere cyanide concentrations could not be associated with apple replant disease (ARD) in *M.* x *domestica*. In 2012 Hofmann *et al.* eluded to the involvement of root exudate and flavonoid phloridzin in *Malus related* RD and the chemotaxis of pathogens toward roots. A further mechanism of action, that had no biotic step, was determined whereby the tricarboxylic acid cycle was disrupted in *Malus hupehensis* (Chinese crabapple) roots due to inhibition of respiratory rates and enzyme activity by phloridzin (Wang *et al.*, 2012).

In addition to this indirect effect of toxins it has been demonstrated that numerous microorganisms can be associated with RD. Bent *et al.* (2009) found that a soil dilution of as little as 1% replant soil mixed with fumigated replant soil induced poor growth in *P. persica* seedlings due to the pathogens therein, possibly due to the lack of competition from of beneficial organisms post treatment. Soil bacteria, pathogenic fungi, actinomycetes, oomycetes and plant parasitic nematodes have been proposed as causes of RD, either as individual components or together as a complex of organisms (Merwin *et al.*, 2001; Eayre *et al.*, 2000; Utkhede and Smith, 1994). The role of micro-organisms can vary according to other extant microbial communities which may either aid in pathogenesis or compete with pathogens for resources. Yang *et al.* (2012) found a positive correlation between numbers of actinomycetes in the Xanthomonadaceae and *P. persica* shoot weights. Meanwhile a study by Otto and Winkler (1976) confirmed that actinomycetes were ubiquitous in the of feeder roots of *Malus* spp. seedlings planted in 'sick' soil. This was further substantiated by Szabó *et al*. (1998) who confirmed actinomycetes could colonise epidermal and cortical tissues by entering the root hairs. Yang *et al.* (2012) and Mulder (1969) demonstrated the presence of oomycete *Pythium vexans*, which was negatively correlated with shoot growth, however this result is not consistently found and is dependent on pathogenicity as demonstrated in a study by Mazzola *et al.* (2002) in which three non-pathogenic isolates (including *P.* MM5 (*aff. vexans*) acted as a biological control of *P. sylvaticum* and *P. ultimum*. Yang *et al.* (2012) isolated fungi *Fusarium oxysporum*, *Ceratocystis fimbriata* and the beneficial fungi *Trichoderma* spp. Caruso *et al.* (1989) found through histological studies that trees affected by RD exhibited the penetration of arbuscules and hyphae of fungi and oomycetes from species in the genera *Rhizoctonia*, *Phytophthora*, and *Pythium*, in the presence of nematodes. This particular set of microorganisms were further noted by Mazzola (1998) and Mazzola and Mancini (2012). These three genera have also been attributed to 'damping off' disease of seedlings which has a similar ability as RD to induce decay causing the stem of a seedling to collapse. From a non-pathogenic perspective Jaffee *et al.* (1982) suggested that RD may be the result of competition with rhizosphere organisms that required the same resources although, this seems an unlikely primary cause. As stated previously, the addition of nutrients has little effect and provides little explanation for root damage that is symptomatic of a diseased tree. Current research suggests that transformations within the soil community, particularly nematodes, fungi and actinomycete bacteria, result in it being conducive to inciting RD (AHDB, 2009). From this information, it can be surmised that the exact organism/s responsible have still not been conclusively determined, and differs between species and locations. It can also be concluded that different groups of pathogens may act together in inciting the disease. The particular bacteria, fungi, stramenophiles and nematodes associated with RD are defined in the immediate sections below (1.5.2.1 to 1.5.2.4).

1.5.2.1 Bacteria and their host range

Several bacteria have been associated with RD (Table 1.2). In 2005, Benizri *et al.* noted the higher incidence of bacteria in replant soils when compared to healthy soil and suggested that saprotrophic phytotoxic microorganisms may be favourably selected for in the presence of rhizodeposits. The study also determined that *Bacillus* spp. were able to produce hydrogen cyanide *in vitro*.

Table 1.2: Bacteria species associated with RD on a range of plants

Biotic factor	Associated plant(s)	Example reference
Actinomycetes	*Malus* spp., *S. aucuparia*, *P. communis*, *P. persica*, *P. avium.*	Otto *et al.*, 1994
	Malus spp.	Westcott *et al.*, 1987; Čatská *et al.*, 1989; Szabó *et al.*, 1998
Bacteria		
Bacillus spp.	*P. persica*	Benizri *et al.*, 2005
Bacillus subtilis	*Malus* spp.	Jackson, 2003
Pseudomonas spp.	Fruit trees	Utkhede, 1996
	P. persica	Yang *et al.*, 2012
P. syringae	*M. domestica*	Mazzola *et al.*, 2002
P. fluorescens	*M. domestica*	Mazzola *et al.*, 2002
P. putida	*M. domestica*	Čatská *et al.*, 1989; Jackson, 2003
Chitinophaga spp.	*Malus* spp.	Franke-Whittle *et al.*, 2015
Hyphomicrobium spp.	*Malus* spp.	Franke-Whittle *et al.*, 2015

Otto *et al.* (1994) demonstrated that actinomycetes in ARD soil could infect *Malus* spp. seedlings, and *S. aucuparia* and *P. communis* rootstocks, and have also been linked with RD in *P. persica* and *P. avium* to a lesser degree. It is possible that there is a link between the Rosaceae tribe and actinomycete infection severity, where severity is greater for species within Maleae in comparison to Amygdaleae (Hummer and Janick, 2009). These filamentous, gram positive and generally anaerobic bacteria damage the root cortex and cause the loss of root hairs (Szabó *et al.*, 1998, cited in Jackson, 2003, p466). They have been suggested as being the primary causal agent (Otto *et al.*, 1994; O'Neill and Wiltshire, 2007; Utkhede and Smith, 1994; Westcott *et al.*, 1987). The role of some bacteria is unclear with some, such as *Sphingomonas* spp. being negatively correlated with plant growth and some aiding pathogen suppression (Franke-Whittle, 2015).

1.5.2.2 Fungi and their host range

Fungi are resilient pathogens that persist in the soil for over 20 years. This can cause difficulty when designing control strategies as some methods such as crop rotation have little success (Easton *et al.*, 1992). The fungi that have been ascribed to RD fall within two taxonomic classes, namely: Ascomycota and Basiodiomycetes. Most cause root rot which leads to a lack of nutrient uptake (Table 1.3). RD has been associated with secondary metabolites, mycotoxins, from fungi (Mancini *et al*, 2017).

Table 1.3: Fungal species associated with RD on a range of plants

Biotic factor	Associated plant(s)	Example reference
Fusarium spp.	*Malus* spp.	Franke-Whittle, 2015
F. equiseti,	*P. persica*	Wensley, 1956; Hine, 1961
F. oxysporum	*P. persica*	Wensley, 1956; Hine, 1961
F. oxysporum	*Malus* spp.	Mancini *et al.*, 2003
F. solani	*Malus* spp.	Mancini *et al.*, 2003
F. solani	*P. persica*	Wensley, 1956; Hine, 1961
F. moniliforme	*P. persica*	Wensley, 1956; Hine, 1961
Rhizoctonia spp.	*Malus* spp.	Mancini *et al.*, 2003
R. solani	*P. persica, Malus* spp.	Yasuda and Katoh, 1987
R. solani	*P. persica*	Browne *et al.*, 2006
R. solani	*S. aucuparia*	O'Neill, 2011
Verticillium dahlia	*P. persica, Malus* spp.	Yasuda and Katoh, 1987
Rosellinia necatrix	*P. persica, Malus* spp.	Yasuda and Katoh, 1987
R. necatrix	*Malus* spp.	Mazzola and Mancini, 2012
Thielaviopsis basicola	*P. avium*	Hoestra, 1965
T. basicola	*P. avium*, *P. domestica*	Sewell and Wilson, 1975
Cylindrocladium spp.	Fruit trees	Utkhede, 1996
Penicillium claviforme	Fruit trees	Utkhede, 1996; Čatská *et al.*, 1989
P. claviforme	*Malus* spp.	Jackson, 2003
P. janthinellum	Fruit trees	Utkhede, 1996
P. janthinellum	*Malus* spp.	Jackson, 2003
Cylindrocarpon spp.	Fruit trees	Utkhede, 1996; Franke-Whittle, 2015
Cylindrocarpon spp.	*Malus* spp.	Mancini *et al.*, 2003
C. destructans	*Malus* spp.	Jaffee *et al.*, 1982; Caruso *et al*., 1989
C. destructans	*S. aucuparia*	O'Neill, 2011
C. macrodidymum	*P. persica*	Schmidt *et al.*, 2014
C. lucidum	*Malus* spp.	Mai and Abawi, 1981; Jaffee *et al.*, 1982a
Peniophore sacrata	*Malus* spp.	
Constantinella terrestris	*Malus* spp.	Jackson, 2003
Acremonium spp.	*Malus* spp.	Jackson, 2003; Franke-Whittle, 2015

In a report of 2007, the AHDB stated that fungicides utilised to treat RD did not have consistent results, therefore suggesting that the aetiology of this disease is not based solely on fungi (O'Neill and Wiltshire, 2007). Furthermore Hoestra (1968) determined that nematicides and fungicides were ineffective.

1.5.2.3 Stramenopiles and their host range

Stramenopile is a Kingdom including diatoms, brown algae and oomycetes (Lévesque, 2011; Adhikari *et al.*, 2013). Oomycetes include some of the most devastating pathogens, including *Phytothphora*. The class of oomycota has been linked to RD on a range of rosaceous stock (table 1.4).

Table 1.4: Stamenopile species associated with RD on a range of plants

Biotic factor	Associated plant(s)	Example reference
Water moulds		
Pythium spp.	Fruit trees	Utkhede, 1996
Pythium spp.	*Malus* spp.	Mancini *et al.*, 2003; Yao *et al.*, 2006
Pythium spp.	*P. persica*	Hine 1961; Schmidt *et al.*, 2014
P. ultimatum	*Malus* spp.	Mulder, 1969
P. ultimatum	*P. persica*	Bent *et al.*, 2009
P. ultimatum	*S. aucuparia*	O'Neill, 2011
P. sylvaticum	*Malus* spp.	Mulder, 1969; Sewell, 1980
P. irregulare	*Malus* spp.	Jaffee *et al.*, 1982a; Braun, 1991
P. intermedium	*Malus* spp.	Mulder, 1969
P. vexans	*P. persica*	Yang *et al.*, 2012
Phytophthora spp.	Fruit trees	Utkhede, 1996
P. cactorum	*Malus* spp.	Mazzola 1998
Diatom		
Sellaphora spp.	*P. persica*	Bent *et al.*, 2009

In 2006, Yao *et al.* noted the presence of *Pythium* spp. in higher concentrations in *Malus* replant soil than *Phytophthora*. Rumberger *et al.* (2004, 2007) found the infestation by the pathogen *Pythium* spp. to be no different irrespective of rootstock. In 2011 ADAS conducted an evaluation of treatments of RD in *S. aucuparia* (O'Neill, 2011), which concluded that *Pythium* is a worldwide (USA, Tunisia, and UK) component of the disease and is often found in a complex with fungi.

1.5.2.4 Nematodes and their host range

Nematodes are free living, microscopic, worm like multicellular animals and can be endoparasitic (parasitic within), semi-endoparasitic, or ectoparasitic (parasitic on the outside of an organism). Nematodes can act exclusively on one host or they can be nonspecific, and are abundant in soil and have various modes of action whereby this occurs, namely: direct cell destruction; creating syncytia; acting as a vector; or facilitation of fungi and bacteria root invasion as seen in *Prunus* spp. (Wang *et al.*, 2007; Hooks *et al.*, 2010). Nematodes have been associated with RD (Table 1.5) however their presence is not always consistent or indicative of their involvement with the disease.

Table 1.5: Nematode species commonly associated with RD on a range of plants

Biotic factor	Associated plant(s)	Example reference
Pratylenchus spp.	*Malus* spp.	Westcott *et al.*, 1986
	Fruit trees	Mai *et al.*, 1981; Pokharel *et al.*, 2015
	Malus spp.	Jaffee *et al.*, 1982b
P. penetrans	*Malus* spp., *P. avium*, *Pyrus*	Mai and Abawi, 1978
P. projectus	spp.	Mai and Abawi, 1978
P. penetrans	*Malus* spp., *P. avium*, *Pyrus*	Utkhede, 1996
Xiphinema spp.	spp.	Utkhede, 1996; Pokharel *et al.*, 2015
Meloidogyne spp.	Fruit trees	Pokharel *et al.*, 2015

Mazzola (1998) found counts of nematodes were comparably higher in healthy soils and lower in replant soil. Westcott *et al.* (1986) determined that steam treatments improved tree growth whilst reducing actinomycetes and nematodes; however there was no indication of whether nematode population decline was responsible for the positive impact of the treatment. The role for nematodes as facilitators of RD is further devalued by the findings that air-drying of soil reduces populations of nematodes but this does not positively impact on tree growth (Jaffee *et al.*, 1982). It is also possible that nematodes could be opportunists that take advantage of a plant compromised by the causal agents of RD. Pokharel *et al.* (2015) noted that nematode community composition (including both plant parasitic and free living) was more diverse and an indicator of a healthy soil in organic *P. persica* and *M. domestica* systems in comparison to a conventional nursery setting.

1.6 Treatment of RD using chemicals and non-chemical alternatives

1.6.1 Methyl bromide as a historical treatment for RD on rosaceous stock

Broad spectrum soil fumigants were introduced in the 1940s as a reliable method of controlling edaphic pest and diseases, resulting in increased quality and yield of crops. Chemical treatments replaced cultural techniques that were used previously as an effective method of boosting growth by lowering disease (Braun and Supkoff, 1994). Crop treatments are therefore orientated towards synthetic chemicals, bringing the inherent risk of environmental contamination and human health problems.

On nurseries RD was previously treated by broad spectrum fumigant MeBr, which allowed for the vigorous growth of trees after fumigation, as was exemplified by experimentation in California by Browne (2002). The improvement in the above ground canopy of peach trees was a result of the growth of extensive healthy roots. Replant roots by comparison appeared fibrous, brittle, discoloured and decayed.

In 1993 the Parties of the Montreal Protocol declared MeBr as a Class I stratospheric ozone depleting compound and MeBr was proposed to be gradually phased out in developed countries by 2005 and in developing countries by 2015 (Appendix I). In the European Community (EC) ozone depleting compounds are regulated with manufacturing, importation, exportation, and marketing legislation under regulation (EC) No. 2037/2000 of The European Parliament and of the Council of 29 June 2000 (Centre for Agiculture and Biosciences International, 2008). In the Netherlands MeBr is classified as a ground, surface, and drinking water contaminant. In Switzerland concerns also centred on the perceived risk of the build-up of bromine in food crops above the recommended daily intake (Braun and Supkoff, 1994; Messenger and Braun, 2000; Trout, 2002).

1.6.2 Alternative chemical treatments to MeBr

Various chemicals were reviewed by Messenger and Braun (2000) to find an alternative to MeBr. Alternatives require a similar capacity to eradicate deleterious organisms and comparable properties such as a low boiling point (36°C) and high vapour pressure allowing effective penetration of soil and minimal residual phytotoxicity. Metam sodium, dazomet, chloropicrin and methyl iodide were all notable broad spectrum biocides that act against fungi, insects, weeds and nematodes, whilst telone exclusively controls nematodes (Messenger and Braun, 2000) and as with any treatment method, each of these have their strengths and limitations (Table 1.6).

Table 1.6: Some of the strengths and limitations of chemical alternatives to methyl bromide

		Strength	Limitation	Reference
Treatment	Metam sodium Dazomet	Acts against a range of pathogens. Reduces RD symptoms by modifying the microbial community.	No effect against fungi *Fusarium* or *Verticillium*. Soil penetration and dispersal potential does not compare to MeBr.	Braun and Supkoff, 1994; Messenger and Braun, 2000; Trout, 2002; Nicola *et al.*, 2017.
	Cis-1,3-Dichloropropene or Telone (cis-1,3-D)	Lasts for up to six years. Acts specifically against nematodes. Effective against pathogens when combined with dazomet.	Needs to be combined this chemical with either MITC or chloropicrin for broad spectrum control.	Duniway *et al.*, 1999; Messenger and Braun, 2000;
	Chloropicrin or Tear Gas®	Works well against RD and its action against fungi is comparable to MeBr	Longer lasting residual phytotoxicity than MeBr	Messenger and Braun, 2000; Trout, 2002
	Methyl iodide	Methyl iodide (MI) was found to be as effective at controlling RD as MeBr. Breaks down readily in the presence of UV light	Concerns regarding toxicity and human health	Eayre *et al.*, 2000 Froines *et al.*, 2010

Chloropicrin has specifically been identified as an effective treatment of RD in a *P. persica* nursery, resulting in healthier trees that were not stunted and had a full canopy (Browne, 2002). Efficacy is dependent on chemical formulation, as demonstrated by Yao *et al* (2006) who stated Telone C-17 (containing chlopicrin) effectively controlled the disease but did not improve tree growth or yield. In 2011, O'Neill trialled the treatment of replant soil on *S. aucuparia* with Custofume (98% chloropicrin), Basamid (98% dazomet), 'Biofence' Caliente mustard meal (*Brassica carinata*) pellets, Novozymes

'MycorrhizaRoots', PlantMate granular root zone starter granules/PlantMate WP (*Trichoderma harzianum*), Agralan Revive (*Bacillus subtilis*), and supplementary nitrogen. Out of these treatments it was found that Custo-fume was the most successful at improving root and shoot growth and reducing bud failure in *S. aucuparia*. Top growth increased by 124% and below ground growth by 113% when applied at a rate of 280 l/ha under sealed conditions. Custo-fume was not deleterious to beneficial *Trichoderma* spp. Growth of *S. aucuparia* after the other treatments were applied was found to be no different to the control group.

The effectiveness of any chemical alternative is dependent on their correct application and thorough mixing with the soil. They also have a residual phytoxicity requiring a period of time called a re-entry interval before an area is deemed safe to enter without protective clothing and equipment (Messenger and Braun, 2000). They have all been found to be pollutants to either the air or waterways, whilst some are considered to be developmental toxins and carcinogens (Braun and Supkoff, 1994; Messenger and Braun, 2000; Trout, 2002; Froines *et al*, 2010). It is possible to use chemicals in limited quantities thus reducing their deleterious impact. For example, Browne *et al* (2013) demonstrated 'GPS-controlled tree spot shank fumigation' resulted in improved vegetative growth and yields of *P. persica* and *P. amygdalus*, when treated with chloropicrin and cis-1,3-D, which is an encouraging result. The use of GPS reduces the quantity of chemicals required and thus is economically beneficial and biorational (Udompetaikul *et al*, 2013). Cabrera *et al* (2015) found that reduced rates of Telone C35 under sealed conditions controlled *P. ultimum* and *V. dahliae*, but had little effect on *Fusarium* spp. and *P. cactorum*. Likewise, Yao *et al* (2006) and Merwin *et al* (2001) found that Telone C-17 and metam sodium were of little benefit to orchards with ARD across a range of soil types, the unreliability was attributed to formulation, dosage, and soil penetration capacity. Use of chemicals therefore yields inconsistent results that may well be dependent on their correct application, and the biotic and abiotic conditions.

1.6.3 Legislation for chemical treatments and move towards organic methods

Plant protection products are tailored to offer protection to plants against harmful organisms such as pathogens, pests and weeds. They also include substances that stimulate or regulate plant growth. The UK has a substantial crop protection industry supplying the agricultural, horticultural, domestic and amenity sectors. According to the latest available figures, dating from 2006, the UK was the fourth biggest user of pesticide consumers in Europe, with 21,251 t of active ingredient being sold in year. Other big users of pesticides include Denmark (31,819 t), France (71,612 t), and Italy (81,450 t). Of the pesticide sales in the UK in 2006 the majority were herbicide products (43%), whilst the rest were fungicides (25%), insecticides (3%), and other plant protection products (28%) (European Commission, 2016).

The regulation of plant protection products plays an important role in their development and approval for use, ensuring that the risk to humans, animals and the environment is minimised, and the benefits to plant production maximised, as stipulated in Regulation (EC) No. 1107/2009, Water Framework Directive (WFD) 2000/60/EC, and Approval of Neonicotinoids Regulation 485/2013. The Sustainable Use Directive (SUD) 2009/128/EC provides a framework for the sustainable use of chemicals within the Agriculture, Horticulture, and Amenity sectors throughout Europe. In 2014, Integrated Pest Management (IPM) was adopted as part of the SUD with the statutory development of a National Action Plan (NAP) outlining Directive implementation (Stark, 2011).

As legislation drives the withdrawal of active substances and the restriction of new products the above mentioned sectors will suffer losses. The only respite from the removal of these products is the AHDB Horticulture Extension of Authorisation for Minor Uses (EAMU) programme which allows growers to apply for short term approval to use a product which is no longer supported for manufacture or use (AHDB Horticulture, 2016). In 2013 the Chemicals Regulation Directorate (CRD) on behalf of the Department for Environment, Food & Rural Affairs (DEFRA) stated that the UK had a five-year research and development programme investigating the use of economically and environmentally sustainable alternatives to chemicals as part of their IPM strategy, including cultural practices, natural active substances and plant breeding measures. IPM lowers reliance on synthetic pesticides and produces comparable yields (Parliamentary Office of Science and Technology, 2009). The research, in this thesis, into the use of green manure as a control of RD on *S. aucuparia* is relevant to this changing ethos. Green manure contains natural constituents that are antimicrobial in nature. The use of a green manure as an alternative to chemical control brings numerous benefits. A broad spectrum pesticide does little else other than non selectively kill both beneficial and deleterious species of microorganisms. Meanwhile the addition of organic matter can condition the soil and improve structure whilst suppressing deleterious microorganisms whilst populations of beneficial microbes increase in numbers.

1.6.4 Treatments used to control RD that do not include synthetic chemicals

1.6.4.1 Soil amendments and suppressive soils

A green manure soil provides a range of benefits from improved soil structure and water holding capacity, to antimicrobial effects and increased soil microbial activity leading to pathogens being out-competed for resources, and long term nutrient availability for plant uptake. Green manures can be used in biofumigation, where natural plant defences or their antimicrobial properties, can be used to benefit a grower in the disinfestation of soil. A well researched plant family used for biofumigation, are the Brassicaceae, which yield toxins due to the glucosinolate-myrosinase defence mechanism (Larkin and Griffin, 2007). Any damage to the tissue of brassicaceous plants results in the release of nitriles, epithionitriles, thiocyanates and isothiocyanates. Oliver *et al* (1999) demonstrated that allyl isothiocyanates and isothiocyanates inhibit *Pythium* sp., *Fusarium* sp. and *V. dahliae* by cessation of germination and mycelial growth. Meanwhile, mulches can be used for the purpose of improving vigour, reducing tree transplant stress, suppressing weed seed germination, and improving colonisation rates of beneficial microbes which out-compete or chemically inhibit pathogenic microbes (Percival *et al*, 2009).

Increase in soil microbial activity induced by organic amendments was established before the 1990's (Fraser *et al*, 1988). Merwin *et al* (2001) demonstrated that a compost treatment (ground leaves/wood chips, vegetable culls, and cattle/horse manure, 40:40:20 respectively) effected soil bacteria and fungal community composition, and increased soil microbial activity. Even before its use as a soil amendment, green manure may be grown as a cover crop that can out-compete weed species and protect the soil from adverse weather and erosion. The stimulation of certain microbes can directly change the microbial composition of a soil by favouring microorganisms that can suppress pathogenic populations. 'Suppressive soils' work by halting establishment of pathogens and/or hindering the growth of deleterious organisms to the point that their presence has no influence upon the host plant. Suppressive soil can be promoted through the use of cover crops and green manure amendments, and has been shown to control pathogens, such as nematodes (Jaffee *et al.,* 1982), bacteria (Bunt and Mulder, 1973) and fungal pathogens (Mazzola, 1998) on a number of woody ornamental species (Merwin *et al*, 2001). Improvements in the soil microbial community can be achieved by simply growing a crop that is later excised before replanting with a rosaceous species, as demonstrated by Gu and Mazzola (2001, 2003) who improved *Malus* sp. seedling growth by cultivating *T. aestivum*. Their results revealed that wheat exudates increased the population of the Plant Growth Promoting Rhizobacteria (PGPR) fluorescent *Pseudomonas putida,* and a decrease in *P. syringae* and *P. fluorescens* bv. III. This change in the microbial composition suppressed *R. solani*, *C. destructans*, and *P. ultimum*. Other organisms that have been noted for their positive association with tree growth or antagonistic activity towards fungi are *Azospirillum*, *Agrobacterium*, *Bacillus*, *Enterobacter,* and *Trichoderma* (Čatská *et al.*, 1982; Čatská *et al.*, 1987; Čatská and Taube-Baab, 1989; Utkhede *et al.*, 1992; Utkhede and Smith, 1993). These natural biostimulant microbes work by producing antimicrobial substances such as antibiotics and chitinases, in

addition to siderophores which deprive pathogens of iron (García-Fraile *et al.*, 2015). They also prime the plant by induction of systematic resistance and mobilise nutrients for plant uptake. Addition of organic matter (OM) can have various effects on the severity of RD. For example rice husk biochar can improve plant height and chlorophyll content in the leaves, and was positively correlated with a reduction of phenolic compounds produced during the decomposition of *Malus* spp. roots and leaves ascribed to RD (Politycka and Adamska, 2003). Green soil amendments, such as seaweed, can also improve plant enzyme activities, leading to a decrease in lipid peroxidisation (Wang *et al.*, 2016), as well as increase soil microbe richness and evenness resulting in a healthy soil quality.

Arbuscular mycorrhizal fungus (AMF) *Funneliformis mosseae* was found to have a similar effect. The addition of AMF aids the growth of *P. persica* seedlings showing RD symptoms which were a result of the activity of cyanogenic compounds against extant mycorrhizae. The availability of nutrients through the 'extended root system of hyphae' resulted in improved growth and chlorophyll concentrations. Much like the fermented OM, mycrorrhizae can also activate catalases and peroxidases (Zhang *et al.*, 2015).

1.6.4.2 Biological controls for RD

The use of biological controls and soil amendments to alter the microbial community can be undertaken. The composition of the microflora can be altered either directly through the addition of a specific species or indirectly via the introduction of a soil amendment that can influence the extant microbial structure. Mazzola (2007) favours the latter, due to the former resulting in inconsistent growth and yields of the crop, due to the introduction of a biological agent into an environment in which it will be out-competed by the native microorganisms and exposed to conditions (pH and temperature) that are not conducive to their population growth. The soil could be considered as suppressive as a result of these changes, and in agreement with this it was demonstrated that antagonists *Agrobacterium radiobacter* (used to treat crown gall) and *Trichoderma harzianum* (used for the control of diseases in crops) were not as effective as fumigation (Messenger and Braun, 2000).

1.6.4.3 Cultural practices – crop rotation, containerisation, steaming and solarisation

Cultural practices are frequently used in the control of RD. The use of crop rotation, manure and inter-cultural planting was suggested by various agriculturalists in the 16th and 17th centuries as a way of reducing the occurrence of RD (Suranyi, 1998). This form of treatment for RD is ideal in an agricultural, soft fruit or gardens and parks scenario but it cannot be applied to nurseries and orchards due to the physical difficulty of rotation and the chronic persistence of RD in the soil. An alternative to rotation is the use of containers which are now manufactured so as to prevent root circling. This practice can have

its limitations however, where the size of the container and need for frequent irrigation and feeding is restrictive to tree growth.

Heat can often be used to control soil disease and can be applied in the form of steam or solar radiation. Steam effectively treats soil infected with pathogens, such as *Fusarium* spp., within half an hour at 50-60 °C (Braun and Supkoff, 1994). Likewise solar heat (soil solarisation) works well in hot climates to control *V. dahliae*, and was found to be more effective against *Verticillium* wilt on *Pistacia vera* (pistachio) than MeBr. Soil solarisation method also controls bacteria and weed species (Messenger and Braun, 2000), and can be used in combination with rice bran compost, after which irrigation to water holding capacity induces anaerobic conditions and stimulates anaerobic decomposers leading to the release of by-products, such as volatile organic compounds that are toxic to pathogens (Strauss *et al*, 2015). Furthermore Browne *et al* (2014) demonstrated that anaerobic soil disinfestation controlled *P. ultimum* at levels comparable to fumigation. Likewise, Shennan *et al* (2014) indicated that this treatment resulted in a change in rhizosphere micro-organisms associated with *Fragaria* spp. A full understanding of how this control influences microbial compositions is not known (Strauss *et al.*, 2015). More recently the use of heat as a means of sterilisation has progressed with recent research by Mao *et al.* (2015) demonstrating that a tractor mounted Flame Soil Disinfestation (FSD) machine can reduce populations of nematodes (>95%), *F. oxysporum* (>44%), *Phytophthora* spp. (>47%) and *Ralstonia solanacearum* (67%). Soil is passed through flames at a temperature of 1200°C and returns to the ground at 50-70°C. This is an expensive method which may not result in the lasting reduction of pathogens. Sterilisation can disrupt the balance of beneficial microbes such as mycorrhizae, and has the potential to release phytotoxic chemicals (Messenger and Braun, 2000), thus the establishment of a new community that may favour re-infestation of pathogens (Jaffee *et al.*, 1982). Conversely Yim *et al.* (2015) demonstrated that heat treatment at 50°C and gamma irradiation improved *Malus* M26 rootstock plants and increased the abundance of bacterial genera antagonistic towards pathogens.

1.6.4.4 Breeding: resistant stock and rootstocks

The breeding of resistant stock and the use of resistant rootstocks are a worthwhile strategy although are not protective against a number of pathogens (Messenger and Braun, 2000). Rootstocks need to be selected carefully, as demonstrated by Rumberger *et al* (2007) on ARD effected sites, where three out of five rootstocks were susceptible to the disease. Rootstock-specific response to RD was also highlighted by Almeida *et al* (2016) and in research on ARD by Zhu *et al* (2014). Browne *et al* (2013) also showed inconsistency in rootstock tolerance and specifically demonstrated that those with *P. persica* parentage were more susceptible to peach RD, where a *P. persica* x *P. amygdalus* rootstock did not significantly improve growth and were susceptible to nematodes. Rumberger *et al* (2007) recommended that RD should be managed by inter-row planting of tolerant rootstocks, a strategy that has been confirmed as an effective method of minimising the affects of RD; however it is required in conjunction with

promotion of suppressive soils (Kelderer *et al*, 2012). At present, the development of rootstocks requires an improved understanding of the molecular response of root plant defences to pathogens, the interaction of a rootstock genotype with scion material, and the soil environment biology and conditions (Zhu *et al*, 2014).

1.6.4.5 Overview of non-chemical treatments for RD

Soil amendments have been shown to be effective on their own and also as a supplement to cultural practices. Their benefits are multidimensional with respect to improving tree vigour and yield, survival through reduction of stressful conditions, and a media that stimulates the colonisation of the soil with beneficial communities of micro-organisms (Section 1.7.4.1).

1.6.5 The use of organic soil amendments and their potential to control RD

This research in this thesis investigated the control of RD on *S. aucuparia* by applying sub-tropical shrub *Phytolacca americana* (pokeweed) as a novel green manure, alongside application of comparison treatments including conventional green manure species used to control soil disease in annual crops (*Triticum aestivum* (wheat); *Brassica juncea* (Indian mustard)), those not traditionally used as biofumigants but known for antimicrobial properties (*Allium sativum* (garlic); and *Tagetes patula* (French marigold)) (Figure 1.6).

a b c d e

Figure 1.4: Green manure species: propagated by vegetative means and seed.

Key: *P. americana* (a), *A. sativum* (b); *T. aestivum* (c); *B. juncea* (d); and *T. patula* (e)

These species have previously demonstrated a range of antimicrobial constituents which have been demonstrated as being effective against a variety of deleterious organisms (Table 1.7).

Table 1.7: Antimicrobial constituents of plants used as green manures

	Major bioactive constituent(s)	Reference(s)
Novel green manure species		
P. americana	Ribosome Inhibiting Protein PAP Ribosome Inhibiting Protein PAP-2	Monzingo *et al.*, 1993; Barbieri *et al.*, 1982; Hudak *et al.*, 2011; Poyet *et al.*; 1998. Ribeiro *et al.*, 2013.
	Phenolic acids	Bae *et al.*, 1997.
	Betacyanins	Schliemann *et al.*, 1996.
	Triterpene saponins	Di Maro *et al.*, 2007.
	Pathogen-related proteins	Ohta *et al.*, 1995a.
	Flavonoids	Hansel *et al.*, 1993 (In Ravikiran *et al.*, 2011); Ohta *et al.*, 1995a.
Species used as comparative soil amendments		
B. juncea	Nitriles, epithionitriles, thiocyanates and isothiocyanates. allyl isothiocyanates	Olivier *et al.*, 1999; Larkin and Griffin, 2007
T. patula	Thiophenes	Wang *et al.*, 2007; Marotti *et al.*, 2010; Gupta and Vasudeva, 2012; Margl *et al.*, 2001.
	Flavonoids	Al-Musayeib *et al.*, 2014; Gupta and Vasudeva, 2012.
	Others: Phenolic compounds; Glycosides; Iodine	Gupta and Vasudeva, 2012.
T. aestivum	Flavonoids	Harborne *et al.*, 1986; Harder and Christensen, 2000; Asenstorfer *et al.*, 2008; Suriyavathana, 2016.
	Glycoside	Asenstorfer *et al.*, 2008; Suriyavathana, 2016.
	Thionins	De Caleya, 1972;
	Antimicrobial peptides	Badea *et al.*, 2009; Odinstova *et al.*, 2013;
	Others: Terpenoids; Steroids; Saponins; Phenols; Tannins; Alkaloids	Suriyavathana, 2016
A. sativum	Allicin (diallythiosulphinate)	Arzanlou and Bohlooli, 2009.
	Diallyl disulphide and diallyl trisulphide	Avato *et al.*, 2000.
	Saponins	Lawson *et al.*, 2007.
	Steroidal glycosides, Flavonoid: Quercetin	Edris and Fadel, 2002

1.7 Aims and Objectives:

Plants produce natural defence chemicals as a response to injury that can be caused by an array of organisms, such as microbes, parasites and herbivores. Plants also produce secondary metabolites as a result of growth related metabolic and biological synthesis activities. The creation of mulch by macerating vegetation and incorporating it into the soil makes these properties more easily available to the rhizosphere of nursery stock. As a result tree growth is healthier and more vigorous.

The aim of this research was to investigate the use of a novel green manure consisting of *P. americana* as a potential control for replant disease on *S. aucuparia*, in comparison to other plants; *B. juncea*, *A. sativum*, *T. aestivum*, and *T. patula*. The research was split into three streams of work involving a three year bioassay running alongside microbial inhibition tests and DNA analysis. Each of these pieces of work contained a degree of novelty as defined below (Figure 1.7).

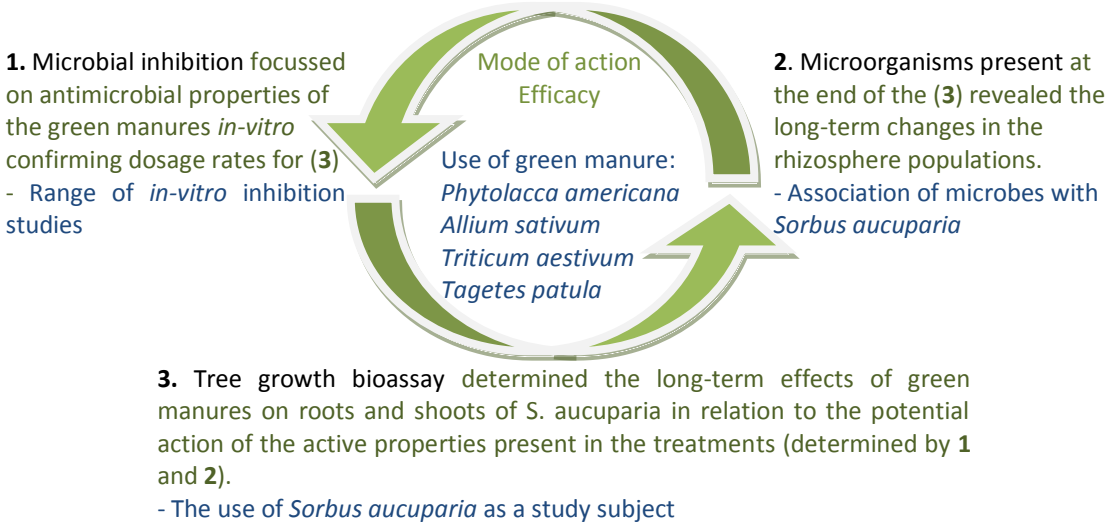


Figure 1.5: The link (green) between the three studies (black) and their novelty (blue).

***S. aucuparia*:** is an amenity/parkland/woodland species that was identified as being sensitive to replant conditions. Much of the research on RD involves fruit trees. Currently there is one study that ascribes a pathogen complex to this species. The question asked here; is this complex always found?

***P. americana*:** has not been used in the context of a green manure before. Its use here was based on the saponin content (antimicrobial) that makes it inedible to humans unless it is processed first.

The use of comparison green manures: have not been assessed in this context before: aerial parts of *A. sativum* have not been utilised as a green manure despite containing allicin content; *T. aestivum* was used as a negative control; and *T. patula* has not been used to treat replant disease previously.

