SOIL MICROBES AS POTENTIAL CONTROL AGENTS FOR PLANT-PARASITIC NEMATODES IN PASTURE

by

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SUMMARY

Induced systemic resistance (ISR) is widely manifest in the plant kingdom, but there are few reports of its occurrence against nematodes and, prior to this study, it had not been reported in white clover. It was decided to investigate the induction of resistance to the clover cyst nematode, *Heterodera trifolii* Goffart, an economic pest in white clover pastures that are a key to high milk yields in dairy cattle in Australia and New Zealand. This study aimed to explore the potential of soil and rhizosphere bacteria to induce systemic resistance in white clover, *Trifolium repens* L.

Salicylic acid (SA) and benzo(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH) are known to induce resistance in both monocotyledonous and dicotyledonous plants against a wide variety of pathogens and pests. In a growth cabinet soil bioassay, both chemicals were applied separately as soil drenches to *T. repens* seedlings, which were subsequently inoculated with infective juveniles of *H. trifolii*. Both chemicals induced resistance to *H. trifolii,* manifest as a reduction in fecundity of the nematode and a higher proportion of abnormal cysts and fewer eggs per cyst, compared to controls treated with water. Resistance was induced in two cultivars of *T. repens*, 'Haifa', and 'Grasslands Huia'. The latter is considered to be very susceptible to *H. trifolii*.

Soil samples and white clover plants were collected from pastures known to be infested with *H. trifolii* in Victoria. These and soil samples from South Australia were examined for the presence of *Bacillus* and *Pseudomonas* spp., both known to be potential inducers of ISR in other plants. *Bacillus* strains were isolated on a medium selective for *B. thuringiensis* and *B. cereus*. Fluorescent *Pseudomonas* strains were isolated on King's B medium, further selected as to colony type on tetrazolium chloride agar, and then selected for pectinolysis on crystal violet pectate agar. Two pectinolytic *Pseudomonas* strains, P29 and P80, applied as nutrient broth (NB) cultures, induced resistance in white clover seedlings equivalent to that resulting from application of SA and BTH. Both live and dead cells of strain P29, resuspended in sterile distilled water and applied as a soil drench, had the same effect as the NB culture of the strain. This suggests that there is some plant

'recognition' of the bacterial cell walls which triggers the plant response. Cell-free culture filtrate of strain P29, applied to white clover seedlings, did not induce resistance to *H. trifolii*, suggesting that the bacterial metabolites did not act as inducing agents. The metabolites had little antagonistic effect against infective juveniles of *H. trifolii in vitro.* The plant response to BTH and P29 as inducing agents was investigated by quantitative biochemical assays for lignin and callose. At 4 weeks after inoculation with infective juvenile nematodes, there was no difference in the concentrations of lignin or callose in the leaves of plants pre-treated with BTH, P29 or water. Similarly, there was no difference in the concentrations of lignin or callose in the roots of plants pre-treated with BTH, P29 or water, 4 weeks after inoculation with the nematodes.

Bacillus strain B1 was also found to induce resistance against *H. trifolii* in the growth cabinet soil-based bioassays, equivalent to that induced by P29 or BTH.

A greenhouse experiment was carried out to investigate whether resistance could be induced to the blue-green aphid, *Acyrthosiphon kondoi*, in white clover and a medic, *Medicago truncatula*. 'Grasslands Huia', and the medic cultivars 'Sephi 6297' (resistant to the blue-green aphid) and 'Jemalong' (susceptible) were treated with strain P29, BTH or water and 4-5 aphids were released onto the plants. Some resistance to the blue-green aphid was observed. Resistance was manifest as greater plant growth in treated than control plants. Tests of strain P29 as a potential growth-promoting bacterium, had shown no increase in growth of white clover plants after 52 days compared to SDW-treated controls. No increase in callose or lignin was found in the leaves and stems of the treated white clover plants.

It is concluded that resistance to the clover cyst nematode can be induced in white clover with a soil drench of BTH, of *Pseudomonas* strains P29 and P80, or of *Bacillus* strain B1 isolated from soil. This is a first report of induced resistance in white clover, and to the clover cyst nematode. These bacterial strains are candidates for further evaluation as biocontrol agents against nematodes in white clover.

STATEMENT OF ORIGINALITY AND CONSENT TO PHOTOCOPY OR LOAN

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VALERIE N. KEMPSTER

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CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

Wherever agricultural use of Australian land for cereal-growing or pasture occurs, the incidence of plant-parasitic nematodes exceeds that found in modified scrub (Yeates & Bird, 1994). In a sense, our use of the land for cropping creates the pest problems we face. It is estimated that the Dairy Industry is worth upwards of $A4 billion per annum to Australia (Hinch *et al.*, 1993). According to Mason (1993), white clover pastures are a key to high milk yields, which in turn leads to a lucrative export market in dairy products. Loss to the industry from damage caused by plant-parasitic nematode infestation in white clover is thought to be in the region of $A16 million per annum in Victoria alone (Berg & Hinch, 1997). A survey conducted in 1993 (McLeish *et al.*, 1997), of 17 sites distributed throughout the permanent white clover pastures of Australia, indicated the widespread occurrence, at 94% of sites sampled, of the root lesion nematode, *Pratylenchus* spp. This nematode was found in Victoria, New South Wales and Tasmania at levels considered by Cook and Yeates (1993) to be economically damaging (McLeish *et al.*, 1997). The clover cyst nematode, *Heterodera trifolii* Goffart was also widespread, being found at 80% of sites. This nematode is estimated to cause up to a 50% loss of yield in mixed white clover and rye grass pastures (Yeates, 1974). Several species of *Meloidogyne* have also been recorded in such pastures: *M. hapla* in New South Wales, Victoria and Queensland; *M. incognita* in Queensland and New South Wales; *M. javanica* in Victoria, New South Wales and Western Australia; and *M. arenaria* in New South Wales (McLeod *et al.*, 1994). Root knot nematodes were found at over 70% of sites in the 1993 survey (McLeish *et al.*, 1997).

Following this survey, the current project was undertaken to investigate a potential biological control strategy against plant-parasitic nematodes in pastures. The investigation was limited to control of *Heterodera trifolii,* thought to be the most economically damaging plant-parasitic nematode in the Australian white clover pastures (McLeish *et al.*, 1997).

Current control strategies for the clover cyst nematode in New Zealand include the use of fungi which parasitise the eggs and cysts of *H. trifolii* (Hay, 1993), and the development of resistant cultivars by classical breeding programs (Mercer, pers. comm.; Grant *et al.*, 1996). The use of resistant cultivars entails the risk of selecting for resistance-breaking isolates of any pathogen (Fisher, 1982; Young, 1992; Rivoal, 1995; Seinhorst, 1995). Kuc (1982; 1987; 1995) suggested that there was evidence that resistance genes were not confined to resistant cultivars, but that resistance could be induced in susceptible cultivars by prior inoculation with non-pathogenic soil and rhizosphere microbes. Thus it was decided to investigate the potential of soil and rhizosphere bacteria to induce resistance to *H. trifolii*, as an alternative biocontrol strategy. It is considered that the non-specificity of such induced resistance augers well for its persistence and low potential for selection of resistance-breaking pathovars (Heath, 1995). The soil and rhizosphere bacteria, *Pseudomonas* spp. and *Bacillus* spp., were widely reported as inducers of resistance, and thus these genera were isolated from some of the locations involved in the 1993 survey (McLeish *et al.*, 1997).

This project sought first to investigate, in a soil-based growth cabinet bioassay, the potential of chemical inducers to elicit resistance to *H. trifolii* in white clover, and then compared that to the response to soil bacteria.

1.2 LITERATURE REVIEW

Plant-parasitic nematode surveys done in other parts of the world (Sasser, 1980; Sasser & Freckman, 1987) indicate the wide distribution and related damage caused by species of the genera *Pratylenchus*, *Heterodera* and *Meloidogyne*. For example, Cook *et al.* (1992) found *Pratylenchus* at 92% of white clover/rye grass sites sampled in England and Wales, *Heterodera trifolii* at 62%, but *Meloidogyne hapla,* which thrives in a warmer climate (Mateille *et al.*, 1995), at only 4% of the sites surveyed. Potter & Townshend (1973) found *P. neglectus* comprised 55-60% of the root lesion nematodes found in forage and cereal plants in a survey of South West and Central Ontario, Canada. In the same survey, they found *M. hapla* at 33% and *H. trifolii* at 21% of forage sampling sites. Willis and

Thompson (1969) found widespread occurrence of *Pratylenchus* sp. on white clover in Prince Edward Island, Canada. In a survey of New Zealand, Skipp and Christensen (1983) found widespread occurrence of *H. trifolii*, *Meloidogyne* spp. and *Pratylenchus* spp. on white clover. Grandison (1963) had first recorded the occurrence of *H. trifolii* on white clover only 20 years before, but considered that the New Zealand pedigree strain of white clover was resistant to it. As discussed in Section **1.1**, these genera of plant-parasitic nematodes are widespread in southern Australia.

1.2.1. Nematode feeding and pathology

The principal plant-parasitic nematodes that infest white clover are: *Pratylenchus* sp., *H. trifolii* and *Meloidogyne* sp. Many authors have reported the detrimental effect of these nematodes on nitrogen fixation by *Rhizobium* (Yeates *et al.,* 1977; Watson *et al.,* 1985; Sarathchandra *et al.*, 1995), leading to yield losses in white clover and legumes. The clover cyst- and root-knot nematodes penetrate the roots and feed on the photosynthetic products of the plant, causing chlorosis and stunting (Sarathchandra *et al.*, 1995). Further, these nematodes have been observed to interfere with the root metabolism and the ability to absorb water and nutrients from the soil (Taheri, 1996).

These three types of plant-parasitic nematodes invade and cause damage to their hosts in somewhat different ways, and each has a unique feeding mechanism. All three penetrate the root behind the root cap as second stage juveniles. *Meloidogyne* spp. migrate intercellularly and induce the formation of specialised giant cells, whereas *Heterodera* spp. migrate intracellularly and induce the formation of syncytia (Bird, 1983; Huang, 1985; Hussey, 1985; Nickle, 1991; Sijmons *et al.*, 1994) due, it is thought in both cases, to secretions from the oesophageal glands of the nematode (Jones, 1981; Bird, 1983; Hussey, 1985; Heinrich *et al.* 1998). Both nematodes become sedentary once a feeding site is established. The syncytia and giant cells develop few plasmodesmata but ingrowths of the cell wall give rise to an extensive plasmalemma, and nutrients are transported across this membrane via a proton pump which provides the energy to co-transport amino acids back across the membrane (Jones, 1981; Dorhout *et al.*, 1992; Atkinson *et al.*, 1994). These specialised sites have a very high metabolic rate and act as a sink for the products of

photosynthesis upon which the nematodes feed (Hussey & McGuire, 1987). The enlarged adult female root-knot nematodes remain within the root surrounded by a gall with only the posterior end protruding from the root. The eggs are laid into a gelatinous matrix (Heinrich *et al.*. 1998). The enlarged mature female of the cyst nematodes breaks through the root surface to the outside and once the female has filled with eggs, it dies and the eggs are protected by the cuticle of the dead female (Heinrich *et al.*, 1998).

The formation of root knots by *Meloidogyne* spp. causes stunting of plants and considerable yield loss depending on the host plant and the population density of the nematode. The presence of *M. hapla* also increases the severity of disease caused by other pathogens: eg. *Pseudomonas caryophylli* on carnations, *Pseudomonas solanacearum* on tomatoes and *Corynebacterium insidiosum* on lucerne (Williams, 1974). *M. hapla* is also reported to break down the resistance to fungal infection of *Fusarium*-resistant cultivars of certain plants, of which tomato is one example (Williams, 1974). There are reports of synergism between *M. hapla* and *Fusarium oxysporum* (Sidhu & Webster, 1977; Webster, 1985; Sarathchandra *et al.*, 1995). Nitrogen-fixation is reduced by up to 50% in nodulated white clover infested with *M. hapla* (Sarathchandra *et al.,* 1995).

Damage due to *H. trifolii* in white clover, such as stunting of roots and knot formation, is reported as severe (Grandison, 1963, Ennik *et al.*, 1965) and yield losses as high (Mulvey & Anderson, 1974). In pot trials with *H. trifolii*-infested plants, nitrogen-fixation (Yeates *et al.*, 1977), phosphorus utilisation (Mulvey & Anderson, 1974) and productivity were reduced (McLeish *et al.*, 1997; Berg & Hinch, 1997), and drought-resistance was diminished (Nickle, 1991). Repeated invasions by infective juveniles in the same region of the root may cause death of the root tip. Additional damage is caused to infested plants by bacteria and fungi which subsequently invade the entry wound caused by the nematode (Mulvey & Anderson, 1974).

In contrast, the root lesion nematode, *Pratylenchus neglectus*, does not form specialised feeding cells. It also invades the root as a second-stage juvenile, and migrates intracellularly through the root tissue. It may exit and re-enter the root and damages roots, initially by external feeding, and subsequently by penetration of the cortex and feeding therein. Damage is also caused indirectly by an additive interaction with root rotting fungi

eg *Rhizoctonia solani* and *Microdochium* sp. (Taheri, 1996) on wheat, and a synergistic interaction with *Verticillium dahliae* f. sp. *menthae* in peppermint plants (Townshend & Anderson, 1976).

1.2.2. Nematode distribution and biology

H. trifolii has a wide host range, and a world-wide distribution (Norton, 1967; Mulvey & Anderson, 1974). *Heterodera* is considered to be among the top ten genera of nematodes in terms of world-wide impact on agriculture (Sasser & Freckman, 1987), ranking in importance after *Meloidogyne* and *Pratylenchus.* It is reported to produce eight generations within 1 year on white clover grown at 10° - 20°C, but the hatching of eggs and emergence of larvae appears to be diapause- and temperature-dependent (Mulvey & Anderson, 1974). *Heterodera* spp. are able to avoid the effect of nematicides in soil through survival of their eggs in cysts, which are dead females detached from the roots, and which are able to persist in the soil. *H. trifolii* reproduces by mitotic parthenogenesis, and produces no males (Wouts, 1978). The second stage juveniles emerge from the cysts which act as a survival stage, sometimes for many years within the soil (Yeates & Visser, 1979). The juveniles enter the root at the elongation zone and migrate intracellularly to the vascular cylinder, causing much cell damage. When the juvenile begins to feed, generally near to the vascular cylinder, it causes the formation of a syncytium by the breakdown of cell walls and continues to feed and grow there. It goes through two moults to become the adult female within 17 - 19 d, which produces eggs by 22 d and is filled with eggs within 26 - 28 d (Mankau & Linford, 1960; Mercer, 1990). The enlarged egg-filled female protrudes through the cortex to the outside of the root. The eggs are retained within the female which eventually drops off into the soil, and the first stage juvenile moults within each egg to become the infective, second stage juvenile. The female dies and its cuticle becomes a dark brown cyst in which the eggs are encased. The cysts are found within the top 5 cm of soil and they may be moved from one site to another by wind, birds, farm machinery or in soil attached to crops (Yeates, 1977).

M. hapla, which tolerates temperatures near to 0°C more readily than other *Meloidogyne* species, invades the host plant and egg-laying by the adult female can commence in about

23 d in the temperature range of 20 - 25°C (Bird & Wallace, 1965). Egg masses of up to 1,337 (average 467) eggs per mass (Williams, 1974) are in a gelatinous matrix, and are known to survive in the field for up to 250 d at 0°C. Infection by second generation juveniles occurs after 39 d in peanut (Williams, 1974). Thus several generations can occur in one year in Mediterranean-type climates, where the seasonal temperatures favour their development. *Meloidogyne* spp. have a wide host range, though not very many grasses or cereals act as hosts (Eisenback & Triantaphyllou, 1991). *M. hapla* has a wide host range, and may exist as several biological races (Netscher & Taylor, 1979; Sasser, 1979; Hussey, 1985). Reproduction is by meiotic parthenogenesis which would enhance the rate of genetic variation over that produced by mutations alone, and thus facilitate rapid evolution of biological races.

P. neglectus is primarily a parasite of grasses, including cereals (Townshend & Anderson, 1976), but also has a wide range of other hosts, including legumes (Goodey *et al.* 1965). This may indicate a species or race complex (Townshend & Anderson, 1976) of this nematode, which has adapted to many plant taxa. The adult is parthenogenetic (Nickle, 1991), an adaptation which enables reproduction of this parasite in sparse populations. Egg-laying starts early in the life cycle, even before the shedding of the fourth-stage cuticle (Townshend & Anderson, 1976). The life cycle may be as short as 28 d on tobacco (Mountain, 1954). Thus many generations can be produced in one season, depending on temperature. The nematode tolerates both low soil moisture levels and temperatures down to 2°C (Townshend & Anderson, 1976). It multiplies readily on white clover. In tobacco, eggs are laid very soon after root penetration, and hatching occurs within 7-9 d (Mountain, 1954).

1.2.3 Current control strategies

1.2.3.1. Chemicals

The use of nematicides is not economical in broad acre and pasture situations, but is discussed here to indicate both its potential and disadvantages.

There are two main categories of chemicals used to control plant-parasitic nematodes in market-garden and greenhouse situations: fumigants (volatile compounds) and non-volatile compounds (Hague & Gowen, 1987, Thomason, 1987). The former include the halogenated aliphatic hydrocarbons, such as methyl bromide and ethylene dibromide, and the methyl isothiocyanate mixtures and compounds, such as metham sodium. The non-volatile compounds include the organophosphates, such as fenamiphos and thionazin, and the oxime-carbamates, such as oxamyl, aldicarb and carbofuran. In turn, the fumigants are divided into nematicides, which kill only nematodes, and general biocides which also control other soil-borne pathogens. The non-volatile compounds are considered to be nematostatic only, in that they do not kill nematodes but change their behaviour (Hague & Gowen, 1987).

Until the early 1980s, the fumigants dominated the nematicide market. In 1983, all pesticides containing ethylene dibromide were banned by the Environmental Protection Agency in the USA., removing one of the most widely used nematicides (Johnson & Feldmesser, 1987). Aldicarb, the active ingredient in Temik® (one of the carbamates), and the dichloropropanes and dichloropropenes (already restricted in their registration for use) may soon be removed due to concerns about environmental damage (Hague & Gowen, 1987; Thomason, 1987; Noling & Becker, 1994). This would then leave only oxamyl, trade name Vydate, which has been reported as an effective nematicide (Kerry *et al.*, 1982b), and some organophosphates and other non-fumigants which are, as stated above, nematostatic rather than nematicidal. Nematicides form only a small part (less than 2.5%) of the total pesticide market. This fact is important for the prospects of development of novel nematicides; the cost of development is itself a deterrent to further research for such a relatively small market (Johnson & Feldmesser 1987). The Environmental Protection Agency of the USA has enforced legislation banning the use of methyl bromide within the USA from the year 2001, citing evidence implicating methyl bromide agricultural emissions as a major contributor to stratospheric ozone depletion (Noling & Becker, 1994). Recently, a new generation of nematicides, the avermectins (produced by *Streptomyces avermitilis)* have been successfully utilised in laboratory tests against *C. elegans*. These have the disadvantage that they are not very mobile in soil and already

resistance has been reported to the avermectins in parasitic nematodes of veterinary importance (Grant et al., 1995). Their lack of mobility in the soil means that they have to be incorporated into the soil on application, adding to the overall cost of use.

One other major disadvantage of specific chemical nematicides particularly after prolonged use is that there is the potential for selection of resistant nematodes. The rapid development of resistance to the endotoxin crystal protein of *Bacillus thuringiensis kurstaki* in the diamondback moth (Adang, 1995) is pertinent to this consideration, as well as the aforementioned resistance of nematodes to the avermectins (Grant *et al.*, 1995).

1.2.3.2 Biological control

The term 'biological control' was first used in relation to plant pathogens by von Tubeuf in 1914 (cited in Baker, 1987). By 1934 the concepts of the rhizosphere (Hiltner, 1904), antagonistic microbes, suppressive soils, cross-protection (inoculation by one organism giving protection against another, Salaman, 1933), production of antibiotics by soil microbes, and the importance of temperature, pH, and the organic content of the soil to the ecological balance of soil microorganisms, were all familiar to plant pathologists (Baker, 1987). However, it was not until 1965 that the body of knowledge was brought together (Baker & Snyder, 1965) and the study and practice of biological control gained momentum. Environmental concerns about the use of chemical pesticides in agriculture and horticulture have increased (Kerry, 1990) and, thus attention has been paid to the use of biocontrol agents (Jutsum, 1988; Weller, 1988; Becker & Schwinn, 1993). In recent years, some of the issues concerning the practice of biological control were aired by Stirling (1991), Sayre and Walter (1991) and Dickie and Bell (1995), writing of the inconsistencies between *in vitro*- and *in vivo*-screening, and laboratory- or greenhouse- and field-screening, of potential control agents. In addition, the need for a clear definition of the economic threshold of pest damage for the target crop was emphasised by Seinhorst (1965) and McSorley and Duncan (1995).

The term 'biological control' has both a broad and a narrow definition. The broad definition is the one used here, and includes classical biological control, i.e. the introduction of parasites or parasitoids to control the pest species, the source of the introduced organism

being generally different from the now-indigenous pest (Hokkanen & Pimentel, 1989; Sayre & Walter, 1991). The broad definition also includes augmentation of an organism which has already co-evolved with the pest, and is providing some 'natural' control, albeit at a level below the economic threshold desired by crop-producers (McSorley & Duncan, 1995). Also included in the definition of biological control is the development and use of resistant cultivars and, more recently, of transgenic plants (Atkinson *et al.,* 1994, Niebel *et al.*, 1994). Further, the term biological control includes the management practices based on the ecology and population dynamics of the host plants and the pests, parasites or pathogens (Yeates & Bird, 1994).

Darwinian theory (Darwin, 1859) tells of competition between individuals of a species to adapt to their environment in order to reproduce their own kind. Van Valen (1973) extended the idea of competition and adaptation of individuals within a species, to that of competition between species, living in an adaptive zone. His hypothesis, the Red Queen Hypothesis, with reference to the Red Queen (Carroll, 1871), who ran constantly in order to stay in the same place, envisaged each species as part of a 'zero-sum game against other species', and invoked the concept of 'mutually incompatible optima within an adaptive zone'. According to this view, all organisms exercise some influence over the resources within their habitat - what Van Lenteren (1989) termed 'natural control'. It is this natural control which we seek to exploit in augmentative biological control. Plant-parasitic nematodes are competing for resources with the plant, in some cases, within the plant; the plant, in turn, may be capable of mounting a defence (induced resistance) against the parasite. There is a dynamic equilibrium involved in which it is selectively advantageous for the plant to decrease its chances of being infested and of being prevented from reproducing, and it is selectively advantageous for the nematode to increase its chance of successful infestation and reproduction within its habitat (the host plant). Plant-parasitic nematodes such as the sedentary endoparasites, *Heterodera* and *Meloidogyne,* become immobile once established at a feeding site but induce the plant to both shelter and feed them and switch their own metabolic activities towards successful reproduction. *H. trifolii* uses such a strategy in white clover. Augmentative biological control, by the introduction of already co-evolved organisms, seeks to push this dynamic equilibrium in favour of the

plant's ability to defend itself from the nematode. An example of this strategy would be the use of soil and rhizosphere bacteria isolated from areas where *H. trifolii* is infesting white clover, to induce resistance to the nematode.

1.2.4 Use of soil microbials in biological control

1.2.4.1 Bacteria

Mankau (1975) reported the actinomycete *Pasteuria penetrans* (Starr & Sayre, 1988), then named *Bacillus penetrans*, as a mycelial bacterium which is an obligate parasite of plant-parasitic nematodes. Stirling and Wachtel (1980), Stirling (1984), Brown *et al.* (1985), Bird (1986) and Kerry (1987), all considered it a potential biocontrol agent, which targets specifically the reproductive capacity of *Meloidogyne* spp. (Bird & Brisbane, 1988; Melki *et al.*, 1995). It does not interfere with the formation of giant cells, nor apparently, with the feeding of the nematodes on the host plant. Starr and Sayre (1988) distinguished between *Pasteuria thornei*, parasitic on *Pratylenchus brachyurus*, and *Pasteuria penetrans*, parasitic on *M. incognita*. Other species of *Pasteuria* have since been reported to parasitise juveniles of *Heterodera* spp., *Globodera* spp. and *Pratylenchus* spp. (Davies *et al.*, 1990) but have not been found in the cysts. Davies *et al.* (1994) found a high degree of heterogeneity among the endospores, enabling them to adhere to different subpopulations of nematodes.

Stirling (1991) cited other examples of bacteria, belonging to the genera *Pseudomonas* and *Streptomyces*, as being antagonistic to plant-parasitic nematodes.

The use of soil amendments to affect the microbiota of soil and ameliorate conditions for antagonists of nematodes is also reported by Rodriguez-Kabana *et al.* (1987). Addition of chitinous material promoted an increase in chitinolytic bacteria which destroy nematode eggs. There is considerable literature on the subject of soil amendments as topic, reviewed by Hasan (1992) and D'Addabbo (1995). Amendments used have included leaves, shoots and roots as green manures, farm effluents and other agro-industrial wastes. Some of these amendments showed a synergistic action when used with nematicides. The other role of

rhizosphere bacteria in biological control is that of induction of systemic resistance, which will be reviewed in Section **1.2.7**.

1.2.4.2 Fungi

Kerry and Crump (1977) and Kerry *et al.* (1982a) observed the frequent occurrence of fungal parasitism in cyst nematode eggs and females. Kerry (1987) has recently reviewed the use of fungi for nematode control, including nematode trapping fungi. Nematode-trapping fungi seem to be relatively non-specific and variable in their ability to trap nematodes. However, only the males and the second stage juveniles of the cyst and root-knot nematodes are potential targets for this type of control. Persistence of these fungi in soils is poor and, overall, they have few characteristics of good biocontrol agents (Mankau, 1980). Cayrol (1983) reported the successful use of *Arthrobotrys irregularis* against *Meloidogyne* spp. on tomatoes. *A. robusta* has been developed for use against nematodes in mushrooms, but its efficacy was inconsistent (Cayrol, 1983). Stirling and Mani (1995) have developed granular formulations of *Arthrobotrys dactyloides* and *Dactylella candida* which performed well in soil against *Meloidogyne javanica*. Use of *Paecilomyces lilacinus,* a fungus which colonises eggs of cyst- and root-knot nematodes, is seen by Kerry (1987) as a promising line of research, with the *caveat* that there have been reports of human disorders associated with this fungus. Notwithstanding, Walters and Barker (1994) reported on the efficacy of *P. lilacinus* against the reniform nematode, *Rotylenchulus reniformis*, in tomato.

In a 3-year study of wheat susceptible to *H. avenae*, Kerry *et al.* (1982b) found that a decline in female fecundity and survival, was due to infestation of eggs by *Nematophthora gynophila* and *Verticillium chlamydosporium*. However, Kerry *et al.* (1982a) mentioned that the efficacy of *N. gynophila* is limited in dry soil as it is a zoosporic fungus and requires free water to disperse within the soil. Hay (1993) and Skipp and Hay (1993) have found fungal parasitism of eggs of both *H. trifolii* and *M. hapla* on white clover in New Zealand. Work is in progress on the effect of both fungi and actinomycetes on *H. trifolii* on white clover in New Zealand (Hay & Skipp, 1993; Hay & Regnault, 1995), and on the potential of *Verticillium chlamydosporium* for the biological control of *Meloidogyne* spp.

