CHAPTER ONE

General Introduction

1.1 Research background

Gorse, *Ulex europaeus* L. (Fabaceae) is a serious weed in pastures and forest plantations (Richardson, 1993). It is now considered as New Zealand's worst weed (Williams and Timmins, 2002) and one of the world's 100 worst invasive weed species (ISSG, 2006). Despite several measures implemented to control this weed, it continues to spread annually with the loss in production to farming estimated at about $22 million per annum (Monsanto, 1984). This research investigated the possibility of using insect species as vectors of the fungal pathogen *Fusarium tumidum* Sherb. to cause infection in gorse.

Gorse originated from Europe and was introduced into New Zealand over a century ago for hedging to constrain livestock. It has been declared a serious weed in many countries where it has been introduced including New Zealand (Richardson and Hill, 1998). It quickly forms dense thickets that can effectively shade out forest plantations, native vegetation and reduce biodiversity. It reduces the productivity of pastures and plantation forests through competition for space, water, light and nutrients. Furthermore, it impedes access for pruning and thinning in forest plantations, and reduces harvestable volumes of timber (Morin *et al*., 1998). It is estimated to occupy about 1 million ha of New Zealand's agricultural land which has production, ecological and aesthetic functions. The ability of gorse to produce large amounts of seed (over 36,000 seeds/m^2/year) that can remain viable in the soil for many years, greatly accounts for its persistence (Rees and Hill, 2001). Moreover, since it fixes atmospheric nitrogen through symbiotic association with *Rhizobium* spp., it is capable of surviving on marginal lands where most plant species can not. These attributes, coupled with favourable climatic conditions in New Zealand and the absence of some of its natural enemies have enabled this weed to grow extremely well.

In an attempt to control gorse, several programmes involving the application of herbicides, mechanical removal, burning, grazing, mulching, over-sowing and classical biological control agents have been implemented (Balneaves and McCord, 1990; Harman *et al*., 1996; Hill and Gourlay, 1989; Prasad, 2002). Each of these control measures has limitations that restrict long-term use. For instance, continuous use of chemical herbicides can result in build up of resistance in weed populations and an accumulation of toxic chemicals in the environment. On the other hand, mechanical control is not always feasible due to inaccessibility of large populations of gorse along hills and in valleys. Some researchers have reported success in using biological control agents for controlling some weeds (Chandramohan and Charudattan, 2001; Charudattan and Dinoor, 2000; Dinoor and Eshed, 1997).

In New Zealand, biological control of gorse has been undertaken mainly by Landcare Research, and has mostly consisted of the release of exotic insect species which feed on the weed. These phytophagous insects, to date, have not produced the high level of control required to prevent the weed from adversely affecting productivity of desirable plant species (Hill *et al*., 2000; Syrett *et al.,* 1999).

Studies of *Fusarium tumidum* Sherb., a foliar pathogen which occurs naturally on gorse and broom in New Zealand (Johnston *et al*., 1995) have shown that this pathogen has the potential to be developed as a mycoherbicide for gorse control (Morin *et al.,* 1998; 2000). Research on the use of *F. tumidum* as a mycoherbicide has shown that the fungus is capable of infecting the soft tissues and seedlings of gorse (Morin *et al*., 1998). Host range tests of *F. tumidum* have shown it to cause significant damage to plants in the same tribe as gorse and broom (*Cytisus scoparius*). These plants include tree lucerne (*Chamaecytisus palmensis*), tree lupin (*Lupinus arboreus*) and one plant from the same subfamily (young white clover) (*Trifolium repens*) (Barton *et al*., 2003). The effect of *F. tumidum* on *Sophora* species (Kowhai); reported as part of the 'core genistoids' (Wojciechowski *et al*., 2004) has not been reported. The pathogen had no significant effect on economic crops such as pine (*Pinus radiata*) (Barton *et al*., 2003).

Attempts to develop an effective *F. tumidum* based mycoherbicide have met with some difficulties as the pathogen requires free water during the initial phases of the infection

process (Fröhlich and Gianotti, 2000). Using gorse-associated insect species as vectors of *F. tumidum* conidia may be an alternative method of delivery as wounds created by the insects on the plant through feeding and oviposition can release water or sap from the plant tissues. Wounds can also provide easy access for penetration by pathogens as reported for *Sclerotinia sclerotiorum* on the weed *Cirsium arvense* (Bourdôt *et al*., 2004).