***In-vitro* inhibition studies:** ranged in terms of application of green manure to agar. Some traditional extractions were used (ethanol extract and distillation); however use of fresh material embedded in agar was designed to reflect its application in the bioassay and a cold pressed extract mirrored current practice for some biostimulants (Section 2.4).

The main aims and objectives of these work streams were as follows:

Chapter 3: Microbial inhibition test - aim and objectives: To determine whether macerated plant material inhibits the *in-vitro* growth of pathogens, *P. ultimum* and *R. solani*.

- Determine the potential of macerated leaves of *P. americana* to inhibit pathogens.
- Demonstrate if volatiles from *P. americana* alone can influence the growth of pathogens.
- Examine the affects of a distillate of *P. americana* on the growth of pathogens.
- Identify if increasing concentration of *P. americana* is correlated with pathogen growth rates.
- Investigate if other proposed green manures are comparable to *P. americana* in their potential to influence the growth rate of pathogens.
- Determine if there is a difference in the inhibition of plant material between pathogens of different biological classifications; fungi and oomycetes.

Chapter 4: Three year bioassay - aim and objectives: To determine the effect of novel green manure applied as a treatment to RD on *Sorbus aucuparia*.

- To demonstrate the influence of green manure on above ground growth parameters, including height, girth, leaf production, leaf chlorophyll content, active buds, and internodal distances, during the course of three years.
- To assess the impact of green manure on the growth of root and shoot biomass, and fruit production, at the end of the trial.

Chapter 5: Determination of microorganisms present - aim and objectives: To determine the difference in rhizosphere microorganisms present in the soils in the diseased sub-set and sterile sub-set in relation to the treatment applied.

- Define the pathogen complex on *S. aucuparia* through analysis of DNA sequencing results from diseased soil.
- Determine the potential long-term effect of *P. americana* and other green manures on the presence of pathogens.
- Examine the presence of pathogens present in the sterile soil sub-set.

Chapter 6: Discussion

To discuss the key findings of each chapter and draw conclusions together.

Chapter 2 – Materials and Methods

2.1 Green manure treatments: used in *in-vitro* studies and tree growth bioassay

Plants that were used as a source of the green manure (Table 2.1) were procured and cultivated during the spring of each research year for use in the tree pot trial or as part of *in vitro* microbial studies.

Table 2.1: Green manure and source

Green manure type	Plant species	Common name	Source
Trial - novel	*P. americana*	Pokeweed	Swines Meadow Farm Nursery, UK
Conventional	*B. juncea*	Indian Mustard	Marshalls Seeds Ltd., UK
	T. patula	French Marigold	Mr Fothergills Seeds Ltd., UK
Unconventional	*A. sativum*	Solent White Garlic	The Garlic Farm, UK
	T. aestivum	Wheat SY30901	Syngenta, UK

P. americana and *A. sativum* were cultivated in 5l pots, to accommodate perennating structures, whilst *B. juncea*, *T. aestivum* and *T. patula* were grown in customised troughs containing 50mm MOT type 1 hardcore base with drainage holes every 100mm and a membrane lining (Figure 2.1).

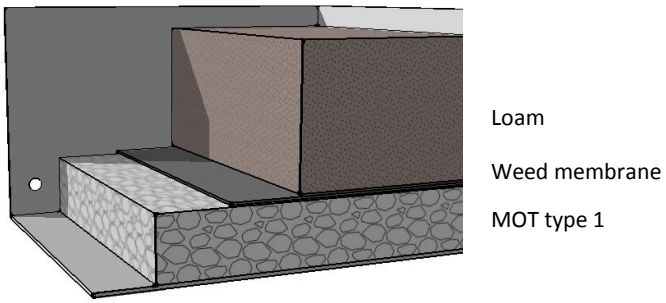


Figure 2.1: Customised trough designed for the growth of green manure.

Loam based John Innis No. 1 compost was used. Plants were started off under protected cover in a glasshouse and moved outside into a sheltered area after establishment with vegetative yield being maximised with appropriate pruning. In the winter *P. americana* plants were protected with frost sheets. Plants were regularly checked for pests and disease and affected plant material was removed, up until pre-flowering spring harvest. The only pests encountered were slugs and snails. Slug pellets were broadcast around the growing area. The vegetative yield of the plants was maximised with the removal of flowering parts with a sharp and clean blade. Once the plants had reached a sufficient bulk they were harvested (for wheat and mustard this was done before the plant reached the flowering stage and whilst the vegetative matter was still soft stemmed).

2.1.1 Tissue analysis: the nutrient content of the green manures

Green manures were cultivated as above and nutrient content/health with nutrient content analysed (Table 2.2; as supplied by NRM methods in Appendix IV).

Table 2.2: Nutrient content of green manure after tissue analysis.

Nutrient	*T. aestivum*	*P. americana*	*A. sativum*	*B. juncea*	*T. patula*
Nitrogen (%)	3.14	2.01	5.05	2.67	4.42
Sulphur (%)	0.38	0.36	1.34*[1]	0.46	0.96*[1]
Phosphorus (%)	0.61*[1]	0.45	0.67*[1]	0.53*[1]	0.92*[1]
Potassium (%)	5.87*[1]	3.82	3.93	4.18*[1]	3.37
Calcium (%)	0.49*[2]	1.48	0.53	1.72*[1]	1.65*[1]
Magnesium (%)	0.16	1.89*[1]	0.46	0.29	1.21*[1]
Manganese (mg/kg)	22.90	422.00*[1]	53.50	18.30	285.00*[1]
Iron (mg/kg)	161.00*[1]	3431.00*[1]	77.70*[1]	857.00*[1]	184.00*[1]
Copper (mg/kg)	8.65*[1]	18.50*[1]	8.81*[1]	5.94	8.10*[1]
Zinc (mg/kg)	78.10*[1]	43.50*[1]	33.30*[1]	80.00*[1]	65.80*[1]
Boron (mg/kg)	6.37*[2]	77.70*[1]	22.00	38.40*[1]	34.40*[1]

*[1] nutrient is excessive *[2] nutrient is deficient

2.2 Green manure application rates

Quantities of green manure applied were comparable to rates used by Cochran and Rothrock (2015) for a *Brassica* based biofumigant bioassay. Application rates of 1.5 tons acre^{-1} (after Sullivan, 2003) were utilised giving 8.33g/l of green manure. The amount of biomass of green manure propagated and applications are detailed in Table 2.3. *P. americana* was tested at three concentrations equating to a low, moderate, and high dosage.

Table 2.3: Yield of green manure

Green Manure	Fresh biomass (g)	Breakdown of application (g per 3 litre pot)	Amount of green manure (g per litre)
P. americana	4500	5.00	1.67
		25.00	8.33
		45.00	15.00
B. juncea	3000	25.00	8.33
T. patula	1500	25.00	8.33
A. sativa	1500	25.00	8.33
T. aestivum	1500	25.00	8.33

2.3 Protocols for *in-vitro* studies

2.3.1 Organisms and culture methods

R. solani AG-5 and *P. ultimum* var *ultimum* were cultured on the Potato Dextrose Agar (PDA; Sigma Aldrich Ltd, UK) from stocks originally acquired from the Warwick Crop Centre in 2013 and stored at 8°C. PDA (Sigma Aldrich Ltd, UK) was prepared in 500ml screw top reagent bottles (39g/l) and autoclaved, using an Astell autoclave, at 121°C for 15 minutes. The molten agar was left to cool to approximately 50°C before being poured into petri dishes (c. 10ml per 90mm dish). Once the agar had cooled sufficiently enough to set the dishes were inverted to avoid condensation on the agar surface. Petri dishes were either used immediately or stored at 3°C. Before commencement of *in-vitro* studies the pathogen cultures were prepared for use by re-plating plugs of the pathogen on PDA and allowing growth to take place over a period of seven days at 23°C. These petri dishes, which were made up with pathogens prior to each set of *in-vitro* studies, were used as the source of pathogen plugs. A 200µl pipette tip was used to consistently cut 5mm plugs of pathogen from seven day old culture stock. These plugs were subsequently placed centrally on agar and incubated, in a Leec incubator, at 23°C in darkness after being sealed with parafilm. The mean radial growth of samples was calculated after taking measurements at cardinal points every 24 hours for a maximum of 168 hours (Charron and Sams, 1999).

The study ceased when the pathogen had reached the edge of the petri dish in the control. Fungal plugs were re-plated following the inhibition studies to determine if effects on growth were fungicidal or fungistatic. Percentage inhibition was calculated using the mean values as defined by Reyes Chilpa *et al.* (1997) and Al-Reza *et al.* (2010): % Inhibition = radial growth in control – radial growth in treatment / radial growth in control x 100. In contrast to Mayton *et al.* (1996), the percentage inhibition of the pathogens was calculated at each time point as a percentage differences in mean radial growth: % Difference = radial growth in control – radial growth in treatment / (radial growth in control + radial growth in treatment)/2 x100. All antimicrobial experiments for each study were undertaken in triplicate, at least three times.

2.3.2 Green manure preparation and use

Spring leaves of green manure were prepared as plant discs or finely macerated matter (<2mm; dried at 24°C for 72 hours; Lazzeri *et al.*, 2004). Various application and extraction methods were used as they influence the liberation of active properties and subsequently their efficacy and mode of action. Details on green manure preparation and use are below. Blank controls were used for each.

Methods for each *in-vitro* study:

Plant discs Section 3.3.1	10mm discs of *P. americana* leaf were cut and soaked in 70% ethanol for 30 seconds, transferred to 10% sodium hypochlorite (5 mins) and then rinsed (4 times; 2 mins per rinse) in sterile water and dried under UV light in a bio-safety cabinet. 10mm disk of plant material discs, cut using a cork borer, were placed centrally on the agar with a plug of *R. solani* on top.
Ethanolic extracts discs Section 3.3.1	An organic solvent extract was made by soaking 12g of *P. americana* leaf matter in 100ml 95% ethanol (on a stirrer for 24 hrs at 24°C) (Quiroga *et al*., 2001; Okigbo and Mmeka, 2008). The contents were filtered (90mm filter paper) and the solvent evaporated off at 78°C. 0.2g of the remaining extract was diluted in 1ml of 95% ethanol and added to a volume of 10ml with a final concentration of 0.02g ml^{-1}. A volume of 10µl was impregnated in sterile filter 10mm grade number 1 qualitative filter paper circle disks with a micropipette (Bayoub *et al.*, 2010). Disks were dried out in a bio-safety cabinet under UV light and refrigerated at 4°C. Ethanol extract disks were placed centrally on PDA with a plug of *R. solani* placed centrally on the disk. Solvent loaded disks were used as controls.
Volatiles Section 3.3.2	10g of finely chopped leaf matter (<2mm) was distributed into each of the nine 500ml beakers (Charron and Sams, 1999). Petri dishes with a 5mm plug of pathogen placed centrally on them were inverted and secured, with parafilm, on top of the beakers (Scientific Laboratory Supplies Ltd., UK) after lid removal.
Distillate infused agar Section 3.3.3	Distillation apparatus including a round bottom steam generating flask, distilling flask, Liebig distillation column, and receiving flask was used to steam plant matter. 50g finely chopped leaf matter (<2mm) was placed in a 1l distilling flask above a steam generating flask containing 500ml of distilled water. Steam was generated using a heating mantle at 100°C at atmospheric pressure. 10% and 20% distillates were stored (4°C) before being added to molten agar (50°C).
Leaf matter Section 3.3.4	Finely chopped leaf matter (<2mm) was placed into molten agar (50°C) at concentrations in line with soil applications (1.67g/l, 8.33g/l, 15.00g/l). The agar was mixed thoroughly by inversion (five minutes). The agar was left on a VWR hotplate stirrer at 50°C until the agar was poured into plates. These were then left to dry before the addition of the pathogen plug. *P. americana* was also tested in increments of 5.00g/l from 15.00g/l to 30.00g/l.
Cold press liquid Section 3.3.5	8.33g/l of finely chopped leaf matter (<2mm) was added to 10ml of sterile distilled water in polypropylene 30mL universal containers (SLS, Nottingham, UK). This cold water extract was then left for 24 hours on a VWR hotplate stirrer at room temperature in line with Nana *et al.* (2015). The solution was then passed directly through a Sartorius Minisart Plus Syringe Filter with a pore size of 0.45µm, using a 10ml Terumo disposable syringe, into 50°C molten agar. The agar-extract mix was inverted (5 mins) and poured into plates which were left to dry.

2.4 Protocols for tree growth bioassay and destructive analysis

This study focussed on the influence of the application of *P. americana* and comparison green manures on tree growth (Section 2.5.1). After a three month establishment period the bioassay began. 'Sick soil' (diseased) and sterile soil in single tree replicates of 25 (Adamopoulos *et al.*, 2012; Akinsanmi and Drenth 2013; Dong and Xiangdong, 2013; Lucia *et al.*, 2013) were amended with green manure. Untreated control groups were used. In total 400 trees were used as study subjects, with 200 in the diseased soil sub-set and 200 in the sterile soil sub-set.

In this bioassay the experimental design trialled *S. aucuparia* in three groups:

1. No treatments applied: to replicate 'normal' soil conditions in terms of RD.
2. Diseased soil: to indicate the influence of green manure on 'sick' soil (Section 2.5.2.1).
3. Sterilised soil: to provide a baseline whereby addition of nutrients to heat sterilised soil could be ruled out as the causal factor for any positive changes in growth of trees (Section 2.5.2.2).

The bioassay ran over three years with initial changes measured three months after the amendment of soil (end of April, 2012). Thereafter, the trees were subsequently measured for a further two years (August 2013, 2014). The AHDB (2007) suggested that RD confers the majority of its deleterious effects in the first two years of tree growth. The chosen time period in this study would therefore cover the time between growth suppression and partial recovery from RD. Measurements of tree development during this time included:

- Primary growth – increase in height and internodal distance
- Secondary growth – stem diameter at a designated height
- Live crown: number of leaves and active buds
- Chlorophyll content

After the last measurement of growth parameters in 2014 destructive analysis was undertaken to quantify:

- Leaf area and dry weight
- Root dry weight
- Shoot dry weight
- Root to shoot ratio
- Presence of nematode cysts.
- Fruit yield and harvest weight

Further detail is provided below in the subsequent sections.

2.4.1 Bare root *S. aucuparia* sapling husbandry and treatment

Bare root *S. aucuparia* saplings (1000 no.) were sourced from James Coles & Sons (Nurseries) Limited (Leicester, UK). The saplings were stored briefly under cover and kept moist to ensure viability until being tagged with Polyplas 150 Micron 191 x 25mm self-ties, and pruned to 100mm above the root crown and where possible trained to a single shoot. Dead material was pruned out if removal of the epidermis did not reveal a green cambium. The potted trees were arranged in four randomised blocks with guard trees placed around them to eliminate edge effects. The whips were heeled in prior to use in late winter in sterile loam and then 400 uniformly healthy trees were transferred to three litre pots containing the test substrates and remaining trees were planted into sterile soil so that they could act as guard trees at the end of April 2012. This was done in closely timed staggered blocks. Close association of tree rhizosphere with the test substrate was achieved.

Test substrates were composed of:

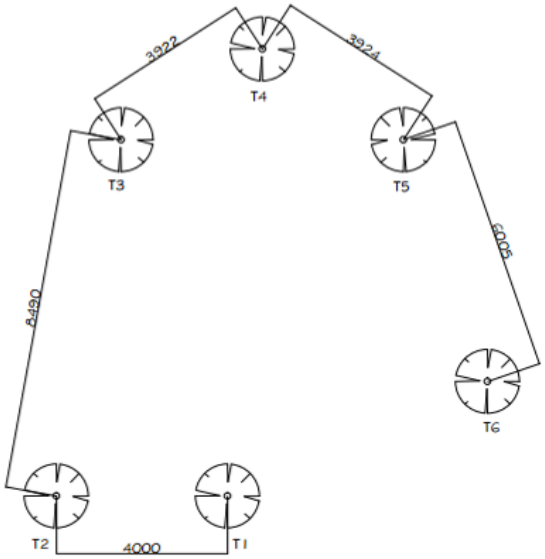
- Sterile soil or diseased soil pre-mixed homogeneously with single species green manure amendments 24 hours prior to the planting.
    - *P. americana* amendments were added to the soil at 1.67g/l, 8.33g/l, 15.00g/l. *B. juncea*, *T. aestivum*, *T. patula* and *A. sativa*, which were added at a concentration of 8.33g/l.
    - All green manures were macerated (c. <5mm) and added immediately uniformly to the rhizosphere (Section 2.1) at rates defined previously (Section 2.2).
- Sterile soil or diseased soil (control groups)

The growing area was constructed at Moulton College in a rabbit proof compound. This area was outdoors and therefore subjected to environmental factors. A weather treated timber frame was constructed, into which a base layer of MOT type 1 hard-core was distributed (100mm) and consolidated. Mypex was then pegged down into place as a weed barrier. The surface was kept clear of weeds and abscised leaves. Trees were monitored throughout the bioassay during fortnightly health checks for pests and disease (Appendix V), and any trees suspected of being dead underwent a cambium layer vitality tested through the removal of a small section of epidermis to check for the presence of living (green) tissue. No regime for fertiliser or pesticide application was planned in to the general maintenance of the trees. Ad-hoc treatment of pests and disease was planned however was not required. Weather data were recorded by the Northampton Moulton Park weather station (DCNN: 4364) two miles from the bioassay site. Local weather station data determined that weather patterns were variable and seasonal (Appendix VI). A Delta T SM300 dielectric soil moisture probe (Cambridge, UK) was used to determine the irrigation schedule, to supplement precipitation, in accordance with the manufacturer. Trees were watered to saturation once or twice a day early in the morning and late in the afternoon to avoid scorching.

2.4.2 Soil

2.4.2.1 Diseased soil: the source

"Sick soil" was soil collected from a mature *S. aucuparia* (c. 60 year old) at SSSI Burbage Common, Leicestershire (grid reference SP 449 948) in a coppiced hazel coup. A site survey (Figure 2.2) demonstrated that the immediate area was dominated by a 25-30% canopy cover of mature *Populus*. The two metre high understory consisted of *Rubus* (bramble), *Corylus* (hazel), and *Sambucus* (elder), with a field layer of grass and sorrel (G. Davies, personal communication, June 2014; Lock, 2015).



Tree	Genus	D.B.H (mm)	Height (m)	Spread (m) N	E	S	W	Comments
T1	*Sorbus*	1600	18					Dead and fallen
T2	*Populus*	940	22	5.5	2.0	3.0	3.8	Good health, dead wood
T3	*Populus*	830	22	4.0	0.5	0.5	4.0	Good health, dead wood
T4	*Populus*	1000	22	4.0	3.5	0.5	4.0	Fair health, basal cavity (N), epicormic growth (3m) and bark cracking (N)
T5	*Populus*	1050	22	0.5	4.0	4.0		Good health, dead wood
T6	*Populus*	1080	22		3.5	3.5	4	Good health, dead wood

Figure 2.2: Tree Survey Burbage Common 07.06.14 (Lock, 2015)

The *Sorbus* had no visible signs of disease (D. Lewis, personal communication, February 2012) and had blown over exposing a 2m root plate (J. Simpson, personal communication, January 2012). Fraser and Simpson (personal communication, November 2011) stated that *S. aucuparia* was planted in this location and in the nearby Sheepy Woods with little success in establishing, survival rates and limited natural regeneration of *S. aucuparia*.

2.4.2.2 Sterile soil: sterilisation of soil as a positive control

Three tonnes of flame sterilised and 3mm screened soil was obtained from manufacturer Boughton Loam (Kettering, UK) and was used as a control to determine if positive changes were the result of nutrients in the green manures. The method of sterilisation was to industry standard.

2.4.2.3 Soil analysis: the physical and nutrient properties of the two soil types

Soil pH for the sterilised loam and sick soil differed dramatically with the former at pH 7.5 and the latter at pH 4.4. Nutrient levels varied between the two soils. The sterilised loam had a soil textural class of sandy silt loam, whilst that of the sick soil was clay loam. Both soils had a medium cation exchange capacity (meq/110g) of 14.2 for the sterile and 14.7 for the sick soil. Other soil characteristics are defined below (Table 2.4; as supplied by NRM methods in Appendix VII).

Table 2.4: Soil characteristics of sterile soil and woodland soil.

Determinand	***Sterile Soil***	***'Sick' Soil***
Dry matter (% w/w)	90.6	84.4
Organic matter (LOI) %	6.7	5.3
Nitrate N (mg/kg)	12.75	63.08
Ammonium (mg/kg)	0.87	6.88
Available N 300mm profile (kgN/ha)	51.1	262.3
Potential N (kgN/ha)	20.0	20.0
Available P (Olsen extraction; mg/l)	51.4[*2]	11.2[*1]
Available K (Ammonium nitrate extractable; mg/l)	61[*1]	75[*1]
Available Mg (Ammonium nitrate extractable; mg/l)	40[*1]	53
Copper (EDTA extractable; mg/l)	7.1	6.2
Boron (Hot Water Soluble; mg/l)	1.5	0.8
Sodium (Ammonium nitrate extractable; mg/l)	9.4[*1]	6.8[*1]
Zinc (EDTA extractable; mg/l)	5.0	4.0
Calcium (Ammonium nitrate extractable; mg/l)	2067.9	433.5[*1]
Iron (DPTA extractable; mg/l)	54.1	347.1[*2]
Sulphate (Phosphate buffer extractable; mg/l)	132.7[*2]	51.9[*2]
Manganese (DPTA extractable; mg/l)	8.7	8.3

[*1] Nutrient is deficient and [*2] nutrient is in excess (DEFRA, 2010)

Differences in soil characteristics determined that results for tree growth were considered separately in treatment sub-sets defined according to diseased and sterile soils and not directly compared.

2.4.3 Bioassay: protocols for monitoring growth of trees

During the course of the three years various growth parameters were measured, including; primary growth, secondary growth, number of leaves and active buds, and chlorophyll content.

Live crown: leaf and bud counts, andchlorophyll Section 4.2.1	Owing to the sparse nature of the canopy in juvenile whips, the numbers of leaves were counted as an expression of the live crown. Active buds were counted as further potential for leaf production. Active buds were identified in the form of a swollen bud (those buds passing from dormancy through to bud burst). Leaf chlorophyll content was assessed using a portable Minolta chlorophyll meter SPAD-502 (Spectrum Technologies, Inc., Plainfield, Ilinois, United States of America) with a method adapted from Percival (2004). After calibration the SPAD-502 was used to collect an average reading from the midpoint of the leaf near to the midrib of seven fully formed compound leaves working down from the apical bud for each of the 400 trees. The mean from the 175 data points per tree group were then converted to chlorophyll content using the Lichtenthaler (1987) regression equation ($5.80 + 0.057x$; r^2 adj = 0.82, p <0.01).
Primary growth Section 4.2.2	Primary growth was measured 10mm from the top of the pot (delineated by a flexible tie around the stem) to the apically dominant bud on the leader stem. Further to this, annual stem growth was measured using a common diagnostic procedure involving the measurement of stem between bud scale scars (internodes). This method was used previously to assess tree health where growth is negatively correlated with increasing stress (Cloyd *et al.*, 2004).
Secondary growth Section 4.2.3	Stem diameter measurement of secondary growth accounted for lateral expansion year on year. Mazzola and Mullinix (2005) suggest marking the trunk/stem with white latex paint 0.7m above soil line, however, due to the height of the bare-root specimens it was decided that girth measurement (as stem diameter in mm) would be completed using electronic callipers at 0.1 from the top of the pot, with a flexible tie delineating this point on the stem. The top of the pot had to be used as a point of reference due to soil erosion experienced at times of high precipitation. Each stem from the multi-stemmed trees were measured and an average taken. Measurements were non invasive with the average of two perpendicular diameters recorded.

Data from the bioassay was considered in two sub-sets with the influence of three concentrations of *P. americana* manure (PaM) being analysed as a separate grouping to the trees which received 8.33g/l amendments of all green manures (AGM). Results from the diseased and sterile groups were also analysed separately due to differences in the soil properties.

2.4.4 Destructive analysis to quantify above and below ground growth responses

At the end of the bioassay in 2014 trees were destructively harvested with measurements on the following being recorded for each tree; leaf dry weight and area (mm2), root and shoot dry weight (not inclusive of leaves), root to shoot ratio, presence of nematode cysts, and fruit yield and harvest weight (2014 was the first fruiting year).

Leaf weight & size Section 4.2.3.1	After the last measurement of growth parameters in 2014 leaves were harvested, weighed (g), and photographed prior to analysis with Imagej. Leaf area was calculated using ImageJ by adjusting the threshold and recording the area of each region of interest (roi). Leaf fresh weight was recorded and the leaves were dried in an oven at 70°C for 24 hours. Once dry the leaves were reweighed.
Roots and shoot Section 4.2.3.2 - 4.2.3.4	Each tree was then removed from its pot and submerged in water in a holding bay for approximately fifteen minutes. Roots were washed in running water to remove all soil over a sieve (2mm), blotted with towelling and the whole tree was photographed next to a scale. Roots were removed at the crown just above the soil line and were assessed for presence of nematodes for a period of five minutes for each root. Roots and shoot were then weighed separately, dried in an oven at 70°C for 24 hours and then reweighed (g).
Root :shoot Section 4.2.1	The root-to-shoot ratios were calculated using the equation: $R = W_{root}/W_{aboveground}$, where R = root-to-shoot ratio; W_{root} = dry weight (g) of root material from root crown to root tips; and $W_{aboveground}$ = Dry weight (g) of transpirable aboveground material (sum of leaf and stem matter).
Fruit Section 4.2.3.5	Fruit production was measured in 2014 (as this was the first occurrence of berries). Upon sighting the development of berries they were covered with pea netting until they were fully formed. In September 2014 the fruit was harvested and weighed fresh as a measure of yield.

2.5 **Pathogen detection methods** (Section 5.2)

2.5.1 Protocols to detect infestation of rhizosphere soil by fungi and oomycetes

In September 2014, 20ml of rhizosphere soil from each cardinal point of 20 trees per treatment (Balci *et al.*, 2013) was sampled using a customised sterile plastic syringe. The samples were sent to Scientia Terrae, Belgium, for soil pathogen composition tests using a DNA array that has been likened to reverse dot blot technique (Tambong *et al.*, 2006). After arriving at Scientia Terrae the soil sample underwent a process of extraction was completed using an UltraClean Soil Plant DNA Isolation Kit (Mo Bio Laboratories, Inc, California) to yield DNA from 0.5g of soil (Figure 2.3). The extracted DNA was then diluted (10 fold) to further eliminate Polymerase Chain Reaction (PCR) inhibitors and stored at -20^{o}C (Lievens *et al.*, 2007).

0.5g of soil ✚ 550µl buffer (bead solution) ⇨ vortex	*Degrades humic acids and separates soil particles*
✚ 60µl sodium dodecyl sulphate	*Aids cell lysis*
✚ 200µl Inhibitor Removal Solution ⇨ vortex 10 mins	*Precipitates off humic acids and PCR inhibitors*
⇨ Centrifuge – 10,000 g – 30 sec.	*Produces pellet and supernatant (approx 800µl)*
⇨ Transfer supernatent to microcentrifuge tube	
✚ 250µl protein precipitation reagent	*Removes of protein contaminants, humic acids and cell debris.*
⇨ Centrifuge – 10,000 g – 60 sec.	
⇨ Transfer supernatent to microcentrifuge tube	
✚ 1.3ml of DNA binding salt solution ⇨ vortex 5 sec.	
⇨ Load 700µl into spin filter	*DNA binds to the spin filter silica membrane.*
⇨ Centrifuge 1 minute 10,000g (Repeat 3x)	
✚ 300µl ethanol based wash	*Removes salt and contaminents*
⇨ Centrifuge – 10,000 g – 30 sec. ⇨ Discard waste	
⇨ Centrifuge – 10,000 g – 60 sec.	*Removes Ethanol based wash*
✚ 50 µl sterile elution buffer 10mM Tris pH 8.	*Releases DNA from silica membrane*
⇨ Centrifuge – 10,000 g – 30 sec.	

Figure 2.3: Procedures for DNA extraction adapted from manufacturer specifications.

In accordance with Lievens *et al.* (2006) amplification and digoxigenin labelling of DNA extracted from soil samples was carried out using the Titanium *Taq* DNA polymerase (Clontech Laboratories, California) and hybridised using the protocol below (Figure 2.4).

Target ITS regions primer sets ITS1-F and ITS4 (fungi) and OOMUP18Sc and ITS4 (oomycete) were used in the reaction (Lievens *et al.*, 2003). A separate PCR reaction control was run using *Saccharomyces cerevisiae* (yeast) labelled with P4501 and P4502.

Master mix reagent

✚ 5µl Titanium *Taq* PCR Buffer
✚ 1µl 50X dNTP mixture (10mM ea.)
✚ 2µl 10µM 5' and 3' primer mix (1µl ea.)
✚ 1µl 50X Titanium *Taq* DNA polymerase
✚ 70µM digoxigenin-11-dUTP
✚ 40µl Sterilised distilled water
✚ 1µl DNA 100ng/µl ⇨ Centrifuge

⇨ Denaturation - 94°C – 2 mins ⇨
⇨ **Three step cycling – 30 cycles**
⇨ Denaturation - 94°C – 45 sec.
⇨ Annealing - 59°C – 45 sec.
⇨ Elongation - 72°C – 45 sec.
⇨ Elongation - 72°C – 10 mins

Hybridisation

✚ 10µl labelled amplicons
✚ 6ml hybridisation buffer
⇨ Chemiluminescence detection – 30 sec. x90
⇨ Quanitification of signal strength

Figure 2.4: Procedures for DNA amplification adapted from manufacturer specifications. PCR cycle information and labelling protocol adapted from Lievens *et al.* (2003), Tambong *et al.* (2006) and Fessehaie *et al.* (2002).

Labelled PCR products were quantified in a two step process of chemiluminescence detection using a digital CCD BioChemi video imaging system and comparison of results using Labworks Image Aquisition and Analysis Software (UVP Inc., California). DNA Multiscan oligonucleotide detector probes were based on primer regions, that are widely known sequence within the rDNA of fungi and oomycetes (White *et al.*, 1990; Lievens *et al.*, 2003). PCR products were sequenced and compared to GenBank to design species-specific probes. Oligonucleotides and a digioxigenin-labeled control were bound to an Immunodyne ABC membrane (PALL Europe Limited, Portsmouth) via a 5'-C6-amino linker (Lievens *et al.*, 2003). The membrane was tailored for the detection of multiple microbial species associated with rosaceous species (Section 1.5.2; Appendix VIII). Levels of infestation were detected and quantified on a numeric scale ranging from 0-3 where 0 = no DNA fragments detected, 1 = low infestation, 2 = moderate infestation, and 3 = high infestation. These ratings were based on bioassays conducted with sets infected with the target pathogen inoculum at known rates (Lievens *et al.*, 2007).

2.6 Protocols for data analysis

All data were assessed for outliers by visual inspection of boxplots and the IQR multiplier approach (Hoaglin and Iglewicz, 1987). Normality was determined by a Shapiro-Wilk's test and Levene's test for equality of variances demonstrated homogeneity of variances. Data complying with assumptions were further tested with parametric methods, including T-test (or Games-Howell) and Analysis of Variance. Those violating the assumption which could not be normalised by logarithmic or square root transformations underwent analysis by non-parametric means, including Mann-Whitney U and Kruskal Wallis H test. Data violating homogeneity of variance, as assessed by Levene's test of equal variance, were treated on a case by case basis. Any significant *p*-values obtained from ANOVA and Kruskal Wallis tests were further interrogated with *post-hoc* tests, Tukey and Benjamini-Hochberg respectively. Spearmans Rank was used to test correlation of non-parametric data and a chi square tested for association between variables. The effect of treatments and pathogen presence on growth factors was determined by Principal Component Analysis. Minitab, SPSS and R packages were used.

Chapter 3 - Microbial Inhibition Studies

3.1 Introduction

The aim of this study was to evaluate the *in vitro* effects of green manure on the saprophytic and parasitic basidomycete fungus *Rhizoctonia solani* (Menzies, 1970) and oomycete *Pythium ultimum* (Trow, 1901). Very few *in vitro* studies using *Phytolacca* leaf material as an antimicrobial for soil borne pathogens have been completed thus far (Bae *et al.*, 1997; Hernandez *et al.*, 2013). The experiments were designed to reflect the procedures used in the bioassay (Chapter 5 and 6), with the effect of volatiles, plant matter, and water extracts being investigated alongside more conventional tests.

R. solani AG-5 and *P. ultimum* var *ultimum* were specifically selected due to their association with root degradation generally, and specifically because they have been linked with a range of rosaceous species suffering from RD (section 1.5.2) and were associated with *S. aucuparia* by DNA analysis (O'Neill, 2011; Lock *et al.*, 2015). The use of these species as model organisms was also chosen in order to determine if fungi respond differently or similarly to oomycete in the presence of plant material, thus indicating the mode of action the plant properties may have.

R. solani (anamorph, telomorph *Thanatephorus cucumeris* (Frank) Donk) was first described in 1858 by Julius Kühn when it was observed on potatoes (Kühn, 1858). This fungus reproduces asexually through vegetative mycelium and sclerotia, rather than through conidia, although still can proliferate through the production of basidiospores in infected tissue. In 2006 Farr and Rossman reported 2589 *R. solani*-host combinations. The globally distributed *R. solani* is sub divided into 13 anastomosis groups (AG) based on the ability for hyphae to fuse and exhibits non-obligate generalist behaviour on a wide host range (Agrios, 2005). In terms of rosaceous stock, the anastomosis groups associated with pathogenicity were AG-5 and AG-6. These *R. solani* groups were isolated from *M. domestica* 'Gala' on west coast of the USA (Mazzola, 1997; Mazzola, 1999). AG-5 was found on *M. domestica* in Austria by Manici *et al.* (2003), and *S. aucuparia* by O'Neill (2011). Unfortunately, there is a dearth of information on other species in the Rosaceae which specifically refer to AGs. From a broader perspective the majority of the anastomosis groups (AG1-5) infect economically important crops (Table 3.1). AG-2 infect these and additionally member of the Amaranthaceae (Herr, 1996). Meanwhile AG-3 is specific to the Solanaceae and Poaceae families (Kodama *et al.* 1982; Bandy *et al.* 1988; Date *et al.* 1984; Ogoshi 1987; Zhang and Dernoeden, 1995). AG-4 hosts include Malvaceae (Rothrock 1996).

P. ultimum is a facultative saprophytic oomycete belonging to the Pythiaceae which was first described by Wager (1931) when it was isolated from *Striga* spp. (witchweed). *P. ultimum* also reproduces asexually when mycelium produces sporangia, the germ tube of which penetrates the host. Sporangia

can give rise to zoospores which are mobile in the presence of water sources (such as irrigation lines or ground water). Sexual reproduction results in the production of thick walled oospores which are resilient in harsh conditions. The oomycete has been reported to be an opportunistic pathogen of seedlings and roots and is associated with root rot and damping off and thus is a problem in production horticulture (Agrios, 2005). In 2006 Farr and Rossman reported 337 *P. ultimum*-host combinations that range from ornamental to crop species found in the families identified for *R. solani* (Table 3.1). *P. ultimum* has been associated with RD in rosaceous stock (Section 1.5.2.3). It is responsible for other diseases such as collar rot in *Malus* spp. (Bielenin, 1976) and has been associated with the oomycete *Phytophora* (Lestari and Arumingtyas, 2013).

Table 3.1: *R. solani* and *P. ultimum* isolated from economically important plant species.

Host	AG	*P. ultimum*	Reference
Fabaceae			
Phaseolus vulgaris (common bean)	1; 2; 4	✓	Muyolo *et al.,* 1993; Nzungize *et al.*, 2012*
	5		Strahnov *et al.*, 1985
Glycine max (soybean)	1	✓	Yang *et al.*, 1990; Marchand *et al.*, 2014*
	2; 5		Nelson *et al.*, 1996
	3		Kuninga *et al.,* 2000
	4		Liu & Sinclair, 1991
Vicia faba (broad bean)	5		Valkonen *et al.,* 1993
Poaceae			
Oryza sativa (rice)	1		Sayler & Yang, 2007; Hashiba & Kobayashi, 1996
	2		Hashiba & Kobayashi, 1996
	3		Kuninga *et al.*, 2000
Zea mays (corn)	3	✓	McCormack *et al.*, 2013. Zhang & Yang, 2000*
T. aestivum	3	✓	Kuninga *et al.*, 2000; Chamswarng & Cook, 1985*
	5		Rush *et al.*, 1994
Pisum sativum (pea)	4; 5	✓	Mathew *et al.*, 2012; Lin *et al.*, 2001*
Hordeum vulgare (barley)	5	✓	Rush *et al.,* 1994; Ingram & Cook, 1990*
Brassicaceae			
Brassica oleracea (cabbage)	3	✓	Kuninaga *et al.*, 2000; Kubota *et al.*. 2006*
Raphanus sativus (radish)	2		Grisham & Anderson, 1983
Brassica napus (canola)	4	✓	Verma, 1996; Gugel *et al.*, 1987*
Solanaceae			
Solanum tuberosum (potato)	2	✓	Chand & Logan 1983; Taylor *et al.*, 2008*
	3		Lahlali & Hijri, 2010; Woodhall *et al.*, 2013
	7; 3		Abd-Elsalam *et al.*, 2009; Carling & Brainard, 1998
	4		Anguiz & Martin, 1989
	5		Bandy *et al.,* 1984; 1988
Solanum lycopersicum (tomato)	2	✓	Kuninga *et al.*, 2000. Rafin & Tirilly, 1995*
	4		Strahnov *et al.,* 1985
Capsicum annuum (pepper)	3; 4; 6	✓	Tuncer & Eken, 2013; Sutton *et al.*, 2006*

Note: *P. ultimum* also shares this host range (reference denoted by *).