(Bourne *et al.*, 1994). Stirling (1991) cited many instances of natural parasitism by fungi on cyst nematodes. Cianco (1995) found mycotoxins from *Fusarium* spp. were nematicidal *in vitro* to *M. hapla* and *P. neglectus*. The presence of fungal endophytes, such as *Acremonium* spp., in pasture grasses, has a varying effect on plant-parasitic nematodes (Van Heeswijck & McDonald, 1992); *Pratylenchus scribneri* and *M. marylandi* were reduced in number, but *M. naasi* was unaffected.

1.2.4.3 Vesicular arbuscular mycorrhizae

Vesicular arbuscular mycorrhizae (VAM) were first described in 1885 by A.B. Frank (Varma, 1995). According to the fossil record, VAM fungi have been associated with vascular plants for 353-462 million years (Simon *et al.*, 1993), ie, there has been a long co-evolution between vesicular arbuscular mycorrhizal fungi and the roots of vascular plants. The effects of this obligate symbiotic association include: improvement in phosphorus uptake in nutrient-poor soils (Fitter, 1989; Barea *et al.*, 1993; George *et al.*, 1995; Abbott *et al.*, 1995), protection against plant pathogens (Cooper and Grandison, 1987; Schenck, 1987; Gianinazzi *et al.*, 1988; Whipps and Lumsden, 1989; Hooker & Black, 1995), improvement in soil aggregation and structure (Schreiner & Bethlenfalvay, 1995), the promotion of root proliferation in micropropagated plants (Varma & Schuepp, 1995), increased plant vigour and yields (Stirling, 1991; Pankhurst & Lynch, 1995) and improvements in rhizobial nodulation and nitrogen uptake (Sequeira *et al.*, 1991; Ahmad, 1995; George *et al.*, 1995). The improvement in phosphorus uptake is thought to be connected with increased disease resistance; mycorrhizal roots showed increased peroxidase activity, which in turn led to increased phenolic content and production of phytoalexins (Mathur & Vyas, 1995). There have been various reports of the stimulating effect of rhizosphere bacteria on the germination of VAM fungi (Azcon *et al.*, 1978; Azcon, 1987,1989; Carpenter-Boggs *et al.*, 1995; Varma, 1995) suggesting that an integrated approach to their use in agriculture may be indicated.

The effect of VAM fungi such as *Glomus* sp. and *Gigaspora* sp. on plant-parasitic root knot nematodes has also been investigated (Marx, 1972; Kellam & Schenck, 1977; Roncadori & Hussey, 1977). Dehne (1982) commented on the antagonistic effect of VAM

fungi on nematodes, noting decreased root penetration and reduced reproduction of *Meloidogyne* sp. in mycorrhizal roots, and Smith (1987) suggested that competition between VAM fungi and nematodes for nutrients was the key factor in nematode deterrence. Cooper and Grandison (1987) studied the effect of VAM fungi on *M. incognita* on tamarillos and reported improved resistance to the nematodes in mycorrhizal plants. A reduction in reproductive rate but not in numbers of adults was noted within roots of acacia in pot studies of plants co-inoculated with *M. javanica* and the VAM fungus *Glomus* sp. (Duponnois *et al.*, 1995).

1.2.5 Resistant cultivars

Parasites, during the course of long co-evolution with their hosts (Mode, 1958; Anderson & May, 1982; Dropkin, 1988), have selectively conserved alternative biochemical and enzymic pathways for some of their key activities (Sturhan, 1971). This factor has particular relevance to both the development of nematicides and transgenic plants which target specific pathways, and to the use of cultivars with resistance to specific nematodes. The large number of offspring and the various phenotypes within populations of plant-parasitic nematodes (Sturhan, 1971; Riggs *et al.*, 1981; Price *et al.*, 1978; Netscher & Taylor, 1979) also have relevance to the use of resistant cultivars, which has been predicted to incur the risk of fairly rapid selection for resistance-breaking pathovars (Fisher, 1982; Young, 1992; Tzortzakakis & Gowen, 1996).

Breeding of plants resistant to *H. trifolii* has been undertaken by Mercer (1996) and to *H. avenae* by Rivoal *et al.* (1995). The genetic transformation of sugar beet to be resistant to *H. schachtii* has also been undertaken (Grundler, 1996; Grundler *et al.*, 1998). However, Rivoal *et al.* (1995) noted resistance-breaking populations of pathovars of *H. avenae* after continuous crops of resistant oats which quickly led to a resurgence of disease on susceptible crops. He also noted that multiplication of another root parasitic nematode, *P. neglectus*, on the *H. avenae*-resistant hosts and emergence of disease due to that parasite, ensued. Fassuliotis (1979), Mai (1985), Kerry (1987) and Roberts (1992) all wrote of the limited availability of host plant resistance to nematodes, particularly *M. hapla*. Sasser and Freckman (1987) also commented that much work needs to be done to identify cultivars

which could be used to control nematodes. Additionally, Roberts (1992) commented on the lack of durability of specific resistance, particularly that which is conferred by a single gene, as in the resistance of peach rootstock to *M. incognita* and *M. javanica* which is considered to involve a single resistance gene to each nematode species. Caveness (1976) and Roberts *et al.* (1990) reported naturally occurring resistance-breaking populations of *M. incognita* on 'resistant' tomato cultivars; and Tzortzakakis and Gowen (1996) reported a similar breakdown of resistance in tomato cultivars to *M. javanica*. Kaloshian *et al.* (1996) documented the breakdown of resistance in tomato plants transformed with the Mi gene. Young (1992) indicated other problems with the use of resistant cultivars: the lower productivity of some resistant- compared to susceptible- cultivars, the occurrence of multiple species of nematodes on the same crop, as well as the selection for resistance-breaking races of nematodes discussed above. Both Fassuliotis (1987) and Steiner and Schonbeck (1995) suggested that the use of genetically altered resistant cultivars could only be a short-term solution if only one gene is altered, since resistance is oligogenically inherited (Fassuliotis, 1987).

With reference to the intra- or infra-specific variation in *M. hapla* (Netscher & Taylor, 1979), this species, as mentioned above, reproduces by meiotic parthenogenesis. Fersht (1981) has suggested that what he termed 'mutator allelles' are advantageous in asexual populations, as these genes would facilitate the development of many individual clones or races within one population and thus lead to resistance-breaking pathotypes*

Riggs *et al.* (1981) reported extreme variation within populations of *H. glycines*, the soybean cyst nematode, finding different pathotypes which could be divided into numerous physiologic groups, within the same field. Price *et al.* (1978) found a dominant multi-allelic series involved in the inheritance of parasitic capabilities of *H. glycines*. This nematode is also amphimictic, and thus wide variation is facilitated within the progeny. *M. arenaria* also showed considerable variability based on molecular analysis of genetic material (Hiatt *et al.*, 1995). Genetic analysis of *P. vulnus* also showed a high level of variation (Pinochet *et al.*, 1994).

***Note:** The varying use of the terms 'race' (Riggs *et al.*, 1981) 'pathotype' (Andersen & Andersen, 1982) and 'biotype' (Sidhu & Webster, 1981) in referring to nematode isolates which vary according to host, is the subject of ongoing discussion in Nematology (Sturhan, 1985; Triantaphyllou, 1987). For this thesis, the term pathotype will be used.

All of these cases, documenting the high levels of genetic variability of plant-parasitic nematodes, point to the potential for development of resistance-breaking pathotypes. The injudicious use of resistant cultivars has the potential to be the source of selection pressure, acting upon high levels of genetic variability of the nematodes, and leading to the emergence of pathotypes capable of breaking resistance. A similar argument can be made against the continual use of nematicides specific to a particular species or to those which target a particular biochemical or physiological pathway in the nematodes.

1.2.6 Tolerance

Cook & Evans (1987) suggested that the use of tolerant, rather than resistant, plants may provide a fruitful avenue of research. Provided that tolerant plants reduced crop damage to the economic threshold level, this could be a strategy to use in biological control. An example of the inadvertent but successful use of tolerant plants appears to have occurred at Ellinbank, Victoria. Mr. J. Stewart (pers. comm., 1996) referred to the persistence of an 'ecotype' of white clover, Dutch Wild White, introduced between 50 and 60 years ago, which out-competes all more modern cultivars under drought- and disease-related stress. In contrast most white clover cultivars only persist for about 5 years (Mr. G. Mitchell, pers. comm., 1996), before reseeding of pastures is needed.

1.2.7. Induced resistance/acquired resistance

Chester (1933) commented on the accumulated documented evidence of acquired resistance in plants and suggested further investigation of this unexploited phenomenon in plants for protection against pathogens. Flor (1942), in discussing the gene-for-gene relationship between avirulence of the rust pathogen and resistance of the wheat host, opened the then new, field further for studies of plant resistance at the genetic level. Ross (1961a & b) reported the induction of localized acquired resistance and subsequent systemic acquired resistance (SAR) by prior infection of tobacco with the tobacco mosaic virus. Further work on induced resistance in plants was reviewed by Cruikshank (1963). Staskawicz *et al.* (1984) demonstrated that the avirulence gene in *Pseudomonas syringae* elicited a hypersensitive response in soybean referred to as an acquired systemic response. These researchers described three possible responses of plants to putative pathogens: none

(plant not affected by the pathogen), hypersensitive response (non-host plant), and pathogenicity or disease (host plant). These three responses were referred to by Linthorst (1991) as non-host interaction, incompatible interaction and compatible interaction, respectively. Linthorst characterised the incompatible or hypersensitive response as an immune response conferring resistance to subsequent infestation by the same or other pathogens. Bell (1981), Hahlbrock and Scheel (1987), Trudgill (1991), Lawton *et al.* (1993), Kuc (1995), Steiner and Schonbeck (1995), and Van Loon (1997) all published reviews of research into the biochemistry and mechanisms of induced resistance in plants. By 1991, it had become clear that there were different types of induced resistance in plants. Kloepper *et al.* (1992) distinguished between systemic acquired resistance (SAR) and induced systemic resistance (ISR). SAR was defined as resistance induced by prior exposure to pathogenic organisms which induced a hypersensitive response, and the production of pathogenesis-related proteins (PR proteins) upon subsequent challenge by pathogens. ISR was defined as resistance induced by non-pathogenic organisms, such as non-pathogenic *Fusarium* sp. or plant growth promoting rhizobacteria, and was not associated with the production of PR proteins (Hoffland *et al.,* 1995; Van Loon *et al.*, 1998). Schonbeck *et al.* (1993), Kuc (1995), Tuzun and Kloepper (1995) and Deverall (1995) reviewed the prospects for the use of induced resistance in plant protection, concluding that its widespread occurrence, persistence and non-specific nature offered very good prospects for biological control. Steiner and Schonbeck (1995) and Kuc (1995) considered the biocontrol prospects to be superior to those offered in the use of parasite-specific resistant cultivars and nematicides which target specific biochemical pathways. In addition, the nonspecificity of induced resistance was considered to make it less likely that random mutations of parasites would circumvent it and lead to the production of resistance-breaking pathotypes (Heath, 1995). Work on the use of plant-sourced chemicals as inducers of systemic resistance and the transferability of induced resistance by grafting, suggested that the instability/plasticity of the plant genome can be disrupted by stress or infection, causing the expression of resistance genes, which are endemic to all plants (Kuc, 1987).

1.2.7.1 Biochemistry and mechanisms of induced resistance

Vidhyasekaran (1988) suggested that plant-parasite interactions can be broken down into the following stages : initial recognition at the plant cell-surface, mediated by lectins binding to pathogen saccharides (occurring within 15-30 minutes of contact) and succeeded by mRNA synthesis, leading to production of proteins which may be involved in signalling the induced resistant response. Localised cell necrosis occurs in some interactions (Ross, 1961) and is considered to be an intermediate event between recognition and disease resistance. The proteins induced at the recognition event may not be inhibitory to the pathogen by themselves, but may trigger the synthesis of defence chemicals (Vidhyasekaran, 1988). Peroxidase and polyphenol oxidase are involved in the pathways to superoxide anions, which are important in cyanide-resistant respiration in the nematode-plant interaction, and to the quinones, lignin and melanin in resistant responses of plants.

Induced resistance involves a cascade of biochemical changes (Ye *et al.*, 1990; Hutcheson, 1998), represented in Figure 1, analogous to the events in vertebrate (Cooper, 1982a) and invertebrate (Cooper, 1982b; Yeaton, 1983) immune responses. Recognition and penetration are followed by an oxidative burst, stomatal opening, lipid peroxidation and raised glutathione levels, all occurring within the first 2 to 6 hours. This leads to a loss of cell viability and to the biosynthesis of ethylene and salicylic acid within the following 9 to 12 hours (Jones, 1996). To counteract the accumulation of superoxide radicals, increased levels of transmembrane superoxide-dismutases (SODs) are produced (Zacheo & Bleve-Zacheo, 1988). These SODs have also been shown to be induced by ethylene, drought and wounding (Perl *et al.*, 1993).

Application to plants of both ethylene and salicylic acid induced the production of various proteins implicated in the hypersensitive and induced resistance response (Enyedi *et al.*, 1992; Malamy & Klessig, 1992). These proteins include chitinases and protease inhibitors (Broglie & Broglie, 1993), glucanases (Linthorst, 1991; Lindgren *et al.*, 1992) and peroxidase enzymes in the phenylpropanoid pathway leading to the synthesis of lignin, suberin, various flavonoids, phytoalexins and coumarins, all of which are involved in plant defence (Dean & Kuc, 1987; Melchers *et al.*, 1993; Edens *et al.*, 1995).

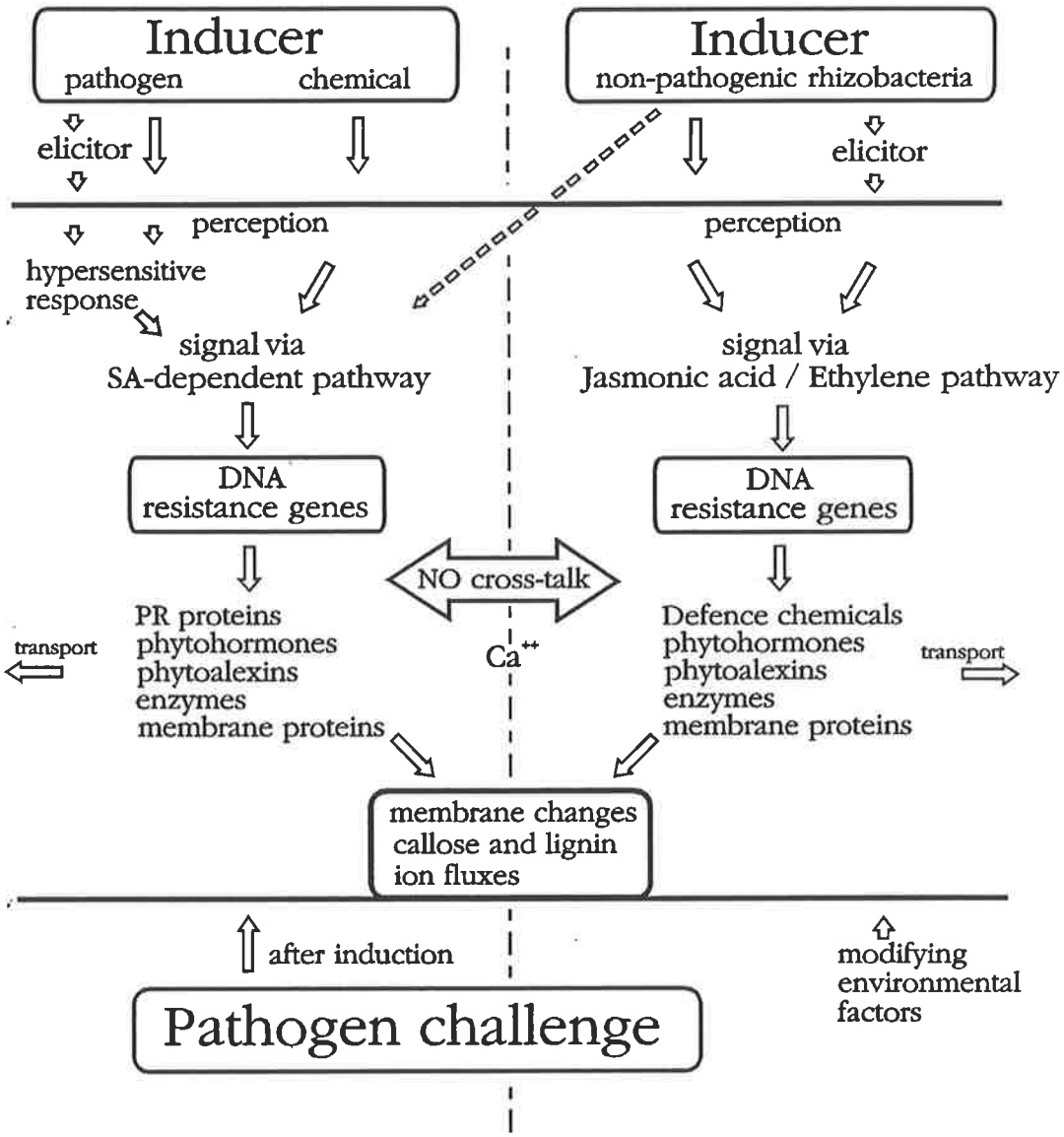

Figure 1. Schematic representation of the cascade of events occurring in induced resistance (SAR and ISR).

1.2.7.2 The use of rhizosphere microbes in induced resistance

Two common genera of bacteria found in the soil and rhizosphere are Gram positive, spore-forming *Bacillus* and Gram negative, non-spore forming *Pseudomonas*. Some of these bacteria promote plant growth (Kloepper *et al*, 1980; Kloepper *et al.*, 1989), and some have been investigated for their ability to reduce plant disease (by the mechanism referred to in Section **1.2.7** as ISR) (Loper, 1988; Ryder & Jones, 1990; Ryder & Rovira, 1993; Mahaffee & Kloepper, 1994). Many researchers have investigated the potential of rhizosphere bacteria as inducers of resistance, for biological control of various plant pathogens (Schippers, 1988; Weller, 1988; Tuzun & Kuc, 1991; Mahaffee *et al.*, 1994; Pieterse *et al.*, 1996; Milus & Rothrock, 1997). Van Loon (1997) has recently reviewed the role of plant growth-promoting rhizobacteria (PGPR), particularly *Pseudomonas* species, in induced resistance. Oostendorp & Sikora (1989) and Hoffmann-Hergarten and Sikora (1995) found that pelleted *Pseudomonas fluorescens* applied to sugar beet decreased the rate of hatch of juveniles of *H. schachtii* and that metabolites of this bacterium were nematostatic *in vitro*. There was a suggestion (Oostendorp & Sikora, 1990) that the reduced root penetration by *H. schachtii*, observed in the presence of *P. fluorescens*, may be due to binding of lectins on the Gram-negative bacterial surface to receptors on the root surface, thereby blocking host recognition by the nematode juveniles. Recently, Van Loon *et al.* (1998) reported that lipopolysaccharides (LPS) extracted from the surface of *P. fluorescens* induced resistance to *Fusarium* wilt in carnation and radish and Rietz (pers. comm., 1999) found that LPS from another Gram negative soil bacterium, *Rhizobium etli*, induced resistance to *Globodera pallida* in potato. An alternative to the explanation of Oostendorp and Sikora (1990) stated above, could be that the binding of the bacterial LPS to the root surface, itself served as the trigger or signal for the response 'cascade', which resulted in induced resistance to the nematode juveniles or fungi, respectively. The use of *Bacillus subtilis* on tomato subsequently infested with *M. incognita* in greenhouse tests has been reported to lead to a significant decrease in gall formation (Keuken & Sikora, 1995), and these researchers found that metabolites of *B. subtilis* were nematostatic *in vitro* to juveniles of *M. incognita*. There appear to be several means of induction of the resistance

response to soil microbials; Van Loon *et al.* (1998) described different bacterial 'determinants' of induced resistance and gave examples of *Pseudomonas* spp. which produced siderophores (Loper, 1988), some in which the determinants were LPS and some which produced salicylic acid. Loper (1988) had correlated the production of siderophores by fluorescent *Pseudomonas* sp. with biological control of *Pythium ultimum*. Morita (1993) suggested that most bacteria exist in nature at the equivalent of stationary phase. Sarniguet *et al.* (1995) found that a factor produced in late log phase of a *P. fluorescens* culture, induced the biosynthesis of antibiotics which have been found to be associated with suppression of disease organisms. Raaijmakers & Weller (1998) noted that the production of the antibiotic 2,4-diacetylphloroglucinol by *Pseudomonas spp.* in Take-All decline soils was associated with the natural protection of wheat against *Gaeumannomyces graminis* var. *tritici.* Antibiotics from *P. fluorescens* Pf-5 have been associated with antifungal activity against *Pythium ultimum* and *Rhizoctonia solani* (Nowak-Thompson *et al.*, 1994; Kraus & Loper, 1995) in roots. Leifert *et al.* (1995) equated antibiotic production by *Bacillus subtilis* and *B. pumilis* with early events in spore formation, which would occur in the stationary phase, and he observed suppression of *Botrytis cinerea* on leaves of *Astilbe* plants in tissue culture, correlated with this antibiotic production. Several researchers have observed that the endophytic colonisation of roots by both *P. fluorescens* and *Enterobacter asburiae* (Groom *et al.*, 1994a, 1994b; Mahaffee *et al.*, 1994; Sturz, 1995) has led to disease suppression and improved plant growth. Leeman *et al.* (1994) found systemic induced resistance to *Fusarium* wilt associated with endophytic *P. fluorescens* in radish. Furthermore, the use of endophytic fungi to induce systemic resistance to other fungi was reported by Meera *et al.* (1995). Elmi *et al.* (1990), Stewart *et al.* (1993) and Hallmann and Sikora (1994) have all shown reduced galling by root-knot nematodes *Meloidogyne* spp., in response to fungal endophytes. Hasky and Sikora (1995) demonstrated in split root tests that resistance against the potato cyst nematode, *Globodera pallida*, was not due to antagonism, but to systemic resistance induced by *Bacillus sphaericus* and *Agrobacterium radiobacter.*

1.2.7.3. The use of chemicals to induce resistance

An enhanced resistance response of tobacco to the tobacco mosaic virus (TMV) following treatment of the plants with acetylsalicylate (Aspirin) was documented by White (1979). This discovery was followed by a report of a similar response to salicylic acid (SA) in tobacco (Antoniw & White, 1980). Since that time, many researchers have investigated the biochemistry and mechanisms of this type of induced resistance (Conrath *et al.*, 1995; Mur *et al.*, 1996). It was found that exogenous application of SA enhanced resistance to bacterial, fungal and viral pathogens by inducing the expression of genes for pathogenesis-related (PR) proteins in plants (Raskin, 1992; Klessig & Malamy, 1994). Ward *et al.* (1991) and Uknes *et al.* (1993) indicated that these were the same PR protein genes as were expressed in SAR induced by prior inoculation with a pathogenic organism and Gaffney *et al.* (1993) established a clear requirement for SA in the induction of PR proteins in SAR.

Benzo(1,2,3)thiadiazole-7-carbothioic acid-S-methyl ester (BTH), which is structurally similar to SA, is also reported as an inducer of resistance in plants (Kessmann *et al.*, 1994; Gorlach *et al.*, 1996). This induction is observed in a variety of plants both monocotyledonous and dicotyledonous angiosperms and in gymnosperms and also against various pathogens (Ward *et al.*, 1991; Kauss *et al.*, 1992; Uknes *et al.*, 1992; Gaffney *et al.*, 1993; Conrath, 1995). Currently, BTH is marketed in Europe and South Africa as Bion WG50®, as a control agent of powdery mildew in wheat in the field (McKenzie, pers. comm., 1999; Gorlach *et al.*, 1996), and is undergoing development trials in the USA. as Actigard®, in a variety of crops against a wide range of pathogens (Novartis, 1997). It is thought that BTH and SA bind to the same receptors in plants as they both cause the expression of the same PR proteins which act against the same array of pathogens (Gorlach *et al.*, 1996). Recently BTH has been found to induce resistance to root-knot nematodes in grape in greenhouse tests (Owen *et al.*, 1998), to bacterial and fungal disease in green bean in a controlled environment room (Siegrist *et al.*, 1997), to *Altenaria* sp., *Xanthomonas* sp., *Verticillium* sp. and *F. oxysporum* in cotton, in field and greenhouse trials (Colson-Hanks, pers. comm., 1999) and to the fungal pathogen *Sclerotina sclerotiorum* in soybean in field and greenhouse studies (Dann *et al.*, 1998). A recent

report also indicated that tomatoes treated with BTH prior to planting in the field, showed resistance to the bacterial spot pathogen, *Xanthomonas* sp., the fungal pathogens *Altenaria solani*, and *Fulvia fulva* and a reduced incidence of leafminer *Lyriomyza* spp. larval infestation (Inbar *et al.*, 1998).

Clearly this chemical inducer, BTH, has a broad range of activity against pests and pathogens when applied as a preventative to plants in the field and greenhouse. It is currently applied as a foliar spray and has yet to be developed as a seed application. Its initial registration in the USA will be for use on tobacco and tomato and it is planned to follow that with registrations for use on bananas in South America and West Africa (McKenzie, pers. comm, 1999).

1.3 SUMMARY

- *H. trifolii* is a widespread pathogen of economic importance in clover pastures in Australia and New Zealand.
- Current chemical management strategies are uneconomic in pasture.
- Genetically modified plants, and resistant cultivars bred by traditional methods are not considered a long-term option in control due to the inherent risk of the selection of resistance-breaking pathotypes.
- Two types of induced resistance are widespread in the plant kingdom: induced systemic resistance (ISR) with endophytic soil or rhizosphere microbes and the related, systemic acquired resistance (SAR) with prior inoculation of pathogenic microbes.
- ISR and SAR result in resistance against a broader spectrum of pests, parasites and pathogens than does a cultivar which is resistant to a specific pest, parasite or pathogen.

The foregoing review of the literature, and the above points led to the design of this project as an investigation of the potential of induced resistance as a strategy for biocontrol of the clover cyst nematode in white clover. Indigenous microbes isolated from the soil and rhizosphere of fields of white clover pasture, were considered suitable organisms to test for induction of resistance to the clover cyst nematode, assuming a long co-evolution with the biotic and abiotic factors within that habitat.