A new concept for biological control of weeds referred to as 'lure-load-infect' has been proposed (Hee *et al*., 2004). In this approach, insects are used as vectors to transmit pathogens to target weeds. The approach is made feasible by using auto-inoculation systems baited with pheromones to attract insects to the inocula. The insects will carry some of the pathogen inocula on their cuticle and deposit it on the target weed upon visit. This research is part of a larger programme on developing smart auto-inoculation systems for weed control. In this study, four potential vectors which are abundant on gorse (*Apion ulicis*, *Cydia ulicetana, Epiphyas postvittana* and *Sericothrips staphylinus*) were assessed. The insect species with the greatest potential for vectoring *F. tumidum* was selected for further evaluation to determine transmission of conidia of the pathogen to infect gorse.

1.2 Literature Review

1.2.1 Invasive weeds in New Zealand

Over 240 species of introduced plants have been listed as actual or potential invasive weeds (Owen, 1997), adversely affecting agriculture, forestry, indigenous biota and ecosystems of lands and water-bodies in New Zealand (Froude, 2002). Some of the major invasive weeds are gorse (*Ulex europaeus* L.), broom (*Cytisus scoparius*), alligator weed (*Alternanthera philoxeroides*), Californian thistle (*Cirsium arvense*) and old man's beard (*Clematis vitalba*). This literature review focuses mainly on biological control of gorse. Both classical and inundative methods including insect-pathogen interactions, are reviewed. Finally the natural surface microflora of insect species and methods for microbial identification are briefly covered.

1.2.2 Origin and distribution of gorse

Gorse belongs to the family Fabaceae, sub-family Faboideae and the tribe Genisteae. It is a thorny, perennial, leguminous shrub. It is native to Great Britain and Central and Western Europe, (Richardson and Hill, 1998). It was deliberately introduced to many countries worldwide, including New Zealand, as a hedge and fodder for livestock. However, gorse escaped from cultivation and has spread in almost every country it was introduced. It has become a serious weed in New Zealand, Australia, USA (Oregon, Northern California, Washington), Chile and Hawaii (Richardson and Hill, 1998). Gorse was recognised as an important weed in New Zealand over a century ago. It is found in both North and South Islands from sea level to an altitude of 800 m (MacCarter and Gaynor, 1980) occupying about 1 million ha (Gourlay, 2005) (Fig. 1.1).

Figure 1.1. Severe gorse infestation in North Canterbury.

1.2.3 Biology of gorse

Gorse usually grows to a height of 2-4 m with a stem diameter of around 22 cm in New Zealand (Lee *et al*., 1986; Shepherd and Lee, 2002). Leaves are present during the seedling stage, but as the plants grow, the leaves are reduced to form spines or

scales (Grime *et al.,* 1988; Roy *et al*., 2004) (Fig. 1.2). Shoots produced during the current growing season are green but turn brown the following season resulting in bushes with a central volume of dry brown vegetation. The growth period typically starts in spring, peaks in early summer and finishes in late summer. Individual plants can live for up to 30 years (Lee *et al*., 1986). Gorse plants start flowering 2-3 years after germination, flowering twice a year, with the heaviest flowering in spring (Shepherd and Lee, 2002). The flowers are yellow and are pollinated by insects (Grime *et al.,* 1988). Seed set occurs about 2 months after flowering. The seeds have hard testa, low germination rate and can maintain viability for more than 30 years (Zabkiewicz, 1976). The seeds are approximately 2-3 mm in diameter and are explosively discharged from the mature pod. Most pods mature and dehisce in mid-summer (Hill, 1982). A small proportion of the seeds are dispersed by wind and water. Several researchers have reported seed production estimates of 500-36,000seeds/m^2/year (Ivens, 1978; Rees and Hill, 2001). This prolific seed production characteristic combined with its ability to germinate at a low rate over a long period of time, if undisturbed, is largely responsible for its persistence as a weed (Ivens, 1978; Rees and Hill, 2001).