The importance of *R. solani* and *P. ultimum* as pathogens associated with the root rot of a range of hosts including those in the Roseaceae made them crucial to this study.

3.1.1 Aim and objectives

Aim:

To determine whether macerated plant material influences the *in-vitro* growth of pathogens, *P. ultimum* and *R. solani*.

Objectives:

- Determine the potential of macerated leaves of *P. americana* to inhibit pathogens.
- Demonstrate if volatiles from *P. americana* alone can influence the growth of pathogens.
- Examine the affects of a distillate of *P. americana* on the growth of pathogens.
- Identify if increasing concentration of *P. americana* is correlated with pathogen growth rates.
- Investigate if other proposed green manures are comparable to *P. americana* in their potential to influence the growth rate of pathogens.
- Determine if there is a difference in the inhibition of plant material between pathogens of different biological classifications; fungi and oomycetes.

3.2 **Results of the microbial study** (Section 2.3; Appendix VII)

3.2.1 ***P. americana*: Effect of plant discs and ethanol extracts on pathogens**

After 96 hours the radial growth of both *R. solani* and *P. ultimum* was statistically different between the plant disc treatment and the respective control. Conversely, growth was not different for pathogens that were amended with the ethanolic extract in comparison to an ethanol based control (Figure 3.1, A and B, respectively).

R. solani was more sensitive to plant matter than *P. ultimum* as the fungus was responded to the presence of green manure quicker than the oomycete. The former exhibited a greater magnitude of inhibition ($p \leq 0.001$) ranging from 38% inhibition after 48 hours ($H(3) = 15.688$, $p=0.002$), to 64% after 96 hours ($H(3) = 20.611$, $p<0.001$). *P. ultimum* displayed a significant inhibition between 72 and 96 hours, ranging from 69% ($H(3) = 8.726$, $p=0.023$) to 66% ($H(3) = 8.634$, $p=0.024$).

Variability in growth increased for both pathogens in the presence of the plant disc indicating that some samples are slower growing than others, resulting in no statistically significant differences between the time points ($p>0.05$).

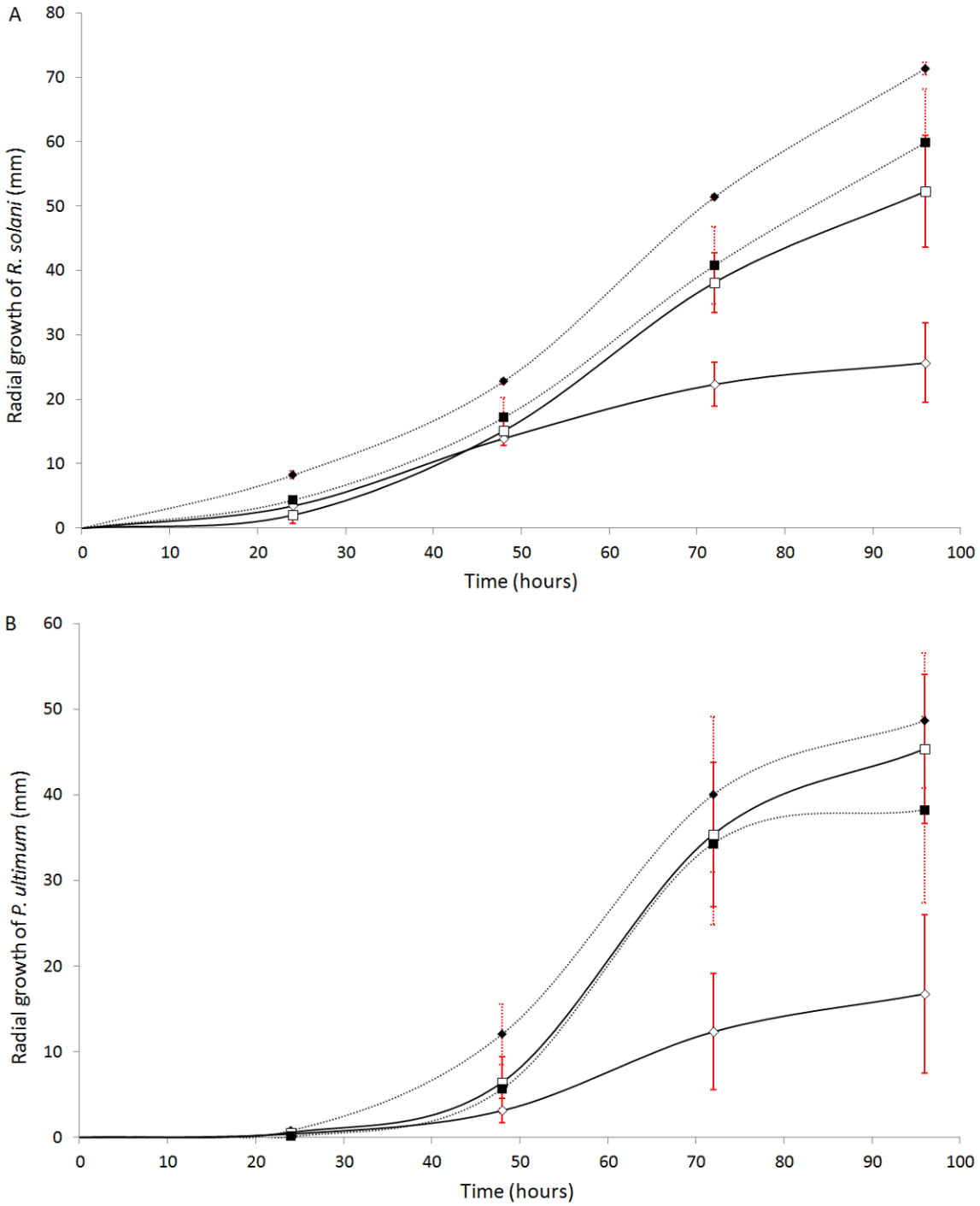


Figure 3.1: Mean radial growth of *R. solani* (A) and *P. ultimum* (B) on agar plates amended with a plant disc and an ethanolic extract disc of *P. americana*. Key and notes: Treatments: Control (no plant matter), ethanolic control (solvent loaded disc), *P. americana* plant disc, *P. americana* ethanolic extract. Error bars are displayed as SEM, n=9.

3.2.2 *P. americana*: Response of pathogens to plant material volatiles

Both *R. solani* and *P. ultimum* were inhibited in the presence of *P. americana* leaf matter with a significant difference, between treated and non treated, noted after 24 hours ($W(1) = 378.000$, $p<0.001$ and $W(1) = 616.500$, $p<0.05$, respectively). Thereafter there was a significant difference between the radial growth on the control in comparison to the presence of plant matter (Figure 3.2, A and B respectively). *R. solani* was more sensitive to the presence of *P. americana* than *P. ultimum*. Percentage inhibition of *R. solani* ranged from 62% to 90% and *P. ultimum* was inhibited by 8% and 46% over the course of 144 hours.

In relation to the growth of the control *R. solani* and *P. ultimum* it was found that there were significant differences over the course of the assay. *R. solani* growth measurements at each time point between hour 24 and hour 96 were significantly different ($F(5) = 4853.413$, $p<0.001$), after which growth slowed. *P. ultimum* followed a similar pattern, but instead of levelling off towards the end, there was a steady and significant increase in the radius of the pathogen ($F(6) = 3639.259$, $p<0.001$).

Volatiles emitted by leaf matter were found to be temporarily suppressive to the pathogens. After re-plating onto fresh PDA *R. solani* and *P. ultimum* recommenced growth at a rate which was similar to that of the control ($P>0.05$).

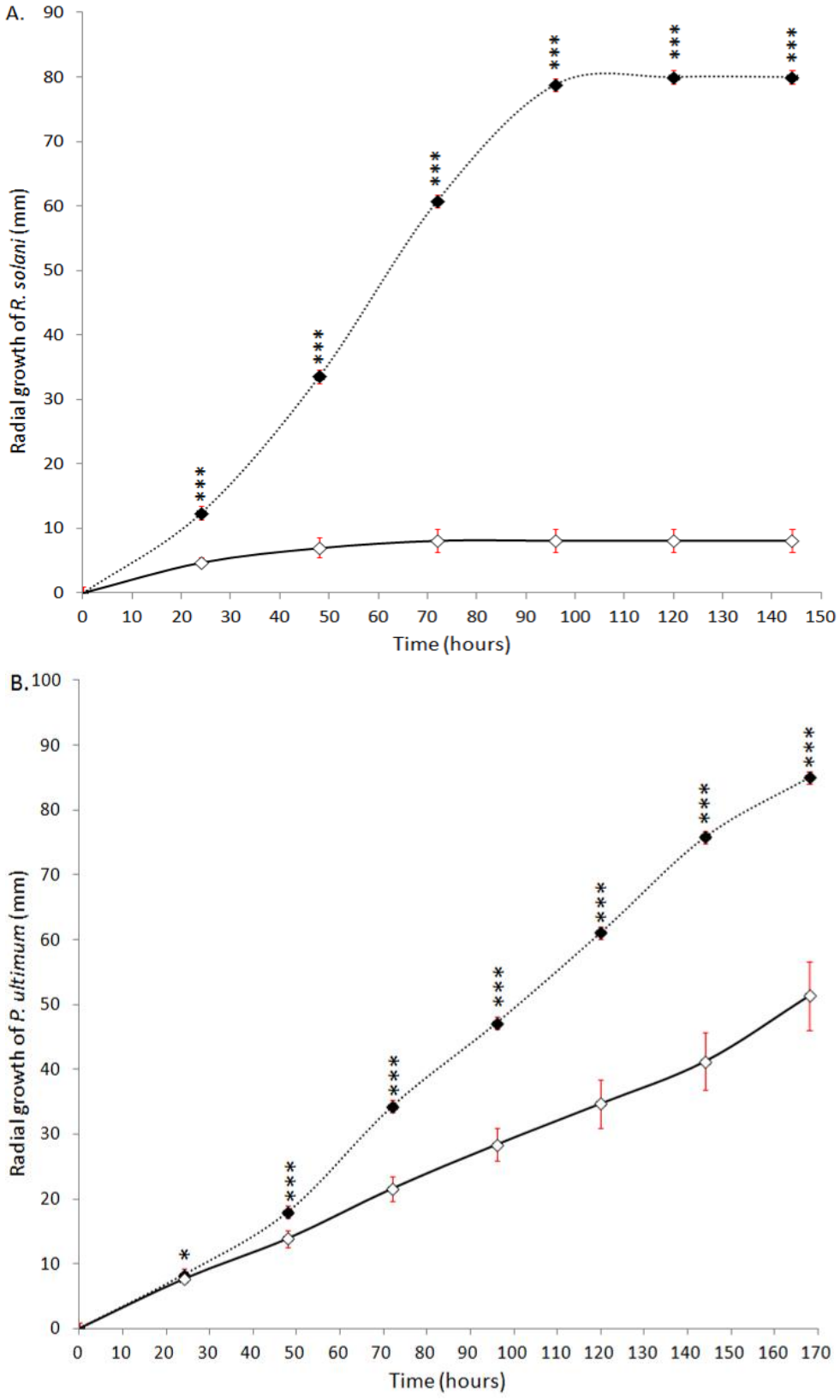


Figure 3.2: Mean radial growth of *R. solani* (A) and *P. ultimum* (B) on agar plates in the presence of *P. americana* volitiles. Key and notes: Treatments: ····◆···· Control (no plant matter) and —◇— *P. americana* plant matter. Error bars are displayed as SEM. Significant differences between radial growth (mm) of pathogen on control in comparison to in the presence of *P. americana*: * $p \leq 0.05$, *** $p \leq 0.001$, n=9.

3.2.3 *P. americana*: Response of pathogens to a distillate

The 20% distillate exhibited the greatest magnitude of pathogen inhibition throughout the duration of the assays for *R. solani* ($F(2) = 29.798$, $p<0.001$), and *P. ultimum* ($F(2) = 33.149$, $p<0.001$), (Figure 3.3, A and B). The distillate had a similar magnitude of inhibition for both pathogens, which is conversely different to studies 3.3.1 and 3.3.2 where *R. solani* was more sensitive to the treatment. The moderate inhibitory action ranged from 36% to 57% for the former and from 38% to 69% for the latter.

Inhibitory activity of the 10% distillate was significantly less than that of the 20% distillate but was no different to the control for both pathogens (apart from an anomaly at hour 24 for *P. ultimum* where growth did not commence until hour 48).

The radial growth between time points of the control and the treated groups were significantly different for each treatment during the course of the assay. The rate of pathogen growth slowed for both the fungus and oomycete ($p<0.001$). The fungal growth did not become biostatic as demonstrated under the influence of volatiles (Section 3.3.2).

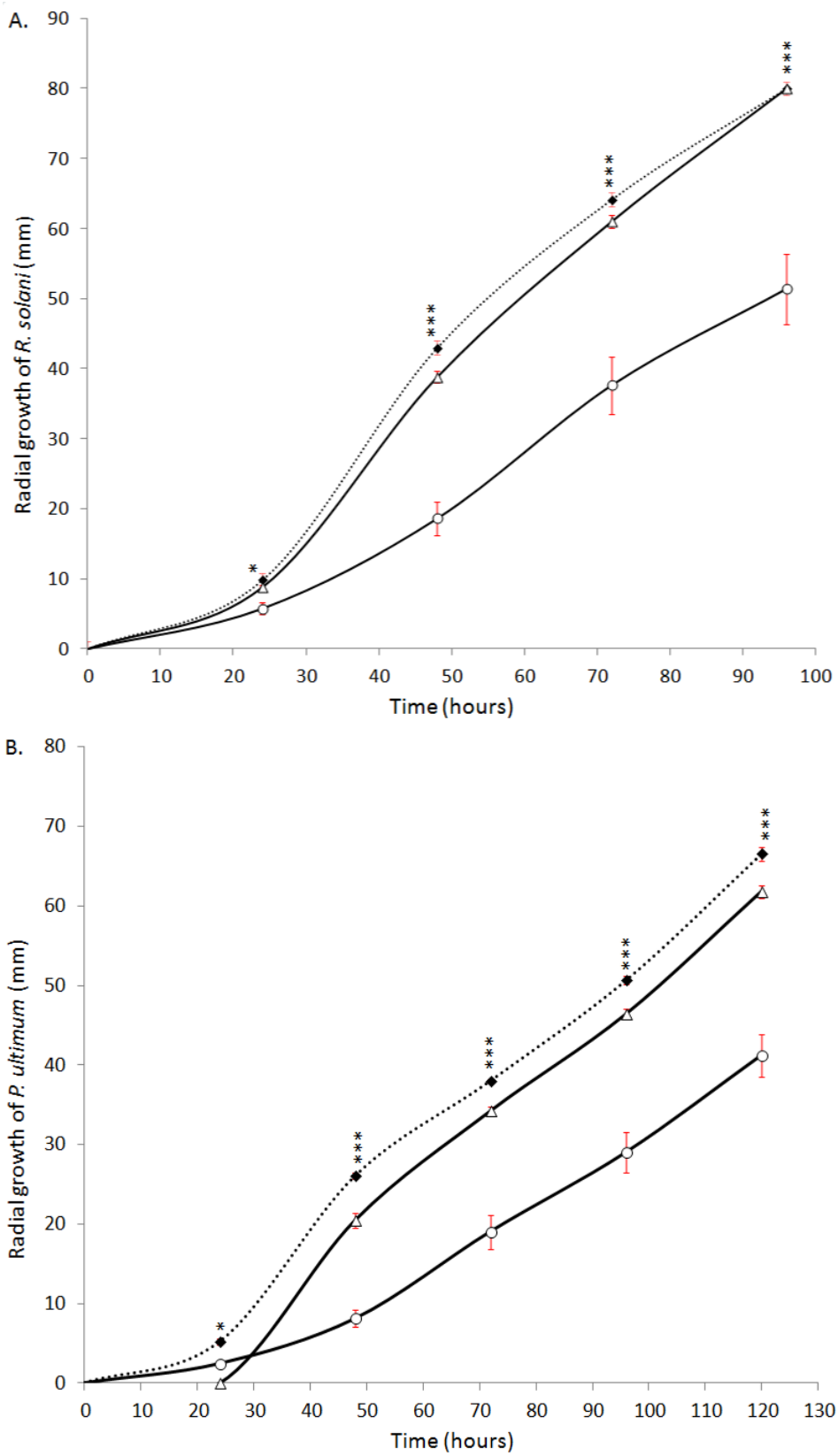


Figure 3.3: Mean radial growth of *R. solani* (A) and *P. ultimum* (B) on agar plates amended with *P. americana* distillate. Key and notes: Treatments: Control, 10% distillate, and 20% distillate. Error bars are displayed as SEM. Significant differences between radial growth (mm) of pathogen on control in comparison to in the presence of 20% *P. americana* distillate: * $p \leq 0.05$, *** $p \leq 0.001$, n=9.

3.2.4 Inhibition of pathogens by agar infused with macerated green manure

3.2.4.1 *P. americana*: application of concentrations relative to field bioassay

The agar infused with the three concentrations of macerated green manure leaves of *P. americana* exhibited very slight (at the lowest concentration) to moderate inhibitory activity (at the two highest concentrations) against the fungus *R. solani* and the oomycete *P. ultimum* (Figure 3.4, A and B). During the course of 168 hours the difference between radial growth of these pathogens on treated agar and the control agar was found to be significant. All *p*-values are in Appendix VI.

The scale of inhibitory activity against *R. solani* ranged from 26% to 39% for 8.33g/l *P. americana* and from 40% to 49% for 15.00g/l *P. americana.* Inhibitory activity generally increased up until hour 120, after which it tapered off. The lowest concentration (1.67g/l) exhibited low inhibition activity ranging from 1% to 17%, increasing by 16% from hour 48 to hour 120 and decreasing by 7% by hour 168.

In the case of *P. ultimum* the two highest concentrations of *P. americana* exhibited a moderate inhibition of 35% or more, ranging from 35% to 45% at 8.33g/l of *P. americana*, and ranging from 40% to 52% at 15.00g/l of *P. americana*. Meanwhile the lowest concentration (1.67g/l) exhibited low inhibition activity against *P. ultimum* between hour 72 and 120 with a percentage inhibition of 13% to 16%.

Although percentage inhibition induced by each concentration was similar for both pathogens, *R. solani* was affected by the low concentration for a longer period of time (144 hours) than *P. ultimum* (72 hours).

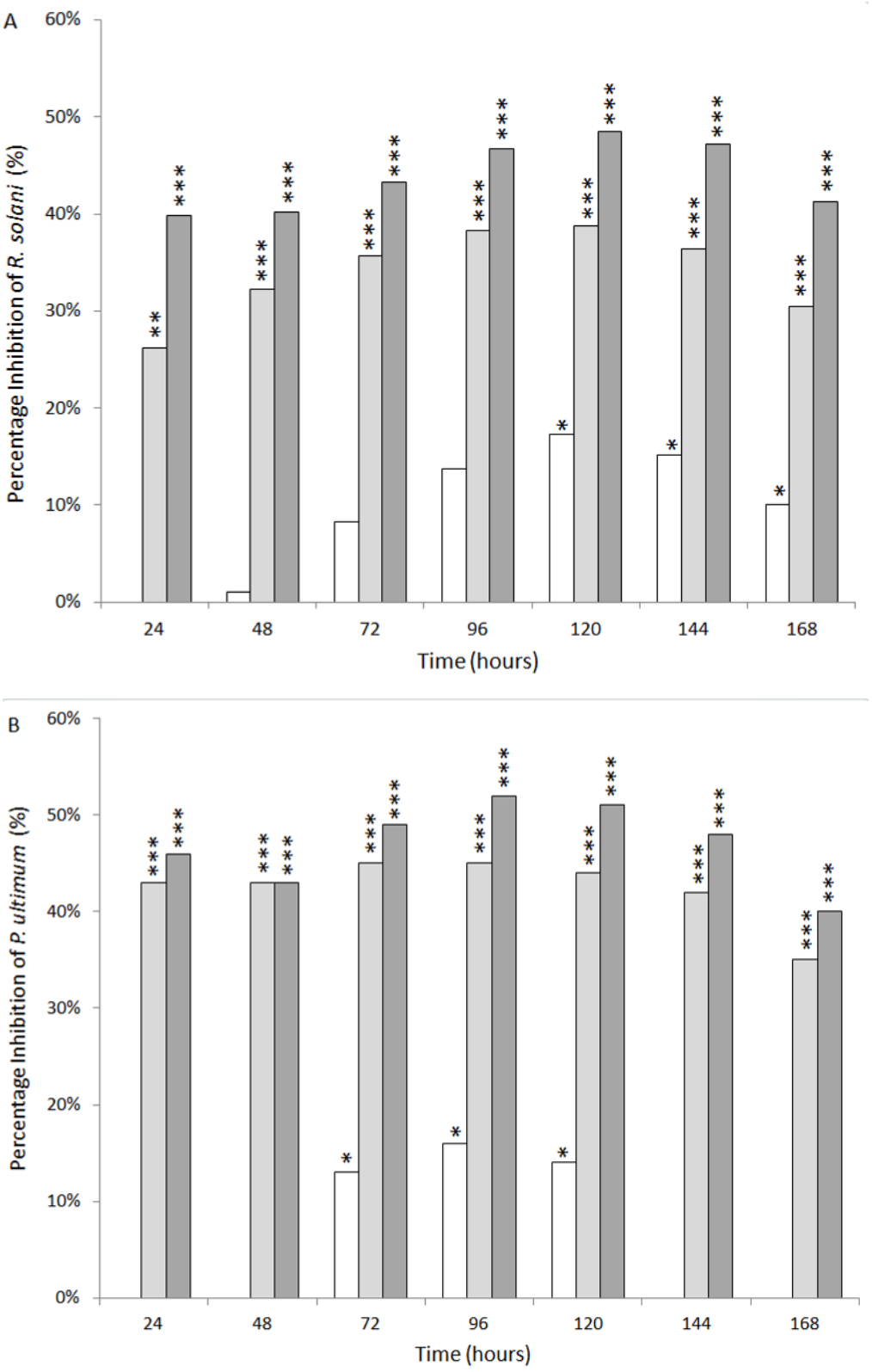


Figure 3.4: Percentage inhibition displayed at each time point where a significant difference found between the radial growth of *R. solani* (A) and *P. ultimum* (B) on agar infused with macerated green manure leaves of *P. americana* in comparison to the control agar. Key and notes: Treatments: □ *P. americana* 1.67g/l, □ *P. americana* 8.33g/l, and ■ *P. americana* 15.00g/l. Significant differences in radial growth: * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, n=9.

The most consistent and sustained suppression of pathogen growth was induced by the two highest concentrations of *P. americana*. Figure 3.5 demonstrates the diffences in radial growth. The influence of these concentrations were not significant.

The growth of *R. solani* on 8.33g/l *P. americana* amended agar was significantly lower than that of the control at hour 24 (p=0.003) by 30% with the reduced growth continuing until the end of the assay (p<0.001), at which point growth was 36% less than the control. Likewise, growth of the pathogen on 15.00g/l *P. americana* amended agar was significantly lower than that of the control throughout the assay (p<0.001), with a minimum difference of 50% and a maximum of 64%.

In relation to the growth of *P. ultimum*, there was a significant difference between the influence of these two concentrations in comparison to the lowest concentration and the control (p<0.001). During the course of the assay the growth of the pathogen is reduced by 55% to 58% by *P. americana* 8.33g/l and by 55% to 71% by *P. americana* 15.00g/l

The lowest concentration of *P. americana* had a delayed influence on *R. solani*. The growth of the fungus was slightly reduced by this treatment from hour 120 (p=0.023) onwards (hour 144, p=0.017; hour 168, p=0.032). In comparison the growth of *P. ultimum* was slightly and temporarily reduced at hours 72, 96 and 120 (p=0.025, p=0.014 and p=0.016, respectively).

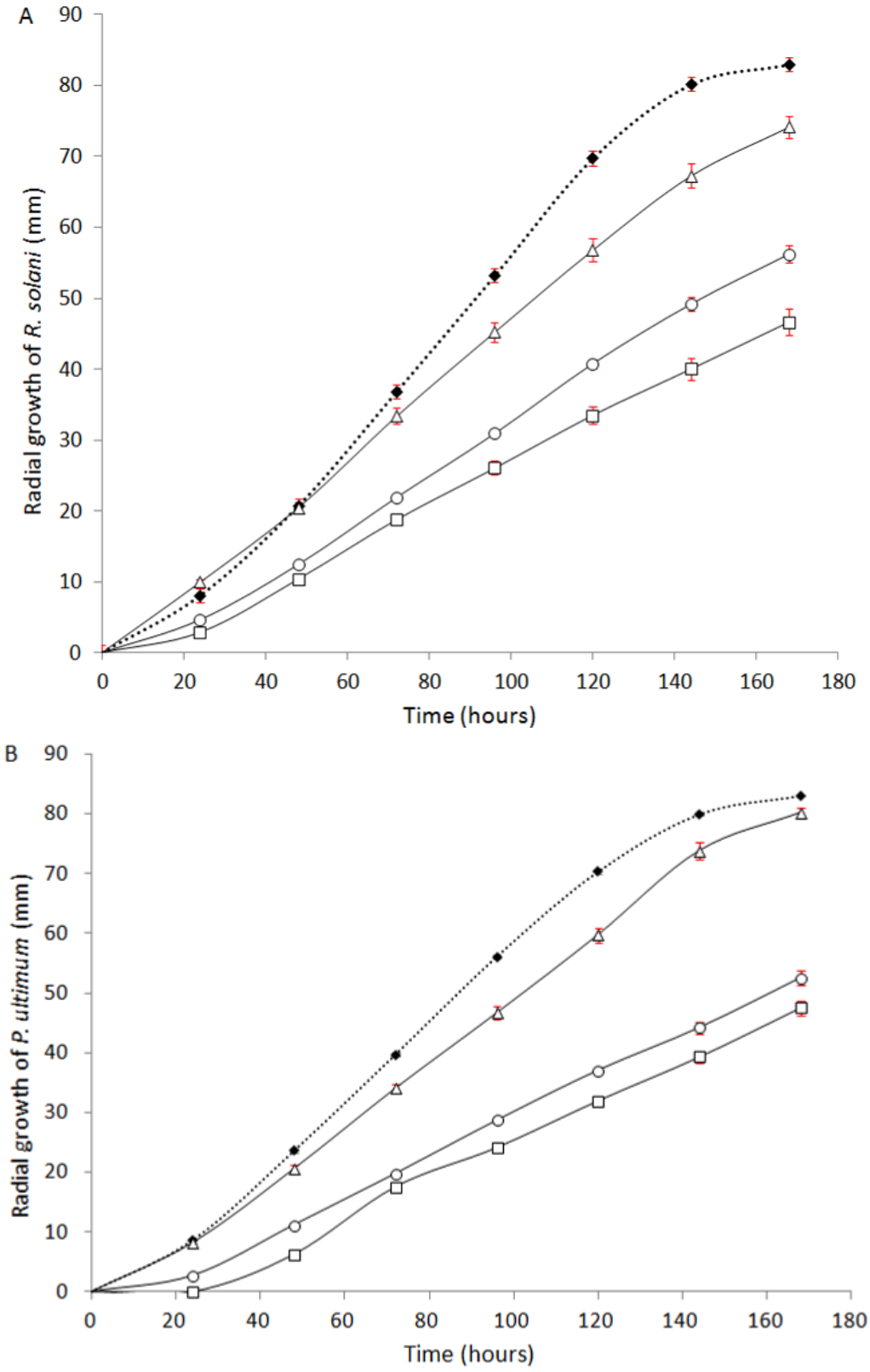


Figure 3.5: Mean radial growth of *R. solani* (A) and *P. ultimum* (B) on agar plates amended with three concentrations of *P. americana*. Key and notes: Treatments: Control, *P. americana* 1.67g/l, *P. americana* 8.33g/l, and *P. americana* 15.00g/l. Error bars are displayed as SEM, n=9.

Further investigations into the effect of higher concentrations (20.00g/l, 25.00g/l, and 30.00g/l) of *P. americana* on pathogen growth were completed. Significant differences in the mean radial growth of *R. solani* and *P. ultimum* were exhibited between each concentration of *P. americana* and the control throughout the assay (p<0.001; Figure 3.6, A and B). The inhibitory activity (>46%) of these three concentrations of *P. americana* against *R. solani* was not significant and was no different to concentrations of 8.33g/l and 15.00g/l *P. americana*.

P. ultimum exhibited suppressed growth at varying degrees. For the first 120 hours 20.00g/l *P. americana* inhibited the pathogen by 44% to 47%. Inhibition was greater with an increase in the amount of *P. americana,* where 25.00g/l inhibited *P. ultimum* by 51% to 54% and 30.00g/l by 58% to 65%. From hour 72 the influence of 25.00g/l was significantly more than that of 20.00g/l (p<0.023), however significantly less than that of 30.00g/l (p<0.002).

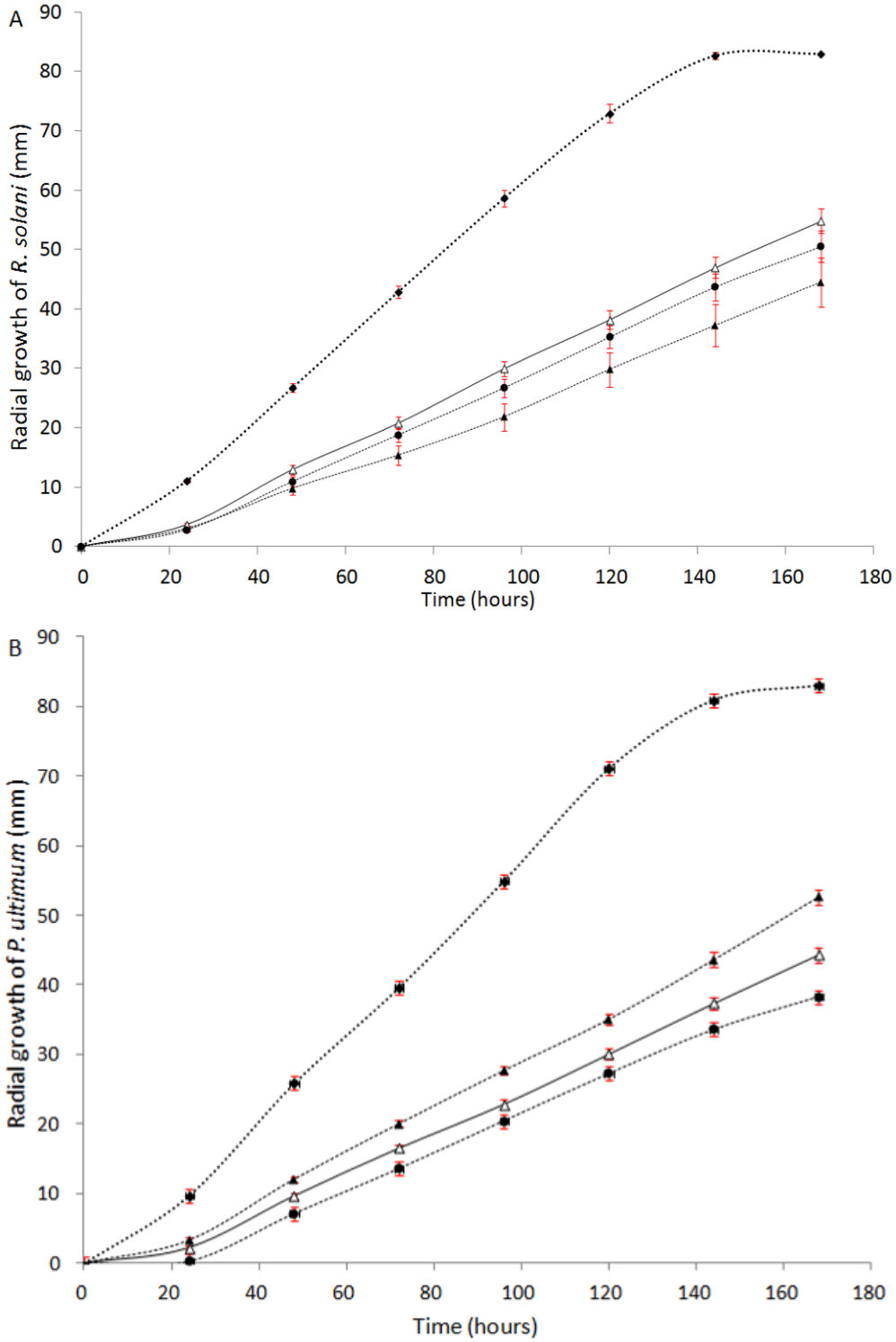


Figure 3.6: Mean radial growth of *R. solani* (A) and *P. ultimum* (B) on agar plates amended with 8.33g/l of macerated green manure leaf matter. Key and notes: Treatments: Control, 20.00g/l *P. americana,* 25.00g/l *P. americana,* and 30.00g/l *P. americana*. Error bars are displayed as SEM, n=9.

3.2.4.2 All green manures: application of 8.33g/l relative to field bioassay

The agar infused with the 8.33g/l of macerated green manure leaves of *P.* americana, *B. juncea*, *T. patula*, *A. sativum*, and *T. aestivum* exhibited inhibitory activity against *R. solani* and *P. ultimum* (Figure 3.7, A and B). The *p*-values representative of a significant difference are in Appendix VII.

From the beginning of the assay *R. solani* was suppressed by *P. americana* with inhibition increasing from 26% by 10%, whilst it fluctuated between 28% and 39% in the presence of *A. sativum*. *B. juncea* and *T. aestivum* but did not display a significant inhibitory effect until hour 48 (26% and 22%) and hour 72 (42% and 32%), which were comparable to *P. americana*. *T. patula* exhibited an insignificant influence on pathogen suppression.

Inhibition of *P. ultimum* was similar to that of *R. solani* in that the effects of *P. americana* increased from 43% by 2%. During the assay the influence of *B. juncea* and *T. aestivum* mirrored one another showing a maximum difference in inhibitory effect of only 3%. Once again these treatments did not display a significant effect until hour 48. Both treatments were as efficient as *P. americana* at suppressing pathogen growth with inhibition increasing from 33% by c. 12%. In contrast *A. sativum* induced lower but consistently significant suppression of pathogen growth with the percentage of inhibition fluctuating between 27% and 34%. The inhibitory effect of *T. patula* was comparatively weaker and did not display a significant suppressive influence until hour 72 exhibiting 24% inhibition (p=0.013).

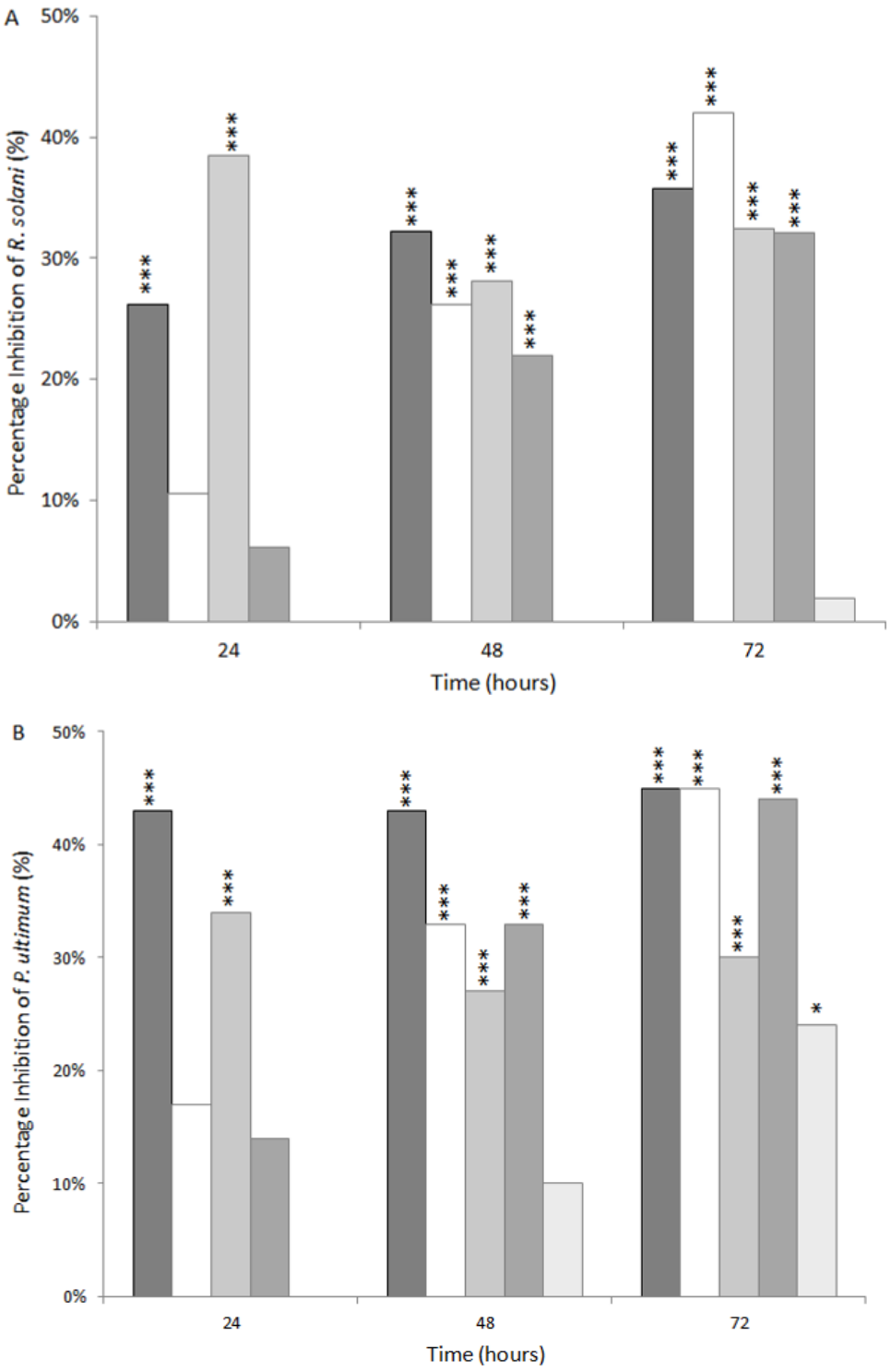


Figure 3.7: Percentage of inhibition of *R. solani* (A) and *P. ultimum* (B) exhibited by the agar infused with macerated green manure leaves in comparison to a control. Key and notes: Treatments: *P. americana* (■), *B. Juncea* (□), *T. patula* (□), *A. sativum* (■), and *T. aestivum* (■). Percentage inhibition has been displayed at each time point where a significant difference in the means was found between the radial growth of *P. ultimum* on treated agar in comparison to the control agar. Significant differences in radial growth: * $p \leq 0.05$, *** $p \leq 0.001$, n=9.