CHAPTER TWO

ISOLATION OF SOIL BACTERIA AND NEMATODES

2.1 INTRODUCTION

As part of the National White Clover Improvement Program, a survey was made in 1993 of 17 sites in the major locations of white clover pastures in Australia (McLeish *et al.*, 1997). The principal plant-parasitic nematodes found at sites in Victoria (Timboon and Ellinbank), were *Meloidogyne* spp. and *Heterodera trifolii*. In South Australia (Flaxley, Monteith and Ponde) the nematodes found were mainly *Pratylenchus* spp.. Berg and Hinch (1997) assessed the impact of the nematodes on pasture health in small-plot trials at Timboon, using Temik® as a nematicide treatment, and found reduced productivity of white clover due to the nematode infestation. These researchers estimated that the cost to the Victorian (Australia) dairy industry alone was A$16 m per annum.
Sampling of plants and soil from nematode-infested locations was undertaken. Subsequently, specific soil bacteria were isolated, and plant-parasitic nematodes were extracted, from these samples. Two bacterial genera, *Bacillus* and fluorescent *Pseudomonas*, were isolated, and the plant-parasitic nematodes, *Meloidogyne* spp., *Pratylenchus* spp. and *Heterodera trifolii* were extracted. The distribution and relative intensity of these were recorded, together with various abiotic environmental variables from each location, such as soil type, soil pH, soil moisture, annual rainfall and average temperature.

2.2 MATERIALS AND METHODS

2.2.1 Location and description of sampling locations

Sampling was done in the autumn of 1996 only. The choice of sampling locations was based in part on the 1993 survey of white clover pastures (McLeish *et al.*, 1997). Samples were taken at the South Australian Research and Development Institute (SARDI) Research Station at Flaxley, South Australia, courtesy of Mr. Greg Mitchell. The field at Flaxley had

been sown in 1991 with perennial ryegrass and in 1992 was oversown with a mixture of the white clover cultivars 'Pitau New Zealand' and 'Haifa'. Irrigation was by centre-point pivot and the paddock was rotationally grazed by dairy cattle in a regime designed to encourage stolonisation of the clover. Fertilisation was 35 units phosphorous and 70-80 units potassium per hectare, per annum. The pasture was 5 years old at sampling (sample codes F-1 and F-36). Sampling was also done on a 3-year-old pasture at the same site (sample code F-E). The sampling codes refer to the tables in the Appendix.

Additional sampling was done at Flaxley at the Watergate Friesian Stud of Mr. David Kuchel, where samples were taken from 10- and 20-year-old pastures (sample codes DK-10 and DK-20). Rotational grazing was practised, but the pasture was a mixture of ryegrass, white clover, paspalum and cocksfoot. Fertilisation was with dairy effluent only. There were a total of 16 samples taken from the Flaxley area, 12 from the SARDI Research Station, and 4 from Mr. D. Kuchel's Friesian Stud. Half of the samples were soil cores, and half were whole plants, including the rhizosphere soil.

Samples were also taken from two flood-irrigated pastures on the Murray River flood-plains. At one location near Ponde, the dairy property of Mr. Mervyn Fran, both 1-year and 5-year-old white clover/ryegrass pastures were sampled (sample codes Fr 1 and Fr 5). These were rotationally-grazed, with 24 h cycles, and fertilised with '1913'® a nitrogen and phosphate fertiliser. The second flood-irrigated location which was sampled was the dairy property of Mr. D. Llewellyn, near Monteith (sample code Ll 5). This was rotationally-grazed with 12 h feeding on rye grass/white clover, followed by 12 h on lucerne. The fertiliser used was '1913'® and the pasture was 5-year-old clover. There were a total of 8 samples taken from the Ponde and Monteith sites, 4 from each site; half as soil cores and half as whole plants.

In Victoria, samples were collected from Timboon and Ellinbank, and both sites were included in the 1993 survey by McLeish *et al.* (1997). These sites were not irrigated, as both are in high rainfall areas. The Timboon site had been used for a small-plot field trial in 1993 and sampling was done in the buffer zone of the mini-plots (sample codes T-P) as well as in the surrounding field (sample codes T-F). The pasture was sown in 1991 and

sampled in 1996, and was a mixture of rye grass and white clover. The field was grazed by a Hereford beef herd.

The Ellinbank location was at the Department of Natural Resources and Environment Dairy Research Institute. Samples were taken from the untreated control plots (sample codes E-P) of a small-scale field trial established in 1993, which had been sown with white clover inoculated with *Rhizobium*, and rye grass. Samples were also taken in the main paddock (sample codes E-F), which was grazed by a dairy herd. This contained a permanent white clover pasture, thought to have been planted in the 1930's with an unknown (now naturalised) cultivar, possibly 'Dutch Wild White'. A total of 18 soil cores (C) and soil plus plant (S) samples were taken at both the Timboon and Ellinbank locations.

The mean annual rainfall and ambient temperature were obtained from the Australian Bureau of Meteorology statistics for the Meteorology Weather Station nearest to each location. The Flaxley figures are from the Mount Barker Station, those for Ponde and Monteith are from the Murray Bridge Station, and Timboon and Ellinbank figures are from their respective stations.

The map coordinates for the South Australian locations were obtained from the Australia 1:250,000 Map Series Gazetteer (Division of National Mapping, 1975), those for Victoria were determined by a portable Global Positioning System taken to the sites.

2.2.2 Sampling methods

Soil samples of approximately 200 g were collected from positions near clover plants using a hand-held auger, of 30 mm diameter by 250 mm length. Whole white clover plants were dug up with a spade and included the soil around the roots. The samples were placed in plastic bags, sealed, labelled, and placed in coolers and transported to the laboratory where they were stored at 10°C until examined for bacteria and nematodes.

The number of samples per site was based on the assumed distribution of soil bacteria. The distribution factor k is an index of dispersion (Goodell, 1982) and a low k corresponds to a non-random or clumped distribution. Many soil bacteria occur in high numbers throughout the soil (Peter Murphy, pers. comm.) similar to the density (1×10^7/g) of bacteria on the

phylloplane, which would correspond to a high *k* factor. Using the formula described by McSorley (1987), $n = (s/Ex)^2$, where x = mean, E = standard error of the mean, and assuming a standard error to mean ratio of 10%, this would allow a lower sample number than would be used for organisms with a clumped or aggregated distribution, and allows a sample number of 10/habitat/site within a location.

2.2.3 Soil characteristics

Soil type was assigned by reference to Northcote's Atlas of Australian Soils, Explanatory Data for Sheets 1 and 2 (Northcote, 1960; 1962). Soil moisture was determined by oven-drying of 1 g soil from each sample at 100°C for 24 h, and calculating the % water loss from the soil. The pH of the soil samples was obtained by mixing 5g soil in 12.5 ml SDW and determining the pH of the supernatant.

2.2.4 Isolation of pseudomonads from soil samples

From each 200 g of stored soil, a sub-sample of 0.5 g was put into 50 ml of sterile minimal salts solution (see **2.2.5**) in a 125 ml baffle flask. This was done in a laminar flow hood to minimise extraneous bacterial contamination. The flasks were placed on a shaker at 22°C for 48 h. Serial dilutions of the supernatant from each of the baffle flasks were made in sterile distilled water (SDW) in 1.5 ml Eppendorf tubes. Dilutions were made to 1×10^{-6}. Two replicate plates for 1×10^{-6}, 1×10^{-5} and 1×10^{-4} dilutions were made by spreading 200 µl sub-samples from each onto about 20 ml Medium B agar in 90 mm Petri dishes (see **2.2.6**). The plates were incubated at 30°C for 48 h, and then examined in UV light at 250-350 nm. The fluorescent colonies were counted, and the number per g soil was calculated. The colonies were dot-transferred (using sterile tooth-picks) to fresh Medium B plates, incubated for a further 48 h at 30°C, and examined under UV light as before. Using aseptic technique, a loopful of each fluorescent colony was transferred into 10 ml sterile nutrient broth (NB, Difco) in each McCartney bottle, and shaken at 28°C overnight. Aliquots of 1 ml, from these nutrient broth cultures, were then 'snap frozen' in liquid nitrogen and stored in a final concentration of 17% glycerol at -80°C (see **2.2.10**) (Lacey, 1997).

2.2.5 Selective medium for fluorescent pseudomonads

The medium of Simon and Ridge (1974) was prepared as follows. To 900 ml nanopure water were added: 1.0 g $NH_4H_2PO_4$, 0.2 g KCl and 0.2 g $MgSO_4.7H_2O$, and the pH was adjusted to 7.0 using 2N KOH. The solution was autoclaved at 121°C for 20 mins at 103.4 kilopascals. Separately, a 2% solution of glucose in nanopure water was autoclaved, and 100 ml of this was added to the 900 ml salts solution.

2.2.6 Culture medium for fluorescent pseudomonads

The Medium B culture medium of Simon and Ridge (1974) based on King *et al.* (1954), was prepared as follows. To 1 L nanopure water were added 20.0 g protease peptone, 10.0 ml glycerol, 1.5 g KH_2PO_4 and 1.5 g $MgSO_4.7H_2O$, and the pH was adjusted to 7.2 with 2N KOH. To this solution, 15 g agar were added and the solution was autoclaved as above for 20 min. The medium was cooled to 45 °C and the following (Sigma) antibiotics added: 75 mg Cyclohexamide (aqueous solution), 12.5 mg Chloramphenicol (alcoholic solution) and 50 mg Ampicillin (dissolved in H_2O:NaOH in the proportion of 9:1).

2.2.7 Isolation of *Bacillus* spp. from soil samples

The isolation, culture and identification of *Bacillus* spp. described here was based on the sodium acetate selection method described by Travers *et al.* (1987), as adapted by Morris *et al.* (1998). A sub-sample of 0.5 g soil from each of the 200 g samples, stored at 10°C, was put into 10 ml of an acetate-buffered Luria broth (see **2.2.8**) in a 125 ml baffle flask. This was shaken at 30°C for 4 h at approximately 240 rpm. The samples were then heat-treated at 80°C (either in an oven or a water bath) for 3 min, allowed to settle for 1 min, and the supernatant was serially diluted with SDW to 1×10^{-1} and 1×10^{-2}. Two replicate plates were made of each serial dilution by spreading 200 µl aliquots on 20 ml L-agar medium (see **2.2.9**) in 90 mm Petri dishes. The plates were incubated for 24 h in the light at 30°C. The next day, individual colonies were picked off using sterile toothpicks and transferred to plates of T3 agar (see **2.2.9**). The T3 plates were incubated for 4 d, until the cells lysed. The location of each bacterial colony was marked on the base of the Petri plates with a felt-tip pen. Samples of the labelled colonies were removed with a sterile

loop and transferred into a drop of SDW on a microscope slide. A thin smear was made, air-dried, fixed by passing through a cool flame, and stained with Smirnoff's (1962) staining method. These slides were examined for the presence of endotoxin crystals of *Bacillus thuringiensis* (*B.t.*). The colony form of *B.t.* is very similar to that of *B. cereus*, and both grow in the selective medium described above, thus the presence of endotoxin crystals characteristic of *B.t.* distinguished between the two species. The aim was to select only *B. cereus* as an experimental organism.

Those colonies which did not produce endotoxin crystals were sub-cultured from the labelled Petri plates, into 10 ml nutrient broth in McCartney bottles and shaken overnight at 28°C. Subsequently, 1 ml aliquots were 'snap frozen' in liquid nitrogen and stored in a final concentration of 17% glycerol at -80°C (see **2.2.10**), as for fluorescent *Pseudomonas*.

2.2.8 Selective medium for *Bacillus* spp.

Luria broth (Morris *et al.*, 1998) was prepared as follows: 10.0g tryptone, 5.0 g yeast extract, and 5.0 g NaCl were dissolved in 900 ml nanopure water, autoclaved for 20 min as above, and stored for up to 2 weeks at 4°C. Sodium acetate buffer at 0.25 M (freshly made) was prepared by adding 2.5 M acetic acid (0.8 ml in 99.2 ml SDW) to 100 ml 2.5 M sodium acetate (34.02 g in 100 ml SDW) until the pH was 6.8, and 100 ml of this buffer was added to the 900 ml Luria broth. This buffered L-broth was used to incubate soil samples as a first step to isolate *Bacillus* spp. (see **2.2.7**).

2.2.9 Culture media for *Bacillus* spp.

L-agar medium (Morris *et al.*, 1998) was made as follows: 15 g Bacto-agar (Difco) was added to unbuffered Luria broth, autoclaved, and cooled to 50°C and about 20 ml was aseptically poured into 90 mm Petri dishes. The dishes were stored for up to 2 weeks at 4°C.

Buffered T3 medium (Morris *et al.*, 1998) was prepared by dissolving 1.5 g yeast extract, 3.0 g tryptone, 2 0 g tryptose phosphate broth, and 0.005 g MnCl, in 900 ml nanopure H_2O. To this was added 100 ml 0.05 M sodium phosphate buffer. The buffer was made by adding a solution of 6.9 g NaH_2PO_4 in 100 ml nanopure water to a solution of 7.1 g

Na_2HPO_4 in 100 ml nanopure water, until the pH was 6.8. Bacto-agar (Difco) was added at 15 g/L and the medium autoclaved as above, cooled to 50°C and about 20 ml aliquots aseptically poured into 90 mm Petri dishes. The dishes were stored for up to 2 weeks at 4°C.

2.2.10 Storage of soil bacteria

Strains of both fluorescent *Pseudomonas* spp. and *Bacillus* spp. were stored in 2 ml cryotubes at -80°C until tested. Into each of a series of cryotubes was put 0.5 ml of a 70% aqueous solution of glycerol (after Lacey, 1997). These were autoclaved for 15 min at 121°C. Each tube was labelled appropriately and 1 ml of an overnight nutrient broth culture of a rhizosphere bacterial isolate was added to each, giving a final concentration of 17% glycerol. The mixture was vortexed and the tubes were 'snap frozen' in liquid nitrogen, and then stored at -80°C in cryoboxes.

2.2.11 Extraction, identification and counting of plant-parasitic nematodes from soil

Second-stage juveniles of plant-parasitic nematodes were extracted from each soil sample using Whitehead trays (Whitehead and Hemming, 1965) with 100 g of soil. The nematodes were collected from the water by passing the suspension through sintered glass filters, and three 1 ml aliquots per sample were examined in Doncaster counting dishes under a dissecting microscope (Doncaster, 1962). From these counts, estimations were made of the number per 100 g soil. Nematodes were also extracted from 100 g samples of plant roots. These were washed and cut into 10 mm segments and put under a mister (Southey, 1986), set at 42°C, for 72 h. The mister was activated for 10 s every 10 min at a pressure of 65 $lb.in^{-2}$, and the second stage juvenile nematodes were collected every 24 h. The nematodes were identified to genus under a dissecting microscope with the aid of the Commonwealth Institute of Helminthology Descriptions of Plant-Parasitic Nematodes (Mulvey & Anderson, 1974; Williams, 1974; Townshend & Anderson, 1976).

2.2.12 Extraction of nematode cysts

H. trifolii cysts were isolated from soil samples from Ellinbank, Victoria using a modified Fenwick can procedure (Fenwick, 1940; Southey, 1986). The soil around the roots of

whole plants was rinsed off, the resulting slurry poured into the Fenwick can and elutriated through a series of sieves with pore sizes 710 µm, 250 µm and 53 µm, respectively. This procedure was repeated with the rinsed roots of the plants, which were placed in water and stirred in the Fenwick can to dislodge most of the cysts. Similarly, soil cores were elutriated through a series of sieves as above. Cysts were washed off the 53 µm sieve, and collected on filter paper in a Buchner funnel. These cysts were stored at 4°C in foil-wrapped Petri dishes for up to 4 weeks before being used to inoculate plants of white clover.

2.2.13 Propagation of *H. trifolii* in white clover *T. repens* 'Haifa' seedlings

T. repens 'Haifa' seeds were obtained from the Adelaide Seed Company, South Australia. The seeds were mixed with a culture of commercial *Rhizobium* Group B (Nitrogerm®, Bio-Care Technology Australasia) in about 0.5 ml SDW and sown onto steam-sterilized Mount Compass (sandy loam) soil in pots, 240 mm diameter by 200 mm deep, in a greenhouse at 19°C.

Each pot contained from 20-30 plants and was inoculated with at least 400 cysts. The cysts were placed in a series of shallow depressions on the soil surface and washed in with sterile tap water from a wash bottle. These plants formed the source of infective juvenile inoculum for subsequent experiments. Plants were also inoculated with second stage juveniles of *H. trifolii*, applied as a drench into depressions on the soil surface.

2.2.14 Statistical analysis

The data presented in this Chapter are expressed as means and standard deviations. They were analysed by parametric analysis using one-way analysis of variance (ANOVA), ($p < 0.05$).

2.3 RESULTS AND DISCUSSION

Tables I - IV (**Appendix**) present the results of the extraction of nematodes and the isolation of soil bacteria from the seven sites within five locations in Victoria and South Australia. Tables I - IV present the raw data obtained from the survey. The data are summarised in Tables 2.1 and 2.2. These show, in broad terms, the widespread occurrence of both fluorescent *Pseudomonas* spp. and *Bacillus* spp. and of plant-parasitic nematodes in the locations sampled. The distribution of soil bacteria, summarised in Table 2.2, shows a variation between locations for both *Bacillus* spp. and fluorescent *Pseudomonas* spp., as determined from the selective plating techniques used. Table 2.1 is a summary of the abiotic characteristics of the sampling sites. It is evident that there are not significant differences in average temperature or soil moisture between locations. The low rainfall figure for the Ponde and Monteith locations is unlikely to be a significant factor as these are flood-irrigated pastures.

Table 2.1 Characteristics of locations sampled in 1996 survey

Location	Soil type#	Soil moisture (Mean % ± s.d.)	Soil pH (Mean ± s.d.)	Annual rainfall (mm)	Average annual ambient temperature (°C)
Flaxley (n = 16)	Podsolic shallow compact loam. Red duplex	24.4 ± 5.5	6.3 ± 0.4	810	13.9
Monteith & Ponde* (n = 8)	Dark clay	37.9 ± 5.0	5.9 ± 0.3	346	16.0
Timboon (n = 18)	Yellow and Yellow-grey duplex	25.9 ± 3.9	4.7 ± 0.1	900	13.4
Ellinbank (n =18)	Krasnozem structured earths smooth-ped fabric	37.6 ± 3.8	4.6 ± 0.3	1046	13.8

n = number of samples from location * Data combined for the two locations
#According to Northcote, (1960; 1962)

There were fewer *Bacillus* spp. than fluorescent *Pseudomonas* spp. isolated from all locations. The distribution of *Bacillus* spp. varied between locations (Table 2.2). Due to the small number of samples from Monteith and Ponde these data were pooled, but a statistical comparison was not made of these data with data from the other locations. A significantly lower count of *Bacillus* spp. was recorded from the Timboon soil samples than from either Flaxley ($p = 0.03$),or Ellinbank ($p = 0.038$) samples. The number of *Bacillus* spp. isolated from Ellinbank was not significantly different ($p=0.7$) from the number isolated from Flaxley. It is difficult to ascribe these differences to any of the measured variables at those locations, such as soil moisture and soil pH, rainfall, or mean annual temperature, but, the soil type may have had an influence on the distribution of *Bacillus* spp.

Table 2.2 Incidence of nematodes and bacteria isolated from samples in different locations in 1996

Location	*Bacillus* (x 10^3 /g soil)	*Pseudomonas* (x 10^6/g soil)	*Heterodera*	*Meloidogyne*	*Pratylenchus*
	Mean ± standard error		Mean ± standard deviation/100g soil		
Flaxley (n = 16)	8.6 ± 4.7	9 ± 4	-	0.06 ± 0.3	61.6 ± 94.4
Monteith and Ponde (n = 8)	9.1 ± 6.5	116± 61	0.13 ± 0.4	-	16.8 ± 12.6
Timboon (n = 18)	5.1 ± 3.1	86 ± 35	123.4 ± 218.4	0.67 ± 1.0	16.2 ± 13.9
Ellinbank (n = 18)	9.2 ± 7.6	62 ± 17	71.8 ± 166.2	4.4 ± 3.5	108.1 ± 82.5

n = number of samples per location

Fluorescent *Pseudomonas* bacteria were found in soil at all the sites sampled. While they had a wide distribution, enumeration of colonies isolated on King's B medium (King *et al.*, 1954; Simon and Ridge, 1974) showed variation in numbers between locations, but in a different way from that of the *Bacillus* spp. Again, statistical comparisons were not made for the Murray flood plain locations. However, there appeared to be more fluorescent *Pseudomonas* spp. isolated from Monteith and Ponde than from Ellinbank, Flaxley or Timboon (Table 2.2). There was no significant difference ($p = 0.54$) between the number of fluorescent *Pseudomonas* bacteria isolated from Timboon and that from Ellinbank. There were significantly fewer *Pseudomonas* bacteria isolated from Flaxley than from Ellinbank ($p = 0.005$) or from Timboon ($p = 0.03$). These differences did not appear to be due to pH or soil moisture content as the variation in these factors seemed to be independent of the variation in bacterial populations between sites (Table 2.1). They did appear to have varied according to different soil types. The podsols of Flaxley yielded the smallest numbers of fluorescent *Pseudomonas*, while the dark clay soils of the Murray flood plain sites at Monteith and Ponde appeared to have more *Pseudomonas* spp. than were isolated from the Krasnozem, podsols or Yellow and Yellow-Grey duplex soils of Ellinbank, Flaxley or Timboon, respectively. This may indicate an influence of the soil type on the populations of fluorescent *Pseudomonas* bacteria.

The distribution of nematode species found in this survey, summarised in Table 2.2, was somewhat regionally specific, and corresponded with the findings of McLeish *et al.* (1997). No *Heterodera* spp. were found in soil samples from South Australia. This may be due to the different soil types sampled in South Australia and Victoria, or to summer soil temperatures which are higher in South Australia than in Victoria. *Pratylenchus* spp. were the predominant nematodes found in the South Australian soil samples (Table 2.2). This skewed distribution may also be due to differences in soil type and summer soil temperatures. Mean annual rainfall at Ellinbank, Victoria is 1046 mm, which is considerably higher than the 810 mm for Flaxley in South Australia. The mean annual rainfall at Timboon (900 mm), however, is not much higher than that at Flaxley. Monteith and Ponde, South Australia, have an annual rainfall of 346 mm. The rainfall effect, if any, is possibly mitigated by irrigation in the South Australian sites sampled, as reflected by the

estimations of soil moisture in soil samples, which showed little difference among all sites (Table 2.1). The only other measured factor which may have had a bearing on the nematode distribution would be the pH, which was more acid for Ellinbank and Timboon, compared to the near-neutral soils of Flaxley, Monteith and Ponde (Table 2.1). Other factors which would influence the nematode distribution are cropping history and fertilisers applied.

From this survey, which was made in one year and one season only, it is not possible to make comparisons between locations sampled nor to determine whether there are correlations between the numerical incidence of plant-parasitic nematodes and intensity of the soil bacteria isolated. It forms a 'snap-shot' of the incidence of nematodes and bacteria in an autumn sampling of those regions for one particular year (1996).

Variation in soil bacterial and nematode counts may also occur between seasons, and between years with different climatic conditions. If soil bacteria are to be applied for biological control in pastures, an investigation will need to be made of the effects of both biotic and abiotic factors for their effects on the population dynamics of the soil bacteria. Such an investigation should also include their compatibility with chemical fertilisers. Biotic factors would include soil amendments which enhance certain bacterial populations, which, in turn, may improve the pasture crop's resistance to disease-causing organisms.

CHAPTER THREE

DEVELOPMENT OF THE BIOASSAY

3.1 INTRODUCTION

The phenomenon of induced resistance was described early in this century (Ray, 1901; Chester, 1933). Interest has recently been revived in this phenomenon (Kuc, 1995; van Loon, 1997), and the potential of induced resistance for biological control of plant pathogens has been recognised (see Section **1.2.7**) (Deverall, 1995). Resistance has been induced, using both chemical and biological agents, against a wide variety of plant pathogens, in both monocotyledonous (Steiner & Schonbeck, 1995) and dicotyledonous plants (Liu *et al.,* 1995; Liu & Kloepper, 1995; Mahaffee & Kloepper, 1994). The target plant-pathogens in these experiments have been fungi, bacteria and viruses, but recently Hasky-Gunther and Sikora (1998) reported that the rhizobacteria, *Agrobacterium radiobacter* and *Bacillus sphaericus*, induced resistance to the potato cyst nematode, *Globodera pallida*, in potatoes. Also, Hallmann *et al.* (1997) induced resistance in cotton inoculated with fluorescent endophytic pseudomonads, to *M. incognita*, and Owen (1998) reported chemically induced resistance against a root-knot nematode, *M. hapla,* in grapevine and tomato. There are no reports, however, of the phenomenon of induced resistance in white clover, *T. repens*, against the clover cyst nematode, *H. trifolii.*

Therefore, the central questions to be addressed here were:

A) Does induced resistance occur in white clover?

and B) If so, is it effective against the clover cyst nematode?

Initially, it was suggested by Dr. B. Staskawicz (pers. comm., 1996) that the question of whether resistance could be induced against *H. trifolii* could be approached by using *Arabidopsis thaliana* as the host plant, since it was known to manifest induced resistance and was also reported to be susceptible to *H. trifolii* (Sijmons *et al.*, 1991). Thus development of the bioassay commenced with *A. thaliana* as the putative host plant. An alternative line of investigation was also pursued, in which two known chemical inducers

of resistance were tested for their effect in *T. repens*, against *H. trifolii*. The chemicals were SA and BTH which have been widely reported in the literature as inducers of resistance (see Chapter One, Section **1.2.7.3**).

A bioassay was first developed to test the effect of these two chemicals on the clover cyst nematode in white clover, and to ascertain how any resistance was manifest in this plant-nematode system. In other parasitic nematode-plant systems, such as *Meloidogyne* spp. in tomato (Kaplan & Noe, 1993), *H. glycines* in soybean (Kim *et al.*, 1998) and *Pratylenchus* spp. in wheat (Holloway *et al.* 1997), resistance to the nematodes is manifest by a reduction in root knots, cysts or lesions, respectively. In contrast, Grant *et al.*(1996), in selective breeding trials for cultivars of white clover resistant to *H. trifolii*, observed no reduction in penetration by the nematodes, nor a decrease in the number of adult females or cysts formed. However, they reported significantly fewer eggs per female and cyst in the resistant lines of white clover. Similarly, in experiments with genetically transformed sugar beet plants resistant to the sugar beet cyst nematode, *H. schachtii*, a greater proportion of distorted, abnormal cysts was found in the resistant plants, compared to the proportion seen in untransformed, susceptible sugar beet plants (Cai *et al.*, 1997).

This chapter describes experiments performed to develop and validate a bioassay for use in screening for induced resistance in white clover.

3.2 MATERIALS AND METHODS

3.2.1 Potting mix for *Arabidopsis thaliana*

The potting mix used for growing *A. thaliana* was prepared according to a protocol developed by A. Vivian-Smith (pers. comm., 1996). The mix consisted of: 1 part white sand, 1 part peat, 1 part Perlite; to this was added a mixture of: 10 g $FeSO_4$, 10 g dolomite, 5 g lime, 5 g gypsum and 30 g Osmocote® (Yates, Cavan, SA). The mixture was autoclaved at 121°C for 20 min, cooled and tested for sterility by streaking a small loopful of the mix onto nutrient agar plates, which were left overnight at 30 °C, and checked for microbial growth. The mix was then put into plastic punnets (125 mm x 70 mm by 4 mm

deep), in which *A. thaliana* was to be sown. Each punnet held approximately 200 ml of the potting mix. The mixture was thoroughly wetted with sterile tap water, before applying the seeds.