The measurement of mean radial growth of *R. solani* and *P. ultimum* at each time point in relation to each agar amendment is displayed in Figure 3.8 (A and B). Throughout the assay the mean radial growth displayed significant differences between the treatment groups (p<0.001).

The mean radial growth of *R. solani* on agar infused with the 8.33g/l of macerated green manure leaves of *P.* americana, *B. juncea*, *A. sativum*, and *T. aestivum* was statistically similar over the 72 hours. The exception to this is at hour 24 when growth on *B. juncea* and *T. aestivum* amended agar was significantly more than growth on *A. sativum* amended agar by 37% (p=0.002) and 42% (p<0.001), respectively). Pathogen growth on *T. patula* amended agar was statistically the same as the control (p>0.05).

Throughout the assay the affect of *T. aestivum* and *B. juncea* on *P. ultimum* growth was statistically similar to that of *P. americana* amended agar (p>0.05). Meanwhile, growth of *P. ultimum* on *P. americana* amended agar was significantly lower that of *A. sativum* by over 15% (p<0.001). At hour 24 the *A. sativum* treatment suppressed growth by 26% in comparison to the *T. aestivum* amendment (p=0.018), whilst the affect of *B. juncea* was similar. By hour 72 however both *B. juncea* and *T. aestivum* inhibited radial pathogen growth more than *A. sativum* by 22% ±1% (p=0.001). As previously stated *T. patula* does not display any inhibitory activity until hour 72 at which point radial growth of the pathogen aligns with that of *A. sativum* whilst remaining distinctly lower than the other treatments (p<0.001). In comparison to *P. americana* pathogen growth on *T. patula* amended agar is over 32% more during the course of the assay.

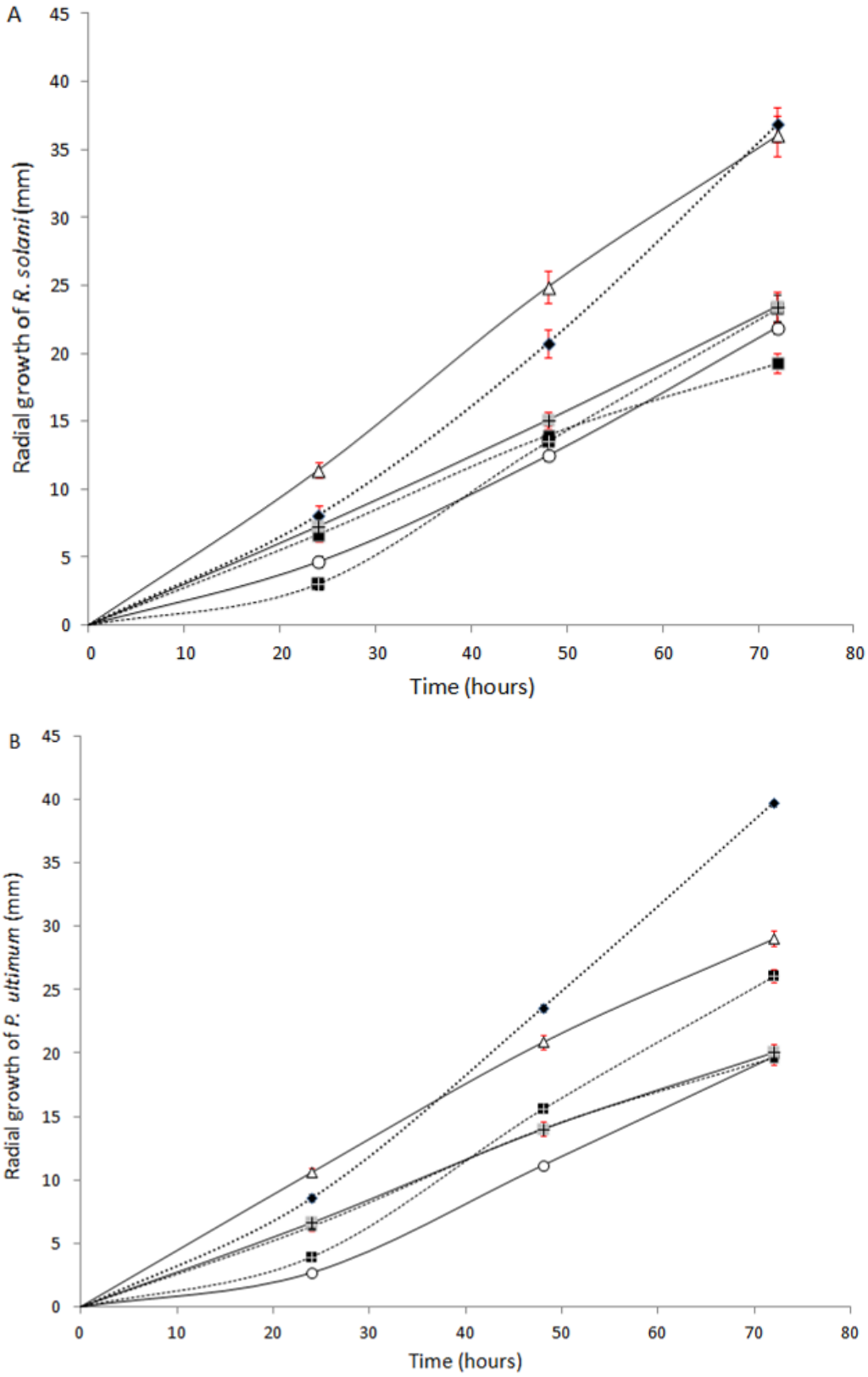


Figure 3.8: Mean radial growth of *R. solani* (A) and *P. ultimum* (B) on agar plates amended with 8.33g/l of macerated green manure leaf matter. Key and notes: Treatments: Control, *P. americana*, *B. juncea* (follows the line of *T. aestivum*), *T. patula*, *A. sativum*, and *T. aestivum*. Error bars are displayed as SEM, n=9.

3.2.5 Inhibition of pathogens with a cold water extract from green manure

3.2.5.1 Inhibition of pathogens by green manure cold water extracts (8.33g/l)

The agar infused with the aqueous extract of *P. americana* exhibited significant inhibitory activity against *R. solani* and *P. ultimum* which increased over the 168 hours by 15% to 34% (p<0.001) for the former and by 25% to 35% (p<0.001) for the latter (Figure 3.9, A and B).

The inhibitory effect of *A. sativum* and *T. aestivum* was minimal and *B. juncea* and *T. patula* was zero. The inhibition of *R. solani* induced by *A. sativum* (5% to 10%) and *T. aestivum* (1% to 2%) was not significant. In comparison the inhibition of *P. ultimum* elicited by *A. sativum* of 12% to 13% (from hour 48) and by *T. aestivum* of 9% to 11% (from hour 96) was significant in comparison to the control (p<0.035 and p<0.011, respectively).

The suppression of *R. solani* and *P. ultimum* by *P. americana* was significantly more than that induced by *A. sativum, B. juncea, T. patula,* and *T. aestivum* throughout the assay (p<0.001; Figure 3.10, A and B). *R. solani* growth on *T. patula, A. sativum* and *T. aestivum* amended agar was not statistically different to that of the control throughout the assay (p>0.05), except during the *R. solani* study at hour 24 when the inhibitory effect of *A. sativum* was not statistically different to that of *P. americana* (p>0.05). The radial growth of the pathogen on *B. juncea* amended agar was in excess of that of the control by 15% (hour 48, p<0.001) and 9% (hour 72, p=0.029), respectively. Radial growth of *P. ultimum* was suppressed by *A. sativum* and *T. aestivum* throughout the assay (p<0.05). Growth of the pathogen on the *T. patula* amended agar was temporarily in excess of that of the control from 12% to 6% between hour 24 to 96 (p<0.035).

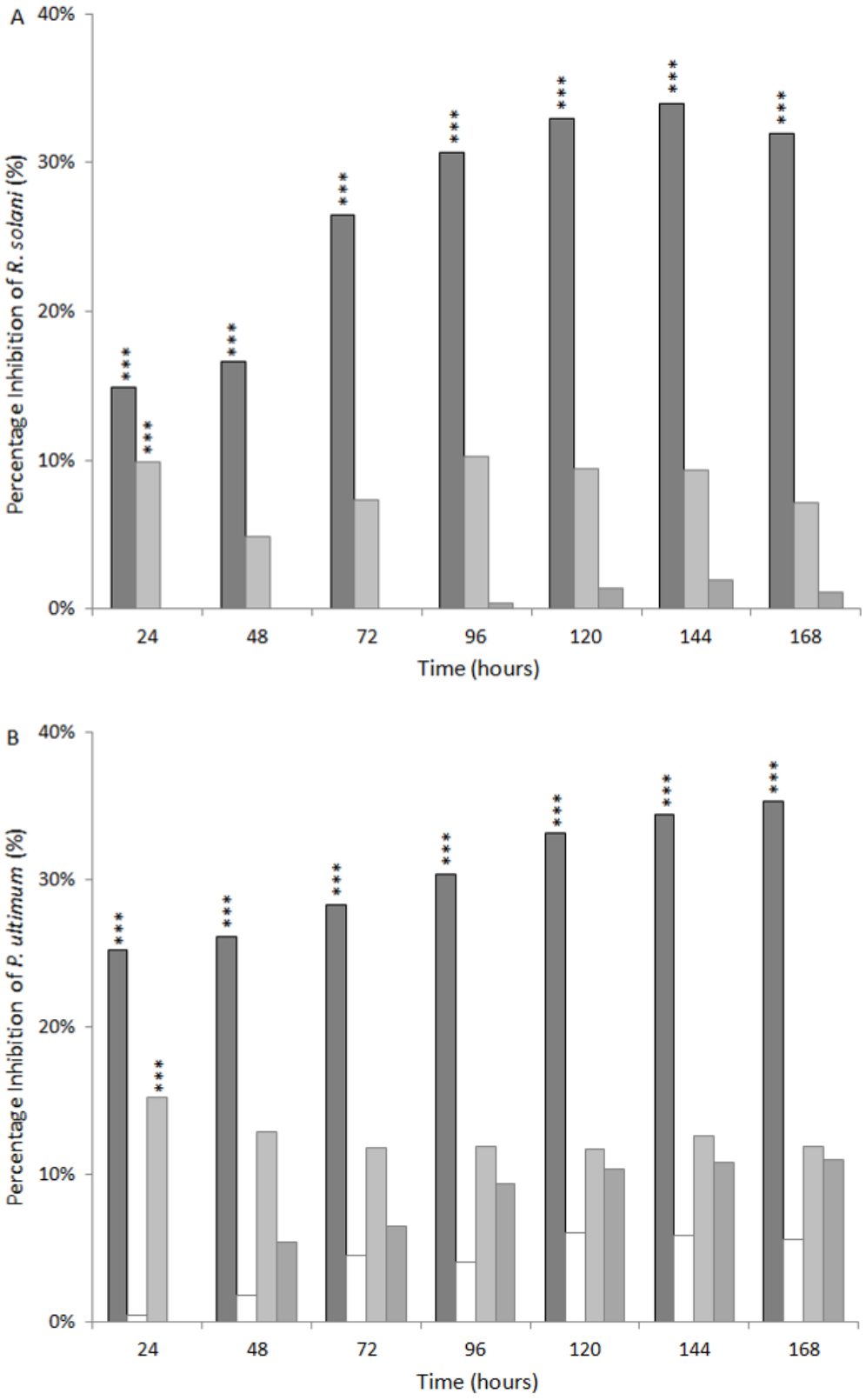


Figure 3.9: Percentage of inhibition of *R. solani* (A) and *P. ultimum* (B) exhibited by the agar infused with cold water extracts. Key and notes: Treatments: *P. americana* (■), *B. Juncea* (□), *A. sativum* (■), and *T. aestivum* (■) in comparison to a control. Significant differences in radial growth: * $p \leq 0.05$, *** $p \leq 0.001$, n=9.

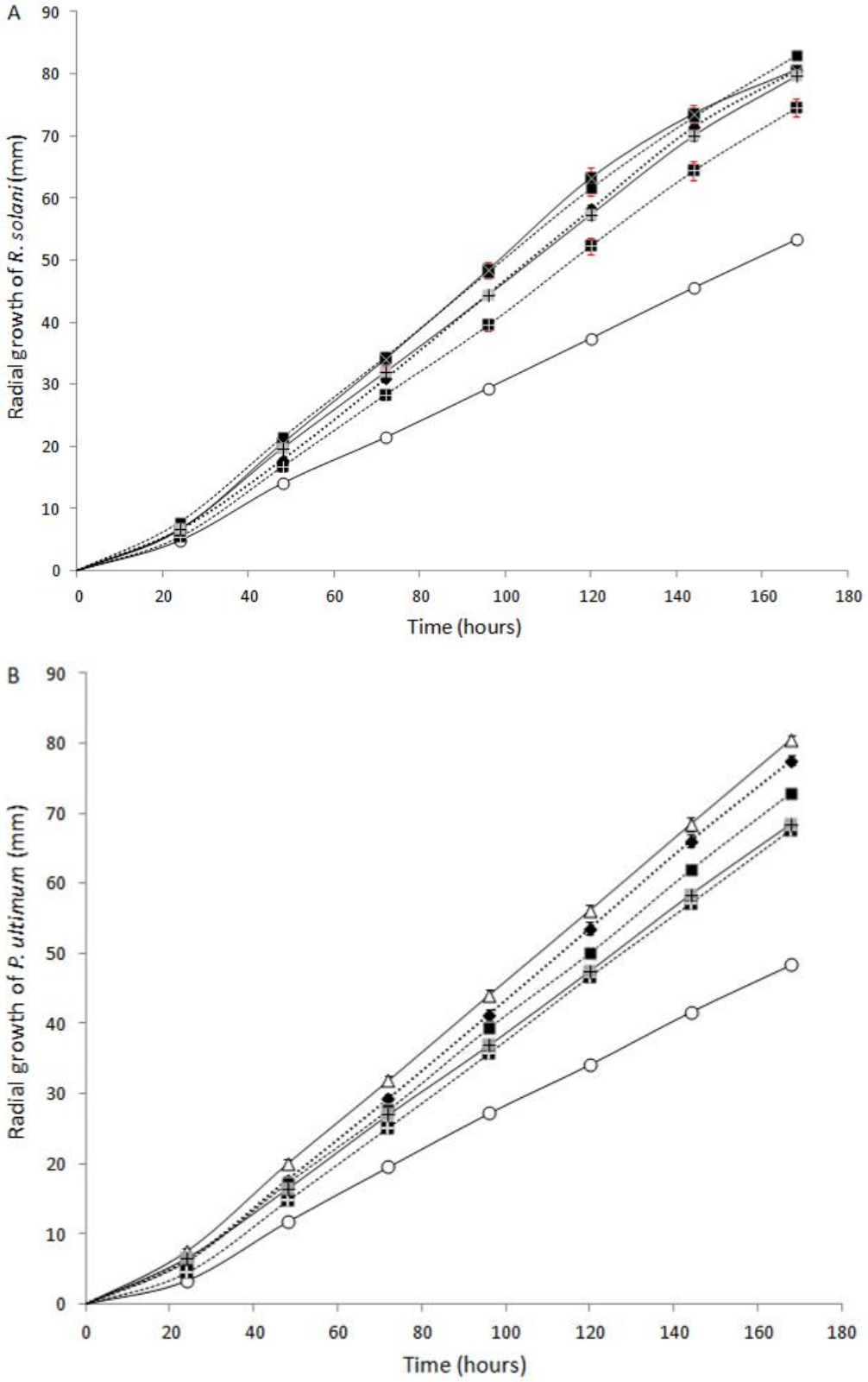


Figure 3.10: Mean radial growth of *R. solani* (A) and *P. ultimum* (B) on agar plates amended with 8.33g/l of cold water extracts. Key and notes: Treatments: Control, *P. americana*, *B. juncea* (follows the line of *T. aestivum*), *T. patula*, *A. sativum*, and *T. aestivum*. Error bars are displayed as SEM, n=9.

3.2.6 A comparison of green manure and cold liquid water extract (8.33g/l)

In comparing the difference in effectiveness of the treatment methods on the radial growth of *R. solani* and *P. ultimum* it was found that the agar infused with macerated green manure leaves and cold liquid water extracts of *P. americana*, *A. sativum*, and *T. patula* had a similar influence on pathogen growth throughout 72 hours. Differences were apparent for pathogens treated with *B. juncea* and *T. aestivum*.

For *R. solani* both *B. juncea* and *T. aestivum* were more effective in the solid form in comparison to the liquid form by 42% and 21% respectively after 48 hours ($H(11) = 229.693$, $p<0.001$) and by 57% and 26% respectively after 72 hours ($H(11) = 265.066$, $p<0.001$) (Figure 3.11).

For *P. ultimum* the macerated green manure of *B. juncea* was 15% more effective than the liquid extract after 48 hours ($H(11) = 293.835$, $p<0.001$). At 72 hours *B. juncea* and *T. aestivum* were both more effective in a solid state than in a liquid state by 28% and 21% respectively ($H(11) = 337.833$, $p<0.001$) (Figure 3.12).

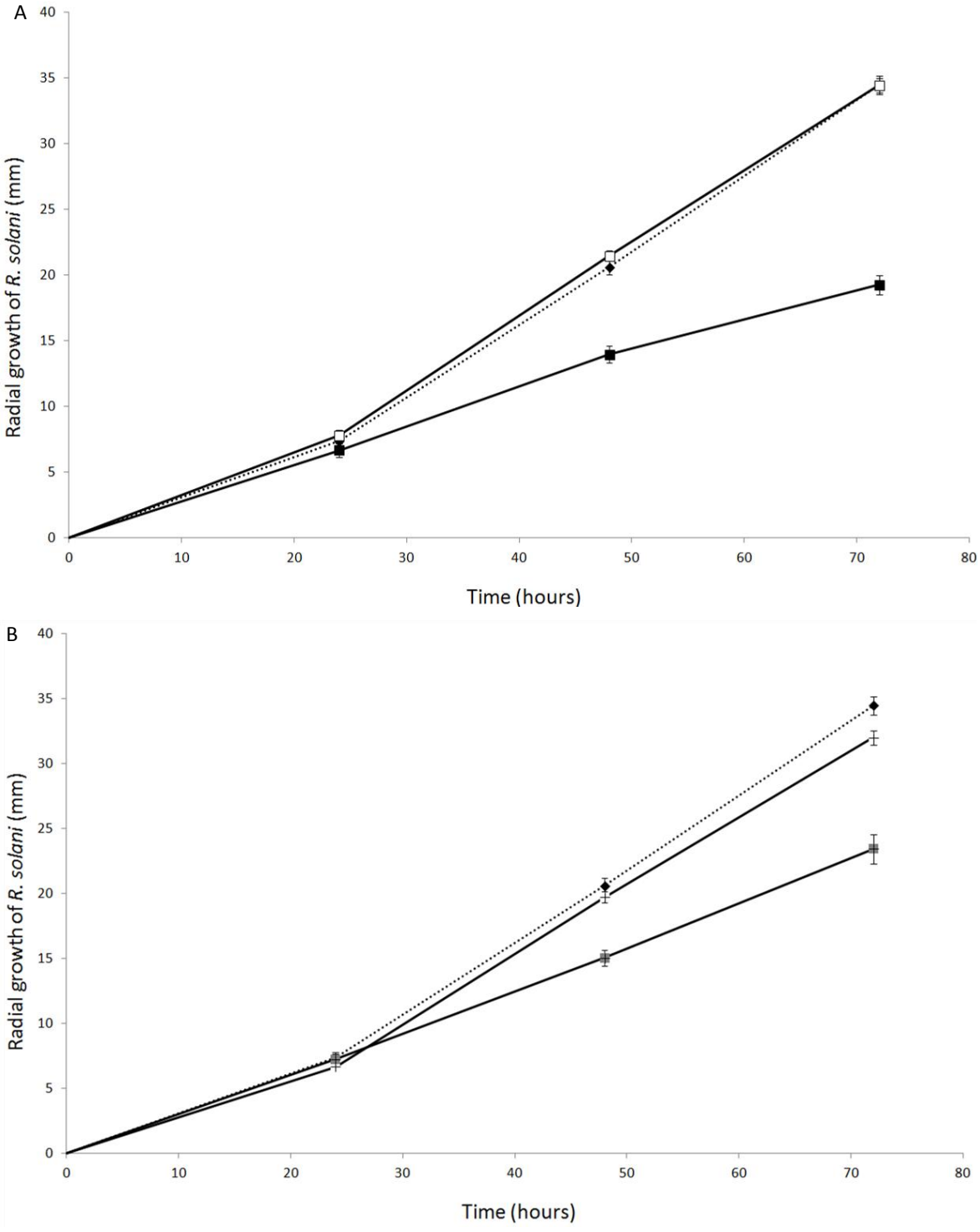


Figure 3.11: Mean radial growth of *R. solani* on agar plates amended with 8.33g/l of (A) *B. juncea* and (B) *T. aestivum* in the form of green manure and cold water extracts. Key and notes: Treatments: ···◆··· Control, —□— *B. juncea* liquid extract, —■— *B. juncea* green manure, —┼— *T. aestivum* liquid extract and —▦— *T. aestivum* green manure. Error bars are displayed as SEM, n=9.

A

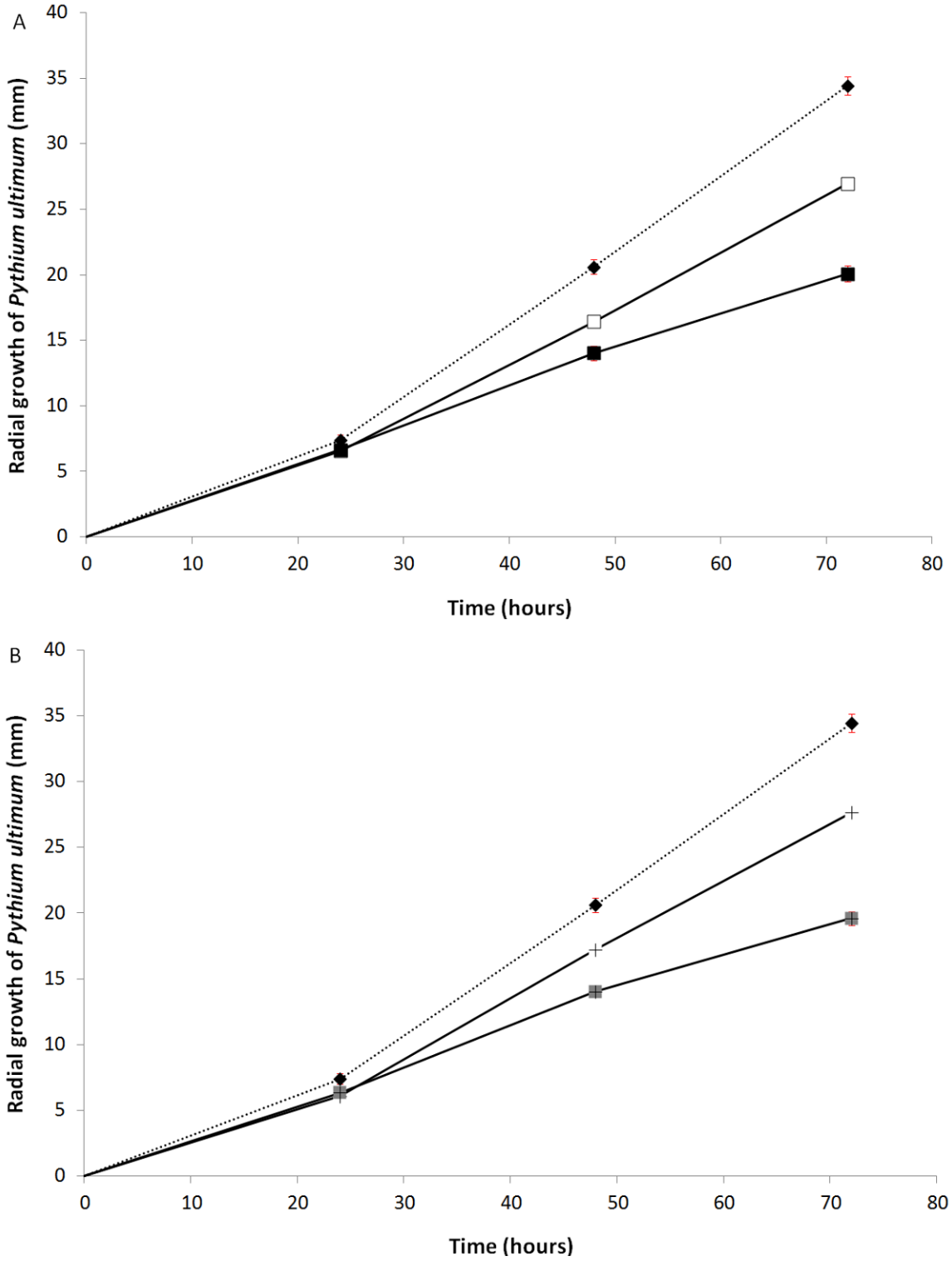


Figure 3.12: Mean radial growth of *P. ultimum* on agar plates amended with 8.33g/l of (A) *T. aestivum* and (B) *B. juncea* in the form of green manure and cold water extracts. Key and notes: Treatments: Control, *T. aestivum* liquid extract, *T. aestivum* green manure, *B. juncea* liquid extract and *B. juncea* green manure. Error bars are displayed as SEM and are on all data points even if not visible, n=9.

3.3 An overview of all *in-vitro* studies

This study demonstrated that the nature of the application method of green manure can influence pathogen growth. The effect on growth was dependent on method of application, concentration, and green manure species used. In terms of *P. americana* all treatment methods significantly inhibited *R. solani* and *P.ultimum*. The variation in percentage inhibition of *R. solani* was lowest when the fungus was treated with plant matter in agar (26-36%). *P. ultimum* was consistently responsive to plant matter when mixed in agar, and additionally when it was applied as a disc (43-45% and 66-69% respectively). The oomycete reduced in growth when treated with the cold water extract (25-35%). Variation in inhibition puts into question the efficacy of the treatments *in-vitro*, however, this study demonstrated that *P. americana* has properties which induce inhibition of pathogen growth, with these properties being liberated from the plant matter directly or as a water based extract, and not as an alcohol based extract (Table 3.2).

Table 3.2: Inhibition of *R. solani* and *P. ultimum* in relation to *P. americana* application.

Treatment type	*Inhibition of R. solani (% mean)*	*Inhibition of P. ultimum (% mean)*
Plant disc	38-64	66-69
Ethanol	-	-
Volatiles	62-90	8-46
Distillate	36-57	38-69
Plant matter in agar	26-36	43-45
Cold water extract	15-34	25-35

This study also determined that *P. americana* induced significant inhibition of *R. solani* and *P.ultimum* when applied at three different concentrations. Inhibition was positively correlated with dose. Of the two pathogens the former was found to be more sensitive to treatments than the latter. Indeed even the lowest concentration inhibited the fungi whilst only transiently limiting to the growth of the oomycete.

In terms of the other green manure species it was clear that the application of most of the green manure as plant matter in agar inhibited *R. solani* and *P.ultimum* growth. *T. patula* was the only plant species that did not inhibit *R. solani* and only temporarily inhibited *P. ultimum* growth (after 72 hours). When the treatment was applied as a cold water extract *P. americana* successfully inhibited both pathogens whilst *A. sativum* and *T. aestivium* only inhibited *P. ultimum* at a significantly lower amount. This is potentially due to the solubility or insolubility of some active ingredients.

Chapter 4 - Bioassay and Destructive Analysis

4.1 Introduction

The addition of organic matter can improve soil structure, increase microbial diversity, provide nutrients and induce a suppressive soil (Bonhilla *et al.*, 2012). The creation of a healthy soil promotes a balanced ecosystem that encourages the growth and improves the health of plant species. Any plant species has a range of metabolites that have evolved to be produced to support plant germination, growth, reproduction, stress tolerance and survival (Calvo *et al.*, 2014). These properties can take the form of amino acids, proteins, vitamins, trace elements, macro nutrients, polysaccharides, phytohormones and defensins or secondary metabolites produced as a response to attack by pests and disease (Chojnacka *et al.*, 2012; Calvo *et al.*, 2014; du Jardin, 2015). As stated in Section 1.7.4.1, plant matter that is incorporated into the soil can directly inhibit pathogens through the release of phytochemicals (biopesticides), and indirectly suppress pathogens through the stimulation of beneficial organisms (biocontrols). They may also contain properties that can promote plant growth by being nutritional (biofertilisers) or through other stimulatory means (biostimulants). There can be a four-fold effect caused by organic matter amendments (Figure 4.1).

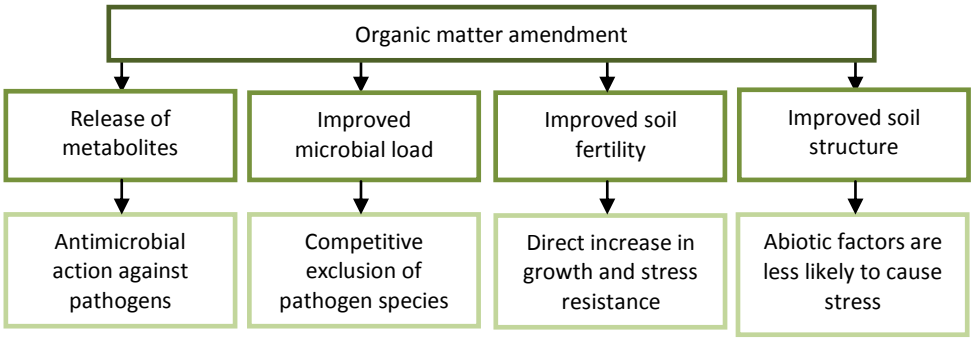


Figure 4.1: The benefits that the addition of organic matter has in relation to soil health and plant growth.

Currently, more emphasis is being put on the use of organic biostimulants, biofertilisers and biopesticides. It would thus seem logical to take advantage of the natural properties of organic material to induce suppression of soil pathogens rather than use a broad spectrum pesticide that will be detrimental to beneficial microbiota even though the intended target is a pathogen. Addition of organic matter can induce a suppressive soil, directly limit the numbers of a pathogen population and/or increase the immune response of a plant (Bonilla *et al.*, 2012). This is possible due to the wide range of bioactive compounds that are created by plants as an evolved part of their defence system. As a result properties of many medicinal plants have been studied for their application in pesticides and also for medical purposes (Dang and Van Damme, 2015).

This study, which ran alongside the microbial study (Chapter 3), investigated the addition of a novel green manure and comparative green manures, chosen on the merit of their antimicrobial properties, as a potential treatment for RD on *S. aucuparia*. The study was designed so that it would establish if the green manure amendments merely stimulated growth generally or if they promoted growth in the presence of disease causing agents.

4.1.2 Aim and objectives

Aim:

To determine the effect of novel green manure applied as a treatment to RD on *Sorbus aucuparia*.

Objectives:

- To demonstrate the influence of green manure on above ground growth parameters, such as height, girth, leaf production, leaf chlorophyll content, active buds, and internodal distances, during the course of three years.
- To assess the impact of green manure on the growth of root and shoot biomass, and fruit production, at the end of the trial.

4.2 Results: three year bioassay and destructive analysis

4.2.1 Results demonstrating limited or no effect

Some growth parameters that were measured demonstrated a limited and insignificant response to treatment and therefore for reasons of clarity this data has not been shown here. Fluctuations in the number of leaves between treatment groups were not statistically noteworthy although overall numbers increased between 2012 and 2013, then decreased to levels comparable to 2012 in 2014 (p<0.001). There was a consistent increase in active buds from 2013 to 2014 for all trees regardless of whether they were grown in the presence of disease or not (p<0.001), with exception to *B. juncea* and *A. sativum* in which active bud number were not statistically different between years. There were no significant differences in the chlorophyll content of leaves in relation to treatment application throughout the bioassay; there was a consistent decrease in chlorophyll content over the course of the three years for both the diseased soil sub-set and the sterile soil sub-set. In addition shoot to root ratio did not reveal any differences between means (p>0.05)

4.2.2 Three year bioassay (Section 2.4.3)

4.2.2.1 Primary growth of *S. aucuparia*

When primary growth was considered in its entirety over the course of the whole three year period (2012 – 2014) mean tree height for all treatment groups increased by 30% to 43% regardless of treatment (in-treatment group growth was significant >0.001 with no mortality; Table 4.1).

Table 4.1: In treatment group difference in height between 2012 and 2014

Treatment group	Percentage increase in in-group mean height 2012-2014	
	Diseased soil sub-set	**Sterile soil sub-set**
No treatment	32%	42%
P. americana 1.67g/l	30%	35%
P. americana 8.33g/l	35%	31%
P. americana 15.00g/l	35%	33%
B. juncea 8.33g/l	30%	33%
A. sativum 8.33g/l	33%	30%
T. aestivum 8.33g/l	37%	31%
T. patula 8.33g/l	43%	40%

The final height of all trees in 2014 was statistically similar. Analysis of internode data demonstrated a comparable result where distances between nodes of all trees in 2012 and 2014 were similar. The only tree group to show stunting was those treated with *P. americana* 8.33g/l. Primary growth did differ between different time points as discussed in Section 4.2.1.1. Throughout the whole bioassay the primary growth of trees in the sterile sub-set was statistically similar regardless of treatment application.

Between 2012 and 2014 trees in *P. americana* 8.33g/l amended soil (AS) did not grow as tall as those in the 1.67g/l and 15.00g/l amendments which were 13% and 11% taller, respectively ($H(3) = 8.765$, $p=0.033$). Likewise, between 2012 and 2013 trees in *P. americana* 1.67g/l AS were 14% taller and those in *P. americana* 15.00g/l AS were 16% taller than those grown in soil amended with *P. americana* 8.33g/l ($H(3) = 11.413$, $p=0.01$).

The initial primary growth of trees treated with *P. americana* 15.00g/l AS was 161% more than that of the diseased group control and different to growth of *P. americana* 1.67g/l AS and 8.33g/l AS treated trees ($H(3) = 9.517$, $p=0.023$; Figure 4.2). Internode data for 2013 also demonstrated an association of stunting with *P. americana* 8.33g/l AS and the positive growth of those treated with *P. americana* 15.00g/l AS. Internodes of *P. americana* 15.00g/l treated trees were 62% and 47% longer than those in the control group and those treated with *P. americana* 8.33g/l AS ($F(4) = 3.680$, $p=0.009$).

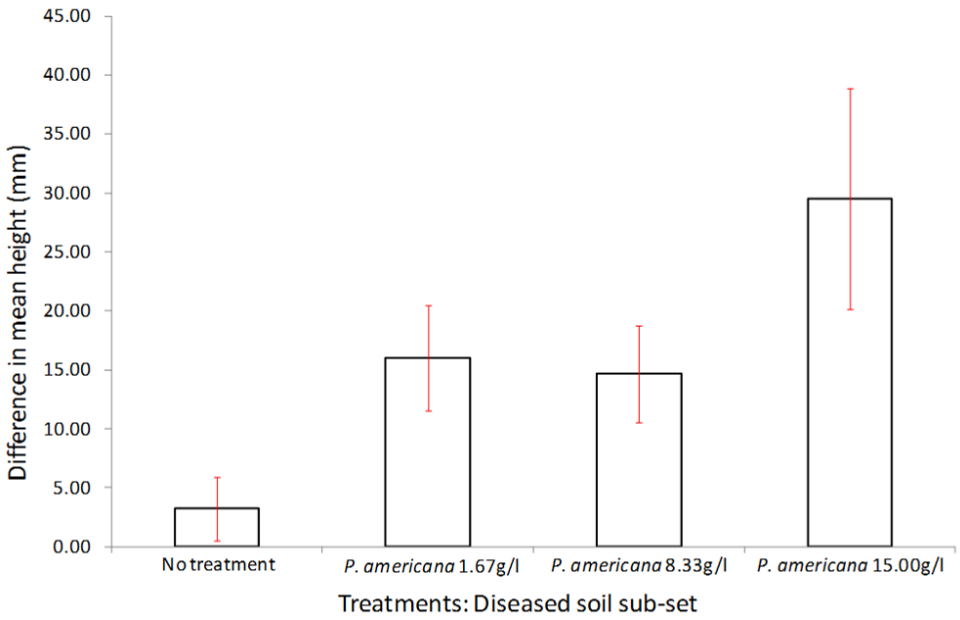


Figure 4.2: Difference in height between spring 2012 and autumn 2012 (increase in growth) for the PaM group in the diseased soil sub-set. Error bars are displayed as SEM, n=25.

In diseased soil there was an initial difference in the rate of growth for all tree groups within the first five months $H(5) = 13.238$, $p=0.021$. The growth of trees within the AGM treatment groups was 130% to 157% more than that of the disease control group (Figure 4.3).

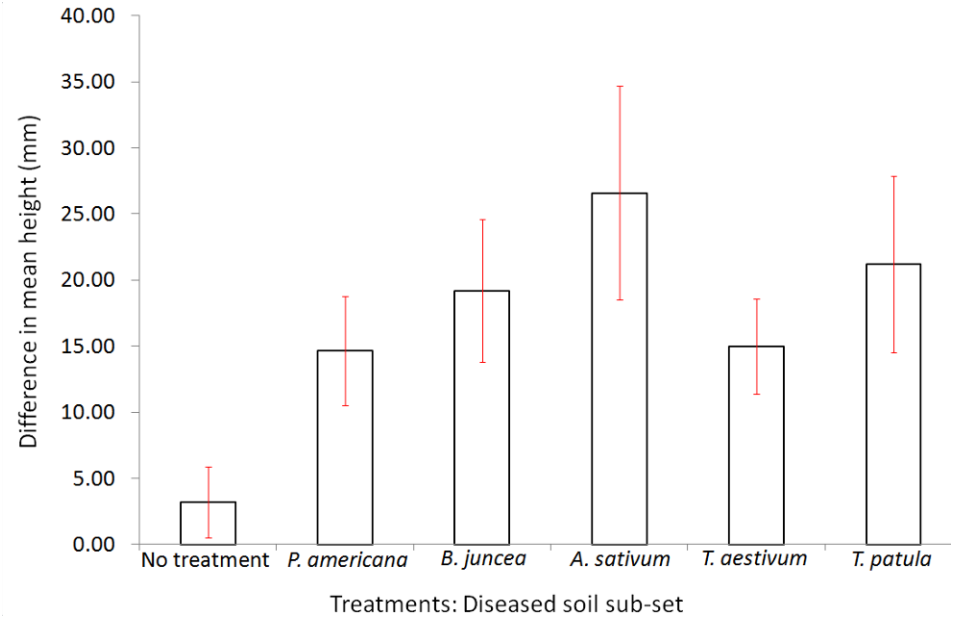


Figure 4.3: Difference in height in the AGM group (diseased soil sub-set) for the first five months (increase in growth). Error bars are displayed as SEM, n=25.

Between 2012 and 2013 primary growth of trees treated with *T. aestivum* AS and *T. patula* AS were found to be 40% to 43% greater than *P. americana* 8.33g/l AS treated trees ($H(5) = 13.512$, $p=0.019$) and 31% to 34% more than those treated with *B. juncea* 8.33g/l AS treated trees (trend; Figure 4.4).

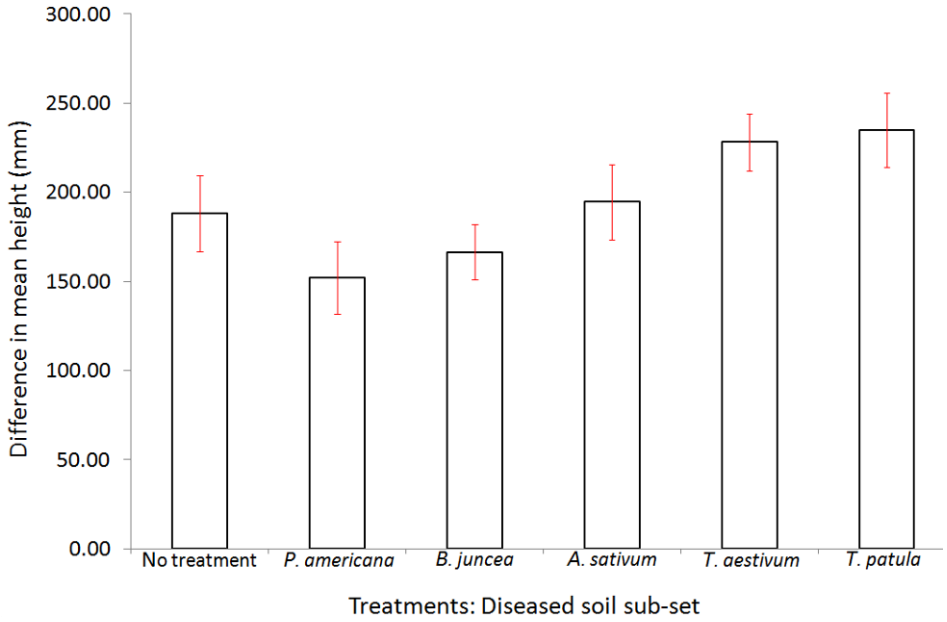


Figure 4.4: Difference in height 2012-2013 for the AGM group in the diseased soil sub-set (increase in growth). Error bars are displayed as SEM, n=25.

4.2.2.2 Secondary growth of *S. aucuparia*

As expected, there was no significant change in mean girth in the initial 2012 growth period. However there was significant in-treatment group growth between year 2012 and 2014 for all tree groups ranging from 12% to 24% (p<0.05, Table 4.2).

Table 4.2: In treatment group difference in girth (secondary growth) between 2012 and 2014

Treatment	Percentage increase in in-group mean stem diameter	
	Diseased soil sub-set	**Sterile soil sub-set**
No treatment	15%	19%
P. americana 1.67g/l	11%	16%
P. americana 8.33g/l	21%	17%
P. americana 15.00g/l	17%	16%
B. juncea 8.33g/l	12%	19%
A. sativum 8.33g/l	12%	15%
T. aestivum 8.33g/l	18%	17%
T. patula 8.33g/l	24%	21%

Between group differences in the sterile soil sub-set were not significant. Differences in the diseased soil sub-group were noted. Analysis of the PaM group demonstrated a significant difference (H(3) = 7.867, p=0.049) in stem diameter growth between 2012 and 2013. *P. americana* 8.33g/l AS treated trees had girths 53% greater than those amended with *P. americana* 1.67g/l AS. This pattern was also observed for growth between 2012 and 2014 (H(3) = 12.579, p=0.006; Figure 4.5). The lowest concentration of *P. americana* was associated with girths smaller in comparison to concentrations of 8.33g/l and 15.00g/l (56% and 41%, respectively). The *P. americana* 8.33g/l AS group had a mean girth that was 33% larger than the no treatment control.

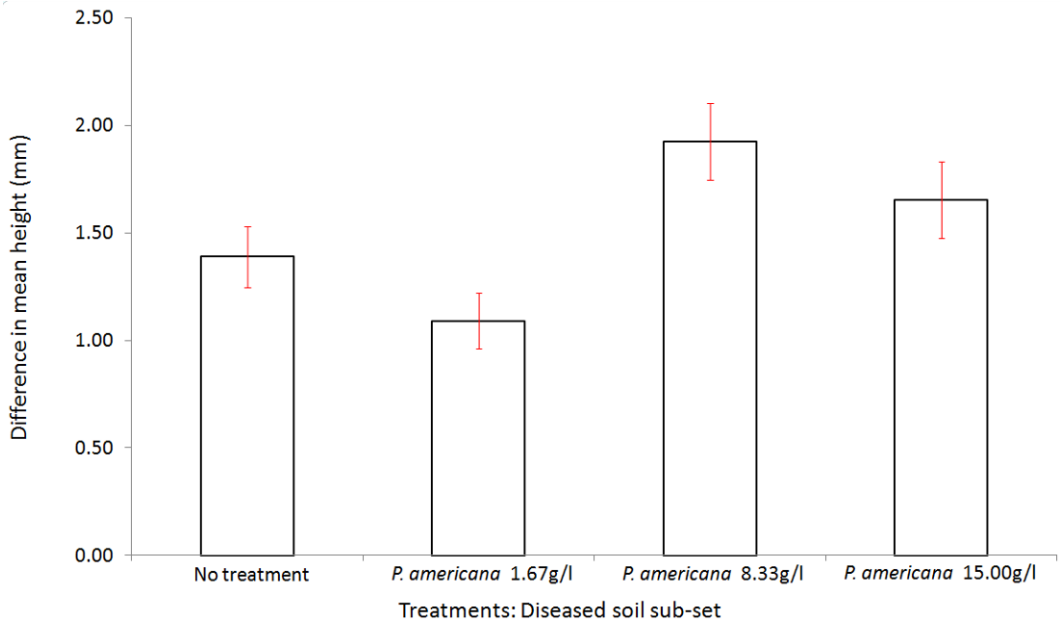


Figure 4.5: Stem diameter difference 2012-2014 for the PaM group in the diseased soil sub-set. Error bars are displayed as SEM, n=25.

The AGM group, in diseased soil, demonstrated a number of statistically significant increases in secondary growth between 2012 and 2013, which are more pronounced when comparing 2012 to 2014. During this timeframe, the significant differences were exhibited by trees treated with *T. patula* AS, *T. aestivum* AS, and *P. americana* AS ($H(3) = 27.581$, $p<0.001$). *T. patula* AS treated tree stem diameters increased by 68%, 57%, and 42% more than those treated with *B. juncea* AS, *A. sativum* AS, and the disease control. *T. aestivum* AS treated trees displayed a 47% and 13% increase in comparison to those treated with *B. juncea* AS and *A. sativum* AS, however was no different to the control. *P. americana* AS treated tree stem diameter increased by 59%, 47%, and 33% more than those treated with *B. juncea* AS, *A. sativum* AS and the disease control respectively (Figure 4.6). Increases in tree girths for those in sterile groups did not differ for the duration of the bioassay.

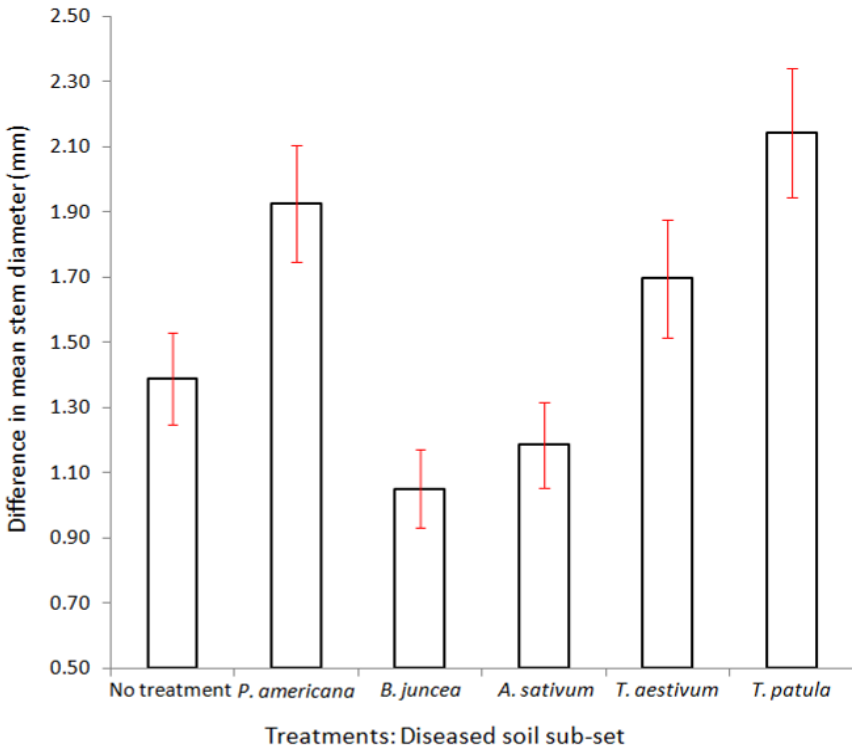
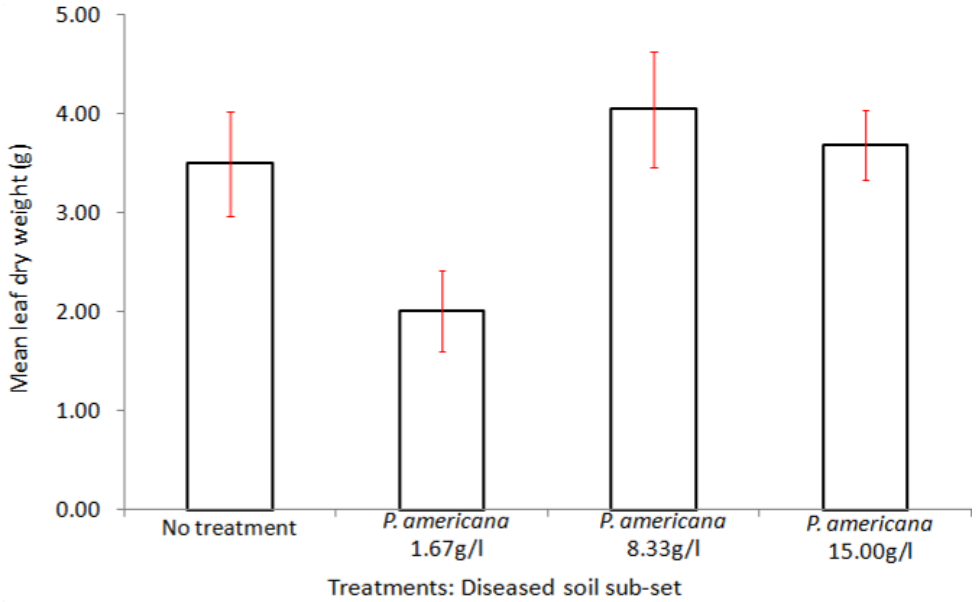


Figure 4.6: Stem diameter difference 2012-2014 for the AGM group in the diseased soil sub-set. Error bars are displayed as SEM, n=25.

4.2.3 **Destructive analysis – end of the bioassay (2014)** (Section 2.4.4)

4.2.3.1 **Leaf dry weight (g) and leaf area (mm^2)**

For the AGM group, leaf dry weight was no different for trees in the sterile soil sub-set and the diseased soil sub-set. When comparing PaM treatments, *P. americana* 1.67g/l AS treated trees demonstrated lower leaf dry weight than those amended with diseased control (54%), *P. americana* 8.33g/l (67%), and *P. americana* 15.00g/l AS (60%) ($H(3) = 11.366$, $p=0.01$; Figure 4.7).



4.7: Mean leaf dry weight per tree of the PaM group in in the diseased soil sub-set. Error bars are displayed as SEM, n=25.

In terms of the different concentrations of *P. americana,* it was determined that 1.67g/l and 15.00g/l had no positive influence on leaf areas in comparison to the disease control. However, 15.00g/l treatments induced larger leaves than the 1.67g/l treatment (by 16%) and the 8.33g/l treatment (by 43%). 8.33g/l was linked to 41% smaller leaves than the control ($H(5) = 39.648$, $p<0.001$; Figure 4.8).

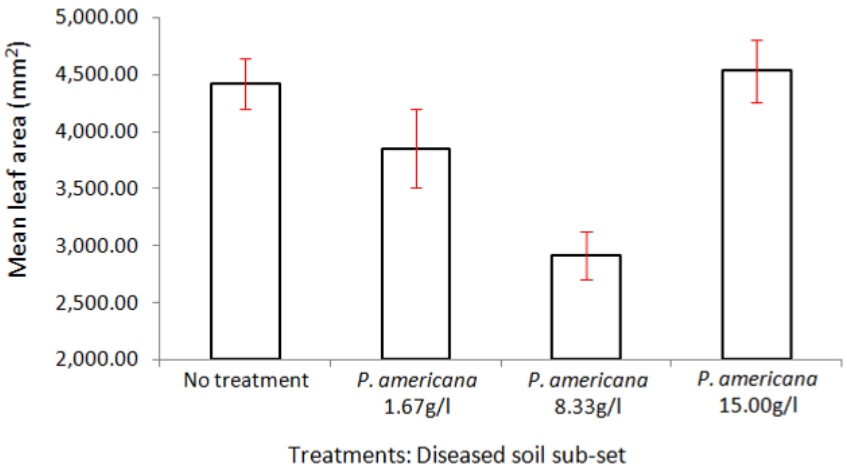
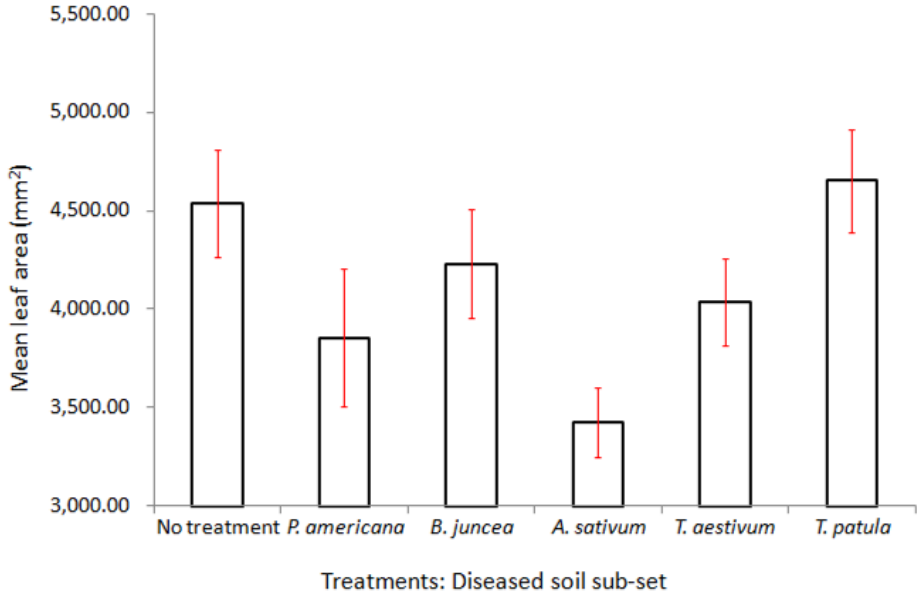


Figure 4.8: Mean leaf areas of the PaM group in in the diseased soil sub-set. Error bars are displayed as SEM, n=25.

Leaf areas differed in the diseased soil AGM group ($H(5) = 22.699$, $p<0.001$). *T. patula* application induced greater leaf areas in comparison to those treated with *P. americana* (by 19%) AS and *A. sativum* AS (by 30%). Untreated trees had bigger leaves than those treated with *P. americana* AS (16%) and *A. sativum* AS (28%; Figure 4.9).



4.9: Mean leaf area the AGM group in the diseased soil sub-set. Error bars are displayed as SEM, n=25.

Leaf areas differed in the sterile soil sub-set ($H(5) = 31.428$, $p<0.001$). *T. aestivum* AS induced the production of leaves that were smaller than those treated with *T. patula* AS (35%), *P. americana* AS (38%), and the sterile control (25%). *B. juncea* AS treated trees were also associated with smaller leaves than those treated with *T. patula* AS, *P. americana* AS, and the sterile control (35%, 38% and 26%, respectively; Figure 4.10).

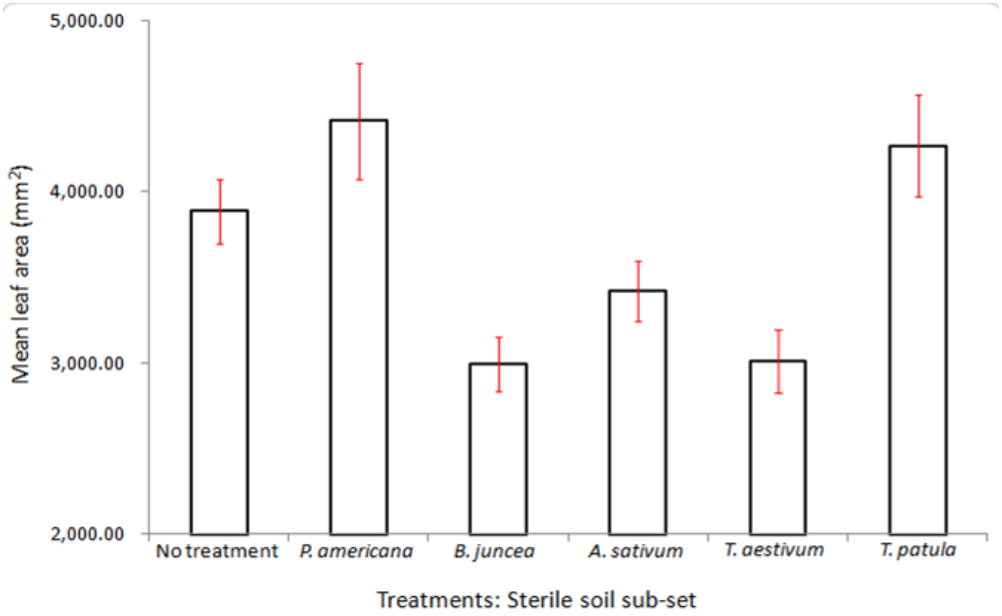


Figure 4.10: Mean leaf area in relation to the AGM group in the sterile soil sub-set. Error bars are displayed as SEM, n=25.

4.2.3.2 Mean dry shoot weight (g)

Mean dry stem weight of the diseased control trees was 22% lighter than trees treated with *P. americana* 8.33g/l AS and 15.00g/l AS ($F(3,96) = 3.188$, $p=0.027$; Figure 4.11) . In the AGM group stem weights were similar with exception to trees treated with *T. patula* AS which was associated with 29% heavier stems than those of the diseased control ($F(5,144) = 3.667$, $p=0.004$; Games Howells $p=0.033$).

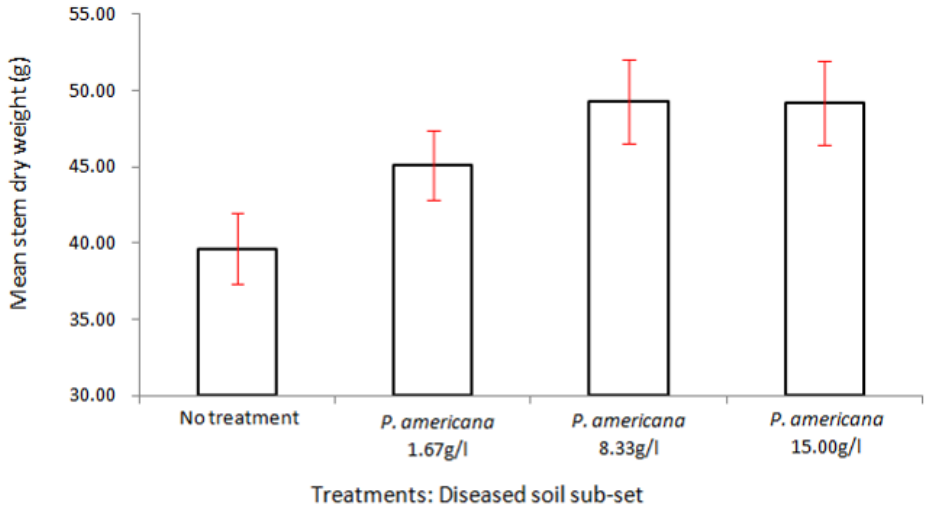


Figure 4.11: Mean weight of stems of the PaM group in the diseased soil sub-set. Error bars are displayed as SEM, n=25.

4.2.3.3 Mean dry root weight (g)

In relation to the PaM group the diseased control demonstrated a trend of having 21% lighter roots than those treated with *P. americana* 1.67g/l and significantly lighter roots than those associated with *P. americana* 8.33g/l (26%), *P. americana* 15.00g/l (36%) ($H(3) = 16.748$, $p=0.001$; Figure 4.12).

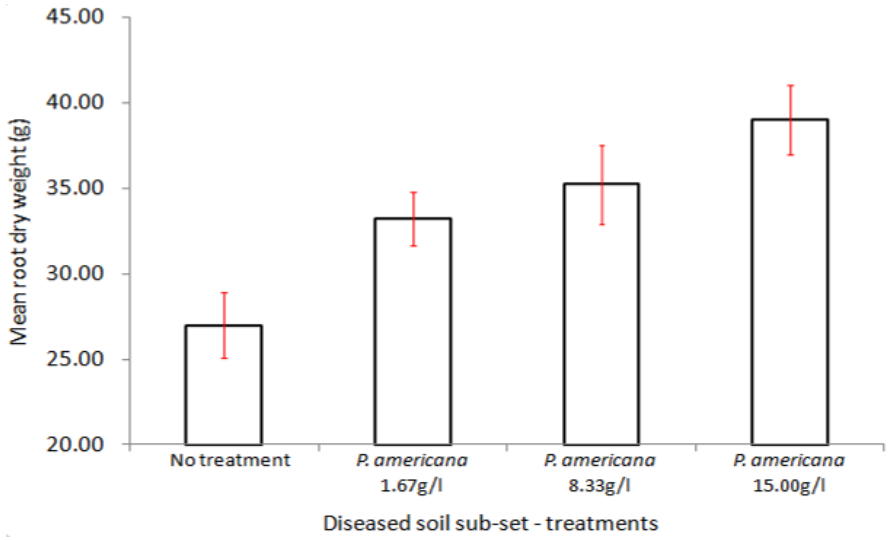


Figure 4.12: Mean dry root weight of the PaM group in the diseased soil sub-set. Error bars are displayed as SEM, n=25.

In the AGM group the diseased control demonstrated lighter roots than *T. aestivum* (by 34%), *T. patula* (by 31%), and *A. sativum* (by 25%). *B. juncea* was associated with lighter roots than *T. patula* (by 17%) and *T. aestivum* (by 20%) ($H(3) = 18.131$, $p=0.003$; Figure 4.13).

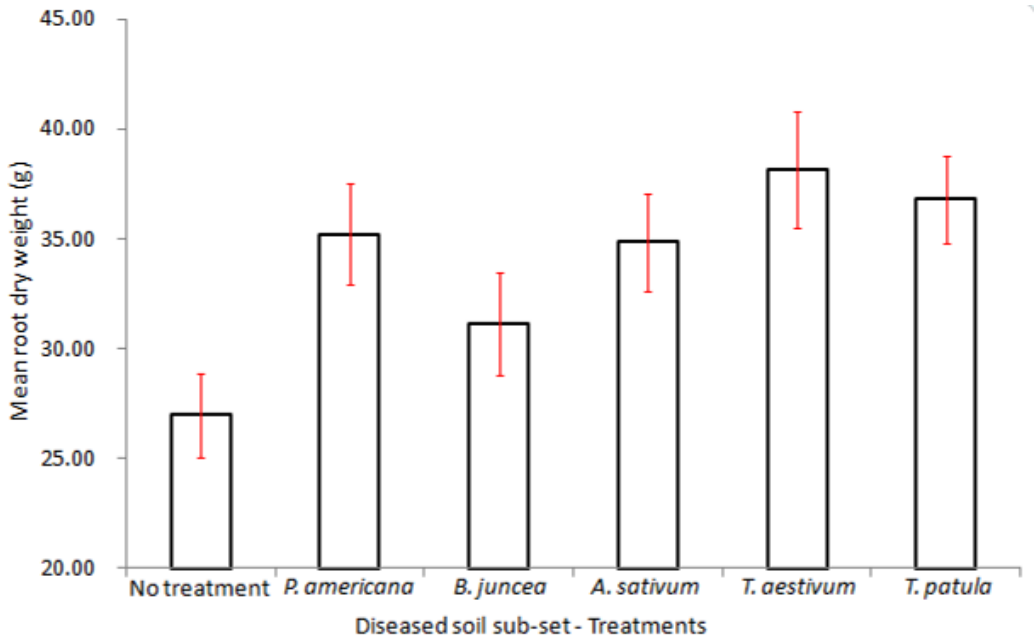


Figure 4.13: Mean dry root weight of the AGM group in the diseased soil sub-set. Error bars are displayed as SEM, n=25.

4.2.3.4 Evidence of nematodes on tree roots

A chi square test for association was conducted between treatment and the count of nematode cysts. All expected cell frequencies were greater than five. There was a statistically significant association within the data set, $\chi 2(15, N=400)$, 31.481, $p = 0.008$. All treatments demonstrated a higher nematode cyst count than the control trees in diseased soil. Trees in sterile soil showed no evidence of nematode cysts. In relation to the bioassay nematode cyst presence was not correlated with trees growth variables; root dry weight ($r_s = 0.013$, $p = 0.793$), stem dry weight ($r_s = 0.014$ (n = 400), $p = 0.408$) and leaf dry weight ($r_s = -0.091$ (n = 400), $p = 0.07$). As a result nematode cyst presence was deemed to be of little importance to tree growth and was not linked to complex formation.

4.2.3.5 Fruit yield (g)

The first harvest of fruit was available in 2014. The PaM group yields were similar. However, in the AGM group the mean number of fruit per tree harvest from *T. patula* treated trees was less than those treated with *A. sativum* AS (by 73%) and *B. juncea* AS (by 49%). *P. americana* application were associated with 66% and 41% less fruit than those treated with *A. sativum* AS and *B. juncea* AS, respectively. There was a trend of *A. sativum* AS treated trees producing 42% more fruit than the disease control (H(5) = 18.543, *p*=0.002; Figure 4.12).

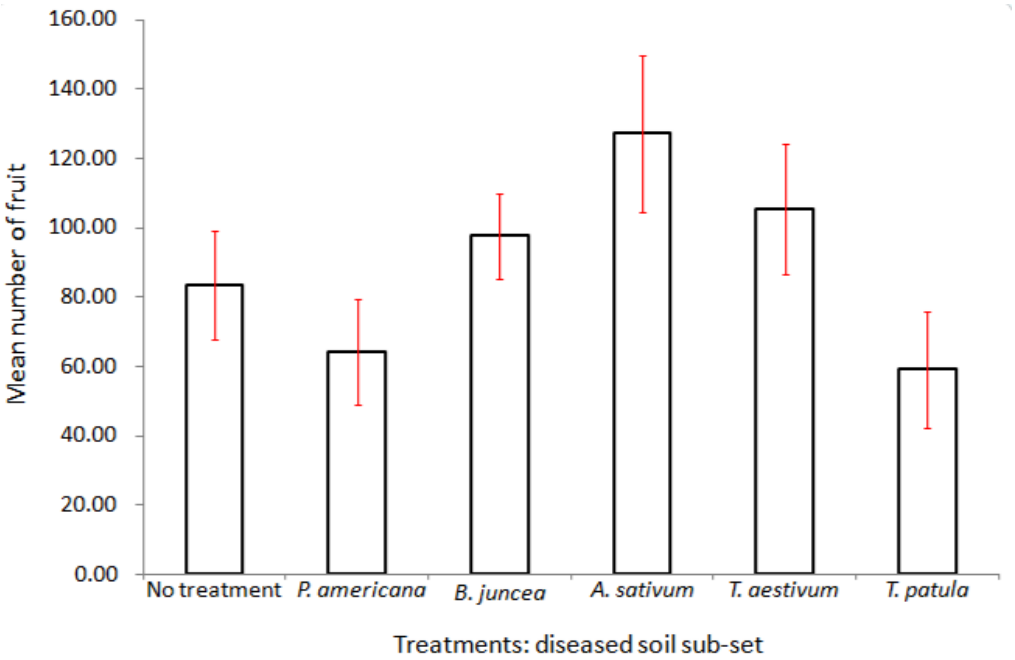


Figure 4.12: Mean number of fruit in relation to the treatment in the AGM group. Error bars are displayed as SEM, n=25.

Mean fresh harvest weight (g) was similar across the treatments in the diseased soil sub-set, with the exception of *A. sativum* AS treated trees which produced a 67% and 66% heavier harvest as compared to those treated with *T. patula* AS and *P. americana* AS, respectively (*H*(5) = 18.871, *p*=0.002).

Irrespective of treatment (diseased soil sub-set), the mean numbers of fruit that failed to develop and the mean fruit weight (per berry) were not statistically different from each other. Fruit variables were no different across the treatment groups in the sterile soil sub-set.

4.3 Overview of findings from the bioassay and destructive analysis

In this study trees were grown in sterile soil to mirror the affects of sterilisation as a treatment for RD soils (Section 1.7.4.4) and to provide an indication of whether growth of trees after green manure application is a result of additional nutrients. It was found that treatments applied to trees did not have an influence on tree growth (such as height, secondary growth, foliage, and fruit production) throughout the three years. There was also no significant difference in root and stem biomass in the sterile sub-set.

All trees used in this study displayed an increase in growth over the three year period although this rate of growth and production decreased in the final year. This may have been due to unseasonal weather and containerisation. The majority of root systems had not undergone girdling and so a deficiency in nutrients is the likely cause (table 2.4).

The use of green manures appears to produce variable results, which is especially true in terms of *P. americana* where improvement in growth parameters was not correlated with an increase in the concentration of the green manure application. This was evidenced by the fact that the primary growth and internodal distances of trees receiving a *P. americana* 8.33g/l AS treatment were stunted in comparison to those receiving the lowest and highest concentrations of the novel green manure, and had a smaller leaf area than those of the control group. Trees treated with *P. americana* 8.33g/l AS did however have a greater girth than those treated with *P. americana* 1.67g/l AS and the control group and destructive analysis indicated that stem biomass was heavier than the control group. In terms of roots, destructive analysis demonstrated that all three concentrations *P. americana* were associated with comparably greater biomass.

The green manures *T. patula* and *P. americana* 15.00g/l were linked to improved growth of trees expected of nursery trees whilst *B. juncea* and *A. sativum* caused stunting. Overall those treated with *T. patula* AS and *P. americana* 15.00g/l AS increased in height significantly. In addition the roots of these trees were more vigorous and biomass was heavier in relation to the control. Growth of trees in *T. aestivum* AS treated soil was significantly more vigorous than trees treated with the other green manures but no different to the disease control due to variability.

Some treatments inhibited growth of trees. Although *A. sativum* AS treatments were linked to the highest initial (first three months) primary growth (157%) in the AGM diseased soil sub-set, which was on par with that of the *P. americana* 15.00g/l AS treatment (161%), it was found that this green manure was associated with stunted growth during the rest of the bioassay. Both *A. sativum* AS and *B. juncea* AS treated trees had small girths and experienced slower secondary growth in comparison to trees receiving other treatments, and also had a significantly lower count of active buds and smaller leaves. The roots of the *A. sativum* AS treated trees were heavier than those of the control on average however

were variable in their vigour. Roots of the *B. juncea* AS treated trees were not vigorous and their weight was statistically similar to the control. *A. sativum* AS treated trees produced the highest quantity and harvest weight of fruit in comparison to the control and *T. patula* AS and *P. americana* 8.33g/ AS treated trees. *B. juncea* AS treated trees also produced a heavier crop of fruit which was in line with that of the diseased trees.

With regards to nematodes, this study did not look at the influence of green manures on the presence of nematodes, but rather their ability to infest the tree roots in the presence of the plant matter. Sterile soils did not demonstrate evidence of nematode presence due to the sterilisation process. There was also no point of ingress due to the trees being grown in a closed pot system. Diseased soils however, would have had a naturally occurring population of nematodes to begin with and it was found that green manure amendment was associated with elevated nematode cyst counts.

Chapter 5 – Infestation by pathogenic organisms

5.1 Introduction

The aim of this study was to determine the pathogen complex in association with the rhizosphere of *S. aucuparia* whips after the addition of green manure. There is very little known about the effects of RD on *S. aucuparia* but one of the few studies by O'Neill (2011) determined that this species is particularly sensitive to soil collected from the vicinity of a *S. aucuparia* stand (three years old) and could be used as an indicator of susceptibility. A complex of pathogens *Cylindrocarpon-Fusarium-Pythium* was detected by a DNA Multiscan and associated with the stand (O'Neill, 2011). *C. destructans* and *F. oxysporum* were specifically identified and have been found previously on *P. persica* and *Malus* spp. (Section 1.4.2.2). The species of *Pythium* was not identified although had accounted for the principle oomycete species found to be associated with RD (Section 1.4.2.3).

Previous studies determined that an entire complex is required to incite the effects of the disease (Section 1.6.2). O'Neill (2011) demonstrated that a treatment of 280 ml/m^3 98% Chloropicrin injected to a depth of 200mm effectively increased the root and shoot growth and bud graft success of *S. aucuparia* under replant conditions. *Pythium* species were eliminated from the soil, whilst infestation levels of *C. destructans*, *F. oxysporum* and beneficial fungi *Trichoderma* populations remained stable. The removal of *Pythium* spp. from the complex alleviated the symptoms of the disease. The consequences of changes in microbial communities are complex with the ecological interactions between micro-organisms requiring further attention. *Trichoderma* spp. are classified as plant growth promoting fungi (PGPF) and are known to control replant associated *F. oxysporum* and *R. solani* in crops (El Komy *et al.*, 2015; Ali and Taha, 2016); their role in RD is not clear.