3.2.2 Culture of *Arabidopsis thaliana*

Seeds of *A. thaliana* ecotype 'Columbia' were obtained from the Arabidopsis Biological Resource Center (ABRC), Columbus, Ohio, USA and stored at 4°C until required. The method used to culture the seedlings was adapted by A. Vivian-Smith (pers. comm., 1996) from the method recommended in the ABRC Seed and DNA Stock List (1995). *Arabidopsis* seeds were spread onto thoroughly wet potting mix in the punnets, as described above, and covered with plastic clingfilm and placed in a growth cabinet for 3-4 d at 20°C, to germinate. The light source used was a mixture of cool white fluorescent tubes and incandescent bulbs (GEC 400 Watt Lucalox) with an overall light intensity of 280 $\mu Em^{-2}s^{-1}$, and there was a 12 h photoperiod. The emergent seedlings were thinned to provide eight seedlings per punnet and, after a further 1-2 weeks, the seedlings were 'hardened' by piercing several holes in the plastic clingfilm daily. The punnets were then covered by a plastic dome to minimise moisture loss (see Fig. 3.1 and 3.2). The dome was made from plastic garden netting, mesh 40 mm^2, which was covered with thin plastic sheeting. The seedlings were watered every 2 d with sterile tap water or a sterile solution of Thrive® (Yates, Cavan, SA) nutrients at 600 mgL^{-1} in SDW. The plants matured in 4 weeks and set seed.

3.2.3 Harvest of seeds of *A. thaliana*

When the siliques were brown and somewhat curled, mature seeds were separated from them, and placed in 2 ml Eppendorf tubes. To begin to dry the seeds, the tubes were left open at room temperature (approximately 25 °C) for 2 d. They were then transferred to a desiccator containing silica gel, at room temperature and left for a further 8 d. Dried seeds which were to be used immediately were sown onto the moist potting mix, and the punnets covered with foil and placed at 4°C for 3 d to meet the requirement for vernalisation. The rest of the dried seeds were stored in small paper seed packets at 4°C, until required.

3.2.4 *Trifolium repens* cultivars

The seeds of *T. repens* were obtained from the Adelaide Seed Company. Two cultivars of white clover were used, 'Haifa', which is considered to be relatively resistant to *H. trifolii* (Mercer, pers. comm., 1996), and 'Grasslands Huia' which had been used as the susceptible 'control' in selective breeding experiments for cultivars resistant to *H. trifolii* (Van den Bosch *et al.*, 1997; Mercer *et al.*, 1992). Before sowing for experimental use they were surface-sterilised.

3.2.5 Surface-sterilization of *T. repens* seeds

White clover seeds were soaked in 1% sodium hypochlorite for 5 min, rinsed a total of seven times in sterile distilled water (SDW) and left in the last rinse for at least 2 h. The water was decanted and a culture of commercial *Rhizobium* inoculant, Type B (Nitrogerm ® Bio-Care Technology, Australia) was added in approximately 0.5 ml SDW, according to the manufacturer's recommendations, and mixed well to coat the seeds.

3.2.6 Culture of *T. repens* seedlings

Using aseptic techniques, the surface-sterilised, *Rhizobium*-coated seeds were transferred to about 20 ml 1% SDW agar in 90 mm plastic Petri dishes, which were sealed with cling film to prevent desiccation, and placed in a growth cabinet at 20°C with a 12 h photoperiod. The lighting was 280 $\mu Em^{-2}s^{-1}$ as above (see **3.2.2**). One week later, the seedlings were transferred to Mount Compass soil, which had been oven-sterilised for 24 h at 120°C. Mount Compass soil is a sandy loam. In early experiments, the seedlings were planted in individual 50 ml wells, which were 45 mm deep, in a 64-well black plastic seedling tray (Yates, Cavan, SA, 'Kwik Pots') and a plastic dome was placed over the tray to maintain the humidity (Fig. 3.1). In later experiments the seedlings were planted in polyvinyl chloride (PVC) tubes of 50 mm diameter x 150 mm long, which held about 150 ml of soil. A plastic bag was secured over the bottom of each tube to prevent soil loss and to effectively isolate the roots of each replicate plant. The tubes were placed in wire racks, with a plastic dome placed over the top as for the *Arabidopsis* seedlings (Fig. 3.2). The seedlings were watered *ad libidum* with sterile tap water or with a sterile nutrient solution

of Thrive® (see **3.2.2**). Validation experiments were carried out to determine the effect of the larger arena size on the size of the seedlings and the consequent effects on penetration of the roots by the nematodes. Seedlings in both arenas were examined 10 d after inoculation with nematodes. Fresh weights and root lengths were recorded. The roots were washed and stained with lactoglycerol acid fuchsin (Bridge *et al.*, 1982) and the number of developing nematodes per root and per gram root, were recorded.

3.2.7 Chemical treatments

BTH was provided by Novartis Crop Protection Australasia Ltd, as an experimental preparation, CGA-245704. Salicylic acid (Sigma) (SA) and BTH were prepared as aqueous solutions in SDW for all experiments with *H. trifolii* in white clover plants or *Arabidopsis*. Initial experiments were designed to test both the phytotoxicity and the dose effect of the chemicals. Three concentrations of BTH and two of SA were tested by applying 2 ml of the chemical solutions to the soil above the roots of the plants. The concentrations of BTH used were 0.5 μM, 5.0 μM and 50 μM; while those of SA were 1 mM and 5 mM. The dose levels used were based on reports of the activity of BTH at 36 μM as a foliar spray and its known phytotoxicity at levels higher than 100 μM (Friedrich *et al.*, 1996) and of the efficacy of SA at 1 mM (Malamy and Klessig, 1992) and its reported phytotoxicity at 10 mM (Mark Potter, pers. comm., 1996). The highest concentration tolerated, and inducing a response of the plants to the nematodes, was then used as the treatment dose in subsequent tests for induced resistance. Controls were treated with 2 ml SDW. All treatments were applied as a drench delivered directly onto the surface of the soil just above the roots of the seedlings. Eight plants were used for each experimental treatment (in a few cases some seedlings died during the period of the experiment) in a randomised block design. Each experiment was repeated at least once to test for phytotoxicity and in subsequent testing for induced resistance.

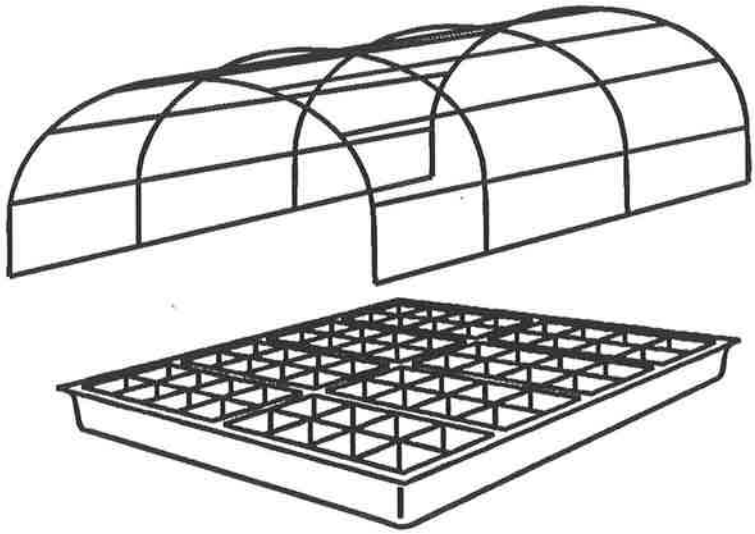

Figure 3.1 Diagram of 64-well Kwikpots® with plastic dome placed over the top to retain moisture, for initial tests with BTH and SA as inducers.

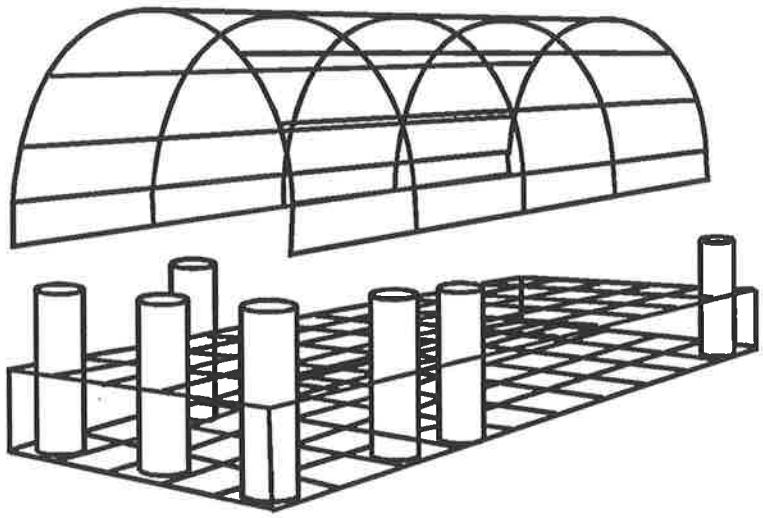

Figure 3.2 Diagram of PVC tubes in wire rack with plastic dome placed over to retain moisture, for tests of microbials for induction of resistance.

3.2.8 Preparation of nematode inoculum

Second-stage juveniles of *H. trifolii* were harvested as required, at least 5 weeks after inoculation, from clover plants grown in the greenhouse (see **2.2.13**). The juveniles were extracted by placing 200 g aliquots of soil from the greenhouse pots on a series of Whitehead trays (Southey, 1986). The juveniles migrated into the water, through two Kleenex® tissues, supported on plastic wide-mesh trays, inside the Whitehead trays. After 48 h, the nematode suspension was concentrated by passing the water from the trays through sintered glass funnels attached to an aspirator. The nematode suspensions were stored in shallow water in small plastic bottles at 4°C.

Second-stage juvenile nematodes were also extracted from washed, 10 mm root segments in a mister (Southey, 1986) for 72 h. The mister was set at 42°C, so that the water temperature is at 25°C when it reaches the roots. The mister was activated for 10 s every 10 min at a pressure of 4.6 Kg cm^{-2} and the second-stage juvenile nematodes were collected every 24 h. The nematodes were concentrated as before, and stored at 4°C for up to 96 h before use.

3.2.9 Surface sterilization of nematodes

Prior to their use in experiments the nematodes were surface-sterilised with a fungicide, Hibitane® (I.C.I. Australia) at 0.5% a.i., and a mixture of the antibiotics penicillin G and streptomycin sulphate (Sigma) at 0.1%, according to the method of Hay (1994). The surface-sterilization was done in a laminar flow hood with a Millipore filter apparatus attached to an aspirator. The nematodes were soaked in Hibitane® for 15 min, rinsed three times with SDW, transferred to the antibiotic solution, and left overnight at 21°C. They were rinsed three times with SDW, soaked for another 15 min in Hibitane®, rinsed three times with SDW and transferred to a sterile 100 ml glass cylinder. The suspension of nematodes was thoroughly mixed by bubbling air through a sterile pipette, plugged with sterile cotton wool to trap dirt and contaminants, with an aquarium aerator and 1 ml aliquots were transferred to modified Doncaster counting dishes (Doncaster, 1962; Southey, 1986) and the nematodes counted under a dissecting microscope. Three 1 ml

aliquots of nematodes were counted and the mean was determined. The volume in the cylinder was adjusted to give 200 infective juveniles per ml.

3.2.10 Inoculation of seedlings with nematodes

Surface-sterilised second-stage juvenile *H. trifolii* were inoculated onto 3-4 week-old seedlings of *T. repens* 'Haifa' or 'Grasslands Huia' from 1 to 3 d after treatment of the seedlings with either SA or BTH. *A. thaliana* seedlings, which are faster growing than *T. repens* seedlings, were treated and inoculated with nematodes after 2 weeks growth, just after 'hardening'. The nematodes were inoculated as a 1 ml suspension of 200 second stage juveniles onto the surface of the soil above the roots of the seedlings.

3.2.11 Assessment of resistance

The effect of chemical treatments was assessed 4 weeks after inoculation with the nematodes. The roots of *A. thaliana* and white clover were rinsed to remove soil and stained for 30 s in boiling lactoglycerol acid fuchsin (Bridge *et al.*, 1982), rinsed in tap water and mounted in glycerol between the nested lid and bottom of a 90mm diameter glass Petri dish. Stained cysts in roots were counted, and their condition classified as normal or abnormal (malformed) under a dissecting microscope. Cysts or females which were empty or contained fewer than 50 eggs, or were malformed, were classified as abnormal (Fig. 3.3). Normal cysts or females were lemon-shaped or rounded and appeared full of eggs (Fig. 3.4).

The soil rinsed from the roots of the white clover was passed through sieves of pore sizes 710 µm and 250 µm, and the cysts and/or females were collected on a 53 µm-pore sieve. Cysts were then washed onto 125 mm diameter filter paper in a Buchner funnel attached to an aspirator, and excess water was removed. The filter paper was transferred to a 150 mm diameter Petri dish and the cysts and females were first examined and counted under a dissecting microscope. An approximation of the volume of individual cysts and females was obtained using a Leitz compound microscope at x100, by measuring the length and breadth of about seven cysts from each replicate. The cysts or females were taken at random from the filter paper bearing each soil sample, mounted individually in drops of

water on a glass microscope slide, covered with another slide and gently squashed (Grant *et al.*, 1996). Cysts or females from the soil only, and not those stained and attached to the roots, were measured. It was considered more informative to make measurements on detached cysts or females because these had presumably completed growth and egg deposition. To estimate the number of eggs per cyst, all cysts or females were picked from the soil on the filter paper and mounted individually in water on a microscope slide as above. Egg numbers were estimated using a Leitz compound microscope at x100 magnification.

3.2.12 Statistical analysis

Owing to the small sample size (eight replicates per treatment) non-parametric statistical analysis was used to analyse the data from these experiments. Non-parametric analysis of variance (ANOVA) of the means, a rank test, was performed for all data in this Chapter. The data presented are mean and standard error and the superscript letters denote statistical similarity or dissimilarity ($p < 0.05$).

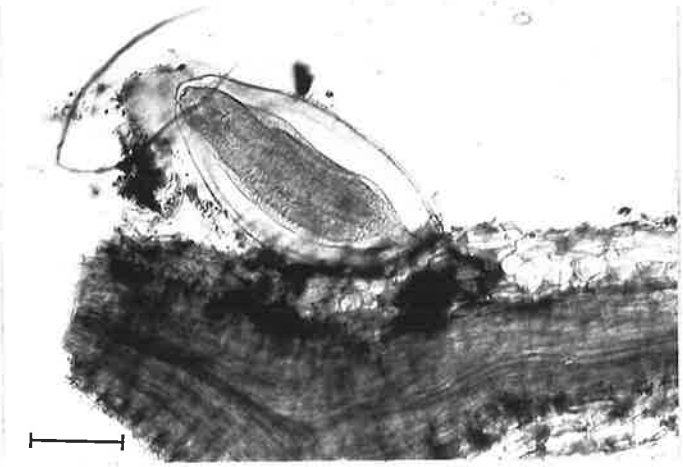

Figure 3.3 Photograph of unstained abnormal female or cyst, note absence of eggs. Scale bar = 100 μm.

Figure 3.4 Photograph of unstained normal female or cyst on root, full of eggs. Scale bar = 100 μm.

3.3 RESULTS

3.3.1 Growth and infestation of *A. thaliana*

The growth conditions for *Arabidopsis* were first optimised, but it was found that even under optimal conditions, the plants were not susceptible to *H. trifolii*. No cysts of *H. trifolii* were found in any of the control or treated plants of *A. thaliana* under the conditions of growth used. The data presented in this chapter, therefore, are for the response of the clover cyst nematode in white clover only.

3.3.2 Phytotoxicity and dose effect

Initial experiments tested for both phytotoxicity of SA and BTH to white clover and response of *H. trifolii*. The maximum concentrations of SA and BTH (which were both effective in inducing resistance and non-phytotoxic) were 5 mM salicylic acid and 50 µM BTH, when applied as a 2 ml soil drench. The plants treated with SA or BTH appeared normal ie no different from those treated with SDW. In these initial experiments, the proportion of abnormal cysts or females was recorded for stained roots only. There did not appear to be a difference in the response to different concentrations of either of the chemicals tested, in terms of number of abnormal cysts or females resulting (Table 3.1). There was no significant difference ($p < 0.05$) between the proportion of abnormal cysts in the roots of plants treated with 2 ml BTH at concentrations of 0.5 µM, 5.0 µM and 50.0 µM, or 2 ml SA at concentrations of 1 mM and 5 mM. However, the proportion of abnormal cysts or females in all treated plants was higher than that in SDW-treated control plants (Table 3.1). The females and cysts are collectively referred to in the Tables and the Results Section as 'cysts'.

Table 3.1 Effect of different concentrations of BTH and SA on *H. trifolii* in roots of white clover

Treatment	Distorted cysts in roots(%)	Total number cysts in roots	Total cysts (normal and abnormal), per pot
	Mean ± s.e.		
1 mM SA	19 ± 7[b]	8 ± 3[a]	24 ± 4[a]
5 mM SA	28 ± 7[b]	9 ± 2[a]	24 ± 3[a]
50 µM BTH	28 ± 7[b]	8 ± 2[a]	17 ± 3[a]
5.0 µM BTH	40 ± 6[b]	12 ± 2[a]	29 ± 3[b]
0.5 µM BTH	27 ± 6[b]	10 ± 2[a]	23 ± 2[a]
SDW	3 ± 1[a]	13 ± 2[a]	21 ± 3[a]

SDW = sterile distilled water. Number of replicates = 8
Within each column, values with the same superscript letter are not statistically significantly different ($p < 0.05$)

3.3.3 Bioassay arena

Comparisons were made of plant growth and nematode penetration of roots in the 64-well arena with 50 ml capacity wells (used for early experiments) and the PVC pipe arena with 150 ml capacity tubes. In an initial experiment with no nematodes, the roots of clover plants grown in the larger arena were considerably longer than those in the 50 ml arena; the mean lengths were 181.8 mm and 76.4 mm, respectively. The root mass of plants grown in the PVC tubes was also much heavier than that in the 50 ml wells, with mean weights of 170 mg and 99 mg, respectively. However, in experiments in which seedlings were inoculated with nematodes, there was no difference in the weights of the roots, but the roots again were significantly longer in the larger arena than in the 50 ml arena. At 10 d after inoculation, the number of developing nematodes per g root was the same for each arena (Table 3.2). The PVC tubes allowed greater elongation of the root and therefore greater nematode infestation per plant than in the 50 ml wells. Later experiments indicated that the same volume of 50 µM BTH or 5mM SA induced an equivalent resistance response of the clover cyst nematode in both arenas (data not shown).

Table 3.2 Root lengths and weights, and nematode numbers at 10 d after inoculation, in two different arenas

Arena	Root length (mm)	Root weight (mg)	Total nematodes per root	No. nematodes per 50 mg root
	Mean and s.e.			
50 ml wells	93.4 ± 3.6	118.6 ± 18.5	24 ± 2.9	11.5 ± 1.7
150 ml PVC tubes	145.9 ± 4.4	153.2 ± 21.9	36.5 ± 2.8	14.1 ± 2.1
Difference between arenas	s ($p = 0.00000001$)	n.s.	s ($p = 0.005$)	n.s.

Number of replicates = 11. Nematode inoculum = 200 juveniles
s = significantly different ($p < 0.05$)
n.s. = not significantly different

3.3.4 Resistance criteria

Assays were conducted to determine the criteria for assessment of resistance to *H. trifolii*. Counts of stained cysts in the roots indicated that, while there was a difference in numbers of cysts between experiments, there was no significant difference ($p < 0.05$) in the numbers of cysts within experiments due to the different treatments including controls (Table 3.3). However, these initial experiments showed a greater proportion of malformed cysts of *H. trifolii* in the roots of chemically treated seedlings compared to the proportion in water-treated control seedlings (data not shown).

Three experiments were carried out to investigate the effect of chemical treatments on the size of nematode cysts isolated from the soil of experimental arenas. Statistical analysis of these data indicated that the area (used as an approximation of volume) of the cysts did not vary consistently with treatment (Table 3.4). In one experiment (I), comparing the effect of sterile distilled water (SDW) with 5 mM SA, there was no difference in the size of cysts in the soil, between treated plants and controls. In a second experiment (II), the cysts resulting from the treatment with 5 mM SA, were significantly smaller than those in the water-treated controls. In a third experiment (III), treatment with SA resulted in significantly smaller cysts than in the controls, but BTH treatment resulted in cysts significantly larger than in controls (Table 3.4).

Table 3.3 Number of cysts of *H. trifolii* in the roots of seedlings of white clover treated with BTH, SA, or SDW in five experiments

Treatment	5mM SA	50 µM BTH	SDW
Experiment	Mean number ± s.e. cysts		
I	20 ± 3^a	-	26 ± 4^a
II	24 ± 3^a	17 ± 3^a	21 ± 2^a
III	14 ± 5^a	-	12 ± 4^a
IV	24 ± 4^a	20 ± 2^a	20 ± 4^a
V	16 ± 2^a	9 ± 2^a	12 ± 4^a

SDW = sterile distilled water. No. replicates = 8
Across the rows, values with the same superscript letter are not significantly different ($p < 0.05$)

Table 3.4 Results of three experiments comparing mean cyst areas of *H. trifolii* in white clover, after treatment with BTH at 50 µM and salicylic acid at 5 mM

Treatment	5 mM SA		50 µM BTH		SDW	
Experiment	No. cysts measured	Mean cyst area ± s.d.	No. cysts measured	Mean cyst area ± s.d.	No. cysts measured	Mean cyst area ± s.d.
I	49	$3.1x10^5 \pm 1.0x10^5$ n.s		NA	49	$3.3x10^5 \pm 1.2x10^5$
II	35	$1.2x10^5 \pm 6.8x10^4$ s		NA	35	$3.8x10^5 \pm 1.1x10^5$
III	54	$1.8x10^5 \pm 7.6x10^4$ s	59	$2.6x10^5 \pm 8.6x10^4$ n·s	49	$2.1x10^5 \pm 5.9x10^4$

SDW = sterile distilled water. Number of replicate plants = 8
n.s = Not significantly different ($p < 0.05$) from SDW treatment, across rows
s = Significantly different ($p < 0.05$) from SDW treatment, across rows

Observations on the number of eggs per cyst extracted from soil indicated that there was a difference in egg numbers between cysts from chemically-treated plants and water-treated controls (Table 3.5). Normal cysts in control plants contained in excess of 50, and generally more than 100, eggs. There was a greater proportion of cysts in the controls

which contained more than 50 eggs, whereas a greater proportion of the cysts from chemically-treated plants contained fewer than 50 eggs (Table 3.5). Additionally, there was a greater proportion of abnormal cysts in the stained roots of chemically-treated plants compared to controls. Plants having a greater proportion of abnormal cysts in stained roots had a greater proportion of cysts in the soil with fewer than 50 eggs.

Table 3.5 Numbers of eggs per cyst recovered from soil and percent distorted cysts in roots, in white clover, cultivar 'Haifa', treated with BTH, SA and SDW

	% of distorted cysts in stained roots	% cysts from soil with fewer than 25 eggs	% cysts from soil with fewer than 50 eggs	% cysts from soil with more than 50 eggs
Treatment	Mean ± standard error			
SDW	8.3 ± 5.5[a]	11.3 ± 5.5[a]	30.6 ± 10.6[a]	46.4 ± 12.6[b]
5 mM SA	59.4 ± 14.9[b]	26.9 ± 9.4[b]	47.6 ± 9.0[b]	29.5 ± 6.9[a]
50 µM BTH	54.2 ± 16.6[b]	24.3 ± 8.0[b]	53.5 ± 15.9[b]	9.0 ± 3.7[a]

SDW = sterile distilled water. Number of replicates = 8
Within columns, values with the same superscript letter are not significantly different ($p < 0.05$)

3.3.5 Cultivar effect

Treatment with BTH induced a greater proportion of cysts with fewer than 50 eggs compared to controls in both 'Haifa' and 'Grasslands Huia' cultivars of white clover. These results were shown to follow the same trend as the data on the proportion of abnormal cysts in the stained roots (Table 3.6A). A comparison was made, in two successive experiments, of the effect of BTH and water on *H. trifolii* in the two cultivars of *T. repens*, 'Haifa' and 'Grasslands Huia'. There was no significant difference ($p < 0.05$) in the response of the nematode between the two cultivars, with respect to either stained cysts in the roots or number of eggs per cyst in the soil (Table 3.6B).

In two further experiments with 'Grasslands Huia' as host plant, carried out to test the effect of 2 ml 50 µM BTH and 2 ml 5 mM SA on *H. trifolii*, there was a significantly

($p<0.05$) greater percentage of abnormal cysts in treated plants than in controls (Table 3.7), but no significant difference between the response to the two chemicals. These results represent a reduction in fecundity of 38-48% for SA treatments and 38-47% for BTH treatments, compared to controls.