The association of this fungal species with RD is inconsistent. Utkhede *et al.* (1992) demonstrated that *Trichoderma* spp. and *B. subtilis* were linked to lower dry root weights of *Malus* spp. seedlings when inoculated seperately. However when these microorganisms were added to the soil together they increased tree heights. The link between *Trichoderma* spp. and reduction in root weight whilst tree heights increase is not logical. *Trichoderma* spp. are known to suppress pathogens by excreting lytic enzymes and antibiotics and therefore it is expected that this genus may reduce root rot, which would in turn lead to healthier and more vigorous roots. Due to these properties, *Trichoderma* spp are now included in commercial formulations for biopesticides and biofertilisers. *T. asperellum* suppress *Phytophthora megakarya*, *F. oxysporum*, *F. lycopersici*, *R. solani*, and nematode *M. javanica* (Yang *et al.*, 2012). Nematodes are known to cause significant crop losses and often facilitate fungal and bacterial root penetration as seen in cherries and peaches (Wang *et al.*, 2007; Hooks *et al.*, 2010). Nematodes were associated with apple RD by Mai and Abawi (1981), with *P. penetrans* (root lesion) being considered as an important factor affecting the growth and yield of apples trees.

5.1.1 Aim and objectives

Aim:

To determine the difference in rhizosphere microorganisms present in the soils in the diseased soil sub-set and sterile soil sub-set in relation to the treatment applied.

Objectives:

- Define the pathogen complex on *S. aucuparia* through analysis of DNA sequencing results from diseased soil.
- Determine the potential long-term effect of *P. americana* and other green manures on the presence of pathogens.
- Examine the presence of pathogens present in the sterile soil sub-set.

5.2 DNA Multiscan pathogen infestation results (Section 2.5)

5.2.1 Infestation of rhizosphere soil by fungi and oomycetes

Of the 65 microorganisms included in the DNA Multiscan array, four genera and five species that are usually considered plant pathogens were detected, along with one beneficial species (Appendix VI). Pathogenic species included *F. solani*, *P. sylvaticum*, *R. solani*, *R. fragariae*, and *Alternaria* spp. Beneficial micro-organisms included an undefined species of *Trichoderma* and specifically *T. asperellum*.

Sterile soil was colonised by *Fusarium* spp. which could not be defined at species level. Diseased soil displayed the presence of a *Fusarium-Pythium-Rhizoctonia* pathogen complex including defined species *P. sylvaticum* and *R. solani*, and an undefined *Fusarium* species. Further analysis determined an association between *Pythium* and *Rhizoctonia* as follows. Principle Component Analysis of all samples demonstrated that 29% of the data is explained by the presence of *Pythium* spp., *P. sylvaticum*, *Trichoderma* spp., *T. asperellum*, and *R. solani* (Figure 5.1). 19% of the data is explained by *Alternaria* spp., *Fusarium* spp., *F. solani*, and *R. solani*. The first group is associated with the species usually ascribed to a replant pathogen complex; whilst the latter is closer to a situation where RD associated pathogens are not present. *Fusarium* spp. occurs both in the absence and the presence of the complex. Nematode cyst counts have no association with the fungi and oomycetes.

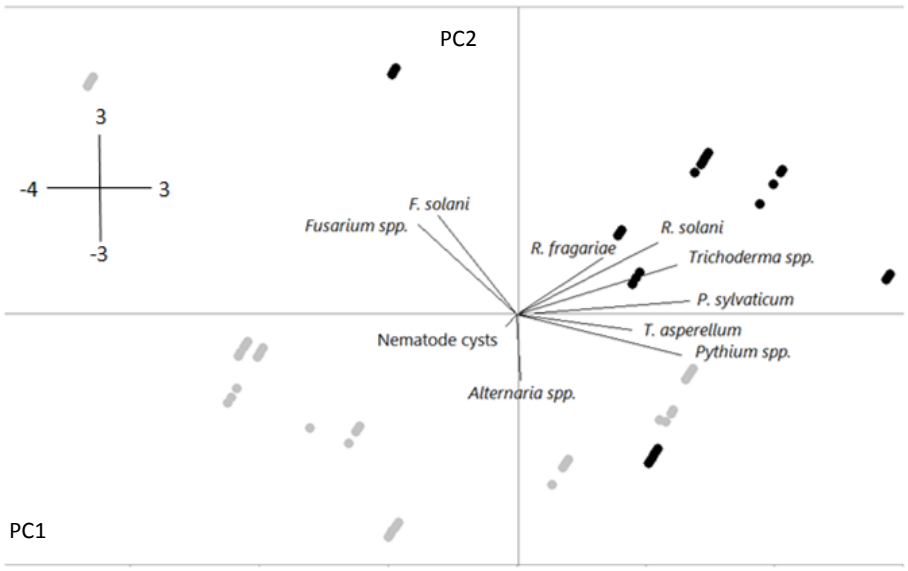


Figure 5.1: PCA-ordination biplot indicating the relationship of DNA signal strength of pathogens and counts of nematode cysts on plant roots in relation to the presence and absence of fungi and oomycete pathogens ascribed to the RD complex.

Note: Eigenvectors are represented by lines and demonstrate the strength of association and the correlation between the different variables (black: diseased samples; grey: sterile samples). The PCA arrows show the weightings of the variables in the first two principal components.

5.2.2 Centroid hierarchical cluster of microbes detected by DNA Multiscan

Hierarchical centroid linkage and agglomeration schedules using squared Euclidean distance identified discrete clusters of infestation in relation to the treatment of soil with amendments (as compared to controls). A dendrogram of the results defined four groups of treatments in relation to the presence of microbes in the soil (Figure 5.2). Group one was composed of diseased soil and soil previously treated with *T. aestivum*, *A. sativum* and *T. patula* and *P. americana* at 8.33g/l. Infestation of microbes in soil treated with *P. americana* at 1.67g/l, *P. americana* at 15.00g/l and *B. juncea* 8.33g/l were grouped as distinct units.

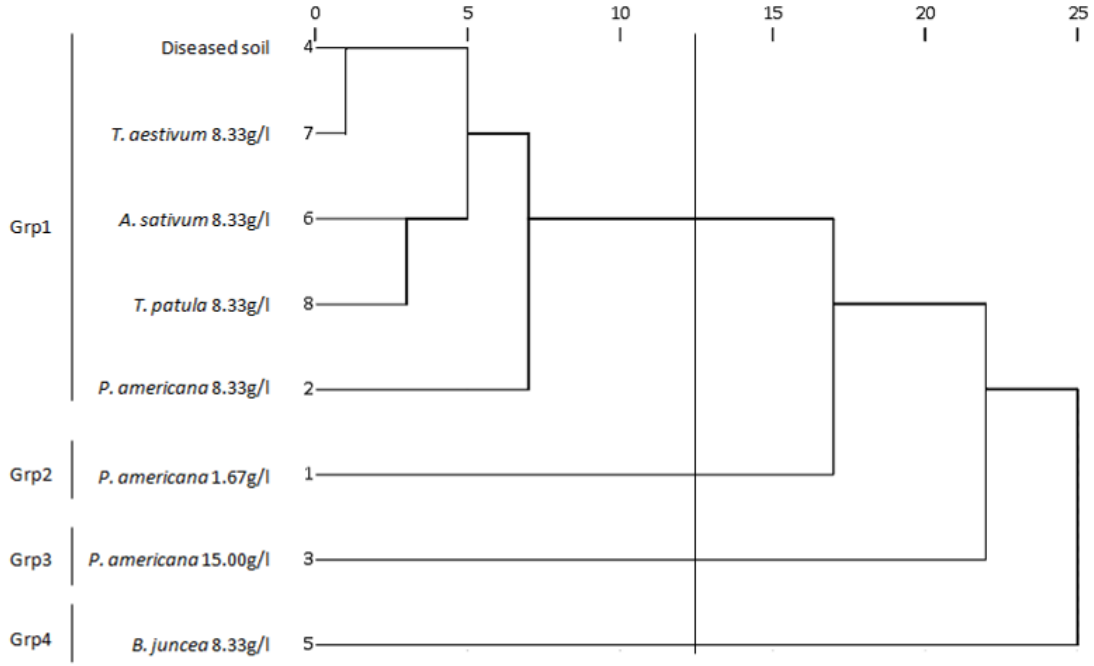


Figure 5.2: Pattern of microbial infestation levels in amended and control soils.

In relation to the sterile soil sub-set analysis identified five clusters. Treated soil that were most similar in infestation rates to the sterile control were *P. americana* at 1.67g/l and *B. juncea* at 8.33g/l. *T. aestivum* and *A. sativum* at 8.33g/l shared similarities, whilst infestation of microbes in soil treated with *P. americana* at 8.33g/l, *P. americana* at 15.00g/l and *T. patula* 8.33g/l were distinctly different to each other and the two other described clusters. Specific differences are defined below in the following sections.

5.2.3 Infestation in relation to the PaM treatment group

PaM treatment groups in the diseased soil sub-set all demonstrated the presence of the pathogens detected in the diseased soil control, with the exception of *P. americana* 15.00g/l, which lacked the presence of *R. solani* (Table 5.1).

Table 5.1: The presence of various microbes and their relative infestation rates of the PaM group (diseased soil).

Treatment: **Diseased soil sub-set**	Usually considered a plant pathogen							Beneficial	
	Fusarium spp.	*F. solani*	*Pythium* spp.	*P. sylvaticum*	*R. solani*	*R. fragariae*	*Alternaria* spp.	*Trichoderma* spp.	*T. asperellum*
No treatment (control)	**2**	**0**	**1**	**1**	**3**	**0**	**0**	**2**	**0**
Autoclaved (control)	0	0	0	0	0	0	0	0	0
1.67g/l *P. americana*	2	2	2	2	2	0	0	3	3
8.33g/l *P. americana*	2	2	2	2	3	2	0	1	0
15.00g/l *P. americana*	3	1	3	3	0	0	0	1	0

Key and notes: ■ Elimination of 'pathogen' ■ Reduction of 'pathogen'/ increase in *Trichoderma* ■ Increase or colonisation of 'pathogen' or reduction in *Trichoderma* ☐ No change. Numbers represent scale of infestation (an increase in number represents going from a state of no microbes detected (0) to an increase in population (1-3). Weak signal (rIOD ≤ 2) l Moderate signal (rIOD > 2 to ≤ 15)).

<u>*P. americana* 1.67g/l treated soil DNA:</u> Infestation by *F. solani* and *T. asperellum*, with undefined *Fusarium* and *Trichoderma* species being eliminated. Infestation rates of *R. solani* were reduced and *P. sylvaticum* rates increased.

<u>*P. americana* 8.33g/l treated soil DNA:</u> reduced infestation rates of *P. sylvaticum* and a persistence of *R. solani* at the same rate as the disease control. Infestation by *R. fragariae* and *F. solani*. Reduced *Trichoderma* spp signal and the *Fusarium* spp. eliminated.

<u>*P. americana* 15.00g/l treated soil DNA:</u> Increased rates of *Fusarium* spp. and infestation by *F. solani*. *P. sylvaticum* displayed a slight increase in infestation rate. *Trichoderma* spp. maintained a presence. *R. solani* was eliminated from the soil.

PaM treatment groups in the sterile soil sub-set demonstrated infestation by microorganisms subsequent to flame sterilisation. The presence of green manure resulted in the colonisation of more microorganisms in comparison to the no treatment control (Table 5.2).

Table 5.2: The presence of various microbes and their relative infestation rates of the PaM group (sterile soil).

Treatment: **Sterile soil sub-set**	Usually considered a plant pathogen							Beneficial	
	Fusarium spp.	*F. solani*	*Pythium* spp.	*P. sylvaticum*	*Rhizoctonia solani*	*R. fragariae*	*Alternaria* spp.	*Trichoderma* spp.	*T. asperellum*
No treatment (control)	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Autoclaved (control)	0	0	0	0	0	0	0	0	0
1.67g/l *P. americana*	1	0	2	2	0	0	0	0	0
8.33g/l *P. americana*	2	0	2	1	0	0	0	2	2
15.00g/l *P. americana*	6	6	1	1	1	0	0	2	0

Key and notes: ■ Elimination of 'pathogen' ■ Reduction of 'pathogen'/ increase in *Trichoderma* ■ Increase or colonisation of 'pathogen' or reduction in *Trichoderma* ☐ No change. Numbers represent scale of infestation (an increase in number represents going from a state of no microbes detected (0) to an increase in population (1-6). Weak signal (rIOD ≤ 2) l Moderate signal (rIOD > 2 to ≤ 15)).

Sterile soil amended with *P. americana* 1.67g/l was associated with colonisation by *Fusarium* spp. at the same rate as the sterile control. Colonisation by *P. sylvaticum* were at the same rates as found in diseased soil treated with 1.67g/l *P. americana*. Amendments of 8.33g/l of *P. americana* demonstrated increased rates of *Fusarium* spp. in comparison to the control, and colonisation by *Pythium* spp., *P. sylvaticum* and *T. asperellum*. The highest concentration of *P. americana* displayed an increased rate of *Fusarium* spp. from low to high. *F. solani* infested the soil at high rates. *P. sylvaticum* and *R. solani*, and beneficial *Trichoderma* spp. colonised the soil at low rates.

5.2.4 Infestation in relation to the AGM treatment group

AGM treatment groups in the diseased soil sub-set all demonstrated the presence of pathogens detected in the diseased soil control, with exception to *B. juncea* which lacked the presence of *Fusarium* spp., *Pythium* spp., *P. sylvaticum*, and *R. solani* (Table 5.3).

Table 5.3: The presence of various microbes and their relative infestation rates of the AGM group (diseased soil).

	Usually considered a plant pathogen							Beneficial	
Treatment: **Diseased soil sub-set**	*Fusarium* spp.	*F. solani*	*Pythium* spp.	*P. sylvaticum*	*R. solani*	*R. fragariae*	*Alternaria* spp.	*Trichoderma* spp.	*T. asperellum*
No treatment (control)	**2**	**0**	**1**	**1**	**3**	**0**	**0**	**2**	**0**
Autoclaved (control)	0	0	0	0	0	0	0	0	0
8.33g/l *P. americana*	2	2	2	2	3	2	0	1	0
8.33g/l *B. juncea*	2	2	0	0	0	0	0	0	0
8.33g/l *A. sativum*	2	1	2	1	1	0	1	2	1
8.33g/l *T. aestivum*	3	0	1	1	3	2	0	2	0
8.33g/l *T. patula*	2	2	1	1	2	0	0	3	0

Key and notes: ■ Elimination of 'pathogen' ■ Reduction of 'pathogen'/ increase in *Trichoderma* ■ Increase or colonisation of 'pathogen' or reduction in *Trichoderma* □ No change. Numbers represent scale of infestation (an increase in number represents going from a state of no microbes detected (0) to an increase in population (1-3). Weak signal (rIOD ≤ 2) l Moderate signal (rIOD > 2 to ≤ 15)).

<u>*B. juncea* treated soil DNA:</u> In addition to the elimination of pathogens described above (Table 5.3), *B. juncea* was associated with colonisation by low levels of *F. solani*.

<u>*A. sativum* treated soil DNA:</u> Lower rates of infestation by *R. solani.* Colonisation by low levels of *F. solani*, an undefined *Pythium* spp., and *Alternaria* spp. Persistence of beneficial species *Trichoderma* and colonisation by *T. asperellum*.

<u>*T. aestivum* treated soil DNA:</u> Presence of the same pathogens and beneficial microbes and at the same infestation rates as the diseased control, with exception to slightly elevated levels of *Fusarium* spp. Colonisation of the soil by *R. fragariae* as seen in *P. americana* 8.33g/l treated soil.

<u>*T. patula* treated soil DNA:</u> *F. solani* colonised soil at a low rate. *P. sylvaticum*, *R. solani*, and *Trichoderma* spp. were persistent in the soil.

AGM treatment groups in the sterile soil sub-set demonstrated infestation by microorganisms subsequent to flame sterilisation. The presence of green manure resulted in the colonisation of more microorganisms in comparison to the no treatment control (Table 5.4).

Table 5.4: The presence of various microbes and their relative infestation rates of the AGM group (sterile soil).

	Usually considered a plant pathogen							Beneficial	
Treatment: **Sterile soil sub-set**	*Fusarium* spp.	*F. solani*	*Pythium* spp.	*P. sylvaticum*	*Rhizoctonia solani*	*R. fragariae*	*Alternaria* spp.	*Trichoderma* spp.	*T. asperellum*
No treatment (control)	**1**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
Autoclaved (control)	0	0	0	0	0	0	0	0	0
8.33g/l *P. americana*	2	0	2	1	0	0	0	2	2
8.33g/l *B. juncea*	1	1	1	0	0	0	0	0	0
8.33g/l *A. sativum*	3	0	2	0	0	0	1	0	0
8.33g/l *T. aestivum*	4	0	0	0	0	0	0	0	0
8.33g/l *T. patula*	7	7	0	0	0	0	0	0	0

Key and notes: ■ Elimination of 'pathogen' ■ Reduction of 'pathogen'/ increase in *Trichoderma* ■ Increase or colonisation of 'pathogen' or reduction in *Trichoderma* ☐ No change. Numbers represent scale of infestation (an increase in number represents going from a state of no microbes detected (0) to an increase in population (1-7). Weak signal (rIOD ≤ 2) l Moderate signal (rIOD > 2 to ≤ 15)).

B. juncea treated soil was colonised by *Fusarium* spp., *F. solani*, and *Pythium* spp. *A. sativum* amendments displayed the persistence of *Fusarium* spp. and the appearance of an undefined *Pythium* spp. and *Alternaria* spp. These DNA results were much like those for diseased soil treated with *A. sativum*. *T. aestivum* was associated with the colonisation of *Fusarium* spp. and much like the sterile control no other pathogens. *T. patula* amended soil was infested by *Fusarium* spp. and *F. solani* at high rates.

5.3 Key findings from detection of microbes in association with untreated and treated soil

This study determined that the common denominators across all treatments in the diseased soil sub-set point towards the presence of a *Fusarium-Pythium-Rhizoctonia* pathogen complex in the presence of beneficial *Trichoderma* species. The presence of *P. sylvaticum* was confirmed, as previously found in Apple RD soils (Tewoldemedhin *et al.*, 2011a), and which O'Neill (2011) expected to find but did not detect. Examination of infestation tables and centroid cluster analysis grouped the treatments in the

diseased soil sub-set according to their similarity. One group that had similar colonisation patterns contained [diseased soil - *T. aestivum* treated soil - *A. sativum* treated soil - *T. patula* treated soil - *P. americana* treated soil 8.33g/l]. These soils all had the same pathogens and *Trichoderma* species. *T. aestivum* and *P. americana* treated soils were both infested with *R. fragariae,* and *T. aestivum* treated soil lacked *F. solani*. This may demonstrate that neither *R. fragariae* nor *F. solani* play a significant role in the presentation of the disease. Further analysis demonstrated that *Fusarium* did not demonstrate a strong association with *Pythium* and *Rhizoctonia* species, which were closely linked in a PCA. It was also clear that the presence of *Trichoderma* species in this instance did not have a bearing on the health of the disease. *A. sativum* treated soils were infested with all pathogens found in the diseased soil in addition to *F. solani* and *Alternaria* species, the latter was also found in sterile soils. *T. asperellum* was also present and *R. solani* numbers were reduced in this particular soil. Soils treated with *P. americana* 1.67g/l, *P. americana* 15.00g/l and *B. juncea* were distinctly different to the group, and to each other [diseased soil - *T. aestivum* treated soil - *A. sativum* treated soil - *T. patula* treated soil - *P. americana* treated soil 8.33g/l] group and to each other. There was an absence of *R. solani* in both *P. americana* 15.00g/l and *B. juncea* treated soils . *B. juncea* treated soils were similar to sterile soil, in that the diversity of microbes detected was minimal and the former only contained a low abundance of unidentified *Fusarium* species and *F. solani* (out of the 65 microorganisms included in the DNA array).

In terms of application of green manure at different concentrations, the results demonstrated that regardless of concentration, *P. americana* did not have a consistent effect on soil microbes. The green manure increases microbial activity, although it is difficult to deduce how the addition of different concentrations of green manure influenced soil interactions and the subsequently altered the microbial community. It appears that soils treated with 8.33g/l stimulated the microbial activity of deleterious species whilst the 15.00g/l stimulated some pathogens whilst supressing *R. solani*. In a sterile environment soil treated with the two lowest concentrations of *P. americana* were infested with undefined *Fusarium* and *Pythium*, and specifically *P. sylvaticum*. *Trichoderma* species were absent from the 1.67g/l treated soil however they were present in the 8.33g/l and 15.00g/l treated soil. The concentration of *P. sylvaticum* may have played a role in this where infestation rates were higher in the 1.67g/l treated soil. The presence or absence of *Trichoderma* species did not influence tree growth rates. *R. solani* was present in 15.00g/l treated soil but did not effect tree growth which may be due to the suppression of this pathogen, which was potentially due to the high infestation rates of *Fusarium* species and *F. solani* specifically (Section 1.7.4.3). Likewise soils treated with other green manures were composed of varying levels of *Fusarium* and *Pythium* species but lacked *R. solani*.

Chapter 6 – Discussion

The primary aim of this study was to evaluate the effectiveness of green manures as control measure for RD through conducting *in vitro*, *in vivo* and DNA sampling.

6.1 *In-vitro* studies

In vitro studies demonstrated that the novel green manure, *P. americana,* was effective in significantly inhibiting growth of *R. solani* and *P. ultimum* at three concentrations (1.67g/l, 8.33g/l, 15.00g/l) when applied either as plant matter or as a cold water extract, whilst an ethanol extract was found to be ineffective. The application of other conventional green manures as leaf matter all proved effective at slowing the growth of the pathogens with the exception of *T. patula*. The cold water extract of *A. sativum* and *T. aestivium* inhibited *P. ultimum* only. *R. solani* was more sensitive to treatments than *P. ultimum,* which was effected by a wider range of treatment methods.

6.1.1 Activity of *P. americana* against *R. solani* and *P. ultimum*

The difference in response to the *P. americana* treatment may relate to phylogenetic differences between *R. solani* and *P. ultimum*. Hernández *et al.* (2013) demonstrated that *in vitro* applications of aqueous and alcohol extracts of the closely related species *Phytolacca tetramera* inhibited the radial growth of fungi *Colletotrichium gloesporiodes*. They proposed that the effect was due to the presence of saponins; indeed *P. americana* contains phytolaccasaponins (triterpenoid saponins) which are antimicrobial (Section 1.7.5). *P. americana* saponins are isolated from the roots, berries and leaves (Wang *et al*., 2008; Takahashi *et al.*, 2001; Di Maro *et al.*, 2007), and are known to protect the plant from fungi (Osbourn, 1996a, 1996b), nematodes (Argentieri *et al.*, 2008) and bacteria (Fons *et al.*, 2004; Edwards, 2006). Saponin hydrolysis product phytolaccagenin (sapogenin) demonstrated strong antifungal activity (Di Liberto *et al*., 2010). Structurally, saponins are diverse, with an aglycone moiety for the phospholipids that sit within the membrane of cells as an insoluble complex. Saponins therefore form pores in the cell membrane, compromising its integrity. The response of pathogens differs according to their ability to degrade or detoxify this glycoside. Oomycetes produce saponinases, glycosyl hydrolases and saponin hydrolases, which mediate the hydrolysis of saponins (Larroque *et al.*, 2012; Gang, 2010; Osbourn *et al.*, 1996b). Unlike fungi, oomycetes are also known to lack sterol groups in the hyphal membrane (Osbourn *et al.*, 1996a; Olsen, 1971; Arneson and Durbin, 1968). This is a problem for plants producing saponins as a preformed defence, as the mechanism of these metabolites rely on the ability to disrupt the membrane structure by binding to a sterol group (Morrissey and Osbourn, 1999). The lack of sterol groups in combination with the ability to degrade metabolites could therefore potentially explain the lessened effect of *P. americana* on *P. ultimum* growth. In addition to saponins *P.*

americana contains other phytochemicals. Flavanol diglycoside, kaempferol-3-O-ß-D-apiofuranosyl-(1,2)-ß-D-glucopyranoside, was extracted from the leaves by Bae *et al.* (1997) and was shown to inhibit fungi *Botrytis cinerea*, *Botryosphaeria dothidea* and *Colletotrichum gloeosporiodes*. *P. americana* also contains chitinases which are known for their antifungal activity (Taira, 2010). In 1995a, Ohta *et al.* purified chitinases PLC-A and PLC-B from leaves of *P. americana*. Chitinases, which are up-regulated in plants in times of stress, are responsible for the breakdown of chitin, a constituent of fungal cell walls (Collinge *et al*., 1993). There are a range of properties contained within the tissue of *P. americana* that have the ability to suppress fungi and oomycetes. They may act independently or in unison to create the effect seen here in this study.

6.1.2 Activity of *P. americana* in relation to extraction and application methods

6.1.2.1 *P. americana*: the effect of an ethanol extract on pathogen growth

The ethanol based extract demonstrated no antimicrobial activity against *P. ultimum* and *R. solani*. This finding is contrary to research which states that alcoholic solvents effectively extract phytochemicals from plants which are successfully used to inhibit pathogen growth (Farias Magalhaes *et al.*, 2003; Sung Og *et al.*, 2007; Pineda *et al.*, 2010; Hernández *et al.*, 2013; Gberikon *et al.*, 2015; Onaran and Sağlam, 2016). Phytochemicals may have been lost during the preparation process or the provision of the extract on a filter disc may not have been sufficient to halt the growth rate of the pathogens. Extraction method, concentration of extract and application method may need to be altered to demonstrate a beneficial effect. Quiroga *et al.* (2001) and Okigbo and Mmeka (2008) recommended an extract made from 120mg/ml resulting in the use of an ethanolic extract with a final concentration of 20mg/ml in this study. However, Onaran and Sağlam (2016) used higher concentrations ranging 50mg/ml to 400mg/ml and demonstrated that all extracts had the ability to inhibit fungi. Bajpai *et al.* (2012) and Onaran and Sağlam (2016) raised the possibility that active ingredients in *P. americana* could be incorporated into agrochemicals when they demonstrated activity against rice blast, tomato gray mould, tomato late blight, and pepper anthracnose, of which the lowest concentration of methanol extract (750mg/l) was most effective against tomato late blight. Furthermore a methanol extract (3000mg/l) of *P. americana* suppressed radial growth of *Botrytis cinerea* (85%), *Magnaporthe oryzae* (63%), *R. solani* (27%), *Puccinia recondita* (27%), *Phytophthora infestans* (82.1%) and *Colletotrichum capsici* (20%) (Bajpai *et al*., 2012). This study (Chapter 3) demonstrated that the *P. americana* at 20,000mg/l was ineffective against the oomycete and fungus which is contrary to findings demonstrated by Bajpai *et al*. (2012). Ngo *et al.* (2017) demonstrated that solvents varied in their ability to extract bioactive compounds and antioxidants, methanol was more effective at extracting saponins whilst ethanol (50%) was more effective at extracting phenolics and flavonoids, thus pointing to the involvement of saponins in the inhibition of pathogens.

6.1.2.2 *P. americana*: fresh leaf matter in comparison to the distillate

When comparing the fresh leaf matter to the distillate it was found that the leaf discs were more effective than the distillate in inhibiting both *R. solani* (38-64% and 36-57%, respectively) and *P. ultimum* (66-69% and 43-45%, respectively). Plant matter in agar however had a lower inhibitory ability than leaf discs and distillate for *R. solani* (26-36%) and *P. ultimum* (43-45%). The inhibition of the pathogens indicates that the distillate still contained an antimicrobial component despite the process of heating, however the lower magnitude of pathogen inhibition may be a result of this component being limited in its activity or due to the elimination of a secondary component of the raw plant material by thermal degradation. Ohta *et al.* (1995a) determined that *P. americana* contains two chitinases; PLC-A and PLC-B, which have the ability to remain active at 40°C, but gradually become inactive at higher temperatures, and are completely denatured by temperatures over 70°C. It is possible to suggest that a secondary component lost in this manner is chitinase; however, this would need to be investigated. The lower inhibitory activity demonstrated by the plant matter in the agar may be indicative that the pathogen needs to be in direct contact with the plant matter.

6.1.2.3 *P. americana*: the effect of a liquid extract on pathogen growth

The agar infused with the aqueous extract of *P. americana* exhibited comparable inhibitory activity against *R. solani* (15-34%) and *P. ultimum* (25-25%). Usually cold water liquid extracts are less effective than solvent extracts. Indeed, the activity of water extracts against pathogens is due to the water solubility of the majority of classes of phytochemicals (Doughari, 2012). Cold water extracts of *P. americana* were more effective than other treatments at controlling the pathogens. Cold water extracts were no different in inhibitory power to fresh leaf material which indicates that phytochemicals with the ability to suppress pathogen growth are water soluble and are released at quantities sufficient enough to have an effect. The antimicrobial activity never completely halted the growth of the pathogens; instead they continued growing regardless of the presence of the aqueous extract. This is likely to be down to the concentration of available phytochemicals in solution and potentially a result of their degradation. For example, Karunamoorthi *et al.* (2008) determined that saponins extracted from *P. dodecandra* were stable for two days and decreased rapidly after day three ($t_{1/2}$ = 15.8 h). By day 10 the constituent was completely biodegraded.

6.1.2.4 *P. americana*: an indirect effect on pathogen growth

In addition to plant material having a direct effect it was also demonstrated that suppression can be induced indirectly with pathogens being exposed to volatiles in a closed environment. *R. solani* was found to be most sensitive to this method with percentage inhibition reaching 90% and remaining at this level for the duration of the assay. This halting effect on growth was evidently temporary as growth

resumed after replating. Meanwhile *P. ultimum* continued to grow despite inhibition of 46%. Charron and Sams (1998) demonstrated that the growth of these pathogens were 27.4% of the control when treated with *B. juncea* Indian mustard. Out of the six *Brassica* species they tested, it was *B. campestris* (Chinese cabbage) that had the highest inhibitory effect (79.6%) against *R. solani*. This study (Chapter 3) demonstrates that *P. americana* (90%) has a higher magnitude of inhibitory activity towards the fungus than the species used by Charron and Sams (1998). In terms of activity against the oomycete *B. campestris* outperformed *P. americana* by inhibiting *P. ultimum* growth (96.3%), whilst *B. juncea* had no effect on the oomycete (0%).

6.1.2.5 Activity of *P. americana* at different concentrations

Application of *P. americana* in concentrations comparable to that of the field study demonstrated that the two highest concentrations (8.33g/l and 15.00g/l) had the same inhibitory affect on the two pathogens *R solani* and *P. ultimum* (26-39% and 40-49%). The lowest concentration of *P. americana* inhibited *R. solani* and *P. ultimum* slightly (1-17% and 13-16%). The inhibitory activity of *P. americana* against *R. solani* did not increase when the plant material was applied at concentrations higher than those used in the field (20.00g/l, 25.00g/l, and 30.00g/l), which was also observed by Hernández *et al.* (2013) who conducted an *in-vitro* investigation into the effect of aqueous and alcohol extracts of *Phytolacca tetramera*. It was concluded that three extracts (15%, 20% and 30%) had the same level of inhibitory activity against fungi *Colletotrichium gloesporiodes*. In comparison, an increase in the concentration of *P. americana* resulted in a 14% increase in the inhibitory activity against *P. ultimum*. This indicates again that *P. ultimum* metabolises phytochemicals in a different manner to the fungi. It could also indicate, as proposed by Hernández *et al.* (2013), that there is more that one antimicrobial compound involved.

6.1.2.6 Activity of *P. americana* in relation to comparative green manure species

8.33g/l of macerated *P.* americana, *B. juncea*, *A. sativum*, and *T. aestivum* plant matter exhibited inhibitory activity against both *R. solani* and *P. ultimum*. The activity of green manure against pathogen growth is a result of their phytochemical constituents (Section 1.7.5). *P. americana* and *A. sativum* were effective immediately; however, some were delayed in their influence. *B. juncea* and *T. aestivum* activity was delayed by 48 hours, which had been noted previously by Charron and Sams (1998) in their study of *B. juncea* volatiles. There is no explaination of why the activity of *T. aestivum* may be delayed other than it relates to the degradation time taken to release antimicrobial properties. The degradation of crop residues is difficult in a field setting in the presence of soil microbes as extra nutrients have to be added to help initiate the breakdown (Rezig *et al.*, 2014). *T. patula* activity was also delayed, however inhibited the oomycete nonetheless by hour 72.

In terms of the cold water liquid extract the suppression of *R. solani* and *P. ultimum* by *P. americana* was significantly greater than that induced by *A. sativum, B. juncea, T. patula,* and *T. aestivum* throughout the assay. In this study *P. americana* was effective as both a solid and a liquid extract. It is known that *P. americana* has toxic phytochemicals that are readily soluble in water and it often requires many rounds of boiling to make it edible. In their study, Ashafa *et al.* (2010), identified that leaf material of *P. dioca* contains water-soluble tannins, alkaloids, glycosides, phenolics, flavonoids, saponins and lectins, all of which have antimicrobial properties (Dang and Van Damme, 2015).

6.1.2.7 Activity of comparative green manure species against pathogens

Macerated *B. juncea* was effective against both pathogens used in the study; however the liquid extract of the plant matter was ineffective. Brassicaceous species have been studied widely for their biofumigant nature (Larkin and Griffin, 2007). Brassicaceae produce glucosinolates (β-D-thioglucosides) which can be classified as aliphatic, aromatic, or indole forms that occur in all parts of the plant and degrade via enzymatic hydrolysis. Tissue damage results in the reaction of glucosinolates with myrosinase to yield nitriles, epithionitriles, thiocyanates and isothiocyanates. Brassicaceous plant matter is normally incorporated directly into the soil whilst both soil moisture and temperature is high to maximise the amount of isothiocyanates that come into contact with the rhizosphere (Gimsing and Kirkegaard, 2009). Macerated *B. juncea* tissue was found to be an effective allelopathic soil amendment, inhibiting 73% of growth in *F. oxysporum* and 100% of growth in *R. solani*, *Phytophthora erythroseptica* and *P. ultimum* (Larkin and Griffin, 2007). It is therefore unsurprising that the macerated *B. juncea* was effective in inhibiting the two pathogens (*R. solani* was inhibited by 42%). In terms of the liquid extract the environmental fate of glucosinolates and their by-products tend to be relatively unstable. Hydrolysis plays a big part in the breakdown of these phytochemicals and so their effects are lost (Kawakishi at Namiki, 1969; Ohta *et al.*, 1995b). The liquid extract of this green manure stimulated pathogen growth (in comparison to the control); this is discussed in Section 6.1.2.8.

Macerated green manure of *A. sativum* was comparable to *P. americana* in its ability to suppress *R. solani*, however the effects on *P. ultimum* were reduced. The green manure was more effective than the cold press liquid which only inhibited *P. ultimum* after 96 hours. It is unknown why there was this delay in inhibition, although it is possible that the mode of action of the active properties is not instantaneous due to their chemical structures. In 1944 Cavallito and Bailey identified a sulphur compound, allicin (diallythiosulphinate), as one of the constituents responsible for giving *A. sativum* its antimicrobial properties. Cavallito and Bailey (1944) defined that allicin is formed within seconds when a clove is damaged or crushed and alliin (S-allyl-l-cysteine sulfoxide) comes into contact with alliinase. Allicin is not only found in the bulb but also found at high concentrations in shoot and leaf extracts at 0.44 ± 0.00 and 0.26 ± 0.1 mg/ml, respectively. Allicin itself reacts with all proteins in the vicinity that have thiol groups, undergoing thiol-disulphide exchange. This action of allicin on thiol groups is widespread and multi-

modal, with many proteins, including enzymes in micro-organisms, being targeted. Allicin (10µg/ml) is also known to inhibit cysteine proteinases, alcohol dehydrogenases and thioredoxin reductases, involved in maintaining the correct redox state within an amoeba parasite of humans (Ankri and Mirelman, 1999). Allicin is potently fungistatic (growth inhibiting) and fungicidal (Gruhlke *et al.*, 2010; Harris *et al.*, 2006; Ankri and Mirelman, 1999). Most activity listed is through the action of allicin, however some of it is attributed to, the allicin condensation product, ajoene. Although *A. sativum* has not specifically been used to treat RD, it has been successfully utilised to overcome *Phytophthora infestans* and downy mildew of (*Cucumis sativus*) cucumber caused by *Pseudoperonospora cubensis*. In terms of *P. infestans* and *P. cubensis* it was found that allicin reduced the germination and growth of sporangia and cysts in *Solanum lycopersicum* (tomato) leaves and *C. sativus* seedlings respectively, with the treatment effectiveness being comparable to commercial copper fungicide (Portz, 2008). When allicin does form its composition is so volatile with a short halflife that it soon degrades to form vinyldithiins and ajoene (Schulz *et al.*, 1998; Ankri and Mirelman, 1999; Gruhlke *et al.*, 2010). In aqueous environments allicin reacts via a monomolecular self elimination reaction or a bimolecular reaction (Block 1986) to produce diallyl trisulphide or diallyl disulphide and allyl alcohol. Lawson and Wang (1994) reported that pure allicin dissolved in water at 23^{o}C has a half life of between 30 and 40 days. Timonin and Thexton (1950) reported that extremely dilute aqueous extracts of garlic inhibited growth of various soil fungi, whilst Shen (1996) determined that diallyl disulphide and diallyl trisulphide were effective against *Cryptoneoformis*. Likewise Abad *et al.* (2007) prepared aqueous extracts of *A. sativum* which inhibited fungal growth and deemed a potential source of antifungal drugs. It was found that a garlic juice preparation can either be applied as a spray to leaves or as a soil drench (the former gaining better results than the latter). These allicin preparations have also been efficacious against other plant diseases (Portz, 2008). The lack of activity against pathogens in this study may be down to the use of leaf matter rather than the cloves which is the norm in most studies. It would have been beneficial if *A. sativum* leaves could have been used as a potential green manure as these are a by-product of field produced garlic bulbs.

Macerated plant matter from *T. aestivum* was more effective than the cold liquid extract. This is contrary to wheatgrass juice which were found to contain saponins, tannins, flavanoids and alkaloids (Ashok, 2011; Sharma, 2016) and extracts made with water, hexane and acetone had varying inhibitory power against Gram positive and Gram negative bacteria, and fungi (Sundarensan *et al.*, 2015). In this study the aqueous extract inhibited pathogen growth after 96 hours. Suriyavathana (2016) characterised the phytochemicals of this plant species and found that an aqueous extract can yield a similar range of compounds as methanol extraction, whilst extraction by ethylacetate and chloroform were less effective. Aqueous extraction appeared to liberate alkaloids, tannins, phenols, saponins, and glycosides which are all known to have antimicrobial activity (Dang and Van Damme, 2015). Likewise acetone and ethanol extracts yielding flavonoids were found to be antimicrobial (Michael *et al.*, 1998). Very little research has considered the use of *T. aestivum* as a green manure. Mazzola *et al.* (2002) discovered *T.*

aestivum to be a useful in terms of Apple RD, however the mode of action was found to be through the promotion of *Pseudomonas putida* populations (strain 2C8) which suppressed anatagonistically by the populations of *Pseudomonas flourescens* bv. III and *R. solani* AG-5 in the soil, whilst reducing root infestation of *R. solani*, *P. ultimum* and *P. penetrans* in *Malus* trees. It is possible that phytochemicals from *T. aestivum* root exudates and tissue have two modes of action where they stimulate beneficial microbes (in accordance with Mazzola *et al.*, 2002) and also directly inhibit pathogen growth as per this study. Commercially it is possible that this particular that this green manure could be cultivated *in-situ* and the residues of plant matter rotovated into the soil. Before this is considered seriously results for soil applications would need to be analysed. The use of wheat as a biofumigant has limited data as thus influence on pathogen growth was unknown. As such it is interesting that the macerated form of *T. aestivum* had an effect.

T. patula was ineffective against the fungus *R. solani*, however displayed an inhibitory effect against *P. ultimum* after 72 hours. Rodino *et al.* (2015) determined that 1% ethanol extracts of *T. patula* flowers inhibited *P. ultimum* growth by 56%. It suggested that chemical constituents; alkaloids, phenolics, flavonoids, benzofurans, carotenoids, saponins and thiophenes were responsible for the antimicrobial activity (Al-Musayeib *et al.*, 2014; Gupta and Vasudeva, 2012; Wang *et al.*, 2007; Marotti *et al.*, 2010; Margl *et al.*, 2001; Lawson *et al.*, 2007; Rodino *et al.*, 2015). Their effectiveness against *P. ultimum* after 24 hours is most likely a result of the extraction method used, the plant parts used, and the slow release of properties during degradation. Picking up on the point that *T. patula* was found to be ineffective against the *R. solani*, this is contrary to other findings (potentially because plant material was used rather than an extract). *T. patula* stimulated pathogen growth (in relation to the control); this is discussed in Section 6.1.2.8.

6.1.2.8 Stimulation of pathogen growth by *B. juncea* and *T. patula*

During the cold water liquid extract assay, lasting 168 hours, it was determined that *B. juncea* stimulated *R. solani* growth temporarily between 48 hours and 72 hours. Meanwhile, *T. patula* stimulated *P. ultimum* growth between 24 hours and 96 hours. This method is relevant however may not be indicative of what may happen in a soil where a petri dish with one pathogen colony does not reflect an environment with millions of microbes which interact with one another. Additionally, cold press liquid extracts contain properties other than antimicrobials, including free amino acids, macronutrients, trace elements and hormones for example. Kirkegaard *et al.* (1996) found that the suppression of *R. solani*, *P. irregulare* and other soilborne pathogens by *Brassica* tissues directly relate to isothiocyanate type and concentration. They stated that *R. solani* was particularly susceptible to antimicrobials whilst *P. irregulare* was less affected. This is in direct contradiction to the results here where *B. juncea* stimulated fungal growth, and may indicate that the process of creating a liquid water extract was ineffective at maintaining isothiocyanates; indeed their precursor glucosinolate is hydrophilic but isothiocyanates are

only slightly soluble in water and often require the presence of myrosinase to form (Tianxin *et al.*, 2012; Brown *et al.*, 2014; National Center for Biotechnology Information, 2017). Meanwhile there may have been a component preserved during extraction that promoted fungal growth temporarily (after 48 hours after plating stimulating growth for 24 hours). In addition to this it is known that *B. juncea* residues have the ability to support *R. solani* and thus explains the growth of the pathogen *in vitro* (Yulianti *et al.*, 2006). *T. patula* also stimulated pathogen growth which may have been influenced by the properties of the aqueous solution. The solubility of phenolics such as flavonoids in water is poor where organic solvents, such as ethanol, methanol and acetone are more effective in the extraction process (Kim and Lee, 2001; Kumar and Pandey, 2013). Likewise this was found for quercetin (Malwade *et al.*, 2013). This may explain why *T. patula* was ineffective in controlling pathogen growth, whilst plant residues acted as a nutrient source.

6.1.3 Limitation of *in-vitro* studies

Inhibition studies were chosen as a standard technique in quantifying effects on growth. There were difficulties in undertaking sporulation studies and a risk of contaminating RNA studies external to this study. The aim of this study was to ascertain the ability of the green manures to suppress growth and given the circumstances growth inhibition studies were justified. Further investigations in the future should quantify the influence of active ingredients on sporulation and thus reproductive viability, in addition to growth.

6.2 Three year bioassay

The three year bioassay demonstrated that trees grown in sterile soil all put on growth at statistically similar rates regardless of treatment. The application of *P. americana* to the diseased soil subset produced variable results in terms of primary and secondary growth, however all three concentrations were linked to a heavier biomass. This study demonstrated that *T. patula* and *P. americana* 15.00g/l improved primary and secondary growth of trees when compared with nursery trees whilst *B. juncea* and *A. sativum* caused stunting. *T. aestivium* treated trees were variable in growth.

6.2.1 Growth of trees in sterile soil

In this study, trees were grown in sterile soil to mirror the effects of sterilisation as a treatment for RD soils (Section 1.7.4.4). It was found that treatments applied to trees did not have an influence on tree growth throughout the three years. It could be argued that this is indicative that the addition of green manure does not have a nutrient effect; however it could also reflect the state of the soil and its inability to provide the resources that a plant needs. This primarily relates to a change in soil chemistry and a decrease in soil microbes responsible for making nutrients available. According to Williams-Linera and

Ewel (1984), steam sterilisation can reduce growth of certain species whilst being correlated to higher rates of growth in others. They alluded to the idea that the plants that grew well in these conditions did not require mycorrhizae, thus supporting the notion that it is loss in the balance of microbes that were responsible for reduced growth.

6.2.2 Vigorous growth of *S. aucuparia*

All trees used in this study displayed an increase in growth over the three year period, although this rate of growth and production slowed down in the final year. This may have been due to unseasonal weather and/or containerisation. The majority of root systems had not undergone girdling, and so a deficiency in nutrients is the likely cause (Table 2.4).

The results in this study suggest that the green manures *T. patula* and *P. americana* 15.00g/l improved growth of trees expected of nursery trees. Overall those treated with *T. patula* and *P. americana* 15.00g/l increased in height significantly. In addition the roots of these trees were more vigorous and biomass was heavier in relation to the control. There was no significant difference in root and stem biomass in the sterile sub-set. Stimulation of tree growth can be attributed to a fertiliser, biostimulant or pesticidal effect. Poor tree growth can be due to an increase in pathogens, a lack of beneficial microbes and an increase in abiotic factors. All trees slowed in growth, and chlorophyll content was reduced, most likely due to the limitations of the pots that they were grown in (Mathers, 2007). In general, all trees increased in height initially that was potentially a result of transplantation and the addition of organic matter (Table 2.2). All green manures contained the nutrients required for plant growth (macro and micro nutrients). *T. aestivum* contained the only tissue that was found to be lacking in terms of calcium and boron. The release and solubilisation of these nutrients and their movement into the plant would only be something of importance had the results demonstrated a nutrient related impact on growth. There is no evidence from the sterile sub-set to suggest a nutrient affect and although this has been ruled out, it is worth considering that green manures can also provide other useful resources that aid plant growth. For example *T. aestivum* contains vitamins (A, C, E, B12) which are metabolically important to a plant (Ashok *et al.*, 2011; Suriyavathana, 2016). Improved growth may have been a result of the stimulation of microbes that are beneficial. Yulianti *et al.* (2006) discovered that the addition of organic matter did not cause a decrease in the inoculum of pathogens although overall microbial activity increased. It is possible that some green manure has a positive effect on the control of RD rather than a nutrient affect.

When a tree is able to grow above ground and below ground structures it is more likely to be resilient. A vigorous root structure gives a tree stable anchorage in the soil and the ability to absorb the required nutrients to maintain metabolic processes and develop structurally. A strong root system gives trees resilience to biomechanical forces and stresses that are imposed on it in the nursery and also where

ever it is translocated to in its new environment. Transplant survival will be dependent on roots which are often lost during the process of moving a tree to another area (Mathers *et al.*, 2007). Economically speaking the loss of trees is a cost that should be minimised. The trees here that were treated with *T. patula* and *P. americana* 15.00g/l had consistently vigorous roots and a vigorous above ground growth. Primary and secondary growth is just important as root growth. A tree that lays down secondary wood and grows in a manner that will ensure it can respond to environmental and biological pressures will have a better survival rate. Meanwhile upward growth combined with substantial roots will reduce time until it is saleable, ensure that the tree can compete for light and provide more opportunity for a larger canopy which will increase photosynthetic activity and the production of sugars.

6.2.3 Variability in growth of trees after amendment with green manure

The use of green manures is linked to variable results, which is especially true in terms of *P. americana* where improvement in growth parameters was not correlated with an increase in the concentration of the green manure application. This was evidenced by the fact that the primary growth and internodal distances of trees receiving a *P. americana* 8.33g/l treatment were stunted in comparison to those receiving the lowest and highest concentrations of the novel green manure, and had a smaller leaf area than those of the control group. Trees treated with *P. americana* 8.33g/l did however have a greater girth than those treated with *P. americana* 1.67g/l and the control group and destructive analysis indicated that stem biomass was heavier than the control group. In terms of roots destructive analysis demonstrated that all three concentrations of *P. americana* were associated with comparatively greater biomass than the control group. This variability in growth may be directly related to the addition of plant matter (organic matter, phytochemicals, nutrients, amino acids, hormones) and the changes in the microbial community. The complexity of soil interations are not yet fully understood. With this study alone it appears that there is no logical explanation as to why *P. americana* 8.33g/l treated trees put resources into secondary growth whilst the trees treated with *P. americana* 15.00g/l allocated resources to primary growth, especially since both had a healthy root system and biomass. It is possible that the addition of *P. americana* 8.33g/l encouraged stressful conditions in the soil and this caused a physiological difference. Indeed Davies and Zhang (1991) suggested that roots are able to send chemical messages that alter the metabolism and physiology of the tree systematically in stressful conditions. The stunting of tree growth by *P. americana* 8.33g/l is a key finding that warrants further investigation due to grower interest in growth inhibitors.

Growth of trees in *T. aestivum* treated soil was highly variable to the point that they were statistically different to other treated trees but no different to the disease control, for which there is no explanation. It is known however, that allelochemicals present in *T. aestivum* has previously reduced the growth of other crops and some grass species (Ma, 2005). The impact on trees is unknown and potentially not transferable due to the physiological differences between monocotyledons and

dicotyledons. Other crops have been incorporated into soil and been beneficial by controlling disease, for example, corn residues can lower the severity of *Verticillium* wilt symptoms (Wiggins and Kinkel, 2005). Although *T. aestivum* demonstrated inhibitory action against *R. solani* and *P. ultimum* it may be that the incorporation of below and above ground residues is required to have an effect in the field.

The inability of a green manure to make an impact on tree growth is not new, in fact composts and organic amendments have not previously been found to control RD (Granatstein and Mazzola, 2001). In terms of RD the trees that were treated with *A. sativum* and *B. juncea* matched with these physiological symptoms, and to a lesser extent so did *P. americana* 8.33g/l. It was noted that *A. sativum* treated trees were linked with stunted growth, as were those treated with *B. juncea*. Both sets of trees had small girths, a lower count of active buds and smaller leaves, and a lack of root vigour. Alongside these results it was noted that both produced a heavier crop of fruit than any other treatment group of trees. The production of a large quantity and a heavy crop of fruit and thus the creation of progeny to continue the species is an evolved mechanism which occurs in response to stress. Stress can be anything that threatens the survival of an organism such as drought or the attack of pests and disease. According to Shelford's law of tolerance biotic and abiotic factors can directly impact on survival rates when individuals in a population move out of the optimal zone and reach the zones of stress and of intolerance. Berman and DeJong (1996) documented an increase in crop load of *P. persica* in water stressed conditions. It was found that a heavier crop load with a low crop weight denoted stress due to limited resources being available for the synthesis of fruit. It can be concluded that *B. juncea* trees were more stressed than those treated with *A. sativum* as the latter demonstrated a heavier fruit weight. It is fair to assume that the production of a heavy crop of fruit is not indicative of stress but rather an indication that other tree groups expended energy on growth (secondary, primary, and leaf production) rather than the production of flowers and therefore fruit. Cross pollination may have been a factor also however this is difficult to substantiate.

6.2.4 Variability in the growth of trees treated with green manure

It was found that the growth of trees in response to the addition of green manure was variable. This variability may have been caused by numerous factors that could not have been controlled. The green manure may not have been consistent throughout in terms of the concentration of components. There is also the fact that the trees were containerised. The use of containers can expose trees to extremes in temperatures and moisture (even though the latter was regulated as much as possible). Changes in temperature and moisture content between pots may have influenced the rate at which the green manure decomposed. The use of containers would have altered the root function and morphology (Mathers *et al.*, 2007). Containerisation was a necessary limitation due to the need to create single tree replicates and provide intact root systems for destructive analysis.

6.2.5 Limitations of the three year bioassay

The three year bioassay was based on a one time application of green manures at the start of the study. Given the constraints on resources it was impossible to trial multiple application alongside single time application; however may be a consideration for future study. Further investigation of root architecture may also be a consideration. Lengths of primary, secondary and teritiary roots, and number of root tips could be quantified to further demonstrate the influence of green manure amendments on growth.

6.3 Microbial study

The microbial study determined the presence of a *Fusarium-Pythium-Rhizoctonia* pathogen complex in the presence of beneficial *Trichoderma* species. Pathogen colonisation was similar for diseased soils and those treated with *T. aestivum*, *A. sativum*, *T. patula* and *P. americana* 8.33g/l. Detection of nematodes, *R. fragariae* and *Alternaria* spp. was noted and disregarded as being pivotal in the incitement of RD and likewise *Trichoderma* spp. could not be correlated with a treatment. *P. americana* 15.00g/l and *B. juncea* treated soils lacked *R. solani* and the latter was similar in composition to the sterile soil. *P. americana* 8.33g/l stimulated the microbial activity of deleterious species whilst the 15.00g/l stimulated some pathogens whilst suppressing *R. solani*.

6.3.1 Pathogens associated with *S. aucuparia*

The association of pathogens with *S. aucuparia* has not been widely researched. Otto *et al.* (1994) found evidence of actinomycetes with the level of infection and damage to the root epidermis and cortex being comparable with that of *Malus* species and higher than that of *Pyrus* species. The only other study to define the pathogens affecting *S. aucuparia* was completed by O'Neill (2011) where the complex of pathogens in untreated soil consisted of *Fusarium* spp., *Fusarium oxysporum*, *Pythium* spp., and *Cylindrocarpon destructans*. This study in contrast determined that the common denominators across all treatments in the diseased soil sub-set point towards the presence of a *Fusarium-Pythium-Rhizoctonia* pathogen complex in the presence of beneficial *Trichoderma* species. This study even indicated the presence of *P. sylvaticum*, previously found in Apple RD soils (Tewoldemedhin *et al.*, 2011a), which O'Neill (2011) anticipated to find but did not detect in their DNA Multiscan results. The difference between the pathogens found in this study and that of O'Neill (2011) indicates that RD is not specific and that there can be overlap of causal agents between rosaceous species in addition to variation within species. This has not been found before for this particular rosaceous species due to limited research, however this has been demonstrated in other species (section 1.5.2).

The interaction within the pathogen complex found in this study is unknown at present and warrants further attention. RD may be the result of just one pathogen, or all three acting in unison where they are

virulent only when acting in synergy (Lamichhane and Venturi, 2015). All three pathogens are causal agents of damping off and are ubiquitous in forest nursery soils (Sneh, 1996) and therefore would not be out of place in a woodland setting. It is likely that these pathogens are linked to RD due to their mechanism of attack on the roots and the persistence of the disease in the soil for over fifteen years. These pathogens can act in unison or independently as demonstrated in a study of bean root rot, by Pieczarka and Abawi (1978), in which *F. solani* elicited severe hypocotyl decay with slight stunting and no root rot, whilst *P. ultimum* species caused root necrosis, limited hypocotyl decay and a loss in dry weight. In combination these two pathogens caused a 74% reduction in dry weight and a greater degree of root rot and stunting than what was observed in plants infected by the individual pathogens. *R. solani* alone restricted hypocotyl and caused root lesions. Pieczarka and Abawi (1978) found that there was no interaction between *R. solani* and *F. solani*, however *R. solani* demonstrated an antagonistic relationship towards, and thus reduced the effects of, *P. ultimum*. Although *Fusarium* has been shown to interact with *Pythium* with deleterious results there is uncertainty regarding the involvement of *Fusarium* species with RD (O'Neill, 2011). In this study *Fusarium* did not demonstrate a strong association with *Pythium* and *Rhizoctonia* species which were closely linked in a PCA. Many studies have implicated *Pythium* and *Rhizoctonia* in the development of the disease and so it is likely that they are acting in unison or with another undetected causal agent (Caruso *et al.*, 1989; Mazzola, 1998; Mazzola *et al.*, 2002; Mazzola and Mancini, 2012).

6.3.2 Long term effect of *P. americana* on the presence of pathogens

Long term suppression of pathogens by green manure is possible through the reduction of deleterious microbes and the promotion of beneficial organisms that can naturally control pathogens. This is something that requires further investigation; however the results demonstrated that *P. americana* can be linked to different populations of microorganism. All three concentrations of *P. americana* were different with regards to the microbes that were present, however all had elevated levels of pathogens where present and reduced rates of the undefined *Trichoderma* species. Soils treated with the lowest concentration of *P. americana* contained all pathogens, with *R. solani* at a reduced rate and also the introduction of the beneficial microbe *T. asperellum*. Soils treated with 8.33g/l were associated with all pathogens and an undefined *Trichoderma*. Meanwhile soils receiving the highest concentration of the green manure had a similar composition of microbes except there was no *Rhizoctonia* species present. These results demonstrate that, regardless of concentration, the addition of *P. americana* does not have a consistent affect on soil microbes. The green manure increases microbial activity although it is difficult to deduce the interactions that have taken place in order to result in the communities present after treatment with different concentrations. It appears that soils treated with 8.33g/l have stimulated the microbial activity of deleterious species whilst the highest concentration has stimulated some pathogens whilst suppressing *R. solani*. It has been found previously that green manures with

antimicrobial properties have the ability to increase the population of pathogens; however this is normally temporary until other microbes increase in numbers (Lazzeri and Mancini, 2001).

6.3.3 Long term effect of comparison green manures on the presence of pathogens

Examination of infestation tables and centroid cluster analysis grouped the treatments in the diseased soil sub-set according to their similarity. One group that had similar colonisation patterns contained [diseased soil - *T. aestivum* treated soil - *A. sativum* treated soil - *T. patula* treated soil - *P. americana* treated soil 8.33g/l]. These soils all had the same pathogens and *Trichoderma* species. *T. aestivum* and *P. americana* treated soils were both infested with *R. fragariae* and *T. aestivum* treated soil lacked in *F. solani*. This may demonstrate that neither *R. fragariae* nor *F. solani* play a significant role in the presentation of the disease. Considering this information with the PCA it is also clear that the presence of *Trichoderma* species in this instance does not have a bearing on the health of the disease. *A. sativum* treated soils were infested with all pathogens found in the diseased soil in addition to *F. solani* and *Alternaria* species. The latter was also found in sterile soils. *T. asperellum* is also present and *R. solani* numbers were reduced in this particular soil.

Soils treated with *P. americana* 1.67g/l, *P. americana* 15.00g/l, and *B. juncea* were distinctly different to this group and the each other. Both P. americana 15.00g/l and *B. juncea* treated soils shared the commonality of the absence of *R. solani.* In fact, *B. juncea* treated soils were similar to sterile soil in that diversity of microbes detected was minimal and the former only contained a low abundance unidentified *Fusarium* species and *F. solani* (out of the 65 micro-organisms included in the DNA array). This finding is in line with Oliver *et al.* (1999) who demonstrated that brassicaceous plants can inhibit *Pythium* spp., *Fusarium* spp. and *V. dahliae*. Out of all the green manure treated soils it was the one associated with *B. juncea* that demonstrated the lowest infestation and lowest diversity whilst all other green manure treated soils were variable in their microbial constituents. Brassicaceous species contain glucosinolates which have previously been shown to amend pathogenic and saprophytic micro-organism communities (Cohen *et al.*, 2005; Ascencion *et al.*, 2015; Mazzola *et al.*, 2015; Zou *et al.*, 2015). Yulianti *et al.* (2006) demonstrated that brassicaceous plant matter can inhibit the growth of soil borne fungi. It could be hypothesised that the activity of this green manure amendment has been indiscriminatory and removed beneficial organisms other than *Trichoderma*.

In a sterile environment soil treated with the two lowest concentrations of *P. americana* were infested with undefined *Fusarium* and *Pythium*, and specifically *P. sylvaticum*. *Trichoderma* species were missing from the 1.67g/l treated soil however they were present in the 8.33g/l and 15.00g/l treated soil. The concentration of *P. sylvaticum* may have played a role in this where infestation rates were higher in the 1.67g/l treated soil. The presence or absence of *Trichoderma* species did not influence tree growth rates. Interestingly *R. solani* was present in 15.00g/l treated soil but did not affect tree growth which

may be due to the suppression of this pathogen, which was potentially due to the high infestation rates of *Fusarium* species and *F. solani* specifically (Section 1.7.4.3). Likewise soils treated with other green manures were composed of varying levels of *Fusarium* and *Pythium* species but essentially in all there was a lack of *R. solani*. The infestation of sterile soil with microbes that are normally associated with being pathogenic may be due to the fact that in isolation these microorganisms are acting as faculatative saprotrophs in the presence of organic matter (green manure amendment).

6.3.4 Green manure amendments and nematode presence

This study did not look at the influence of green manures on the presence of nematodes but rather their ability to infest the tree roots in the presence of the plant matter. Sterile soils did not demonstrate evidence of nematode presence due to the sterilisation process and the difficulty of entering a closed pot system when there is no point of access into the water of the soil pores. Diseased soils however would have had a population of nematodes to begin with and it was found that green manure amendment was associated with elevated nematode cyst counts. Regardless of this there was no association between tree growth and the presence of these organisms. Jaffee *et al.* (1982) determined that nematode removal has no link to reduced disease expression. This may be due to the stimulation of plant resistance to nematodes. This has been observed before on *Solanum tuberosum* where data analysis of potato cyst nematodes population increases showed that there was no loss of the vigour, growth and yield as a result of the infestation (A. Barker, personal communication, February 2017). Barker (personal communication, March 2017) suggested that the crop may have up-regulated its defences against the parasite. Indeed Kim *et al.* (2005) noted that phenolic compounds increased seedling biomass of *Cassia mimosoides* (artillery plant) and potentially change root tip physiology allowing the vacuoles to store protective compounds.

6.3.5 Limitations of molecular methods

The use of molecular methods allows the detection of these pathogens to either a genus or species level, whilst eliminating the problem of contamination by fast growing saprophytic fungi and bacteria and the expense of antibiotics encountered with *in vitro* isolation studies. Sampling DNA using molecular techniques does not come without its pitfalls and care must be taken in evaluating the results. There is the potential of misclassifying pathogens due to similar DNA sequences in their genomes. It is also possible to find species that could be pathogenic or non-pathogenic (Lievens and Thomma, 2005). This is exemplified by the many AG groups of *R. solani* which can have specific hosts (Section 3.1). In line with O'Neill (2011) samples were collected using methods that accounted for variability and the occurrence of 'hot-spots' within the soil Balci *et al.* (2013) and were a robust method of detection for samples that have multiple population targets (Lievens *et al.*, 2007; Lievens *et al.*, 2010). The microarray method has been used previously to quantify legume yield depression syndrome (Fuchs *et al.*, 2014); evaluate

chlorine disinfestation (Cayanan *et al.*, 2009); detect *Pythium* spp. (Tambong *et al.*, 2006); and detect fungi and oomycetes in Solanaceae species (Zhang *et al.*, 2008).

6.4 Links between the three studies and conclusion

The overall outcome of the studies is displayed (Table 6.1); in relation to the diseased soil sub set and grouped in accordance with the dendrogram (Figure 4.2). As anticipated there is no clear connection between treatment and the effects on growth and composition of microbes; however some links can be inferred. DNA Multiscan results indicated that the long term changes in the soil microbial composition could be ascribed to four groupings of green manure (Section 5.2.2). Trees that displayed variable growth were associated with a varied inoculum of pathogens. As previously stated the trees that demonstrated significant growth were those that were treated with *P. americana* 15.00g/l and *T. patula*, whilst those receiving applications of *B. juncea* and *A. sativum* were stunted. Those receiving all other treatments were variable in growth.

Table 6.1: Overall results demonstrated by each study.

Treatment *(Grouped in accordance with DNA Multiscan)*	*Inhibition study* *Chapter 3*	*Bioassay study* *Chapter 4*	*Microbial study* *Chapter 5*
Diseased			**F, P, PS, RS, T**
T. aestivum	Moderate inhibition solid	Variable	F, P, PS, RS, T, **+ RF**
A. sativum	Moderate inhibition solid	Stunted, stressed	F, **P**, PS, **RS**, **T**, **+ FS**, **A**, **TA**
T. patula	Slight inhibition/stimulant	Vigorous	F, P, PS, **RS**, **T**, **+ FS**
P. americana 8.33g/l	Good inhibition liquid/solid	Variable	F, **P**, **PS**, RS, **T**, **+ FS**, **RF**,
P. americana 1.67g/l	Slight inhibition	Variable	F, **P**, **PS**, **RS**, **T**, **+ FS**, **TA**
P. americana 15.00g/l	Good inhibition liquid/solid	Vigorous	**F**, **P**, **PS**, **T** **+ FS**
B. juncea	Moderate inhibition/stimulant	Stunted, stressed	F **+ FS**

Inhibition study key: moderate: where inhibition is significantly different to the control but not to other treatments; slight: inhibition is temporary; stimulant: growth of pathogen is more than the control; good: inhibition of pathogens is significantly more than other treatments. Bioassay study key: variable: variability around the mean is high enough that range of growth is not significantly different to the control or other treatments; stunted, stressed: tree growth is significantly less than other tree groups; vigorous: tree growth is statistically more than the majority of other tree growth. Microbial study key: Green: decrease in pathogen inocula and an increase in beneficial microbes. Red: increase in or the introduction of pathogen inocula and a decrease in beneficial microbes. F = *Fusarium*, P = *Pythium*, PS = *P. sylvaticum*, RS = *R. solani*, T = *Trichoderma*. Introductions: RF = *R. fragariae*, TA = *T. asperellum*, FS = *F. solani*, A = *Alternaria* species.

Table 6.1 demonstrates that 1. *In-vitro* results do not necessarily relate to what may happen in 'real world' circumstances; 2. Plant growth can vary in different situations and do not necessarily correlate with a prescribed complex of pathogens; 3. Addition of green manure, specifically *P. americana*, at different concentrations may not share consistent results in terms of short term inhibition of pathogens and long term microbial composition; 4. Improved tree growth may be related to green manure soil amendments but the mode by which they act requires further investigation.

The amendment of soil with organic plant matter is known to improve the overall health of a soil, both biologically and physically. Organic matter stimulates population growth of microbes which can in turn create an environment which is suppressive to disease through the stimulation of plant defence mechanisms, in soil competition for space and resources, and antagonistic behaviour towards pathogens (Garbeva *et al.*, 2004; Mazzola, 2007; Larkin *et al.*, 2011; and Bernard *et al.*, 2012). Organic matter is also beneficial due to its by-product humic acid which chelates nutrient ions and improves the structure of the soil binding soil particles, improving water retention and stabilising pH.

It was found that two treatments were associated with improved growth; however it is unclear how this has been achieved. This lack of clarity is down to the converse difference in their activity. *T. patula* was unsuccessful *in vitro* where it induced a slight inhibition of microbes, whilst in terms of soil microbes it was associated with a reduction in *R. solani* and an increase in *F. solani* and *Trichoderma* species. It was determined earlier that *R. solani* plays a key role in the expression of the disease whilst *Trichoderma* presence does not make a vast difference. Meanwhile *P. americana* induced to a totally different response in the soil. *In vitro* this treatment successfully inhibited the two pathogens, however *in vivo* the incidence of pathogens was elevated. *P. americana* treatment was previously correlated with increased microbial activity which may explain this (Lock, 2010; unpub). The key difference between *T. patula* and *P. americana* 15.00g/l and the other treatments is that the former reduced the inoculum of *R. solani* and latter removed *R. solani* from detection, whilst soils treated with other green manures still contained this pathogen often in the presence of additional pathogens (with exception to *B. juncea* treated soils). This goes back to the principle as discussed in previous chapters that *R. solani* is required for this disease to be expressed (Section 5.4.2.1). These green manures may act differently to each other due to their properties (Table 1.7).

The lack of *R. solani* is not always related to an improvement in tree growth. *B. juncea* was associated with a loss of diversity in both pathogens and beneficial species (Sections: 4.6.4; 5.4.5). *In vitro* the plant matter either provided moderate inhibition or stimulation of pathogen growth in certain circumstances. Here the addition of this green manure was associated with colonisation by *F. solani* and the loss of other pathogens associated with diseased soil. As stated previously this loss in pathogens and beneficial microbes may be also linked to the loss of other organisms that were not measured in the DNA multiscan (such as mycorrhizae). Indeed the inoculum in the *B. juncea* treated soil was much like that of a sterilised environment that had been recolonised. The removal of diversity in the soil in this case has been detrimental to the growth of trees. Lacey (2000) found that *Streptomyces scabies* was negatively correlated with microbial diversity. *B. juncea* soils may have favoured a pathogen that was not detected by the DNA Multiscan and caused a stunting in tree growth. It is possible that the *in-vitro* activity of these green manures could not translate from the laboratory to the field. This is potentially due to the loss of active properties due to biodegradation, sorption, and leaching (Kim *et al.*, 2005). In fact Kim *et*

al. (2005) suggested that concentrations of phytochemicals would be higher *in vitro* than in the soil. It is also possible that the properties within plant tissues can have an allelopathic affect whereby plant growth is inhibited. For example Kim *et al.* (2000) demonstrated that phenolic acids from *P. americana* could inhibit 35 plant species and also nine fungal species. This is due to this particular substances ability to reduce nutrient fixation and mineral uptake, and inhibit metabolic pathways in photosynthesis and respiration. Limited tree growth was not only connected to a loss of microbial diversity but also the presence of diversity. Although *A. sativum* demonstrated an ability to suppress pathogens *in vitro* the growth of trees was stunted. Soils were associated with higher amounts of *Pythium* and the addition of *F. solani* and *Alternaria* species. The benefit of this green manure relating to the reduction of *R. solani* and the stimulation of colonisation of *T. asperellum* did not mean that tree growth was improved as a result. It is possible that the role of *R. solani* in the complex was replaced by another unknown causal agent. It appears that RD is not specific as demonstrated here and the complex detected can vary between diseased tree stands.

In addition to demonstrating that plant matter from one green manure can stimulate growth whilst another can stunt growth it was found that the concentration of green manure can have unexpected results. This was displayed by the trees that had received a treatment of *P. americana* 8.33g/l which had a smaller primary growth and larger secondary growth in relation to the lower and higher concentrations of the green manure. It is clear from the overview in Table 5.1 that each green manure concentration was in its own group as defined by centroid hierarchical clustering. Although *P. americana* 8.33g/l demonstrated inhibitory power against pathogens *in vitro* it was found that the soil associated with this treatment had the same composition of pathogens as the diseased soil in addition to the occurrence of *F. solani* and *R. fragariae*. *Trichoderma* species were reduced and the inoculum of most pathogens was elevated. P. americana 15.00g/l as stated above was not associated with the presence of *R. solani*. Trees treated with *P. americana* 8.33g/l may well have reacted to the presence and infestation rates of the pathogens. It is possible for a tree to undergo a response producing secondary growth as part of a defensive reaction. When a tree is under tangential stress it can result in an increase in the diameter of the stem due to cell division, cell elongation, formation of dilatation tissue and frequent creation of cambium (Romberger and Mikola, 1970). In the event of a vascular attack a tree can compartmentalise any decay (CODIT) and initiate a reaction in RAP cells (ray and axial parenchyma). The physiological activity and spatial arrangement of these cell types in response to pests and disease is not fully known and the differentiation of their function in defence and tree mechanics has not been defined. It is known that trees that are poor at compartmentalisation respond to wounding by laying down thicker bark and wide dilating rays (Morris *et al.*, 2016). Indeed ray and axial parenchyma actively divide producing reaction wood to overcome wounding as part of CODIT so it is not farfetched to suggest some involvement of these cells here in response to any detrimental conditions caused by the addition of *P. americana* 8.33g/l. In addition to this response, it is possible for growth inhibitors to be present in the green manure being used. Allelopathic chemicals, such as flavonoids, phenolics,

cyanogenic glycosides, and terpenoids are used in forestry to inhibit growth of some unwanted species (Fisher, 1980; Chick and Kielbaso, 1998; Li *et al*. 2010). However; this would need to be investigated further for confirmation.

6.5 Conclusion

Vigourous and structurally sound trees are more likely to survive disease events and transplantation to a new environment. Indeed, stress from transplanting trees alone results in mortality rates of 30-50% in the first growing season due to a loss of root mass of up to 98% from bare root stock, due to the sub-optimal conditions in which the tree is placed (Hirons and Percival, 2012; Schaffert and Percival, 2016). RD is well known to cause stunting and necrosis of roots and thus, suppression of the causal agents for RD is an important factor in securing the survival of trees, which are placed in an orchard, previously occupied by the same species. In adding a substance to the soil a certain amount of care needs to be taken to ensure that any changes that occur are going to be positive and consistent for the growth of amenity trees such as *S. aucuparia*. It is important to provide a solution to disease that suppresses pathogens in the long term, by providing a situation where these deleterious organisms are in balance with and controlled by beneficial organisms. Disease control is facing difficulties, with active ingredient (AI) availability fluctuating, due to new registrations approved for market use and withdrawal of key products which contain chemicals that are no longer approved. Two recent examples of this are: the withdrawal of ipriodione, which was used extensively by the sports amenity turf sector, and; and the review of gyphosate by a European board to determine if its registration should be renewed. With chemicals and biocontrol agents (currently), and biostimulants (as of 2019) being party to regulatory registration, it is important that there are other options available to the horticulture sector. An IPM approach takes into account the methods of biological control, alongside land use management, use of resistant stock, and cultural practices. It is a preferred strategy for disease control, and should be considered over and above the exclusive use of a chemical control (or even an organic control) that results in a sterilised soil environment, in which both pathogen and beneficial organism has been removed. This approach would need to be consistent in action, so as to yield healthy stock that is fit for purpose.