Table 3.6A Numbers of eggs per cyst recovered from soil in pots, and percent distorted cysts from roots, for two cultivars of white clover, treated with BTH and SDW

Treatment and Cultivar	% of distorted cysts in stained roots	% of cysts from soil with fewer than 25 eggs	% of cysts from soil with fewer than 50 eggs	% of cysts from soil with more than 50 eggs
Grasslands Huia	Mean ± standard error			
SDW	19.1 ± 4.8^{a}	25.5 ± 6.8^{a}	54.0 ± 5.6^{b}	52.9 ± 6.2^{b}
50 µM BTH	44.9 ± 7.7^{b}	47.8 ± 6.6^{b}	79.1 ± 5.5^{b}	20.9 ± 5.5^{a}
Haifa	Mean ± standard error			
SDW	27.8 ± 5.9^{a}	9.3 ± 1.6^{a}	28.6 ± 4.7^{a}	70.3 ± 4.4^{b}
50 µM BTH	72.8 ± 2.6^{b}	52.8 ± 13.4^{b}	76.4 ± 9.1^{b}	23.6 ± 9.1^{a}

SDW = sterile distilled water
There were 7 replicates of 'Grassland Huia' and 6 replicates of 'Haifa'
Within columns, values with the same superscript letter are not significantly different ($p < 0.05$)

Table 3.6B Effect of BTH and SDW treatment on all *H. trifolii* cysts (from roots and soil) in two cultivars of white clover, 'Grasslands Huia' and 'Haifa'

Treatment	50 µM BTH		SDW	
	Abnormal cysts (%)	Total number of cysts	Abnormal cysts (%)	Total number of cysts
Cultivar	Mean ± standard error			
'Grasslands Huia' (n=7)	75 ± 3^{a}	59 ± 8^{x}	41 ± 3^{b}	36 ± 9^{y}
'Haifa' (n=6)	56 ± 9^{a}	63 ± 9^{x}	21 ± 4^{b}	60 ± 6^{x}

SDW = sterile distilled water. n = number of replicates
Values with the same superscript letter are not significantly different ($p < 0.05$), within columns and between rows

Table 3.7 Mean percentages of abnormal cysts of *H. trifolii* in response to treatment of white clover seedlings with BTH at 50 µM and SA at 5 mM concentration, compared to SDW-treated controls in two experiments

Treatment	5 mM SA	50 µM BTH	SDW
	Mean ± standard error		
Experiment I[1] % Abnormal cysts	83 ± 12[a]	73 ± 10[a]	35 ± 13[b]
Total No. cysts[1]	5 ± 1[x]	6 ± 1[x]	4 ± 1[x]
Experiment II[2] % Abnormal cysts	69 ± 6[a]	78 ± 12[a]	31 ± 9[b]
Total No. cysts[2]	16 ± 2[x]	9 ± 4[x]	12 ± 4[x]

[1] Cysts in roots only
[2] Cysts in roots and soil
Across the rows, values with the same superscript letter are not significantly different ($p < 0.05$)
SDW = sterile distilled water. Number of replicates = 8

3.4 DISCUSSION

A. thaliana was not susceptible to *H. trifolii* under the conditions used in these experiments, even though it has been reported as susceptible by European researchers (Sijmons *et al.*, 1991). In 1997, *H. trifolii* was recorded in soil collected from fields of sugar beet, *Beta patellaris*, in Italy, and was described as 'a host race of *H. trifolii* reproducing on beet' designated *H. trifolii forma specialis beta* (Maas *et al.*, 1982; Ambrogiani *et al.*, 1999). Also, *H. trifolii* has commonly been found in the Netherlands on sugar beet (Maas and Hejibroek, 1982; Maas *et al.*, 1982). It may be that the *H. trifolii* recorded by Sijmons *et al.* (1991) as reproducing on *Arabidopsis,* which like sugar beet is a brassica, has adapted to these hosts in Europe. However, it may also be that the *H. trifolii* recorded by Sijmons *et al.* (1991) was, in fact the *H. trifolii* referred to above, (Maas *et al.*, 1982). Alternatively, the conditions of infestation described by Sijmons *et al.* (1991) were quite different from the potting mix which was used in this project. Furthermore, the *H. trifolii* used in the assays described here was isolated from white clover in Victoria and

may not be adapted to brassica hosts, and for this reason would not develop in *Arabidopsis*.

Therefore, in this project, a bioassay was developed using white clover seedlings and two chemicals, BTH and SA, which have been reported as inducers of resistance to a variety of plant pathogens. Tests of the phytotoxicity of the two chemicals initially were carried out to establish a maximum level of treatment on white clover. No toxic effects were noted in the white clover seedlings, at any of the concentrations tested. Direct effects of SA and BTH on *H. trifolii* were not tested *in vitro* or in the soil, but the fact that there was no significant difference between the numbers of nematodes which penetrated and formed cysts in treated and control plants, indicated that there was not a direct, antagonistic effect of either chemical on the nematodes in the soil. The possibility still remains, however, that the chemicals could have had a delayed effect on the reproductive capability of the nematodes, manifest once they had entered the host plant. This effect would, however, be minimal as the half-life of BTH at 2 ppm in loamy soil is reported to be less than 1 d, when it is degraded to its corresponding acid which, in turn, degrades in 16.5 d (Novartis, 1997). Nematodes in this bioassay were applied at least one full day after application of the BTH, and took about four to five weeks to become adults, similar to the length of time to reach maturity in white clover reported by Mercer (1990).

In early experiments to test the effect of BTH and SA on white clover infested with *H. trifolii*, no consistent differences were found between the size of cysts developing in soil associated with chemically-treated plants, compared to the size of those in soil from water-treated controls. It was expected that not only would there be a difference in numbers of cysts between treated and control plants, but also that the size of cysts would be correlated with a difference in fecundity or egg numbers, but, instead the percentage of distorted cysts of *H. trifolii* on roots was significantly greater in chemically-treated, inoculated white clover roots than in plants treated with water and inoculated with nematodes. These distorted cysts were similar in appearance to those found on sugar beet which had been genetically transformed to be resistant to the sugar beet cyst nematode, *H. schachtii* (Cai *et al.*, 1997). Moreover, the percentage of cysts of *H. trifolii,* in soil, containing fewer than 50 eggs was significantly greater than the percentage in soil associated with control plants,

where cysts typically contained more than 100 eggs. The finding by Grant *et al.* (1996) and Mercer & Grant (1993), of reduced fecundity of this nematode in resistant cultivars of white clover, was thus corroborated. Grant *et al.* (1996) had also found no difference in the size of cysts, nor in the proportion of nematodes entering the white clover plant roots. The two phenomena, distorted cysts and fewer eggs per cyst, were combined in the data descriptors as percent abnormal cysts and became the criterion of induced resistance in white clover to the clover cyst nematode in these experiments. It became clear that, in any assessment of response to the chemical treatments, it was important to examine both the cysts in the soil and those stained in the roots. The lengthy process of assessment of the bioassay, coupled with the technical difficulties of obtaining large numbers of inoculant nematodes, necessitated the relatively low replicate number of eight plants per treatment in the experiments described in this thesis.

Chemical induction of resistance by SA was equivalent to that induced by BTH, compared to water-treated controls. Furthermore, there was no significant difference between the induced response to BTH in 'Haifa', and that in 'Grasslands Huia'. Since the latter is reported (Mercer *et al.*, 1992) to be more susceptible to *H. trifolii* than the relatively resistant 'Haifa', it was decided to test 'Grasslands Huia' only in subsequent experiments, so as to eliminate any putative inherent resistance effect in 'Haifa'. There was no difference in the response to chemical treatments between the smaller 50 ml-well arena and the 150 ml PVC tube arena, thus the latter was adopted for all subsequent experiments. Moreover, the PVC tubes served to isolate each experimental unit, as the bottom of each tube was encased in a small plastic bag.

The results of this study indicated that, in soil bioassays in a growth cabinet, white clover plants can be chemically induced, with either 50 μM BTH or 5 mM SA, to show resistance to the clover cyst nematode. This is the first report of induced resistance to nematodes in white clover, one of the first reports of chemically-induced resistance to nematodes and the first report of induced resistance in roots of white clover to *H. trifolii*.

The use of Bion WG50® (BTH) in Europe, to induce resistance to powdery mildew on wheat in the field (Gorlach *et al.*, 1996), has indicated the potential of BTH as an inducer of resistance to fungal pathogens on a commercial scale in the field. This report suggests it

may have potential also as an inducer of resistance against the clover cyst nematode in white clover. In the past few years, there have been numerous trials in the USA of BTH in approximately fifty different crops, and against bacterial, viral and fungal pathogens (Friedrich *et al.*, 1996, Lawton *et al.*, 1996, Kessmann *et al.*, 1994), prior to its registration as a control treatment to be known as 'Actigard'. Those trials have revealed the wide range of efficacy and relative lack of phytotoxicity of this chemical as a foliar spray (Novartis, 1997). There are reports that BTH is taken up and translocated throughout the plant and induces the accumulation of chitinase, a PR protein (Smith-Becker *et al.*, 1998).

The finding of chemically-induced resistance in white clover, and the development of an indicative soil bioassay, was followed in this project by tests of the effect of putative biological inducers of resistance, using BTH-treated and nematode-inoculated plants as positive controls.

CHAPTER FOUR

SELECTION OF BACTERIA FOR TESTS OF INDUCED RESISTANCE

4.1 INTRODUCTION

The use of nematicides to control clover cyst nematodes in pastures is uneconomic, and alternative approaches are being investigated in New Zealand. These include biological control with fungi which parasitise cysts and eggs of *H. trifolii* (Hay and Skipp, 1993; Hay, 1993), and breeding for resistance in *T. repens* (Mercer *et al.*, 1992; Mercer & Grant, 1993; Grant *et al.*, 1996). However, the use of cultivars resistant to a specific pathogen carries with it the risk of selecting for resistance-breaking pathotypes (see Section **1.2.5**). As only one migratory stage of *H. trifolii* occurs in the soil, the second-stage juveniles (Mulvey and Anderson, 1974; Wouts, 1978; Heinrich *et al.*, 1998) other options for biological control in the soil and rhizosphere are limited.

However, the use of the natural defence systems of plants is considered to be a viable means of biological control (Kuc, 1990; Kloepper *et al.*, 1992a; Deverall, 1995; Sticher *et al.*, 1997). The potential of rhizosphere bacteria as inducers of resistance, for biological control of various plant pathogens has been suggested and is being actively investigated (see Section **1.2.7.2**). Rhizosphere *Pseudomonas* strains and species are reported to induce resistance to bacterial, fungal and viral plant pathogens in a range of crop species (Maurhofer *et al.*, 1994; Liu *et al.* 1995a; Liu *et al.* 1995b), and to pest insects feeding on plants (Zehnder *et al.* 1997). Kloepper *et al.* (1992b) reported antagonism of rhizobacteria, including *Pseudomonas* strains, against the plant-parasitic root-knot nematode, *M. incognita* and the soybean cyst nematode, *H. glycines*, in pot trials. Oostendorp and Sikora (1989) observed the antagonistic activity of rhizosphere fluorescent pseudomonads against *H. schachtii* in both greenhouse and field trials. Moreover, bacterial endophytes in the roots of cotton plants have been shown to control *Fusarium* wilt (Chen *et al.*, 1995), and in potato tubers have been shown to improve resistance to *Fusarium* spp. and *Phytopthora*

infestans (Sturz *et al.,* 1999). Hallmann *et al.* (1997) found that bacterial endophytes in cotton roots reduced the incidence of the root-knot nematode, *M. incognita*. However, until now, neither endophytic nor rhizosphere fluorescent *Pseudomonas* strains have been tested as biocontrol agents for the induction of resistance or antagonism against the clover cyst nematode, *H. trifolii,* in white clover.

The first part of this project established that resistance to *H. trifolii* in white clover could be chemically induced with both BTH and SA (see Chapter Three). The second part of the project investigated the ability of fluorescent *Pseudomonas* strains P29 and P80 and a *Bacillus* strain B1, isolated from the soil and rhizosphere of white clover, to induce resistance to *H. trifolii* in two cultivars of *T. repens*, 'Haifa' and 'Grasslands Huia'.

4.2 MATERIALS AND METHODS

4.2.1 Further selection of *Pseudomonas* strains

Soil samples had been taken from both the rhizosphere and the surrounding soil, as outlined previously (Section **2.2.2**) from sites in five locations known to be infested with *H. trifolii* or other plant-parasitic nematodes (McLeish *et al.*, 1997). A total of 89 strains of fluorescent *Pseudomonas* was isolated from the soil samples using selective media (Simon and Ridge, 1974), and stored at -80 °C until tested (Sections **2.2.4 - 2.2.10**). The bacteria were further characterized on the basis of colony type on tetrazolium chloride (TZC) agar prepared after the method of Kelman (1954). The 89 strains were grown overnight in NB, as in Section **2.2.4**, streaked onto TZC agar in 90 mm Petri dishes, and incubated for 48 h at 32°C. Four distinct colony types grew on TZC agar, and one strain from each colony type was selected at random and tested for the ability to break down pectin in crystal violet pectate (CVP) agar (Cuppels and Kelman, 1974).

4.2.2 Determination of a growth curve

A growth curve was determined for one of the pectinolytic *Pseudomonas* strains, P1, of the same colony type as P29 (See Table 4.1A). The strain was inoculated into NB in a series of

McCartney bottles, shaken at 30°C, and sub-samples of 1 µl were taken every 2 h, serially diluted, and the CFU/ml determined by the drop-plate method (Miles and Misra, 1938). Additionally, at each 2-h time point, a 5 ml aliquot was taken and its optical density (O.D.) was determined on a Turner spectrophotometer at 550 nm wavelength. Sampling continued until two successive O.D. readings remained the same. The CFU/ml readings were checked 24 h later to ascertain that these also remained the same. A growth curve was determined from these data.

4.2.3 Further characterisation of *Pseudomonas* strain P29

After selection of the pectinolytic strain P29, biochemical tests were conducted using the Biolog Microplate® system (Biolog, Hayward, USA). P29 was grown overnight at 28°C on Tryptic Soy Agar (TSA). A single colony was streaked onto TSA and incubated for 18 h at 28°C. Using a sterile cotton bud, gently rolled over the surface of the colonies, this subculture was emulsified in approximately 20 ml sterile 0.85% saline. The concentration (CFU/ml) was adjusted to 3 x 10^8 CFU/ml, using the optical density at 550 nm as a correlative gauge, and the test wells in 2 plates were inocluated with the bacterial suspension and incubated at 28°C, according to the protocol of the manufacturers. The plates were read in a microplate reader at 6 h, 24 h and 48 h and the biochemical responses compared to the Biolog database of bacterial species in the MicroLog computer program MR3. A second set of 2 plates was inoculated with a bacterial suspension which was 20 times more dilute. This is recommended by the manufacturers for bacteria with surface polysaccharides which may be secreted into the medium.

4.2.4 Test of plant growth-promoting effect of strain P29 on white clover plants

Seedlings of white clover were treated at 3 - 4 weeks after germination with 1 ml of a NB culture of strain P29 or with SDW, and grown in the growth cabinet at 19°C, as described in Chapter Three. After 52 d, the above-ground biomass was determined as fresh weight and a comparison was made between the P29-treated and SDW-treated plants.

4.2.5 Test of growth-promoting effect of strain P29 on seeds of white clover

Seeds of *T. repens* 'Grasslands Huia' were surface-sterilised, mixed with *Rhizobium* inoculum and placed on 1% water agar in Petri dishes, as in Chapter Three. When the roots began to develop, 1 d later, each seedling was treated with one drop (approximately 0.1 ml) of P29 culture, and the plates re-sealed to retain moisture. A second set of three Petri plates with untreated seeds were set up at the same time. There were three Petri plates per treatment, each containing five or six germinating seeds. The root lengths were determined at 5 and 7 d after treatment, and a comparison made between the measurements of treated and untreated seedlings. A comparison was also made of the breadth of the leaves 7 d after treatment.

A second test on seeds of 'Grasslands Huia' compared the effect of a drop of strain P29 with the effect of a drop of SDW as a control, on each seed. The aim was to compare the effect of moisture alone, with that of strain P29, on growth of the seeds. The roots were measured in this experiment at 4 and 5 d after treatment, and both the root length and the breadth of the leaves were determined at 8 d.

4.2.6 Effect of cell-free culture filtrate of strain P29 on infective juveniles of *H. trifolii*, *in vitro*

Stock cultures of strain P29 were inoculated into fresh NB and grown overnight on a shaker at 30°C. In a laminar flow hood, the bacterial cells were removed by passing the NB cultures through a 0.45 µm filter (Millipore®). The filtrate was stored at 4°C and used within 24 h. The nematodes were isolated from white clover plants grown in the greenhouse (Section **2.2.11**), surface-sterilised, according to the method of Hay (1994), and then concentrated. Suspensions were 'bubbled' with an aquarium aerator for 10 s just before observations were made at each time interval. For controls, nematodes were suspended in sterile tap water (STW). Two experiments were carried out to test for an *in vitro* antagonistic effect of the culture filtrate, at room temperature without shaking.

In an initial experiment, the culture filtrate was tested in the proportion of 2 ml filtrate to 1 ml suspension of 200 infective juveniles in STW, in 90 mm Petri dishes. For this experiment, observations were made at time zero, and 1-, 3-, 5-, 7-, 24-, 26- and 30-h. In a second experiment, the filtrate was tested in two different ratios: 6 ml filtrate to 1 ml, and

9 ml filtrate to 1 ml, nematode suspension of 1,000 infective juveniles in STW. In this experiment observations were made at time zero, 3-, 21-, 24-, and 48-h.
Sub-samples of 1 ml were taken at each of these time intervals, and examined in a modified Doncaster counting dish (Doncaster, 1962) under a dissecting microscope. The number of juveniles which were inactive, and the total number of juveniles per ml, were recorded for each time, and the proportion of juveniles which were inactive was determined as a cumulative percentage at 30 h and 48 h, respectively, for the two experiments.

4.2.7 Preparation of bacterial inoculum for testing *in planta* against *H. trifolii*

Two pectinolytic *Pseudomonas* strains (P29 and P80) and a non-pectinolytic strain (P37) from the cryopreserved cultures were cultured in NB overnight at 30°C, as before. A further two bacterial strains, *Pseudomonas putida* strain 879 from wheat rhizosphere, which suppresses *Pythium*, *Rhizoctonia* and *Gaeumannomyces graminis* var. *tritici* (*G.g.t.*) in wheat (Maarten Ryder, pers. comm.), and *B. cereus* (B1), which was isolated from soil in white clover pasture (Sections **2.2.7 - 2.2.9**), were similarly prepared for testing against *H. trifolii* in *T. repens* plants. A single test with eight replicate white clover plants was conducted with *Pseudomonas* strains P80 and 879, and two tests of *Bacillus* strain B1, each with eight replicate plants. The inoculum density (CFU/ml), was determined by the drop-plate method (Miles and Misra, 1938). The optical density (O.D.) of each culture suspension was determined at 550 nm using a Turner spectrophotometer, before applying to the plants as a 1 ml culture suspension.

4.2.8 Test of viability of strain P29 bacterial cells in various media

The aim of this experiment was to test the optimal medium for application of bacterial cells to the white clover plants. *Pseudomonas* strain P29 was grown overnight at 30°C on a shaker, in about 10 ml NB in each of three 125 ml baffled flasks. The bacterial cells were filtered from two of the cultures as described (**4.2.6**). One batch of cells was re-suspended in 10 ml SDW, and the other batch in 10 ml sterile Ringer's solution, each in a 125 ml flask. The third culture was tested as a 10 ml NB culture of the cells. The CFU/ml was

determined by the drop-plate method (Miles and Misra, 1938) for each of the cell suspensions at the 24 h, stationary phase, point of culture (time zero). The suspensions were left at room temperature (approximately 25°C) for 48 h and re-sampled and the CFU/ml again determined for each. Viability tests were repeated once for each medium.

4.2.9 Preparation of live and dead bacterial cells from P29 NB cultures

Bacterial cells were filtered from an overnight NB culture of strain P29 as above (see Section **4.2.6**), washed well through the Millipore filter with two or three rinses of SDW and then washed from the Millipore filter membranes with SDW, re-suspended in SDW and the CFU/ml determined as before. To prepare dead cells, the bacteria were grown as above, then boiled for 3 min and examined microscopically (x1000) to check that the cell walls were intact. Aliquots of the boiled suspensions were spread (1 ml) onto nutrient agar plates and incubated overnight at 30°C to check that all cells had been killed.

4.2.10 Treatment protocol *in planta*

Three to four week-old white clover plants were aseptically inoculated with bacteria (*Pseudomonas* strains P29, P80, P37, 879 or *Bacillus* strain B1) as 1 ml of culture suspension at concentrations between 10^5 and 10^6 CFU/ml NB. Bacteria were applied to the soil surface above the seedling roots using a Gilson® 1 ml pipettor, as for the application of chemical inducers (see Section **3.2.7**). Control plants were treated with 1 ml sterile NB or SDW. Culture filtrate was obtained as described (see Section **4.2.6**) and, after storage for 24 h at 4°C, was tested *in planta* as a 1 ml soil drench for its effect against *H. trifolii* cysts in white clover seedlings. Live and dead bacterial cells prepared as above (see Section **4.2.6**) were applied as 1 ml suspensions in SDW, and culture filtrate as a 1 ml drench to the soil. All experiments included a separate treatment with 2 ml BTH at 50 µM for comparison of the response.

Surface-sterilised infective juveniles of *H. trifolii* were prepared as for the chemical treatment experiments (see Sections **3.2.9; 3.2.10**), and inoculated onto the soil above the roots from 1 to 3 d after treatment with the bacteria. The plants were assessed as in Section **3.2.11**, for response to treatment, 4 weeks after nematode inoculation.

4.2.11 Statistical analysis

The data for the experiments described in this Chapter are presented as the mean percentage and standard error of the mean. The difference between the means was analysed by non-parametric analysis of variance (ANOVA), a rank test, and the superscript letters denote statistical similarity or dissimilarity, ($p < 0.05$).

4.3 RESULTS

4.3.1 Selection of colony types of fluorescent *Pseudomonas* strains

There were four distinct colony types detected on the TZC agar plates, characterised respectively as "pale beige matt", "pink matt", "shiny red smooth" and "red matt". Of these four colony types, the "pink matt" and "shiny red smooth" colony types were pectinolytic, and the "pale beige matt" and "red matt" types were not (Table 4.1A).

4.3.2 Distribution of colony types within and between the sampling sites

Further examination of the sampling data showed that the pectinolytic and non-pectinolytic pseudomonads were found in both soil and rhizosphere samples in approximately equal proportions (Table 4.1B). The weighted proportion of pseudomonad samples from each site which were pectinolytic were: 31%; 26%; 34% and 31%, from Flaxley, Ponde and Monteith, Timboon and Ellinbank respectively, effectively showing no difference in the incidence of pectinolytic pseudomonads between the sites. The distribution among the sites, of bacteria classified according to colony type (Tables 4.1A and 4.1B), indicates a predominance of red and pink matt and shiny red smooth colonies overall, with proportionately fewer pale beige matt colonies isolated from Timboon and Ellinbank compared to Flaxley, Ponde and Monteith. There appeared to be neither a positive nor negative correlation of the incidence of clover cyst nematodes and that of pectinolytic pseudomonads among the sites sampled.

4.3.3 Determination of a growth curve for a pectinolytic *Pseudomonas*, strain P1

The growth curve for the pectinolytic strain, P1, is shown in Figure 4.1, and indicates that maximum population density for the medium used (NB) was reached after 14 h at 30°C. The O.D. readings at 550 nm corresponded well with the CFU/ml, and could be used subsequently as a preliminary check of population density, prior to applying bacterial treatments to white clover seedlings, and confirmed by checking CFU/ml after 24 h on NB agar plates.

Table 4.1A Characterization of colony types of 89 strains of rhizosphere fluorescent *Pseudomonas* on tetrazolium chloride agar

Colony type	Number of strains	Sample codes	Pectinolysis*
Red matt	31	P4, P10, P11, P13, P14, P15, P22, P34, P35, P39, P45, P47, P48, P50, P52, P54, P57, P58, P61, P63, P66, P69, P71, P72, P75, P76, P79, P82, P83, P84, P86	N
Pink matt	26	P1, P2, P3, P7, P12, P17, P29, P30, P32, P36, P38, P40, P41, P42, P43, P53, P59, P62, P68, P70, P77, P78, P85, P87, P88, P89	P
Pale beige matt	8	P8, P9, P18, P19, P25, P26, P27, P37	N
Shiny red smooth	24	P5, P6, P16, P20, P21, P23, P24, P28, P31, P33, P44, P46, P49, P51, P55, P56, P60, P64, P65, P67, P73, P74, P80, P81	P

N = non-pectinolytic, P = pectinolytic
*One strain taken at random from each colony type, tested for pectinolysis

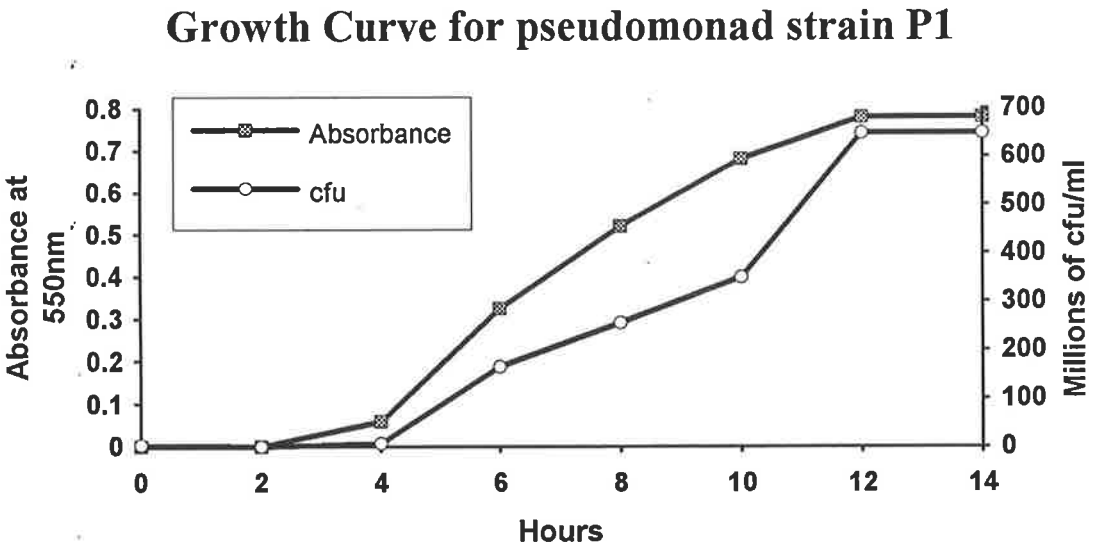

Figure 4.1 Growth curve for pectinolytic *Pseudomonas* strain P1, optical density at 550 nm plotted against CFU/ml (x 1 m) and time in hours.

Table 4.1B Distribution of colony types of pectinolytic and non-pectinolytic fluorescent pseudomonads within sampled area

	Number of colony types per site				
Colony type (P/N)*	Flaxley	Ponde & Monteith	Timboon	Ellinbank	Ratio of S : R#
Red matt N*	7	1	11	12	14S : 17R
Pink matt P*	7	1	10	8	13S : 13R
Pale beige matt N*	2	5	1	0	4S : 4R
Shiny red smooth P*	4	4	9	7	11S : 13R

* P = pectinolytic; N = nonpectinolytic
# S = soil; R = rhizosphere.

4.3.4 Biochemical tests of *Pseudomonas* strain P29

The results of the biochemical assay on the Biolog Microplate, indicating utilisation of a selection of 95 carbon sources, are shown in Table 4.1C. The table serves as a 'metabolic fingerprint' of P29. The MicroLog computer program MR3 is designed to compare the test results with a species database and indicate the species which most closely matches strain P29. The reading indicated the nearest species as *Enterobacter gergoviae* with a similarity index of 0.523. However, the Biolog Microplate system of identification is limited by the range of bacteria included in the MR3 program database. Moreover, comparison with Bergey's Manual of Systematics (Krieg, 1984) indicated a closer similarity to Group V *Pseudomonas* spp. commonly found in soil. Additional investigation to characterise strain P29 should include a test for oxidase, a GC FAME test and a microscopic examination involving staining of the flagella.

Table 4.1C Tables of carbon sources and optical density readings at 590 nm for 95 carbon sources for strain P29 in two replicate Biolog Microplates.

Table of Biolog Microplate carbon sources

	1	2	3	4	5	6	7	8	9	10	11	12
A	water	α-cyclodextrin	dextrin	glycogen	tween 40	tween 80	N-acetyl-D-galactosamine	N-acetyl-D-glucosamine	adonitol	L-arabinose	D-arabitol	cellobiose
B	i-erythritol	D-fructose	L-fucose	D-galactose	gentiobiose	α-D-lactose	m-inositol	α-D-lactose	lactulose	maltose	D-mannitol	D-mannose
C	D-mellibiose	β-methyl-D-glucoside	D-psicose	D-raffinose	L-rhamnose	D-sorbitol	sucrose	D-trehalose	turanose	xylitol	methyl pyruvate	mono-methyl succinate
D	acetic acid	cis-aconitic acid	citric acid	formic acid	D-galactonic acid lactone	D-galacturonic acid	D-gluconic acid	D-glucosaminic acid	D-glucuronic acid	α-hydroxy-butyric acid	β-hydroxy-butyric acid	χ-hydroxy-butyric acid
E	p-hydroxy phenyl-acetic acid	itaconic acid	α-keto butyric acid	α-keto glutaric acid	α-keto valeric acid	D,L-lactic acid	malonic acid	proprionic acid	quinic acid	D-saccharic acid	sebacic acid	succinic acid
F	bromo succinic acid	succinamic acid	glucuron-amide	alaninamide	D-alanine	L-alanine	L-alanyl-glycine	L-asparagine	L-aspartic acid	L-glutamic acid	glycyl-L-aspartic acid	glycyl-L-glutamic acid
G	L-histidine	hydroxy L-proline	L-leucine	L-ornithine	L-phenylalanine	L-proline	L-pyroglutamic acid	D-serine	L-serine	L-threonine	D,L-carnitine	χ-amino butyric acid
H	urocanic acid	inosine	uridine	thymidine	phenyl ethylamine	putrescine	2-amino ethanol	2,3-butanediol	glycerol	D,L-α-glycerol phosphate	glucose-1-phosphate	glucose-6-phosphate

Tables of 24 h O.D. readings at 590 nm

rep 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.000	0.117	0.974	0.254	0.422	0.432	1.753	1.824	0.306	1.548	0.305	1.512
B	0.087	0.658	0.656	1.531	0.960	0.562	0.519	1.320	0.358	1.517	1.083	1.776
C	1.532	1.627	0.438	1.136	1.186	1.573	0.519	1.522	0.394	0.284	1.821	0.904
D	0.234	1.727	1.236	0.562	1.523	1.560	1.608	0.350	1.463	-0.015	0.982	1.250
E	0.266	-0.091	-0.092	0.877	0.010	1.555	1.507	0.609	1.440	1.897	0.296	1.346
F	0.534	-0.076	0.148	0.072	0.652	0.784	0.327	1.915	1.930	1.952	0.647	0.600
G	1.263	1.988	0.152	0.396	0.259	1.453	0.736	1.507	0.934	0.433	0.610	2.084
H	0.530	0.941	1.781	0.596	0.645	1.618	0.422	0.415	1.440	1.227	1.583	1.710

rep 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.000	0.094	1.005	0.243	0.452	0.478	1.647	2.017	0.198	1.218	0.196	1.404
B	0.079	0.696	0.487	1.268	0.923	0.610	0.485	1.333	0.175	1.226	0.737	1.607
C	1.287	1.821	0.534	0.774	1.157	1.347	1.577	1.350	0.268	0.091	1.321	0.855
D	0.432	1.402	1.402	0.542	1.484	1.841	1.750	0.003	1.798	0.023	0.791	1.935
E	0.279	-0.041	-0.050	1.065	-0.008	1.627	0.136	0.402	1.564	1.738	-0.044	0.923
F	0.384	0.001	0.060	0.042	0.668	0.678	0.666	1.715	2.259	1.780	0.473	0.184
G	1.262	2.310	0.161	0.283	-0.003	1.292	1.079	1.651	0.737	0.030	0.600	1.895
H	0.396	0.922	1.803	0.519	0.630	1.315	0.348	0.070	1.582	0.901	1.650	1.803

4.3.5 Effect of strain P29 on plants and seeds of white clover

Tests of the growth-promoting effect of strain P29 on seedlings of white clover showed no significant difference between the above-ground biomass of white clover treated with P29 or SDW, after 52 d in a growth cabinet at 20°C (Table 4.2A).

Treatment of germinated seeds with P29 resulted in a significant increase ($p < 0.05$) in root length at days 5 and 7, and in leaf breadth at day 7, compared to untreated seeds (Table 4.2B). When this experiment was repeated with an equal volume of SDW on the control seeds, there was no significant difference between root lengths of plants with the two treatments at days 4, 5 and 8, nor in leaf breadths at day 8 (Table 4.2C). It was evident that treatment with SDW alone induced an effect on early growth similar to treatment with strain P29 of germinating seeds, or it may have been ineffective because of the low number of CFU delivered by the drop of bacterial culture

Table 4.2A Comparison of above-ground biomass of white clover plants treated with P29 or SDW, 52 d after treatment

	Above-ground weight (mg) at 52 d after treatment
Treatment	Mean ± standard error (n = 8)
P29	2.63 ± 0.10^{a}
SDW	2.84 ± 0.14^{a}

SDW = sterile distilled water; Number of replicates = 8
Values with the same superscript letter are not significantly different ($p < 0.05$)

4.3.6 *In vitro* effect of cell-free filtrate of strain P29 on infective juveniles of *H. trifolii*

In the first *in vitro* experiment, there was not a significant increase in the proportion of inactive nematodes, compared to those exposed to NB or sterile tap water, after 30 h exposure to a cell-free filtrate from a P29 culture, in the proportion 2 ml filtrate to 1 ml nematodes (Table 4.3). At the higher concentrations of 6 ml and 9 ml of filtrate to 1 ml nematode suspension, there was a significantly higher proportion of inactive nematodes

after 48 h, compared to the controls in STW. There was, however, no significant dose effect between the two levels of filtrate in this second experiment (Table 4.3).

Table 4.2B Comparison of root length and leaf breadth at 5 d and 7 d after treatment of seeds of white clover with P29

	Root length at 5 d (mm)	Root length at 7 d (mm)	Leaf breadth at 7 d (mm)
Treatment	Mean ± standard error (n = 17)		
P29	12.24 ± 0.54[a]	13.06 ± 0.6[a]	4.03 ± 0.22[a]
No treatment	9.41 ± 0.76[b]	10.00 ± 0.79[b]	2.26 ± 0.20[b]

No treatment = seeds treated with *Rhizobium* only, (P29-treated seeds with *Rhizobium* plus P29)
Within columns, values with the same superscript letter are not significantly different ($p < 0.05$)

Table 4.2C Comparison of root length and leaf breadth at 4 d, 5 d, and 8 d after seeds treated with P29 or SDW

	Root length at 4 d.	Root length at 5 d.	Root length at 8 d.	Leaf breadth at 8 d.
Treatment	Mean ± standard error (mm) (n = 18)			
P29	11.61 ± 1.04[a]	12.11 ± 1.19[a]	15.72 ± 1.35[a]	2.08 ± 0.17[a]
SDW	10.44 ± 1.09[a]	11.94 ± 1.16[a]	14.83 ± 1.48[a]	1.75 ± 0.11[a]

Within columns, values with the same superscript letter are not significantly different ($p < 0.05$)

4.3.7 Effect of NB cultures of pectinolytic strains P29 and P80 on *H. trifolii* in white clover, 'Haifa' and 'Grasslands Huia'

In an initial experiment, a pectinolytic *Pseudomonas* bacterium, strain P29, was found to induce a response similar to that to the chemical inducer, BTH. Treatments with a nutrient broth culture of strain P29 at a concentration of $> 1 \times 10^5$, or an aqueous solution of BTH at 50 μM concentration, applied as a 2 ml soil drench, increased the proportion of abnormal cysts in both 'Haifa' and 'Grasslands Huia' white clover inoculated with *H. trifolii* (Table 4.4), with no cultivar effect. The response to both treatments, an average of

experiments I and II, of 80% abnormal cysts for 'Grasslands Huia' and 65% abnormal cysts for 'Haifa', was significantly greater ($p < 0.05$) than the proportion of abnormal cysts in response to treatment with SDW, which were 41% and 21% for 'Grasslands Huia' and 'Haifa', respectively. A single bioassay using a second pectinolytic strain, P80, with a "shiny red smooth" colony form, and 'Grasslands Huia', yielded a mean of 80% ± 5% (s.e.) abnormal cysts (n = 8), compared to a mean of 70% ± 5% (s.e.) for BTH treatment (n = 5), and a mean of 38% ± 5% (s.e.) abnormal cysts for NB treatment (n = 4), a response similar to that obtained with strain P29. In both experiments, there was no significant difference between the total number of cysts developing in the plants in response to all treatments.

Table 4.3 Effect of cell-free filtrate from a nutrient broth culture of *Pseudomonas* strain P29 on infective juveniles of *H. trifolii*, *in vitro*

Experiment I	Filtrate @ 2:1	NB	STW
% juveniles inactive @ 30 h	35.1 n.s.	17.0 n.s.	7.5 n.s.
Cumulative % inactive @ 30 h	20.6 n.s.	22.4 n.s.	15.2 n.s.
Experiment II	**Filtrate @ 6:1**	**Filtrate @ 9:1**	**STW**
% juveniles inactive @ 48 h	65.2 n.s.	70.2 n.s.	10.7 s
Cumulative % inactive @ 48 h	41.2 n.s.	31.2 n.s.	16.7 s

STW = sterile tap water; NB = nutrient broth
n.s. = not significantly different; s = significantly different ($p < 0.05$)
Sub-samples of 1 ml examined at 1 - 2 h intervals for nematode activity, and cumulative % nematodes inactive recorded for 30 h and 48 h respectively in experiments I and II

4.3.8 Effect of an NB culture of non-pectinolytic *Pseudomonas* strain P37 on *H. trifolii* in white clover , 'Grasslands Huia'

In two experiments with cultivar 'Grasslands Huia', following treatment with the non-pectinolytic *Pseudomonas*, strain P37 (> 1 x 10^5 CFU/ml in NB), the proportion of abnormal cysts of *H. trifolii* was not significantly different ($p < 0.05$) from that in controls consisting of 1 ml NB in the first experiment or 1 ml SDW in the second experiment (Table 4.5). However, the average proportion of abnormal cysts in the two experiments was 75% in plants treated with NB cultures of strain P29 and 91% in plants treated with BTH, significantly greater ($p < 0.05$) than in plants treated with NB cultures of strain P37, which had a mean proportion of 22%. This lesser proportion of abnormal cysts in response to treatment with P37, is statistically comparable ($p < 0.05$) to that of *H. trifolii* in response to treatment with SDW or NB in control plants (Table 4.5). The total number of cysts developing in the white clover plants did not vary in a significant manner ($p < 0.05$) across treatments.

Table 4.4 Effect of pectinolytic *Pseudomonas* strain P29 on *H. trifolii* in two cultivars of white clover compared to BTH at 50 µM and water control

Treatment	P29		50 µM BTH		SDW	
	Abnormal cysts (%)	Total cysts	Abnormal cysts (%)	Total cysts	Abnormal cysts (%)	Total cysts
	Mean ± standard error					
'Grasslands Huia' (*n*=7)	84 ± 4^{a}	50 ± 5^{x}	75 ± 3^{a}	59 ± 8^{x}	41 ± 3^{b}	36 ± 9^{y}
'Haifa' (*n*=6)	73 ± 4^{a}	51 ± 7^{x}	56 ± 9^{a}	63 ± 9^{x}	21 ± 4^{b}	60 ± 6^{x}

Within columns, means with the same superscript letter are not significantly different ($p < 0.05$)

Table 4.5 Effect of a pectinolytic *Pseudomonas* strain, P29, and a non-pectinolytic *Pseudomonas* strain, P37, on *H. trifolii* cysts in white clover, compared to BTH or controls (SDW or NB)

Treatment	P29 (n = 8)	P37 (n = 8)	50 µM BTH (n = 5)	Control (n = 8)
Experiment I	Mean ± standard error			
% Abnormal cysts	75 ± 3^{a}	23 ± 4^{b}	-	28 ± 4^{b} (SDW)
Total cysts	36 ± 4^{x}	30 ± 7^{x}	-	17 ± 3^{y}
Experiment II	Mean ± standard error			
% Abnormal cysts	74 ± 7^{a}	21 ± 6^{b}	91 ± 4^{a}	31 ± 4^{b} (NB)
Total cysts	13 ± 3^{x}	24 ± 8^{y}	12 ± 2^{x}	18 ± 3^{xy}

Within rows, values with the same superscript letter are not significantly different ($p < 0.05$).

4.3.9 *In vitro* viability of strain P29 bacterial cells in SDW, NB and Ringer's solution

Prior to treatments with live bacterial cells, two experiments were carried out to determine the effect of different diluents on live cells of *Pseudomonas* strain P29 *in vitro*. In the first experiment, no significant difference was found between the initial and 48 h counts of cells suspended in either SDW or Ringer's solution. In the second experiment, there was a significant increase in cell numbers of P29 in SDW, but not in Ringer's solution (Table 4.6). There was a significant ($p < 0.05$) increase in CFU/ml in the controls in NB culture medium.

4.3.10 *In planta* effect of live and dead cells of strain P29 in SDW on *H. trifolii* in white clover

The total number of cysts of *H. trifolii* in the white clover seedlings did not differ significantly ($p < 0.05$) across treatments, within experiments. The number of cysts did vary between experiments where different batches of *H. trifolii* would have been used, perhaps due to differences in viability or infectivity of the juveniles. However, the

proportion of abnormal cysts formed in response to the various treatments was not significantly different ($p < 0.05$) between experiments.

Both live and dead cells of *Pseudomonas* strain P29 suspended in SDW induced the formation of a greater proportion of abnormal cysts of *H. trifolii* in white clover 'Grasslands Huia' (Table 4.7), than in water-treated controls. Treatment with live cells of P29 in SDW induced an average of 81% abnormal cysts, between experiments I and II, which was significantly greater ($p < 0.05$) than for the SDW, the control treatment. Similarly, treatment with dead cells in SDW induced an average (between experiments I and II) of 82% abnormal cysts, which was significantly greater than for the SDW, the control treatment, with 43%. Treatments with both live and dead cells resulted in the formation of a proportion of abnormal cysts not significantly different ($p < 0.05$) from those resulting from treatment with 50μM BTH, which yielded an average between experiments of 84% abnormal cysts.

Table 4.6 Viability of cells of *Pseudomonas* strain P29 in SDW, Ringer's solution and NB, *in vitro*

Treatment	SDW		Ringers solution		NB	
	Mean ± s.e. (CFU/ml)		Mean ± s.e. (CFU/ml)		Mean ± s.e. (CFU/ml)	
Experiment	24 h culture#	+ 48 h*	24 h culture#	+ 48 h*	24 h culture#	+ 48 h*
I	1.6×10^9	1.7×10^9	2.4×10^9	2.4×10^9	2.5×10^9	2.7×10^9
(n = 12)	$\pm 2.3 \times 10^7$	$\pm 1.1 \times 10^8$ n.s.	$\pm 2.3 \times 10^7$	$\pm 6.5 \times 10^7$ n.s.	$\pm 6.0 \times 10^7$	$\pm 5.8 \times 10^7$ s
II	1.0×10^9	2.0×10^9	1.6×10^9	1.8×10^9	1.5×10^9	2.0×10^9
(n = 12)	$\pm 5.6 \times 10^7$	$\pm 5.0 \times 10^7$ s	$\pm 2.9 \times 10^7$	$\pm 8.4 \times 10^7$ n.s.	$\pm 4.9 \times 10^7$	$\pm 8.9 \times 10^7$ s

#Counts made at time zero *Counts made after 48 h at room temperature, in normal daylight, not shaken
s = significantly different ($p < 0.05$) from time zero; n..s. = not significantly different ($p < 0.05$) from time zero; n = number of replicates

Table 4.7 Effect of live and dead cells from NB culture of *Pseudomonas* strain P29, suspended in SDW, compared with BTH, on cysts of *H. trifolii* in white clover

Treatment	Live cells	Dead cells	50 µM BTH	SDW
	Mean ± standard error			
Experiment I				
% Abnormal cysts	81 ± 4^{a}	78 ± 3^{a}	84 ± 3^{a}	41 ± 4^{b}
Total cysts	33 ± 4^{x}	38 ± 4^{x}	45 ± 6^{x}	34 ± 6^{x}
Experiment II				
% Abnormal cysts	80 ± 5^{a}	88 ± 4^{a}	84 ± 8^{a}	45 ± 8^{b}
Total cysts	13 ± 3^{x}	16 ± 3^{x}	28 ± 5^{x}	13 ± 3^{x}

Number of replicates = 8. Data presented are mean and standard error
Within rows, values with the same superscript letter are not significantly different ($p < 0.05$)

4.3.11 Effect of cell-free filtrate of strain P29 on *H. trifolii* in white clover

The response to treatment with cell-free culture filtrate of strain P29 did not differ significantly from the response to treatment with SDW in the first two experiments (Table 4.8). Treatment with 2 ml of 50 µM BTH, however, resulted in an average of abnormal cysts between the first two experiments of 82%, and this effect was significantly greater ($p < 0.05$), than the average proportion of abnormal cysts, 50% and 51%, from plants treated with SDW and strain P29 filtrate, respectively. In a third experiment, treatment with cell-free filtrate of strain P29 elicited a response in *H. trifolii* which was not significantly different from the response to BTH, which was somewhat lower in that experiment, nor to the control treated with NB. Within all three experiments, the total number of cysts of *H. trifolii* did not vary significantly between treatments (Table 4.8).

Table 4.8 Effect of cell-free filtrate of *Pseudomonas* strain P29 on *H. trifolii* cysts in white clover in three growth cabinet soil bioassays, compared to the effect of BTH and controls

Treatment	P29 filtrate	50 µM BTH	Control
Experiment I	Mean ± standard error		
Abnormal cysts (%)	52 ± 7^{b}	80 ± 6^{a} (n=6)	54 ± 10 (SDW)^{ab}
Total cysts	21 ± 3^{x}	30 ± 5^{x}	11 ± 2^{y}
Experiment II	Mean ± standard error		
Abnormal cysts (%)	50 ± 5^{b}	84 ± 5^{a}	45 ± 8 (SDW)^{b}
Total cysts	15 ± 2^{x}	28 ± 5^{x}	13 ± 3^{x}
Experiment III	Mean ± standard error		
Abnormal cysts (%)	64 ± 8^{a}	67 ± 9^{a} (n=7)	36 ± 5 (NB)^{ab}
Total cysts	14 ± 4^{x}	16 ± 3^{x}	18 ± 3^{x}

There were 8 replicates (n) per treatment, unless otherwise stated in the table
NB = nutrient broth, SDW = sterile distilled water
Within rows, values with the same superscript letter are not significantly different ($p < 0.05$)

4.3.12 Effect of *Bacillus cereus* strain B1 on *H. trifolii* in white clover

The response of *H. trifolii* in white clover to treatment with *Bacillus cereus* strain B1 was the development of a greater proportion of abnormal cysts compared to the response to SDW, and did not differ significantly from the response to P29 or to BTH. The total numbers of cysts developing in the white clover plants, however, did not differ significantly ($p < 0.05$) across all treatments (Table 4.9).

4.3.13 Comparison of the effect of *P. putida* strain 879 and *Pseudomonas* pectinolytic strain P80 on *H. trifolii* in white clover

There was no significant difference ($p < 0.05$) between the total numbers of cysts of *H. trifolii* which developed in plants treated with strain 879 (*P. putida*) isolated from the rhizosphere of wheat, and those treated with 2 ml BTH at 50µM, NB or with the pectinolytic *Pseudomonas* strain P80 isolated from the soil of a white clover pasture.

However, the proportion of abnormal *H. trifolii* cysts which developed in response to strain 879 was not significantly different from that formed in response to NB, but was significantly less ($p < 0.05$) than that formed in response to BTH. The proportion of abnormal cysts of *H. trifolii* formed in response to treatment with the pectinolytic strain P80 was not significantly different from that in response to BTH ($p < 0.05$) (Table 4.10).

Table 4.9 Effect of *Bacillus cereus* strain B1 on *H. trifolii* cysts in white clover, compared to the effect of *Pseudomonas* strain P29 or BTH or SDW

Treatment	*B. cereus* (1)* strain B1 (n = 8)	*B. cereus* (2)* strain B1 (n = 8)	*Pseudomonas* strain P29 (n = 8)	BTH (n=3)	SDW (n=3)
	Mean ± standard error				
% abnormal cysts	72.5 ± 7.9^{a}	61.0 ± 4.4^{a}	58.7 ± 10.0^{a}	56.5 ± 7.2^{a}	27.2 ± 8.9^{b}
Total cysts	16.9 ± 4.3^{x}	25.6 ± 4.7^{x}	19.3 ± 5.5^{x}	24.0 ± 5.5^{x}	18.0 ± 5.2^{x}

SDW = sterile distilled water; n = number of replicates
*(1), (2) two experiments with 8 replicates each
Within rows, values with the same superscript letter are not significantly different ($p < 0.05$)

Table 4.10 Effect of a pectinolytic *Pseudomonas* strain P80, and *P. putida* 879*, on cysts of *H. trifolii* in white clover, compared to the effect of BTH and NB

Treatment	P80 (n = 8)	879* (n = 8)	BTH (n = 5)	NB (n = 4)
	Mean ± standard error			
% abnormal cysts	80.4 ± 5.2^{a}	37.5. ± 3.7^{b}	70.1 ± 5.5^{a}	37.5 ± 4.7^{b}
Total cysts	17.4 ± 3.5^{x}	23.3 ± 4.1^{x}	20.2 ± 3.6^{x}	22.3 ± 5.2^{x}

n = number of replicates
Within rows, values with the same superscript letter are not significantly different ($p < 0.05$)
* 879 isolated from wheat rhizosphere, suppressive to *Pythium*, *Rhizoctonia*, and *G.g.t.* in wheat

4.4 DISCUSSION

This is the first report of induction of resistance to *H. trifolii* in white clover, in a growth cabinet soil bioassay, by a fluorescent *Pseudomonas* strain, isolated from the soil associated with nematode-infested white clover pastures. Application to clover roots, of nutrient broth cultures or washed live or dead bacterial cells, but not cell-free metabolites, of *Pseudomonas* strain P29, 1 - 3 d prior to inoculation with *H. trifolii,* caused an increase in the proportion of abnormal cysts in both roots and soil. Thus, resistance was manifested by a reduction in fecundity of the nematodes compared to water-treated controls. The presence of abnormal cysts and cysts with fewer eggs than normal have been used previously as criteria of resistance to clover cyst nematodes in white clover (Grant *et al.*, 1996). This resistant response to soil bacteria was equivalent to that reported in Chapter Three, after treatment of white clover plants with the chemical inducers, SA and BTH. There is experimental evidence that resistance induced by chemical means, for example, by BTH, acts through the pathway activated in pathogen-induced systemic acquired resistance (SAR) with the production of pathogenesis-related (PR) proteins (Lawton *et al.*, 1996; Friedrich *et al.*, 1996; Siegrist *et al.*, 1997). Resistance induced by nonpathogenic rhizobacteria, for which the term induced systemic resistance (ISR) was used by Kloepper *et al.*, 1992a, does not result in the production of PR proteins (Zdor and Anderson, 1992; Pieterse *et al.*, 1996; Van Loon, 1997), nor does it depend upon the endogenous production of salicylic acid (Knoester *et al.*, 1999). In the experiments described here, the manifestation of resistance induced by both chemical and biological means, was similar biologically, even though there is well-documented experimental evidence that the biochemical pathways are different.

Resistance was induced in both cultivars of *T. repens*, the relatively resistant, 'Haifa' and the susceptible 'Grasslands Huia' (Mercer *et al.*, 1992; Mercer & Grant, 1993). The latter was used in all subsequent experiments. Further characterisation, on tetrazolium chloride agar, of the 89 *Pseudomonas* strains selectively cultured from the soil samples, had indicated there were four colony types. Following reports of the inducing activity of endophytic rhizosphere bacteria (Mahaffee *et al.*, 1994; Hallmann *et al.*, 1997), it was

decided to select a pectinolytic *Pseudomonas* strain for initial screening, since it was considered that the ability to break down pectin may be a characteristic of an endophyte. A pectinolytic pseudomonad, strain P29, of colony type 'pink matt', induced a level of resistance equivalent to that induced by BTH. An additional pectinolytic strain, P80, which was a 'shiny red smooth' colony type, induced a similar level of response. In contrast a non-pectinolytic, fluorescent pseudomonad, strain P37, did not induce resistance. However, it appeared that pectinolysis was not a prerequisite for the induction of resistance, as the proportion of abnormal cysts of *H. trifolii* was similar in plants treated with suspensions of both live and dead cells in SDW, as well as with viable nutrient broth cultures of strain P29. Additionally, cell-free culture filtrate of strain P29, which may have contained pectinolytic enzymes, was not effective in inducing resistance. It would, therefore, appear that the cell-free metabolites were not involved in the induction of resistance.

An alternative hypothesis is that a 'recognition event' is involved, as described by Keen (1982) and De Wit (1997) in gene-for-gene host-parasite interactions, and by Sequeira and Gaard (1977) in relation to bacteria and host cell walls. This recognition event may be mediated by lectins, as has been suggested by Inbar and Chet (1997). It is here suggested that the recognition event itself acts as a trigger (Kloepper *et al.*, 1992) to initiate induction of the chain of events which leads to resistance against the clover cyst nematode, manifest only upon challenging the plant with *H. trifolii*.

There are reports of 'quorum sensing' in bacteria (Salmond *et al.*, 1995; Loper, 1996; Pierson *et al.*, 1998), thought to be mediated by homoserine lactones (Kaiser, 1999). Quorum sensing is defined by Swift *et al.* (1996) as 'a population density-dependent modulation of bacterial phenotype'. Raaijmakers *et al.*, (1998) indicated that a minimal population density, or concentration, of > 1 x 10^5 *Pseudomonas* bacterial cells per g of root was necessary for the induction of systemic resistance to *Fusarium* wilt. For this reason, in this project, test bacteria were applied at concentrations of greater than 1 x 10^5 CFU/ml, ensuring that the requirement was met for a minimum population density of the bacteria to allow change from a free-living to an endophytic mode. The bacteria used in these experiments were derived from cultures in stationary phase and hence they had

undergone a change in metabolism which may be reflected in cell surface changes. Such changes are known to be associated with metabolic changes in Gram negative bacteria (Costa *et al.*, 1999), such as *Pseudomonas*, but the mechanism may not be the same for Gram positive bacteria. Recent work by Reitz has indicated that lipopolysaccharides (LPS) extracted from the surface of a Gram negative bacterium *Rhizobium etli* were observed to induce resistance to the potato cyst nematode *G. pallida* (Reitz, pers. comm., 1999) and Van Loon *et al.* (1998) documented the induction of resistance to *Fusarium* wilt in radish and carnation by LPS extracted from fluorescent *Pseudomonas* sp. It is proposed that altered structures on the bacterial cell wall interact with root cell surface receptors (Hahn, 1996) such as lectins and that this is the recognition event referred to above which acts as a trigger to 'prime' the plant for induced resistance.