The use of organic amendments on a soil ecosystem is not that clear cut. Indeed, this study has highlighted that different concentrations of the same green manure can have a varied response. *In-vitro* effects on pathogens do not always relate to what is expected. *P. americana* applied at 15.00g/l had a positive impact on root and shoot growth as well as having encouraging results *in-vitro* and in relation to the long term microbial composition. However, the variability between the two lower concentrations of the green manure put into question the efficacy of this as a solution to RD. *T. patula* yielded positive root and shoot growth, although performed poorly in *in-vitro* due to stimulating pathogen growth. It is unknown whether *T. patula* would demonstrate, as seen with *P. americana*, a variable affect on tree

growth at different concentrations. Likewise, *B. juncea* action was inconsistent, as evidenced by this green manure stimulating pathogen growth *in-vitro*, despite reducing the diversity of pathogen infestation *in-vivo*. The potential stimulation of deleterious rhizobacteria and fungi and related/unrelated stunting of tree growth by green manures is someting that needs to be avoided. In a seasonal disease this stimulation of deleterious pathogens could be avoided, however in a system in which the pathogen is present throughout the year this may not be possible. Green manure is often applied when it has the most concentrated AIs (before flower set), however it is unknown if this is the optimum time of application in terms of disease progression. The concentration of properties available in the plant matter can change during the life cycle of the green manure and are not always retained subsequent to harvest, and can also vary between species of a medicinal plant. It may also be that the green manure needs to be applied in yearly applications rather than a single application due to the long term persistence of RD causal agents in the soil which build up as a result of tree root exudates (Section 1.5.2). To add complication to the matter, abiotic soil properties can play their own role; with pH, OM, texture, water availability and temperature all becoming a factor affecting the release and action of properties in the green manure. Indeed, even a tree can have an impact on microbial communities within the soil due to the release of metabolites in the form of exudates. Yulianti *et al.* (2006) demonstrated that the use of green manures as a disease break can be fairly inconsistent in practice; and is unlikely to be a practicable approach in an orchard setting. Likewise, Granatstein and Mazzola (2001) and Neilsen *et al.* (2004) found that composts or green manures are ineffective at controlling RD in *Malus*. This variability is down to the microbial communities that are extant in the soil. Populations of microbes can occur in hot spots and move towards root exudates via chemotaxis. As stated earlier plants and micro-organisms are continuously interacting with each other and the environment that they occupy. For example, this complexity even extends to the use of beneficial microbes, where *Trichoderma* spp. and *B. subtilis* were linked to lower dry root weights of *Malus* spp. seedlings when inoculated separately. However when these micro-organisms were added to the soil together they were positively associated with increased tree heights (O'Neill, 2011). In terms of abiotic factors, the production system can also have an influence on the ability of a green manure to perform. For example Mazzola (2007) found that compost was much more effective in treating containerised plants than those planted directly into the soil. But then as stated previously containerisation can present its own problems by exposing the trees to extremes in moisture and temperatures.

These interactions need to be understood more fully to tailor a specific treatment to a situation. The variability in the results obtained from using green manure as a single time application demonstrates that this is not a viable option for treating RD. The problem with using green manure, as demonstrated here, is that there is no specificity in its activity and the suppression of disease is inconsistent (Hoitink and Boehm, 1999), as too is tree growth (Section 4.6.3).

The results demonstrated here have determined that *P. americana* and *T. patula* can both impact positively upon tree growth in a replant situation and also, in relation to, *P. americana* can stunt growth.

It is therefore possible to conclude that there are AIs available that may have potential as biocontrol agents and also plant growth regulators, both of which are important in amenity horticulture and forestry. In order to positively utilise these AIs they need to be applied in a more consistent manner. This research determined that green manure does influence tree growth in a replant situation, but further investigation should be undertaken to specify which actives (one or several) suppress deleterious organisms, and even which actives act as plant growth regulators (as an interesting side line). As an alternative of using green manure it may be that soluble phytochemicals with inhibitory effects, as found in *P. americana*, are better applied as a cold press liquid (which would be organically certifiable) or as another liquid extract. It may also be possible to apply properties as pure extracts however it is likely that it will be economically active as a synthetically made product. As previously stated it has been suggested before that *P. americana* constituents could be incorporated into agrochemical formulations (Bajpai *et al.*, 2012; Onaran and Sağlam, 2016). The extraction of antimicrobial constituents from plant matter would need to be refined to produce consistent results and avoid delayed action as noted previously (Sections: 3.4.2.2; 3.4.2.6). Degradation of these compounds would also need to be investigated to define if they can be stable for long periods of time as formulated biopesticides especially when place into a soil situation where factors such as temperature, leaching, and biodegradation can affect concentration of actives (Section 3.4.2.3). The solubility of some of the active properties (Section 3.4.1) is a useful attribute when it comes to mixing them with other chemicals (Sharp, 2017; pers comms). It is recommended that the efficacy of extracts of phytochemicals as possible treatments for RD is tested thoroughly due to potential for pathogens to be stimulated (Section 6.1.2.8; Table 6.1). Likewise further investigation of a cold water extract may prove fruitful in the development of an effective biopesticide for use against RD or as a growth inhibitor where above ground growth can be limited.

If an organic route is pursued and if a product has potential but cannot be used alone it may be worth considering that it would have to be applied alongside a biopesticide, biofertiliser and biostimulant in order to give the plant the resources to grow at the same time providing the necessary constituents to either tolerate disease or up-regulate disease resistance. In addition application of a cold press liquid or green manure could be followed by inoculation of the soil with a healthy balance of micro-organisms that would act much like a probiotic for the soil.

Any solution that is found for RD will have to pass the rigours of legislation to allow it to be released to the market. In addition to this products that claim to be pesticidal in nature need to be registered under government regulations. Sometimes the movement of a product from factory to shelf can take up to ten years (R. Sharp, pers communication, April 2017) and also will require a CE mark backed by trial results by 2024 (R. Cannings - EBIC, pers communication, November 2017). This makes the creation of solution for tree diseases very labour intensive and therefore a confidence in the consistency of a product needs is required before investment even at the early stages. For these reasons, and based on current results

this study does not advocate the use of green manures made of *P. americana*, *T. patula*, *B. Juncea*, *A. sativum*, and *T. aestivum* as one time soil amendments to treat RD.

6.6 Recommendations for future research

- There should be more time dedicated to exploring the interations between the pathogen complex, *Fusarium-Pythium-Rhizoctonia*, identified by the DNA results.
- A long term study on the effects of green manure, *T. patula*, when applied at different concentrations, to demonstrate if this species demonstrates consistency. In addition, an exploration of the effect of changing application timing for green manures, *T. patula* and *P. americana*.
- The investigation of active ingredients in *P. americana* which regulates plant growth, and those that suppress pathogens *in-vivo*. Likewise, a study to identify useful biocontrol actives in *T. patula*.
- Once useful active ingredients have been determined, a study should be conducted to: define an extraction or synthesis method; identify their environmental fate; conclude if they act independently or synergistically; and find the optimum application method and timings. Bioassays should investigate root growth in addition to interactions with the rhizosphere and a consideration of changes in the soil phenolic profile, the impact on the soil community, and as a result of the rootstock being present and also any influence that an amendment may have, conducted as a metabolomic study.
- In addition, an exploration of the influence of potentially useful AIs on pathogen sporulation and growth should be conducted to define if they are fungistatic or fungicidal.
- As replant disease is not species specific, in terms of the composition of the complex of pathogens, then trials with any proposed treatments should be conducted across a number of orchards globally, to account for variation.
- Other *Phytolacca* species should be considered for their ability to positively impact on tree growth in replant conditions. Active ingredients can vary according to species.
- An appraisal of the use of active ingredients as part of an IPM approach.
- The economic consequences of any replant disease treatment should be considered to quantify the costs versus the benefits. Economic losses in the amenity sector are not considered in as much detail as the top-fruit horticulture sector. This disparity in information should be removed.
- Studies have demonstrated that the defence reactions are inadequate to mitigate biotic stresses (Weiß et al, 2017). Molecular and physiological studies into the use of active ingredients to elicit and up-regulate a defence response should be undertaken.

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Appendices

I Commission Decision 2008/753/EC

FOOD AND ENVIRONMENT PROTECTION ACT 1985
CONTROL OF PESTICIDES REGULATIONS 1986

Commission Decision 2008/753/EC – Methyl Bromide – professional products

Notice is hereby given that in exercise of the powers conferred by Regulation 5 of the Control of Pesticides Regulations 1986 (SI 1986/1510) (as amended) and of all other powers enabling them in that behalf, the Secretary of State, and the Scottish Ministers (as regards Scotland) and the Welsh Ministers and the Secretary of State (acting jointly as regards Wales) have:

(a) revoked approval for the advertisement, sale, supply, storage and use of the pesticide products listed in the Schedule.

(b) Given approval until 18 March 2009 for the advertisement, sale and supply by any persons for the pesticide products listed in the Schedule.

(c) Given approval until 18 March 2010 for the storage and use by any persons for the pesticide products listed in the Schedule.

Date of issue: (a) 17 October 2008

Dates of revocation: (b) 18 March 2009

(c) 18 March 2010

ALL OTHER CONDITIONS OF APPROVAL REMAIN UNCHANGED

SCHEDULE

Product Name	MAFF/ MAPP Number	Approval Holder	Notice of Revocation Number
Mebrom 100	M04869	Mebrom NV	2360 of 2008
Methyl Bromide 100%	M01336	Bromine & Chemicals Limited	2361 of 2008
Methyl Bromide 98%	M01335	Bromine & Chemicals Limited	2362 of 2008

Signed by: susan d ponting
Signing time: Friday, October 17 2008, 12:18:56 GMT
Location: York
Reason to sign: For the Pesticides Safety Directorate

PSD Digital Signature

II Income in 2013 in relation to the top three producing countries in the world

Table 1.2: Income (US dollars/tonne) in 2013 in relation to the top three producing countries in the world (data sourced from FAOSTAT, 2016).

Produce	*Producing country*	*Income (US dollars/tonne)*
Almond	Spain	1984.53
	Iran (Islamic Republic of)	5111.19
	United States of America	7077.00
Apple	China, mainland	621.45
	United States of America	668.00
	Italy	753.86
Cherry	Spain	2037.26
	Iran (Islamic Republic of)	2141.33
	United States of America	2877.00
Sour cherry	Ukraine	742.15
	United States of America	791.00
	Turkey	1064.12
Peach and nectarine	United States of America	680.00
	Spain	955.61
	China, mainland	1155.73
Pear	United States of America	541.00
	Spain	819.74
	China, mainland	863.57
Quince	Morocco	580.70
	China, mainland	849.04
	Iran (Islamic Republic of)	1591.25
Strawberry	Spain	1293.89
	China, mainland	1565.72
	United States of America	1887.00

III Records of *S. aucuparia* in the UK

	Ancient	*Veteran*	*Notable*
Arable	1 Highland	0	0
Bridle path/ footpath	4 Monmouthshire Northumberland Lancashire North Yorkshire	15 Highland Northumberland North Yorkshire Lancashire Conwy Powys Denbighshire Shropshire Monmouthshire Gloucestershire Dorset	15 South Lanarkshire Northumberland Gwynedd Conwy Denbighshire Nottinghamshire Shropshire Herefordshire Hampshire
Common/health	2 Shropshire Herefordshire	0	0
Deer park	0	8 Highland Northumberland Cumbria Shropshire Herefordshire Hampshire Berkshire East Sussex	5 Durham Conwy Herefordshire Hampshire East Sussex
Domestic garden	0	0	1 East Ayrshire
Field	14 Dumfries and Galloway Cumbria Durham Lancashire North Yorkshire Denbighshire Powys Herefordshire	16 Cumbria North Yorkshire Conwy Gwynedd Powys Denbighshire Shropshire	16 Cumbria Durham Conwy Powys Denbighshire Shropshire
Hedgerow	5 North Yorkshire Shropshire Monmouthshire	5 Powys Monmouthshire	7 Powys Shropshire
Landscape garden	0	3 Herfordshire West Sussex Surrey	0
Moorland	24 Highland Scottish Borders Northumberland Cumbria North Yorkshire Durham Conwy Gwynedd Powys Flintshire	27 Highland South Lanarkshire Northumberland Cumbria North Yorkshire Durham Conwy Gwynedd Powys Derbyshire	8 Gwynedd Powys Conwy
Parish boundary	1 Northumberland	1 Northumberland	0
POS	0	6 Perth and Kinross North Yorkshire Lancashire Dorset Surrey	1 Lancashire
River/ canal bank	16 Northumberland North Yorkshire	12 Northumberland North Yorkshire	5 Gwynedd Durham

	Cumbria Durham	Cumbria Durham	Conwy
Roadside	1 Derbyshire	2 Gwynedd West Sussex	0
Upland	63 Highland Northumberland Cumbria North Yorkshire Durham Shropshire Herefordshire	41 Highland Northumberland Cumbria Durham North Yorkshire Conwy Gwynedd Powys Shropshire Denbighshire Derbyshire Herefordshire	13 Northumberland Durham Conwy Powys Shropshire Herefordshire
Wood Pasture	1 Gwynedd	14 Stirling West Dunbartonshire Dumfries and Galloway Scottish Borders Durham Gwynedd Conwy Powys Shropshire Monmouthshire	3 Stirling Hampshire Greater London
Woodland	23 North Yorkshire Durham Tyne and Wear Norfolk Suffolk	42 Highland Perth and Kinross West Dunbartonshire Stirling Dumfries and Galloway Cumbria Durham North Yorkshire Lancashire Conwy Powys Shropshire Monmouthshire Gloucestershire West Midlands Surrey West Sussex East Sussex Kent	29 Angus South Lanarkshire Cumbria Durham Northumberland Tyne and Wear Lancashire Shropshire Nottinghamshire Powys Herefordshire East Sussex Surrey
Other	13 Cumbria North Yorkshire Durham Argyll and Bute	11 Highland Durham North Yorkshire Conwy Herefordshire	5 Durham Tyne and Wear Gwynedd Herefordshire

IV NRM methods for nutrient content study of leaf tissue

Determination of nitrogen and carbon

Matrix: Samples dried and ground to pass 0.5mm screen

Samples are totally combusted in an oxygen enriched atmosphere in a reaction tube. Nitrogen and carbon products are carried by a constant flow of carrier gas (helium) through an oxidation catalyst, and then through reduced copper wires, where excess oxygen is removed and nitrogen oxides are reduced to elemental nitrogen. The nitrogen and carbon products are separated through a chromatographic column. As the products are eluted from this column they pass through a T.C.D detector, which generates an electrical signal proportional to the amount of nitrogen and carbon present. Various products can be eliminated if required using various traps, such as a magnesium perchlorate trap to eliminate hydrogen. Peak elimination reduces the risk of overlapping peaks and shortens run times. The procedure is known as the Dumas Technique.

Determination of elemental content of an aqua regia digest

Matrix: Samples are oven dried and ground to pass 1mm screen

A representative portion of the prepared sample is digested in an open vessel with concentrated nitric and hydrochloric acid (reverse aqua-regia) using a temperature controlled digestion block. The formation of strong oxidising agents will destroy organic matter and break down the mineral matrix of the sample. The elements dissolved in the acid are analysed by ICP-OES / ICP-MS which gives an estimation of the 'total' content. Silicates present in the sample are not solubilised and are left as an insoluble residue in the digest. The elements in solution are then determined either by Inductively Coupled Plasma Mass Spectrometery (ICPMS), Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) or Atomic Fluorescence Spectroscopy (AFS). Elements determined by OES are Phosphorus, Potassium, Magnesium, Calcium, Sulphur, Sodium, Manganese, Zinc, Boron and Copper with Chromium, Lead, Arsenic, Cadmium, Molybdenum, Nickel and Cobalt by MS and Mercury and Selenium by AFS.

V Agents that can affect *Sorbus*

Main agents of concern

Agent	Problems caused	Solution/treatment
Pests		
Mammals: deer, hares, rabbits, field mice, voles	Reduced establishment of the young tree. Damage to seedling or bark.	Research area located in a compound with a rabbit proof fence, and away from the natural habitat of these pests.
Insects: *Ottiorynchus sulcatus* (Vine weevil grub)	Occurring mostly in autumn and winter, these pests eat the bark of roots to 5mm above ground level. The affected plant comes into leaf slowly in Spring, is easily removed from the soil and has woody roots. O. sulcatus prefer humus rich peaty soils.	It is recommended that whips are planted into garden soil, as natural predators would control *O. sulcatus* larvae. This however is not a possibility in this instance as the soil needs to remain consistent for research purposes. Area quarantined from other plants not used for the study. Closest source: garden centre.
Diseases		
Fireblight *Erwinia amylovora*	*Sorbus* (especially those stressed) are particularly prone to this disease. Causes wilt and acute die back.	Removal of infected area.
Honey fungus caused by *Armillaria* spp.	Causes mortality to Sorbus that are not being grown in optimum conditions where stress to the tree is likely.	Excavate and burn infected material
Coral spot fungus caused by *Nectria galligena*	Can result in die-back where trees are stressed by an overload of nutrients. A symptom of this disease is the wilting of flower clusters and in the worst cases the spread of the disease to the previous year's growth. Coral red fructifications can develop.	Avoid drought stress and high nutrient levels.
Other	Other pests and diseases to be identified during the course of the study.	

McAllister, 2005

VI Weather data

Weather in terms of temperature (°C) and rainfall (mm) was variable throughout the duration of the bioassay (Figure 5.3).

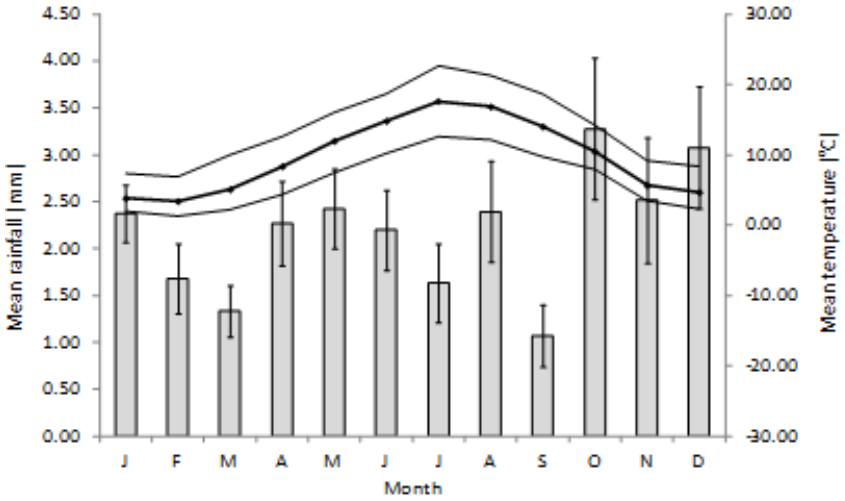

Figure 5.3: Mean rainfall and air temperature (including maximum and minimum) for the duration of the bioassay from 2012-2014.

Rainfall was consistent across the year. October, December and January had more rainfall on average than September and July ($H(11) = 927.402$, $p<0.001$). Mean air temperatures were cooler from November to March in comparison to April to October. The temperature rose to a peak of 18°C in July ($H(11) = 764.743$, $p<0.001$). Mean ground and soil temperatures had similar patterns (Figure 5.4).

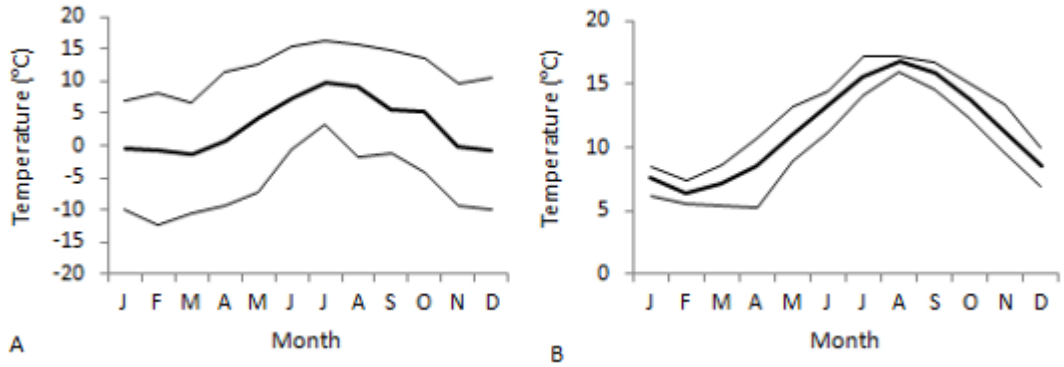

Figure 5.4: Mean temperature (including maximum and minimum at ground level (A) and in the soil at a depth of 1000mm (B) for the duration of the bioassay from 2012-2014.

Ground level temperatures reached extreme lows in the minus from November to March with a peak of 10°C in July ($H(11) = 477.663$, $p<0.001$). Soil temperatures showed a lag with colder temperatures from January to March and a high of 17°C in August. Temperatures did not drop below zero.

On comparing between year differences it was demonstrated that weather variables were found to be statistically different (Table 5.1).

Table 5.1: Between year comparison of weather conditions (mean temperature and rainfall)

	Mean temperature (°C)		
Month	Air (bulb)	Soil (1000mm)	Mean rainfall (mm)
Jan 13-14	-	↑**12%** ($H(5) = 32.509$, $p<0.001$)	↑**147%** ($H(5) = 15.682$, $p<0.001$)
Feb 13-14	↑**173%** ($H(5) = 17.939$, $p<0.001$)	↑**20%** ($H(5) = 55.221$, $p<0.001$)	-
Mar 13-14	↑**327%** ($H(5) = 46.116$, $p<0.001$)	↑**35%** ($H(5) = 63.297$, $p<0.001$)	-
Application in 2012 and entry into weekly monitoring and measurement phase			
Apr 12-13	-	↓**27%** ($H(5) = 62.685$, $p<0.001$)	↓**75%** ($H(5) = 14.893$, $p=0.001$)
Apr 13-14	↑**34%** ($H(5) = 21.498$, $p<0.001$)	↑**46%** ($H(5) = 62.685$, $p<0.001$)	↑**8%** ($H(5) = 14.893$, $p=0.001$)
May 12-13	-	-	-
May 13-14	↑**19%** ($H(5) = 6.492$, $p=0.039$)	↑**15%** ($H(5) = 37.524$, $p<0.001$)	-
Jun 12-13	-	↓**6%** ($H(5) = 21.632$, $p<0.001$)	↓**82%** ($H(5) = 8.466$, $p=0.015$)
Jun 13-14	↑**16%** ($H(5) = 13.899$, $p=0.001$)	↑**6%** ($H(5) = 21.632$, $p<0.001$)	-
Jul 12-13	↑**17%** ($H(5) = 22.581$, $p<0.001$)	-	-
Jul 13-14	-	-	-
Aug 12-13	-	-	-
Aug 13-14	↓**8%** ($H(5) = 7.477$, $p<0.024$)	-	-
Sep 12-13	-	-	-
Sep 13-14	↑**9%** ($H(5) = 8.866$, $p=0.012$)	-	-
End of weekly monitoring and measuring phase – End of study 2014			
Oct 12-13	↑**31%** ($U = 739.500$, $p<0.001$)	↑**7%** ($U = 806.500$, $p<0.001$)	-
Nov 12-13	-	↑**12%** ($U = 755.000$, $p<0.001$)	-
Dec 12-13	-	↑**21%** ($U = 926.000$, $p<0.001$)	-

During the first year, from 2012 and 2013, soil temperature and rainfall decreased significantly in April and June. Temperatures between October and December 2013 were higher than those experienced at the same time of the year in 2012. 2013-2014 data demonstrated that rainfall and soil temperatures in January 2014 were higher than January 2013. Temperatures were milder from February to the end of March 2014 and these higher temperatures continued until July at which point they were statistically similar to 2013. Rainfall was lower than previous years in June 2014.

VII NRM methods to characterise soil properties

Matrix: Sample air-dried at a temperature not greater than 30°C and sieved to pass a 2mm screen, excluding stones and any fibrous material from roots, etc.

Determination of the pH and Lime Requirement of Soil

The pH of soil is defined as the pH, measured potentiometrically, of the suspension obtained by stirring soil with water. The ratio of soil to water is 1:2.5. Temperature is one of the factors that affect the measurement of pH so the measurement is carried out in a temperature controlled environment.

Determination of Olsen's Extractable Phosphorus in Soil

A variety of chemical extractants have been developed to mimic the soil situation, thereby obtaining an assessment of the potentially plant-available phosphorus. One of the most commonly used extractants is 0.5M sodium bicarbonate known as Olsen's Reagent. The available phosphorus is extracted from the soil at 20°C by shaking with 0.5M sodium bicarbonate solution at pH 8.5. Inorganic phosphorus then reacts with acid ammonium molybdate to form the phosphomolybdate ion, which, when reduced with ascorbic acid, forms a blue coloured complex. The blue colour is measured spectrophotometrically at 880nm.

Soil Tests for Potassium, Magnesium and Phosphorus

A variety of chemical extractants have been developed to mimic the soil situation, thereby obtaining an assessment of the potentially plant-available potassium. One of the most commonly used extractants is Molar Ammonium Nitrate. The available potassium is extracted from the soil by shaking with M ammonium nitrate at 20°C for 30 minutes. After filtration, the concentration of potassium in the extract is determined by flame photometry. There are no significant interferences from other elements in the determination of potassium.

Determination of Ammonium Nitrate Extractable Magnesium in Soil

A variety of chemical extractants have been developed to mimic the soil situation, thereby obtaining an assessment of the potentially plant-available magnesium. One of the most commonly used extractants is Molar Ammonium Nitrate. The available magnesium is extracted from the soil by shaking with M ammonium nitrate at 20°C for 30 minutes. After filtration, the concentration of magnesium in the extract is determined by atomic absorption spectroscopy. The addition of a releasing agent to the sample before analysis eliminates interference by phosphate.

Particle size distribution

Determined using a laser diffraction particle sizer. The soil sample is suspended in water and passed through a flow cell. The flow cell is positioned in the path of a laser beam and the particales of soil passing through the cell cause the laser light to be defracted. The amount the light is defracted is dependant upon the size of the particle in its path. Small particles cause greater defraction than large particles. The soil is then given a textural class using UK classification.

Determination of elements zinc, manganese, iron and copper

The available elements zinc, manganese, iron and copper are extracted from the soil at 20^{o}C with DTPA solution, ratio 1:2. In theory the DTPA extraction is the basis for equilibrium of the metal in the soil with the chelating agent. A pH level of 7.3 enables DTPA to extract iron and other metals.

Determination of available sulphate

The available sulphate is extracted from the soil under controlled conditions, using a phosphate buffer extracting solution ratio 1:2. The filtered extract of the sample is analysed by Inductively Coupled Plasma Emission Spectroscopy.

Determination of elements zinc, copper, nickel, chromium, lead and cadmium

The available elements zinc, copper, nickel, chromium, lead and cadmium are extracted from the soil at 20^{o}C with 0.05M EDTA solution at pH 7.0 , ratio 1:5. The concentration of the elements in the extract are determined using ICP-OES (Inductivley Coupled Plasma Optical Emission Spectroscopy).

Determination of calcium and sodium

A variety of chemical extractants have been developed to mimic the soil situation, thereby obtaining an assessment of the potentially plant-available calcium and sodium. One of the most commonly used extractants is Molar Ammonium Nitrate. The available calcium and sodium is extracted from the soil by shaking with M ammonium nitrate at 20°C for 30 minutes. After filtration, the concentration of calcium in the extract is determined by Atomic Absorption Spectrophotometry.

Determination of boron

The available boron is extracted by hot water extraction. The concentration of boron in the extract is determined using ICP-OES (Inductively Coupled Plasma Optical Spectroscopy).

Determination of organic matter content

The organic matter is destroyed by dry combustion at 430^{o}C and the loss in weight of the sample is reported as % of the original sample as the organic matter content.

VIII DNA Multiscan results

	PAM 1.67g/l	PAM 8.33g/l	PAM 15.00g/l	BJMD 8.33g/l	ASMD 8.33g/l	TAMD 8.33g/l	TPMD 8.33g/l	Diseased (D)	PAMS 1.67g/l	PAMS 8.33g/l	PAMS 15.00g/l	BJMS 8.33g/l	ASMS 8.33g/l	TAMS 8.33g/l	TPMS 8.33g/l	Sterile (S)
Plant Pathogens																
Alternaria	0	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0
Athelia (Sclerotium) rolfsii	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Bipolaris spp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Botryosphaeria spp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Botrytis spp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Botrytis cinerea	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Colletotrichum spp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C.gloeosporioides	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
C. lindemuthianum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Coniothyrium fuckelii	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cylindrocarpon destructans	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cylindrocladium spp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Fusarium spp.	2	2	3	2	2	3	2	2	1	2	6	1	3	4	7	1
Fusarium oxysporum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Fusarium sacchari	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Fusarium solani	2	2	1	2	1	0	2	0	0	0	6	1	0	0	7	0
Gaeumannomyces graminis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Geotrichum candidum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Gnomonia comari	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Leptosphaerulina spp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Myrothecium roridum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Olpidium bornovanus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Olpidium brassicae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Olpidium virulentus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Penicillium spp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Penicillium expansum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Phoma destructiva	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Phomopsis obscurans	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Phomopsis sclerotioides	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Phytophthora spp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
P. cactorum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
P. citricola	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
P. cryptogea	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
P. drechsleri	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
P. infestans	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
P. ramorum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Plectosphaerella cucumerina	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Podospora leucotricha	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pythium spp.	2	2	3	0	2	1	1	1	2	2	1	1	2	0	0	0
P. aphanidermatum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
P. dissotocum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

P. graminicola	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
P. irregulare	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
P. polymastum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
P. sylvaticum	**2**	**2**	**3**	0	**1**	**1**	**1**	**1**	**2**	**1**	**1**	0	0	0	0	0
P. ultimum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Rhizoctonia fragariae	0	**2**	0	0	0	**2**	0	0	0	0	0	0	0	0	0	0
R. solani	**2**	**3**	0	0	**1**	**3**	**2**	**3**	0	0	**1**	0	0	0	0	0
Sclerotinia spp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
S. minor	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
S. sclerotiorum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Stemphylium spp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Thielaviopsis basicola	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Venturia inaequalis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Verticillium spp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
V. albo-atrum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
V. dahliae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pseudomonas marginalis	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
P.s syringae pv. porri	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
P. viridiflava	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
P. fluorescens	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Rhizobium radiobacter	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Erwinia amylovora	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E. carotovora atroseptica	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E. carotovora carotovora	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E. chrysantemi	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
E. pyrifoliae	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Ralstonia solanacearum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Beneficials																
Trichoderma spp.	**3**	**1**	**1**	0	**2**	**2**	**3**	**2**	0	**2**	**2**	0	0	0	0	0
T. asperellum	**3**	0	0	0	**1**	0	0	0	0	**2**	0	0	0	0	0	0
T. hamatum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
T. harzianum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

VIIII P-values for Chapter 3

Radial growth (mm) of *R. solani* on a plant matter disc and ethanolic disc in comparision to that of the control. Mean ± SEM are listed against the percentage inhibition of the treatment calculated using the control as a baseline.

Hour	Control	Plant matter	*Inhibition*	Ethanolic extract	*Inhibition*
24	8.22 ± 0.62	3.44 ± 0.85	**36%**	2.00 ± 1.26	**47%**
48	22.78 ± 0.47	13.93 ± 1.17	**32%**	15.07 ± 1.48	**28%**
72	51.37 ± 0.48	22.33 ± 3.42	**52%**	38.11 ± 4.70	**24%**
96	71.33 ± 0.98	25.67 ± 6.16	**64%**	52.26 ± 8.70	**27%**

Radial growth (mm) of *R. solani* on *P. americana* amended agar in concentrations of 1.67g/l, 8.33g/l and 15.00g/l over 168 hours Independent-samples Kruskal Wallis test

Hour	Total N	Test statistic	DF	Adjusted *P*-value
24	144	68.524	3	0.000
48	144	82.768	3	0.000
72	144	92.210	3	0.000
96	144	106.767	3	0.000
120	144	109.230	3	0.000
144	144	111.920	3	0.000
168	144	111.937	3	0.000

Radial growth (mm) of *R. solani* on agar amended with 8.33g/l of *P. americana*, *B. juncea*, *T. patula*, *A. sativum*, and *T. aestivum* amended agar over 72 hours. Independent-samples Krukal Wallis test

Hour	Total N	Test statistic	DF	Adjusted *P*-value
24	216	92.255	5	0.000
48	216	102.060	5	0.000
72	216	118.910	5	0.000

Radial growth (mm) of *R. solani* on *P. americana* amended agar in concentrations of 20.00g/l, 25.00g/l and 30.00g/l over 168 hours Independent-samples Kruskal Wallis test

Hour	Total N	Test statistic	DF	Adjusted *P*-value
24	144	104.339	3	0.000
48	144	104.499	3	0.000
72	144	105.958	3	0.000
96	144	107.356	3	0.000
120	144	105.777	3	0.000
144	144	108.479	3	0.000
168	144	103.253	3	0.000

Radial growth (mm) of *R. solani* on agar amended with a liquid percolation of *P. americana*, *B. juncea*, *T. patula*, *A. sativum*, and *T. aestivum* amended agar over 72 hours. Independent-samples Kruskal Wallis test

Hour	Total N	Test statistic	DF	Adjusted *P*-value
24	216	50.759	5	0.000
48	216	97.306	5	0.000
72	216	108.276	5	0.000
96	216	114.242	5	0.000
120	216	111.325	5	0.000
144	216	107.569	5	0.000
168	216	131.100	5	0.000

Radial growth (mm) of *P. ultimum* on *P. americana* amended agar 1.67g/l, 8.33g/l and 15.00g/l over 168 hours. Independent-samples Kruskal Wallis test

Hour	Total N	Test statistic	DF	Adjusted *P*-value
24	144	98.847	3	0.000
48	144	108.429	3	0.000
72	144	118.602	3	0.000
96	144	123.489	3	0.000
120	144	123.616	3	0.000
144	144	113.355	3	0.000
168	144	119.730	3	0.000

Radial growth (mm) of *P. ultimum* on agar amended with 8.33g/l of *P. americana*, *B. juncea*, *T. patula*, *A. sativum*, and *T. aestivum* amended agar over 72 hours. Independent-samples Kruskal Wallis test

Hour	Total N	Test statistic	DF	Adjusted *P*-value
24	216	136.049	5	0.000
48	216	153.122	5	0.000
72	216	164.535	5	0.000

Radial growth (mm) of *P. ultimum* on *P. americana* amended agar in concentrations of 20.00g/l, 25.00g/l and 30.00g/l over 168 hours Independent-samples Kruskal Wallis test

Hour	Total N	Test statistic	DF	Adjusted *P*-value
24	144	189.999	3	0.000
48	144	203.880	3	0.000
72	144	200.596	3	0.000
96	144	198.329	3	0.000
120	144	199.766	3	0.000
144	144	194.586	3	0.000
168	144	193.651	3	0.000

Radial growth (mm) of *P. ultimum* on agar amended with a liquid percolation of *P. americana*, *B. juncea*, *T. patula*, *A. sativum*, and *T. aestivum* amended agar over 72 hours. Independent-samples Kruskal Wallis test

Hour	Total N	Test statistic	DF	Adjusted *P*-value
24	216	76.762	5	0.000
48	216	125.722	5	0.000
72	216	147.230	5	0.000
96	216	158.985	5	0.000
120	216	164.775	5	0.000
144	216	160.735	5	0.000
168	216	177.275	5	0.000

X Conference proceedings and papers

Major achievements:

- **Shortlisted (1 of 59 in UK):** Presented at SET for Britain, Parliament, Postcullis House, Westminster - March 2016
- **Speaker: audience of 70 experts:** Australasian Plant Pathology Conference, Perth, Australia - Sept 2015
- **Shortlisted (1 of 3 in UK):** Prince of Wales Trophy – Sustainable Horticulture - May 2015
- **Poster presentation:** European Conference of Arboriculture, Turin - May 2014
- **Poster presentation:** Presented at International Congress of Plant Pathology, Beijing, China - Aug 2013
- **Poster presentations:** BSPP: University of Birmingham and University of Reading. TPBE II: University of Aston

Publications:

- Lock, J.J., Phillips, C., Sharp, R. & Littlemore, J. (2016) Getting to the root of the problem - replant disease in a rosaceous tree. Poster submission for SET for Britain, House of Commons.
- Lock, J. J., Phillips, C., Littlemore, J. & Sharp, R. (2015) Novel Green Manure: Control of Specific Replant Disease on the rosaceous ornamental tree Sorbus aucuparia. Oral paper presented at the *Australasian Plant Pathology Conference, Fremantle, Western Australia 14th September 2015*.
- Lock, J. J (2015) Fake Plastic Trees. The Axe – The Official Quarterly Journal of the Municipal Tree Officer Association **10**(2): pp.15-16
- Lock, J. J., Phillips, C., Littlemore, J. & Sharp, R. (2014) The potential of a Novel Green Manure Radial growth of pathogens associated with replant disease. Poster Submission presented at *Trees, People and the Built Environment Conference, University of Birmingham, UK, 2nd April 2014*.
- Lock, J. J., Phillips, C., Littlemore, J., and Sharp, R. (2014) The Potential of a Novel Green Manure - Radial growth of pathogens associated with replant disease. In: Giordano L., Ferrini, R., and Gonthier P. (eds) *Conference and abstracts book of the European Conference of Arboriculture - Planning the Green City: Relationship between trees and infrastructures. Turin 26th-28th May 2014*, DISAFA Editions, 100 pp.
- Lock, J. J., Phillips, C., Littlemore, J., and Sharp, R. (2013) The efficiency of a novel green manure in controlling replant disease on mountain ash (*Sorbus aucuparia*). In: Liu, P., Yang, J. Wang, H., Zhang, G., Jian, H., Fan, Z., Wu., Yang, A., Li, H., Zou, J., Yu, J., and Han, C. (2013) *10th International Congress of Plant Pathology - Acta Phytopathologica Sinica. Beijing 25th-30th August 2013, Chinese Society for Plant Pathology*. 376 pp.