P. putida, strain 879, which was isolated from the rhizosphere of wheat and showed suppressive activity against *Pythium*, *Rhizoctonia* sp. and *G.g.t.* in laboratory and greenhouse tests (Maarten Ryder, pers. comm.), was not suppressive to the clover cyst nematode in our experiments. This is of interest as it is a bacterium from a microhabitat different from the rhizosphere of white clover, and is possibly not 'recognised' by the latter as a compatible endophyte. Alternatively, if its suppressive activity against *Rhizoctonia*, *Pythium* and *G.g.t.* is due to biochemical antagonism in the soil, it simply may not have the same effect on the clover cyst nematode. On the other hand, *B. cereus* strain B1 was isolated from the soil in a white clover pasture (see Chapter Two) and this strain showed the ability to induce resistance to the clover cyst nematode in the growth cabinet soil-based bioassay. The resistance induced was statistically comparable to that induced by chemical inducers, and by the biological agents tested, *Pseudomonas* strains P29 and P80. Further testing of this *Bacillus* strain would seem to be warranted.

Seed applications of both *Bacillus* and *Pseudomonas* endophytic bacteria have been found to induce resistance to nematodes in field crops (Hallmann *et al.*, 1997), and to root rot caused by *Pythium* sp. in wheat (Milus and Rothrock, 1997). Such technology would be the preferred method of application for further testing of *Pseudomonas* strains P29 and P80 in greenhouse and field trials. Furthermore, the use of endophytic bacteria as seed applications would have the advantage of using an agent in such a manner that rhizosphere

competence of the bacteria would not be a critical issue. Tuzun and Bent (1999) have emphasised that the performance of soil and rhizosphere bacteria is dependent upon a variety of abiotic factors, such as: soil structure, type, moisture and pH, as well as biotic factors such as competition from other soil and rhizosphere microbials. Further evaluation of these bacteria in greenhouse trials should include the use of pasteurised field soils of several different soil types. Field-plot trials should include a variety of soil types located in different microclimates. The distribution of soil and rhizosphere bacteria noted in this study, and reported in Chapter Two, may indicate the influence of both soil type and local microclimatic conditions on bacterial population dynamics. These factors would be an important consideration in a biocontrol strategy with any soil bacteria. The ultimate aim is to provide a viable alternative biological agent as an augmentative application in dairy pastures for the control of the clover cyst nematode. The findings reported from this project indicate that *Pseudomonas* strain P29 is one potential candidate for further investigation in white clover, with the ultimate aim of reducing the incidence of the clover cyst nematode in dairy pastures.

CHAPTER FIVE

INVESTIGATION OF RESISTANCE TO BLUE-GREEN APHIDS IN MEDICS AND WHITE CLOVER

5.1 INTRODUCTION

There are numerous reports that plants, in which a hypersensitive response to one pathogen has been induced by prior inoculation with a virulent or avirulent pathogenic organism, are then resistant to an unrelated pathogen (Dong *et al.*, 1991; Strobel *et al.*, 1996; Raupach & Kloepper, 1998). This phenomenon has been termed cross-resistance (Schuster *et al.*, 1995; Inbar *et al.*, 1998; Warren *et al.*, 1998) and has been observed to confer resistance across different taxa, even kingdoms, of pathogens (see Section **1.2.7**).

In 1995, the tight linkage between the tomato Mi gene for resistance to the nematode *Meloidogyne incognita* and the Meul gene for resistance to the potato aphid *Macrosiphum euphorbiae* was reported (Kaloshian *et al.*, 1995). Reports of resistance in tomato plants transformed with the Mi gene to both *M. incognita* and *M. euphorbiae* followed (Kaloshian *et al.*, 1997; Rossi *et al.*, 1998).

In the light of these reports, it was decided to investigate whether resistance to the clover cyst nematode, *H. trifolii*, induced by treatment with strain P29 or BTH, also conferred resistance to the blue-green aphid, *Acyrthosiphon kondoi*, which attacks the leaves and stems of *T. repens*. The investigation was also designed to address the question of whether the resistance, induced by a root drench with strain P29 or BTH, against a root-parasitic nematode, was systemic in nature and would confer resistance against a foliar pest, thus spatially separated from application of the inducing agent.

The blue-green aphid feeds on *T. repens* and *Medicago truncatula* Gaertn. var *truncatula*, a pasture medic commonly grown in South Australia, and is considered to be one of the causes of medic decline (Mathison, 1978). Dr. Andrew Lake, of SARDI, has run extensive tests on the resistance of cultivar 'Sephi 6297' (Reg. No B-9a-10) to *A. kondoi*. 'Sephi 6297' was developed by Quigley *et al.* (1983). Experiments reported in this chapter involved a

comparison between the activity of *A. kondoi* on *T. repens* 'Grasslands Huia', pre-treated with strain P29 or BTH, and SDW-treated control plants. It also compared the behaviour of *A. kondoi* on a susceptible medic cultivar *M. truncatula* 'Jemalong', with its behaviour on the resistant medic cultivar 'Sephi 6297'. The blue-green aphid had previously been observed to settle on the resistant cultivar 'Sephi 6297', start feeding, then to leave the plants, which then recovered from the attack, but on susceptible medic cultivars such as 'Jemalong', the blue-green aphid fed and reproducesd and the plants remained stunted (A. Lake, pers. comm.).

5.2 MATERIALS AND METHODS

5.2.1 Bioassay design

The blue-green aphids were cultured on 'Paravivo' Lucerne, *Medicago sativa* , by Mr. Steve Robinson in a greenhouse at 19 °C, with natural daylight. Seeds of *M. truncatula* 'Jemalong' and 'Sephi 6297', which had been stored at 4°C, were obtained from Mr. Robinson. *T. repens* seeds of the cultivar 'Grasslands Huia' were used for the white clover experiments. Both the white clover and the medic seeds were surface-sterilised, germinated and the plants were grown in a growth cabinet at 20°C, with a 12 hr photoperiod, as for the experiments with *H. trifolii* (see Section **3.2.5; 3.2.6**). After germination, the *T. repens* seedlings were transferred to sterilised Mount Compass soil, and the *M. truncatula* seedlings to a medic potting mix (described in Section **5.2.3**), in 150 ml plastic pots. The seedlings were watered *ad libidum* with sterile tap water or a sterile solution (600 mg/L) of Thrive® (Yates, SA) nutrients. The plants were treated with BTH, SDW or strain P29 at 3-4 wk, and later transferred to the greenhouse described above. The plants were exposed to aphids at times ranging from 1 to 10 days after treatment. Unless otherwise stated, there were eight replicates per treatment. The experimental design was a complete randomised block. A total of six experiments was set up with the blue-green aphids, four with white clover and two with medics, as described in Sections **5.2.2** to **5.2.4.**

5.2.2 Effect of BTH, strain P29, or SDW on blue-green aphids feeding on white clover

In the first aphid experiment, the white clover plants were transferred to soil in 150 ml plastic pots when approximately 2 weeks-old. They were treated at 3-4 weeks-old with 2 ml BTH or 1 ml of an overnight NB culture of strain P29 (at > 10^5 CFU/ml) or 1 ml SDW, as described in Sections **3.2.7** and **4.2.6**. Five days later the plants were transferred to the greenhouse and four to five aphids were released onto each plant. The aphids were a mixed population of all instars. Observations were made on the plants two to three times per week for up to 61 days after exposure to aphids. Notes were made on the presence of aphids and their exuviae as indications of reproduction and development. At 61 days, the leaves and stems of five of the plants were cut at approximately 2 mm above the crown of the root and the above-ground biomass was measured as fresh weight. The length of the longest stem of the five plants was measured. The leaves of the othert hree plants were frozen in liquid nitrogen and stored at - 80°C until lignin and callose estimations were made (see Chapter 6).

The protocol for the other three experiments involving aphids and white clover was as described for the first, with the timing of the exposure to aphids varied as described below.

In the second aphid experiment, the plants became infested with peach aphids after transfer to the greenhouse, and were gassed with Dichlorvos ® (BOC Gases, SA), a contact, non-persistent aphicide, and left for 2 days. Blue-green aphids were released onto the plants 5 days after the inducing treatments. No leaf samples were taken.

In the third aphid experiment, the plants were exposed to aphids 1 day after treatment with P29 or SDW. The plants were observed as before and aphid behaviour was noted up to 54 days after release of aphids. No leaf samples were taken.

The fourth aphid experiment included a BTH treatment as well as strain P29 and SDW and the plants were exposed to the aphids 6 days after treatment. There were seven replicates per treatment. Observations were made up to 55 days after exposure to aphids, and the above-ground biomass was measured for all plants.

5.2.3 Potting mix for medic culture

The following potting mix, developed for the greenhouse culture of medics at SARDI, was obtained from Mr. Robinson. To a mixture of: 150 L Mount Compass (coarse) grey sand, 150 L Gawler River (fine) white sand, and 20 L Perlite, was added the following nutrients: 35 g potash, 50 g ammonium nitrate, 100 g super phosphate, 35 g complete mineral mix, 150 g calcite (AgLime). This mix was sterilised by heating in an oven at 120°C for 12 h, and cooled.

5.2.4 The effect of strain P29, BTH or SDW on the feeding of blue-green aphids on medic cultivar 'Jemalong' compared to SDW-treated 'Sephi 6297'

The medic seeds were germinated as for the white clover seeds (see Section **5.2.1**). After 8 - 10 days the seedlings were transferred to medic potting mix (see Section **5.2.3**) in 150 ml square plastic pots. Eight, 2-week old 'Jemalong' medic seedlings were treated with strain P29 (at > 10^5 CFU/ml) or SDW. The eight 'Sephi 6297' replicates were treated with SDW. All plants were placed in the greenhouse at 19 °C, 7 days after treatment, for exposure to blue-green aphids. In this experiment, one of the 'Sephi 6297' plants, two of the P29-treated, and one of the SDW-treated 'Jemalong' plants died. Observations were continued on surviving plants for 54 days of aphid exposure.

In the second medic experiment the 'Jemalong' cultivar was treated with BTH, an overnight NB culture of strain P29 or SDW. The eight replicates of 'Sephi 6297' were treated with SDW. 'Jemalong' germinated poorly, so that five replicates per treatment of this cultivar were used. The plants were exposed to blue-green aphids in the greenhouse 10 days after treatment. Observations were made as before for up to 55 days, when above-ground biomass was measured for all plants.

5.2.5 Statistical analysis

The data from these experiments are presented as mean and standard error and were analysed by non-parametric analysis of variance of the mean using a non-parametric Wilcoxon ANOVA and Kruskal-Wallis Tests (Rank Sums).

5.3 RESULTS

5.3.1 Effect of P29, BTH or SDW on feeding of blue-green aphids on white clover

Blue-green aphids were still feeding and reproducing, on all plants, at the end of all four experiments on white clover.

In the first experiment, at day 61, there was a significant difference ($p < 0.05$) in both the above-ground biomass (fresh weight in mg) and the height (mm) of plants, in response to treatment with strain P29 and with SDW (Table 5.1). The P29-treated plants were both heavier and taller than the SDW-treated plants. There was also a significant difference ($p < 0.05$) between weight and height of BTH- treated plants and the SDW-treated controls. The difference between the height and biomass of P29- and BTH-treated plants was not significant ($p < 0.05$).

In the second experiment, in which plants were initially accidentally infested by peach aphids, and consequent treatment with Dichlorvos ®, there was no discernible difference between any of the treated plants after 47 days.

Table 5.1 Above-ground biomass and height of white clover plants exposed to blue-green aphid attack 5 days after treatment with P29 or BTH, compared to SDW-treated controls

	Fresh weight (mg)	Height (mm)
Treatment	Mean ± standard error	
BTH	1150 ± 150[a]	107 ± 13.4[a]
P29	1612 ± 200[a]	124 ± 12.1[a]
SDW	826 ± 260[b]	72 ± 13.2[b]

Number of replicates measured = 5; SDW = sterile distilled water
Within columns, values with the same superscript letter are not significantly different ($p < 0.05$)

In the third experiment, where the plants had been exposed to aphids less than 24 hr after treatment, there was no discernible difference between any of the treated plants, after 54 days of exposure to aphids.

In the fourth experiment, there was a significant difference ($p < 0.05$) in the above-ground biomass, between plants treated with BTH, and the SDW-treated controls, and between the P29-treated plants and the SDW-treated plants (Table 5.2).

Table 5.2 Above-ground biomass of white clover exposed to blue green aphid attack 6 days after treatment with BTH or P29, compared to SDW-treated controls

Treatment	BTH	P29	SDW
	Mean ± standard error		
Fresh weight (mg)	720 ± 220[a]	820 ± 260[a]	470 ± 90[b]

Number of replicates measured = 7; SDW = sterile distilled water
Values with the same superscript letter are not significantly different ($p < 0.05$)

5.3.2 Effect of BTH, P29 or SDW on feeding of blue-green aphids on medic cultivars 'Jemalong' and 'Sephi 6297'

In the first medic experiment, 'Jemalong' plants treated with P29 were smaller than 'Sephi 6297' plants by day 6 after release of aphids. Blue-green aphids were feeding and reproducing on all 'Jemalong'. By day 12, both SDW- and P29-treated 'Jemalong' plants appeared stunted compared to 'Sephi 6297'. At day 54, when the experiment was ended, there was no visual difference between the 'Jemalong' plants treated with P29 or with SDW. The 'Sephi 6297' plants were healthy and producing flowers and were largely free of aphids. The 'Jemalong' plants, by contrast, did not produce flowers during these experiments.

In the second medic experiment, 'Sephi 6297' plants were taller than the 'Jemalong' plants by day 6, and very few aphids remained on 'Sephi 6297' from day 18 onwards. 'Jemalong' plants treated with BTH or P29 were larger than the SDW-treated controls by day 6 after aphid release, but aphids were feeding and reproducing on all plants. At day 6 and until day 18, the SDW-treated plants were visually smaller than either the P29- or BTH-treated 'Jemalong'. On day 18, new growth began to appear on all 'Jemalong' plants and by day 25 there was no appreciable difference between the size of the 'Jemalong' plants from each of the three treatments. By day 39, the P29- and BTH-treated 'Jemalong' appeared to have

more leaves than the SDW-treated controls. Aphids continued to feed and reproduce on these plants. There was a significant difference between the above-ground biomass of the 'Jemalong' plants treated with P29, and those treated with BTH, compared to SDW-treated controls at day 55 (Table 5.3). 'Sephi 6297' plants were flowering and free of aphids, from day 33 onwards.

Table 5.3 Above-ground biomass of medic 'Jemalong' exposed to aphid attack 10 days after treatment with BTH, P29 and SDW, compared to medic 'Sephi 6297' treated with SDW

Cultivar	Jemalong[1]			Sephi 6297[2]
	Mean ± standard error			
Treatment	BTH	P29	SDW	SDW
Fresh weight (mg)	612 ± 190[a]	644 ± 80[a]	408 ± 30[b]	750 ± 110
Number of replicates	5	5	5	7

SDW = sterile distilled water
[1] cultivar susceptible to blue-green aphid; [2] cultivar resistant to blue-green aphid
Values with the same superscript letter are not significantly different ($p < 0.05$)

5.4 DISCUSSION

In the first experiment using white clover with blue-green aphids, the plants were treated 5 days before challenge. The values for both fresh weight biomass and height of the BTH-treated plants appeared to be intermediate between those of P29-treated plants and the SDW-treated controls. There was, however, no significant difference ($p < 0.05$) in those parameters between P29- and BTH-treated plants. Differences in size were evident by 54 days after challenge with the aphids and, thus, it was decided to assess subsequent experiments at around 54 days. Plants in the second experiment suffered an infestation by peach aphids and subsequent aphicide treatment; two of the P29-treated plants died and the rest looked weakened. After 47 days, there was no discernible difference between plants in the various treatments, and the experiment was terminated. In this experiment, release of the aphids was preceded by a Dichlorvos ® treatment and the plants took some time to

recover. It is possible that the Dichlorvos ® treatment interfered with the plant response to either or both the BTH- and the strain P29- treatments.

In the third experiment, the aphids were released less than 24 hours after the plants were treated with strain P29, BTH or SDW. There are reports that the timing of a pathogen challenge following treatment with an inducing agent is of critical importance (Hammerschmidt, 1993; Kessmann *et al.*, 1994; Dann *et al.*, 1996; Lawton *et al.*, 1996). Given that there was no evidence of a resistance response in this experiment, it would seem that the 'priming' of the white clover and medic plants, to a state of resistance to pest or parasite attack, takes at least 24 h. This timing effect was also found by Uknes *et al.* (1992) in studies of the effect of chemical induction of resistance to *Peronospera parasitica* with 2,6-dichloroisonicotinic acid in *A. thaliana*, and by Ward *et al.* (1991) who induced resistance in tobacco to tobacco mosaic virus with SA and with isonicotinic acid. In studies of the events following resistance-inducing treatment, Anderson *et al.* (1991) found that many changes at the molecular level occurred within the first 6 h after treatment of a suspension of bean cells with an elicitor derived from *Colletotrichum lindemuthianum*. Atkinson (1993) in a review of the HR-associated resistance in plants indicated there was a general consensus among plant pathologists that recognition of elicitors from pathogens by plant receptors occurs, but that at that time there was less consensus about recognition of non-host pathogens. Nevertheless, it is hypothesised that the molecular and biochemical events connected with, and following, initiation of induction by non-pathogens such as strain P29, necessitate a 'waiting period' before resistance to a pathogen challenge can be manifested.

In the fourth experiment, where the aphids were released onto the plants 6 d after treatment of the white clover plants with BTH, P29 or SDW, there was a significant difference ($p < 0.05$) between the biomass of plants treated with BTH or P29 and that of control plants treated with SDW. The above-ground biomass of BTH- or P29-treated plants was consistently greater.

In the white clover experiments with blue-green aphids, there was a growth differential between plants treated with strain P29 and those treated with BTH. This difference in response to BTH and P29 could have been due to a growth-promoting effect of strain P29.

Many soil and rhizosphere *Pseudomonas* strains have been found to promote growth (Sturz, 1995; Tuzun & Kloepper, 1995; Kim *et al.*, 1997). However, comparisons of the effect of strain P29 and SDW on the growth rate of white clover had been examined (see Chapter Four), and there was no evidence in the mature plants, 52 d after treatment, of greater growth due to P29 treatment, and the response of seeds to treatment with P29 or SDW did not show evidence of a growth-promoting effect.

In the first medic experiment, the resistant 'Sephi 6297' plants still had aphids on them at day 54, but were much taller than the 'Jemalong' plants which showed no visual difference between treatments. Only a visual assessment was made of this experiment.

In the second medic experiment, there appeared to be some early difference between treatments (day 6), and after new growth appeared (day 21) there was little visual difference between 'Jemalong' plants treated with BTH and those treated with P29, and only SDW-treated plants showed signs of loss of vigour due to aphid attack. The above-ground biomass of the P29- and BTH-treated 'Jemalong' plants was significantly ($p < 0.05$) greater at day 54 than that of the control plants treated with SDW.

The results of these experiments provided evidence for a systemic resistance response to treatment with strain P29 or BTH on both white clover and medics. The inducing agents were applied to the roots but the aphids fed on the above-ground parts of the plants. These results also provided evidence of the non-specificity of the induced response to P29 and BTH, in that the plants were resistant to pests or parasites from two different taxa, blue-green aphids and nematodes.

CHAPTER SIX

INVESTIGATION OF THE DEFENCE RESPONSE

6.1 INTRODUCTION

There are many reports in the literature of lignification as a means of defence in plants (Bell, 1981; De Leeuw, 1985; Collinge and Slusarenko, 1987) in both monocotyledonous (Ride, 1975; Maule *et al.*, 1976) and dicotyledonous plants (Vance *et al.*, 1980; Hammerschmidt and Kuc, 1982; Hammerschmidt, 1984; Dean & Kuc, 1987). There are also reports of increases in suberin and suberin-like substances produced as 'vascular coating' (Newcombe and Robb, 1988; Robb *et al.* 1989) in plants resistant to bacterial infections. Aist (1976), Vance *et al.* (1980), De Leeuw (1985), Cahill and McComb (1992) and Benhamou *et al.* (1996) all recorded histochemical and electron-microscopic evidence for an increase in callose and lignin in plants in response to phytopathogenic fungi. There are also reports of an increase in lignin in response to herbivory by insects (Karban and Myers, 1989). Southerton and Deverall (1990) found an increase in lignin during a hypersensitive response to the leaf rust fungus, *Puccinia recondita* f.sp. *tritici* in wheat, and Podile and Laxmi (1998) reported an increase in phenylalanine ammonia-lyase in a response to fusarial wilt in pigeonpea, induced by seed bacterisation with *Bacillus subtilis*. Jones and Northcote (1972) and Jones (1981) reported an increase in lignin in syncytial cell walls of cyst nematodes, and Fogain and Gowen (1996) found evidence of lignin and suberin as defence molecules against *Radopholus similis*, the burrowing nematode, in banana. While Sobczak (1996) did not find an increase in lignin or other phenolics in response to infection with the sugar beet cyst nematode, *H. schachtii*, Jones and Payne (1977) noted callose in the feeding sites of the sedentary nematode, *Nacobbus aberrans*, in tomato. Kobayashi *et al.* (1995), on the other hand, considered callose increase to be a basic plant defence response to any pathogen infection. There are reports of an increase in callose-like material in host plant roots in response to the endoparasitic cyst nematodes, *H. schachtii* and *H. glycines* (Grundler, 1997; 1998), and to the root-knot nematode, *M. hapla*

(Hallmann *et al.,* 1997). There is also evidence for the production of callose (Hussey *et al.*, 1992) in plants, as a response to infection with *Criconomella xenoplax*, an ectoparasitic nematode.

In this project, a possible increase in lignin and/or callose production was investigated in plants which had been induced with chemical or bacterial agents, and subsequently inoculated with infective *H. trifolii* and which were expressing resistance responses, such as reduced fecundity and a higher proportion of abnormal cysts in the nematode than in the SDW-treated controls.

In order to answer the question of whether the resistance observed was systemically induced or local, the roots of white clover plants were treated with *Pseudomonas* strain P29 or BTH, and the plants exposed to a foliar pest, *A. kondoi*. Subsequently, the response, in terms of lignin and callose content was measured.

6.2 MATERIALS AND METHODS

6.2.1 Preparation of leaf material for lignin estimation

White clover plants which were untreated and not inoculated, and white clover plants which had been treated with strain P29, SDW or BTH and subsequently inoculated with *H. trifolii* (as described in Sections **3.2.7** and **4.2.7**) were used for the estimation of lignin in leaves. Four weeks after the nematode inoculation of the treated plants, the stems and leaves from five plants were snap frozen in liquid nitrogen and stored at -80°C. The roots of all eight replicates of the treated plants were stained with lactoglycerol acid fuchsin (Bridge *et al.*, 1982) and examined for signs of induced resistance to the nematodes (as in Section **3.2.11).**

The following method for preparation was adapted from Iiama and Wallis (1990). The frozen leaves and stems of both treated and untreated plants were pulverised in a mortar with liquid nitrogen, washed with 80% ethanol into 50 ml volumetric flasks and placed on a shaker at 28°C, to remove the chlorophyll. They were rinsed several times over the course of about an hour until the supernatant was clear. The suspension was then filtered

using Whatman No. 42 filter paper in a filter funnel, and the residue on the filter paper was placed in 55 mm diameter Petri dishes and dried overnight at 40°C to remove the ethanol. These samples were stored at -80°C for up to 3 months, until the estimations were made.

6.2.2 Preparation of root material for lignin or callose estimation

Root material was taken from a soil bioassay experiment in which the white clover plants were treated with BTH, P29 or SDW, and subsequently inoculated with *H. trifolii* (as in Sections **3.2.7** and **4.2.9**), or treated with SDW but not inoculated with *H. trifolii*. There were eight replicate plants for each treatment as above, but the roots of three plants were examined for the presence of abnormal cysts, and the roots of the other five plants were used for the lignin and callose estimations. The roots were cut from the plants 4 weeks after inoculation of the nematodes, and the procedure for preparation was as for the leaves (Section **6.2.1**), except for the removal of chlorophyll. These samples were stored at -80°C for up to 3 months.

6.2.3 Lignin extraction from leaves and roots of white clover

The procedure used for estimation of lignin was after the method of Iiama and Wallis (1990). About 10 mg of the pulverised, frozen plant material (roots, or leaves and stems) was weighed out and placed in 10 ml reaction vials (Pierce, Rockford, Illinois) with PTFE-coated silicone caps. To this was added 2.5 ml of a 25% solution of acetyl bromide in 100% glacial acetic acid, and 0.1 ml of 70% perchloric acid. The reaction vials were sealed and placed in an oven at 70°C for 30 min, shaken once during that time, and then cooled in ice for at least 15 min. The contents of the vials were then transferred to 50 ml volumetric flasks containing 10 ml of 2M NaOH and 10 ml 100% glacial acetic acid. The vials were washed out with 100% glacial acetic acid, to make the total volume in each flask up to 50 ml. Blank solutions were made up in 50 ml volumetric flasks with 2.5 ml of 25% acetyl bromide in 100% glacial acetic acid, 0.1 ml 70% perchloric acid, 10 ml 2M NaOH, and approximately 37.5 ml acetic acid, to make the total volume to 50 ml.

6.2.4 Assay of lignin from leaves and roots of white clover

The estimation of lignin was done using a Varian spectrophotometer with a UV lamp at 280 nm wavelength. The blank solution (prepared as in **6.2.3**) was also used to rinse out the crystal cuvette (which had a light path of 10 mm) between each reading. There were two replicate samples per plant, and each replicate was assayed in the spectrophotometer twice. The absorbance of the blank solution was checked at frequent intervals.

6.2.5 Estimation of percent lignin in roots or leaves of white clover

Estimation of the percent lignin in samples was done as follows:

The absorbance value for lignin is 20.0 g/L/cm, which is the extinction coefficient for lignin. The formula used to estimate the percent lignin was $250 \times A/W$, where A is the absorbance at 280 nm, and W is the weight of the sample in milligrams.

The equation from which this formula is derived is: $A = ECL$,

where A is the absorbance at 280 nm, E is the extinction coefficient for lignin (as above), C is the concentration of the sample (W(mg)/50 ml or W/50 g/L), and L is the length of the light path of the spectrophotometer cell (10 mm).

[$E = A/C = A/W/50 = A \times 50/W = 50A/W$

% lignin $= E/E_0 \times 100 = 50A \times 100/W/20 = 250 \times A/W$]

6.2.6 Callose extraction from roots of white clover

The procedure for estimation of callose was based on the method of Kauss (1989). The frozen root material was pulverised in a mortar with a pestle, with liquid nitrogen, washed in 80% ethanol to remove any extraneous fluorescent material and air-dried in an incubator at 40°C overnight. A subsample of 10 mg was put into 5 ml 1N NaOH in plastic, capped centrifuge tubes, vortexed about 30 s and heated in an oven at 80°C for 15 min. The mixture was centrifuged at room temperature (about 25°C) at 380 G (approximately 1,000 rpm for 100 mm radial distance) for 5 min. The supernatant was used for the assay.

In plastic centrifuge tubes, 800 µl of a 0.1% (w/v) aqueous solution of aniline blue was added to a 400 µl aliquot of the alkali supernatant. The solution turned a violet-red colour. To this solution was added 420 µl of 1M glycine at pH 9.5. This was mixed well, by

vortexing for about 10 s. The solution was decolourised after incubation in a water bath at 50 °C for 20 min and a further 30 min at room temperature. Blanks were prepared for the assay with 400 µl 1N NaOH in place of the supernatant, 800 µl 0.1% aqueous solution of aniline blue, 420 µl 1M glycine at pH 9.5, and with the same treatment as the samples. The assay was done using a Turner-Sequoia (Sigma) fluorometer with wavelengths set at 356 nm excitation and 450 nm emission. The fluorometer was zeroed at frequent intervals with the blank solution.

The roots of plants treated with P29 or BTH, and subsequently inoculated with *H. trifolii*, were compared to control plants which had been treated with SDW and inoculated with *H. trifolii*. There were five plants per treatment and three treatments, and two replicates were assayed per plant, with two readings per replicate.

6.2.7 Statistical analysis

The data from these experiments are presented as mean and standard error and were analysed by non-parametric analysis of variance of the mean using a non-parametric Wilcoxon ANOVA and Kruskal-Wallis Tests (Rank Sums).

6.3 RESULTS

6.3.1 Lignin content of leaf and stem material from treated white clover, 4 weeks after inoculation with *H. trifolii*

Assays comparing the percentage of lignin present in the leaves and stems of white clover, indicated that there was no significant difference ($p < 0.05$) between the amount of lignin present in the leaves and stems of plants pre-treated with BTH or with *Pseudomonas* strain P29, at 4 weeks after inoculation with *H. trifolii* (Table 6.1). Moreover, there was no significant difference ($p < 0.05$) between the lignin content in the leaves and stems of treated and inoculated plants, and leaves and stems from plants which had been neither treated with inducing agents nor inoculated with *H. trifolii* (Table 6.1).

Table 6.1 Estimations of lignin (as % of dry matter) from leaves and stems of white clover pre-treated with P29 or BTH, at 4 weeks post-inoculation with infective juveniles of *H. trifolii*, compared to leaves from untreated, uninoculated plants

Treatment	P29 (n=10)	BTH (n=10)	Untreated (n=9)
	Mean % ± standard error		
% lignin	6.30 ± 0.35	6.84 ± 0.43	6.92 ± 0.23
Significance	n.s. (p = 0.17)	n.s. (p = 0.89)	n.s.^{x} (p = 0.42)

n = number of samples assayed (2 per plant)
Untreated = plants not treated or challenged with *H. trifolii*
n.s. = not significantly different from untreated plants
n.s. ^{x} = not significantly different from BTH- or P29-treated plants

6.3.2 Lignin content of root material from white clover, treated with BTH, P29 or SDW and inoculated with *H. trifolii*

Assays on root material from white clover, comparing the lignin content from plants treated with the chemical inducer, BTH, the biological inducer, strain P29, or control plants treated with SDW detected no significant difference ($p < 0.05$) between the percentages of lignin measured in the roots of plants in response to any of the treatments (Table 6.2).

Table 6.2 Estimations of lignin (as % of dry matter) from roots of white clover plants pre-treated with BTH, P29 or SDW, at 4 weeks after inoculation with infective juveniles of *H. trifolii*

Treatment	P29	BTH	SDW
	Mean % ± standard error (n = 10)		
% lignin	3.64 ± 0.25	4.55 ± 0.42	4.44 ± 0.42
Significance	n.s. (p = 0.12)	n.s. (p = 0.85)	n.s.^{x} (p = 0.19)

n = number of samples assayed (2 per plant)
n.s. = not significantly different from control plants with SDW ($p < 0.05$)
n.s.^{x} = not significantly different from BTH or P29 treated plants ($p < 0.05$)

6.3.3 Callose content of root material from white clover, 4 weeks after inoculation with *H. trifolii*

Assays on root material from plants treated with BTH, strain P29 or SDW, and inoculated with *H. trifolii* 3 d later, indicated that there was no statistically significant difference ($p < 0.05$) in the absorbance values of the extracted material, between the three treatments (Table 6.3).

6.3.4 Callose content of leaf and stem material from treated white clover, 54 d after exposure to blue-green aphid infestation

There was no significant difference ($p < 0.05$) between the absorbance values for material extracted from the leaves and stems from plants treated with BTH, strain P29 or SDW, and exposed to blue-green aphid attack 5 days later (Table 6.4).

Table 6.3 Absorbance values in callose assay for roots of white clover plants treated with BTH, P29, or SDW, 4 weeks post-inoculation with infective juveniles of *H. trifolii*

Treatment	P29	BTH	SDW
Absorbance	Mean ± standard error (n = 10)		
@ 365nm excitation, 455nm emission	122.40 ± 10.27	119.65 ± 10.82	129.8 ± 6.65
Significance	n.s.	n.s.	n.s.[x]

n = number of samples assayed (2 per plant)
n.s. = not statistically significantly different from SDW treated plants
n.s.[x] = not statistically significantly different from BTH or P29 treated plants

6.3.5 Lignin content of leaf and stem material from treated white clover, 54 d after exposure to blue-green aphids

After 54 days of exposure to *A. kondoi*, there was no significant difference ($p < 0.05$) between the percentage of lignin detected in leaves and stems taken from white clover which had been treated 5 d previously with BTH, strain P29 or SDW (Table 6.5).

Table 6.4 Absorbance values from callose assay for leaves and stems of white clover, treated with BTH or strain P29 and infested with a foliar pest, *A. kondoi*

Treatment	P29 (n = 4)	BTH (n = 5)	SDW (n = 6)
Absorbance	Mean ± standard error*		
@ 365nm excitation, 455nm emission	332.25 ± 17.38	321.60 ± 43.13	349.83 ± 47.15
Significance	n.s.	n.s.	n.s.^{x}

n = number of replicates * values are fluorometer readings at 365 nm excitation, 455 nm emission
n.s. = not significantly different from SDW-treated plants
n.s.^{x} = not significantly different from BTH- or P29-treated plants

Table 6.5 Lignin estimations (as % of dry matter) for stems and leaves of white clover, treated with BTH or strain P29, and infested with *A. kondoi*

Treatment	P29	BTH	SDW
	Mean % ± standard error (n = 10)*		
% lignin	9.34 ± 1.37	8.21 ± 0.82	10.87 ± 0.71
Significance	n.s.	n.s.	n.s.^{x}

* Five replicate plants treated and inoculated (2 samples per replicate)
n.s. = not significantly different from SDW treated plants
n.s.^{x} = not significantly different from BTH or P29 treated plants

6.4 DISCUSSION

No difference was found between the levels of lignin and callose in the roots or leaves and stems of plants treated with putative inducers of resistance and those treated with sterile distilled water, 4 weeks after challenge with either the root-parasitic nematode, *H. trifolii* or the foliar pest, *A. kondoi*. Moreover, there was no difference between levels of lignin detected in leaves and stems of untreated, uninoculated plants and those of treated, inoculated plants.

Some plants have a constitutively high level of lignin, suberin or callose, which renders them resistant to attack by certain pathogens, and which has a selective advantage for

survival in the plant's natural habitat (Bowers *et al.*, 1994; Boots and Haraguchi, 1999). Without that selective advantage, the production of defence molecules in the absence of a pathogen would not occur, as the production of any defence molecule has a cost to the plant (Chapin *et al.*, 1990; Karban, 1993; Boots and Begon, 1993; Antonovics and Thrall, 1994) in terms of reduced ability to grow or reproduce (Smedegaard-Petersen and Stolen, 1981; Herms and Mattson, 1992). In studies of induced resistance, the terms 'potentiated' (Kauss *et al.*, 1992), and 'primed' (Katz *et al.*, 1998) have been used to describe plants which have been treated with biotic or abiotic agents to induce resistance to a pathogen, for a more rapid response to pathogens than plants which were untreated prior to pathogen challenge (Hammerschmidt, 1984; Dean & Kuc, 1987; Schweizer *et al.*, 1989). This 'priming' could be an instance of 'poised genes' (Crossley, 1998), which is described as the 'readiness' of a gene without transcription of that gene. The priming itself does not entail the production of defence-related chemicals but the readiness to produce these more rapidly, upon challenge by a pest, parasite or pathogen, than in a non-primed plant.

Many researchers have mentioned the timing of events connected with induced resistance (Dean & Kuc, 1987; Uknes *et al.*, 1992; Ward *et al.*, 1991; Kessmann *et al.*, 1994; Wong *et al.*, 1998) and emphasised this important point. Induced resistance would seem, otherwise, a counter-intuitive strategy for plants to have evolved, involving a needless 'cost' to the plant. However, it is evident from the above-mentioned research that a 'primed' plant does not express active resistance to a pathogen until challenged, and then only in the parts of the plant affected by the pathogen attack.

In this current project, the evidence from estimations of the lignin and callose content of plant material taken from roots, leaves and stems at the end of the experiments indicates that the overall cost to the plant of defence against *H. trifolii* or *A. kondoi* is no greater after pathogen challenge in a 'primed' plant than in a water-treated plant. The key may lie in the timing and the location (only in roots in those challenged with *H. trifolii*) of production of defence chemicals, rather than in the quantity of such chemicals produced. Moreover, this would indicate a net gain to the plant in terms of a more effectively timed defence against the pathogen, parasite or pest.

In the example of *H. trifolii*, the reduced fecundity most likely results from the females' reduced ability to utilise the nutrients of the host white clover plants, due to development of callose in the syncytium, similar to that observed by Grundler *et al.* (1997) for *H. schachtii*. This is somewhat different to the defence of wheat plants resistant to the cereal cyst nematode, *H. avenae* (Williams and Fisher, 1993; Williams, 1994), where there was evidence of vacuolation and breakdown of the syncytium by day 15, preventing the formation of adult females.

In the case of pre-treated white clover plants exposed to blue-green aphids, where estimations of lignin and callose in leaf and stem material indicated no difference between primed and water-treated plants at the end of the experiment, the same argument about cost-effectiveness could be applied. Alternatively, in this particular host-pest interaction, defence may even involve chemicals other than lignin or callose, such as pheromones or feeding deterrents. The ineffectiveness of treatments applied immediately prior to challenge with the blue-green aphids was taken as evidence for a series of biosynthetic events, such as signal transcription and translation, and the production of B-1,3-glucanase and chitinase associated with induced resistance responses (Dann & Deverall, 1999) which would have occurred after priming and before defence was manifest in the plant.

The apparent resistance to the blue-green aphids noted in these experiments was measured by the parameters of above-ground biomass and height of the plants. It could be argued, in the case of treatment with strain P29, that what was being measured was due to a growth-enhancing effect of the *Pseudomonas*. There are many examples of soil-borne bacteria which are referred to as plant growth-promoting rhizobacteria (PGPR) (Yan *et al.*, 1998; Van Loon, 1997; Tuzun & Kloepper, 1995; Mahaffee *et al.*, 1994). However, the results from the PGPR experiments (reported in Chapter Four) seem to indicate that strain P29 does not have a detectable growth-promoting effect on seeds or seedlings of white clover. From this we concluded that both BTH and strain P29 were able to enhance the ability of the white clover, *T. repens* 'Grasslands Huia' and the medic, *M. truncatula* 'Jemalong' to resist the blue-green aphid *A. kondoi*, but that it was due to a mechanism other than plant growth-promotion and increase in total lignin and callose.

CHAPTER SEVEN

FINAL DISCUSSION AND CONCLUSIONS

The work presented in this thesis provides evidence for the salicylic acid (SA)-dependent pathway, known as systemic acquired resistance (SAR) (Ross, 1961b; Dempsey *et al.*, 1999). Resistance was induced in white clover to the root cyst nematode *H. trifolii* and to the foliar pest *A. kondoi*, in response to treatment with BTH. It also shows evidence of the SA-independent pathway known as induced systemic resistance (ISR) (Kloepper *et al.*, 1992), by the induction of resistance using soil microbials against *H. trifolii* and *A. kondoi*. Prior to this project, there was no documented evidence of these pathways in white clover, nor of their effectiveness against a plant-parasitic nematode or an aphid.

The results of this investigation, therefore, have provided further evidence of the ubiquity of induced resistance in the plant kingdom. Kuc (1995) held the view that resistance genes were present in susceptible as well as resistant plants. From this he argued that resistance to pests, parasites and pathogens could be induced in cultivars of all species with appropriate elicitors. There was ample evidence (Steiner & Schonbeck; 1995, Deverall, 1995; Sticher *et al.*, 1997) of the widespread occurrence of induced resistance in the plant kingdom, and this project has added to that evidence.

An important aspect of this project is that it investigated the feasibility of inducing resistance by the potentiation (Kauss *et al.*, 1992) or priming (Katz *et al.*, 1998) of endogenous, non-specific, resistance genes, rather than by the introduction of exogenous genes for resistance to specific pathogens. Research on the introduction of exogenous genes has had among its aims (Honee, 1999): the introduction of genes for activating the hypersensitive response (HR); the insertion of a key regulator of SAR (Jakobek *et al.*, 1998; Cao *et al.*, 1998), and the introduction of genes encoding particular PR proteins (Broglie *et al.*, 1991; Tabei *et al.*, 1998), of which there are now 12 known families (Van Loon *et al.*, 1994, 1997; Gamas *et al.*, 1998). In all these lines of research, there are potential disadvantages. In the first and second lines, a permanent state of HR or activation of SAR would entail a high energy cost to the plant, potentially reducing yield. With

respect to the third line of research, it should be noted that each class of PR-protein is only effective against a narrow range of pathogens, parasites or pests, and there is also the potential for pathogens, parasites or pests to develop 'avoidance' strategies to such a single-gene transformation. The risk of selection for resistance-breaking pathotypes in the case of nematodes, has been documented by Rivoal *et al.* (1995) for *Pratylenchus* sp. and *H. avenae* in cereals, by Kim *et al.* (1998) for *H. glycines* in soybean, and by Tzortzakakis and Gowen (1996) for *M. javanica* in tomatoes. In the case of pathogens like *B. thuringiensis*, resistance-breaking strains of the target insect, the diamondback moth, *Plutella xylostella*, have rapidly developed on *Bt*-altered crops (Roush, 1998; Tabashnik *et al.*, 1997). In contrast, the induction of resistance using microbial or chemical inducers involves the potentiation of endogenous genes, which are activated only upon challenge by a parasite, pathogen or pest, and have a wider spectrum of efficacy. Moreover, because there are multiple genes involved, induced plants do not provide a high level of selection pressure for the development of resistance-breaking organisms. For example, in recent work on BTH-treated peppers, Romero *et al.* (1998) showed a reduced occurrence of 'race-change mutants' of *Xanthomonas campestris* pv *vesicatoria*, the causative agent of bacterial spot of peppers used to challenge the plants, compared to that observed in response to the use of cultivars bred for resistance to that specific pathogen.

New molecular and genetic data, further elucidating the SAR and ISR pathways and the genes involved, are being generated rapidly. Many parallels have been uncovered between the "immune response" of plants and that of both vertebrate and invertebrate animals (Low and Dwyer, 1994; Durner and Klessig, 1996; Baker *et al.*, 1997). Whitham *et al.* (1994) and Hutcheson (1998) reported that the involvement of nitric oxide, hydrogen peroxide, the oxidative burst and protein kinases, in the cascade of events which constitute the immune response, is common to the three groups of organisms. This should give momentum to the research on plant resistance responses and provide cross-disciplinary hypotheses to the field of plant immunology.

In the current climate of caution about the use of chemicals and genetically modified (GM) foods, a 'natural enhancement' idea is timely, and in keeping with the clean, green image of Australian agriculture. The world-wide use of pesticide chemicals is costly to the

environment, and is increasingly being proscribed. Moreover, the development and deployment of GM seeds with 'terminator genes' is considered to be a potentially serious threat to biodiversity due to the relative ease of cross-pollination with wild plants. The GM 'terminator gene' technology also is a source of considerable concern and potential hardship to farmers in developing countries who traditionally have relied upon seed-saving for the following year's crops.

This research project has shown the potential of the soil bacteria, *Pseudomonas* strains P29 and P80 and *Bacillus cereus* strain B1, as inducers of resistance to a cyst nematode and an aphid in a controlled (growth cabinet or greenhouse) experimental environment. To move from the perceived potential of these bacterial strains in experimental conditions, to their use in field conditions and ultimately to their deployment for biological control will involve a great deal more investigation. Molecular characterisation of the bacteria would be an essential prerequisite to further work in an uncontrolled experimental environment. This would facilitate the following investigations involving application and subsequent recovery of the bacteria. First, testing of the putative inducing bacteria in a non-sterile field soil in greenhouse conditions would indicate their competence and persistence in the presence of a normal soil biota. Second, testing in various soil types and microclimates would indicate some of the abiotic limitations to growth of the bacterial strains, such as temperature, nutrients and residual pesticides in the soil and the moisture, pH, texture and type of soil. Third, testing on a variety of host plants would give an indication of the range of potential host plants which recognise the inducing agents. Fourth, tests against a variety of plant parasites and pathogens would give an indication of the range of specificity of the induced plant response (Stout & Bostock, 1999).

The application of the research outcomes of this project will be greatly facilitated by the seed coating technology already extant and tested in greenhouse (Alstrom, 1991) and field conditions (Rivas-Davila *et al,*, 1998; Murphy & Zehnder, 1998) with both *Bacillus* spp. and *Pseudomonas* spp. PGPR. Efficacy with the chemical inducer BTH using foliar sprays has been shown (Kessmann *et al.*, 1994); an investigation of the reported synergism between modes of application of BTH (D. MacKenzie, pers. comm.) should also be

undertaken, to test whether synergism also occurs using bacterial treatments as both seed treatment and foliar spray.

Investigations of induced resistance in different microenvironments, as outlined above, should be followed up with research on the use of soil amendments to provide ameliorated microhabitats in which the beneficial microbials could proliferate. Optimal formulation for both shelf-life and application of putative agents would also be important considerations in their further development for biocontrol.

In order to test further the hypothesis of 'recognition' by plants of endemic, coevolved soil bacteria, the putative inducing agents should be isolated from the soil and rhizosphere of the specific crops to be investigated. These trials should include a variety of crops, such as annual medics, white clover and wheat, to investigate the potential of induced resistance using microbials for protection against nematodes, aphids and fungi. The degree of resistance achieved should also be assessed in terms of yield, to ensure that such use of microbials is economic.

Initial survey data from white clover pastures in South Australia and Victoria, Autumn 1996

TABLE I **Flaxley** **South Australia**

Latitude: 35° 08' Longitude: 138° 50'

Sample identification		Soil		Bacterial genera/g soil		Storage codes		Number of nematodes /200g soil		
Letter	Code	% moisture	pH	*Bacillus* (X 1000)	*Pseudomonas* (X 1m)	*Bacillus*	*Pseudomonas*	*Pratylenchus*	*Meloidogyne*	*Heterodera*
A	F-1-U	21	6.44	5.80	0.55	1,2,3,4,5	P1,P2	0	1	0
B	F-E-H	22	6.52	4.60	0.10	6	P3	50	0	0
C	F-36-H	29	6.44	9.20	1.35	0	P4	92	0	0
D	F-S-1-H	30	6.85	5.20	25.00	0	P38,P39	12	0	0
E	F-E-U-S	31	6.58	10.40	2.75	7,8,9	P6	34	0	0
F	F-E-U	19	6.1	5.90	0.05	10,11	P5,P7	46	0	0
G	F-36-U	23	5.6	7.80	1.05	12,13	P8	52	0	0
H	F-1-U-S	16	5.83	4.50	0.10	14	P9	3	0	0
I	F-1-H	18	5.5	4.90	0.00	15,16	0	7	0	0
J	F-E-H-S	19	6.42	3.35	0.40	17,18	0	37	0	0
K	F-36-U-S	21	6.3	10.80	30.00	19,20	P10 - P12	47	0	0
L	F-36-H-S	34	5.9	22.10	60.00	21,22	P13 - P15	41	0	0
M	DK-10-H	23	6.41	8.95	10.00	23,24	P16	58	0	0
N	DK-20-H	28	6.41	10.50	0.00	25	0	41	0	0
O	DK-10-H-S	25	6.42	8.30	10.00	26	P17	404	0	0
P	DK-20-H-S	31	6.28	14.80	2.75	27	P20	61	0	0

Soil type: Podsolic, shallow, compact loamy soil. Red duplex (Northcote, 1960)

Mt Barker Region Bureau of Meteorology Station figures

Annual rainfall: 810mm Average temperature: 13.9°C Range: 4.3°C - 27.1°C

Codes 36 = pivot plot No. 1,10,20 = age of pasture (years) S = spade (whole plant dug up)
DK = D. Kuchel E = Echunga Rd. F = Flaxley U = unhealthy plant H = healthy plant

TABLE II **Ponde** **and** **Monteith** **South Australia**

Latitude: 34° 57' Longitude: 139° 19' Latitude: 35° 11' Longitude: 139° 23'

Sample identification		Soil		Bacterial genera/g soil		Storage codes		Number of nematodes/200g soil		
Letter	Code	% moisture	pH	*Bacillus* (X 1000)	*Pseudomonas* (X 1m)	*Bacillus*	*Pseudomonas*	*Pratylenchus*	*Meloidogyne*	*Heterodera*
Q	LI-5-U	35	5.52	12.00	0.00	28	0	4	0	0
R	Fr-5-H	32	6.43	7.80	30.00	0	P18,P19	14	0	0
S	Fr-1-H	36	5.96	6.00	47.50	29,30	P21,P22	10	0	1
T	LI-5-H-S	44	5.93	15.50	2.50	31,32	P23	29	0	0
U	LI-5-H	45	6.03	9.50	47.50	33	P24,P25	38	0	0
V	Fr-1-H-S	42	5.76	19.50	365.00	34,35	P26	7	0	0
W	Fr-5-H-S	34	6.08	0.45	420.00	36,37	P27	26	0	0
X	LI-5-U-S	35	5.66	1.80	12.50	38	P28,P29	6	0	0

Soil type: Dark clay soils (Northcote, 1960)

Murray Bridge Bureau of Meteorology Station figures

Annual rainfall: 346mm Average temperature: 16.0°C Range: 13°C - 18°C

Codes Fr = Fran LI = Llewellyn H = healthy plant U = unhealthy plant S = spade (whole plant)

1,5 = age of pasture

TABLE III **Timboon** **Victoria**

Latitude: 38° 33.47' Longitude: 142° 59.558' Altitude: 125 m

Sample identification		Soil		Bacterial genera/g soil		Storage codes		Number of nematodes/200g soil		
Number	Code	% moisture	pH	*Bacillus* (X1000)	*Pseudomonas* (X 1m)	*Bacillus*	*Pseudomonas*	*Pratylenchus*	*Meloidogyne*	*Heterodera*
AT-1	T-P-C-4	23	4.63	2.50	10.00	39,40	P40,P41	11	0	104
AT-2	T-P-S-3	29	4.72	1.30	0.50	41,42	P30,P31	8	0	18
AT-3	T-P-S-2	33	4.75	4.50	130.00	43,44	P42,P43	15	0	25
AT-4	T-F-S-2	26	4.89	1.55	14.00	45,46	P32,P33	44	0	64
AT-5	T-F-S-1	34	4.96	2.30	2.00	47,48	P34,P35	17	0	59
AT-6	T-P-S-1	26	4.56	9.50	24.50	49,50	P36,P37	23	1	2
AT-7	T-P-C-1	28	4.55	2.00	430.00	51,52	P47,P48	7	0	4
AT-8	T-P-C-3	23	4.54	7.50	475.00	53,54	P49,P50	25	0	9
AT-9	T-P-C-2	21	4.7	5.50	45.50	55,56	P44,P45	54	1	27
AT10	T-P-C-5	27	4.7	4.00	31.00	57,58	P46	12	2	62
AT-11	T-F-C-4	24	4.79	6.00	5.00	59,60	P51	19	0	289
AT-12	T-F-C-5	24	4.86	9.50	270.00	61,62	P52,P53	13	3	19
AT-13	T-F-C-1	24	4.53	4.00	11.50	63,64	P54	13	3	4
A-T-14	T-F-C-2	25	4.86	6.50	80.00	65,66	P55,P56	11	0	24
A-T-15	T-F-C-3	18	4.53	6.50	8.00	67	P57,P58	18	0	200
A-T-16	T-F-S-5	27	4.72	11.50	3.00	68	P59,P60	1	1	196
A-T-17	T-F-S-4	30	4.71	6.00	6.50	69,70	P61	1	0	187
A-T-18	T-F-S-3	25	4.72	11.50	2.50	71	P62	0	1	929

Soil type: Yellow and Yellow-Grey duplex soils (Northcote, 1962)

Annual rainfall: 900mm Average temperature: 13.4°C Range: 4.3°C - 26.0°C

Codes P = plots F = field S = spade C = core

TABLE IV **Ellinbank** **Victoria**

Latitude: 38° 14.979' Longitude: 145° 55.554' Altitude: 240 m

Sample identification		Soil		Bacterial genera/g soil		Storage codes		Number of nematodes/200g soil		
Number	Code	% moisture	pH	*Bacillus* (X 1000)	*Pseudomonas* (X 1m)	*Bacillus*	*Pseudomonas*	*Pratylenchus*	*Meloidogyne*	*Heterodera*
AE-1	EI-F-S-4	39	4.98	21.00	35.00	72,73	P63	50	0	73
AE-2	EI-F-S-3	38	4.79	9.00	160.00	74	P64,P65	69	0	715
AE-3	EI-F-S-1	41	4.93	23.50	205.00	75,76	P66,P67	296	10	118
AE-4	EI-F-S-2	42	4.69	8.00	20.00	77	P68,P69	76	4	81
AE-5	EI-P-C-5	35	4.53	18.00	225.00	78	P70	43	3	5
AE-6	EI-P-C-4	36	4.52	16.00	125.00	79,80	P71,P72	11	1	34
AE-7	EI-P-S-4	41	4.67	7.00	15.00	81	P73	148	4	14
AE-8	EI-F-C-2	35	4.48	1.60	0.15	82	P79	34	1	2
AE-9	EI-F-S-5	38	4.58	0.85	0.00	83	N/A	227	7	4
AE-10	EI-F-C-4	36	4.28	7.50	1.05	84,85	P80	56	5	4
AE-11	EI-F-C-3	32	4.13	0.65	65.00	86	P74 - P76	33	2	25
AE-12	EI-F-C-5	34	4.60	3.50	100.00	87	P77,P78	151	10	1
AE-13	EI-F-C-1	34	4.28	1.25	85.00	88,89	P81,P82	172	3	146
AE-14	EI-P-S-1	45	4.47	13.50	45.00	90,91	P83	188	6	7
AE-15	EI-P-S-5	40	4.76	17.00	3.00	92,93	P84	28	1	11
AE-16	EI-P-C-3	31	4.29	2.65	1.00	94,95	P85	32	4	1
AE-17	EI-P-C-1	38	4.22	1.65	9.50	96	P86,P87	162	10	8
AE-18	EI-P-S-2	42	5.03	13.50	15.00	97	P88,P89	170	9	43

Soil type: Krasnozem structured earths, smooth ped fabric (Northcote, 1962)

Annual rainfall: 1046mm Average temperature: 13.8°C Range: 3.8°C - 26.2°C

Codes P = plots F = field S = spade C = core

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