1.2.4 Economic importance of gorse

Gorse forms dense, impenetrable monocultures that compete and reduce the productivity of pastures and plantation forests. In the early establishment phase of a plantation forest, gorse can out-compete and kill tree seedlings, reducing stand numbers (Richardson and Hill, 1998). Later in the rotation, it can compete with trees for water, light and nutrients, impede access for pruning and thinning, and ultimately reduce harvestable volumes of timber (Morin *et al*., 1998). Due to this thorny and impenetrable characteristic, useful pasture plants growing under or beside gorse are often avoided by grazing livestock, thus reducing the utilisation of pasture plants (Matthews *et al*., 1999). In 1984, the loss in production to farming was estimated at about $22 million per annum and the cost of controlling this weed was estimated at approximately $27 million per annum to the forestry and farming industries (Monsanto, 1984). Gorse can also pose a serious fire risk to forests and invade braided riverbeds, destroy open feeding and nesting sites of native wading birds, and provide cover for predators (Balneaves and Hughey, 1990).

Figure 1.2. Gorse plants at flowing; spine with scales at its base (insert).

1.2.5 Gorse control options

Gorse control programmes include the application of herbicides, mechanical removal (i.e. cutting), mulching, burning, grazing, over-sowing and introduction of both insect and fungal biological control agents (Balneaves and McCord, 1990; Fröhlich *et al*., 2000; Harman *et al*., 1996; Hill and Gourlay, 1989; Prasad, 2002; Zabkiewicz and Balneaves, 1984).

Continuous application of chemical herbicides can result in build up of resistance in weed populations and an accumulation of toxic chemicals in the environment, especially, in the underground water. High levels of herbicides can be toxic to humans, animals including aquatic life and soil fauna as they accumulate in groundwater. In both the United States and in Europe there are now detectable levels of chemicals in the domestic water supply (Deacon, 2006). The development of effective biocontrol agents could provide at least a partial solution to some of these environmental problems (Deacon, 2006). Until recently, fire was the most important technique used to control gorse and reduce the number of seeds in the seed bank. However, burning often leaves stumps that can re-sprout. Moreover, soon after burning a gorse

infestation, a thick cover of gorse seedlings appears because fire helps to break the dormancy of the seeds and provides nutrients for growth. Studies consistently show that the seedlings compete poorly with perennial pasture plants such as ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*) (Davies *et al*., 2005; Ivens, 1983). As a result of this, gorse control in pastures after burning is often achieved by over-sowing with perennial pasture plants and fertiliser to promote competition with the emerging gorse seedlings.

Grazing with sheep and goats can also reduce gorse establishment (West and Dean, 1990). Ivens (1979) recorded 70-100% reduction in the number of gorse seedlings because of competition, grazing and trampling by grazing livestock. Mechanical control such as cutting is effective when combined with either mulching or herbicide (e.g. triclopyr) application (Prasad, 2002). However, cutting is not always feasible due to inaccessibility of large populations of gorse growing along hills and in valleys. Recently, much attention has been focused on biological control of weeds as this method is relatively safe to both humans and the environment. This study focuses on biological control of gorse using both classical and inundative approaches.

1.3 Biological weed control

Biological control is a broad term used to describe the ability of a biological agent to lower the size or vigour of the population of another organism. Biological agents commonly used for weed control are insects and pathogens, especially, fungi. The two broad approaches are inundative and classical biological control.

1.3.1 Inundative biological control (Mycoherbicide)

Inundative biological control involves the repetitive application of a large quantity of a biological agent as a bioherbicide in order to create a disease epidemic in a weed population. A mycoherbicide is a bioherbicide with a fungus as the active ingredient and is used as a weed control agent through inundative and repeated applications of its inoculum. The development of mycoherbicides has received much attention worldwide in the last three decades. DeVine® (Kenney 1986) and Collego® (Bowers 1986) were the first to be registered in 1981 and 1982, respectively. Recently registered

mycoherbicides include Smolder (2005) in USA and Myco-Tech™ paste (2004) in Canada (Table 1.1). In New Zealand, mycoherbicide research for gorse control included the development of GOB-stopper, based on *F. tumidum* and Chonquer, with *Chondrostereum purpureum* as the active ingredient (Fröhlich and Gianotti, 2000). Research on GOB-stopper was abandoned due to problems with producing a formulated product for commercial use.

Mycoherbicides are often more selective in their mode of action so the risk of damage to other plants is reduced. However, the potential of these products is often limited as the growth, sporulation, dispersal and infection capability of the pathogen can be affected by changes in the environment. McRae and Auld (1997) noted that the dew period requirement of most fungi is perhaps the over-riding constraint to the development of mycoherbicides. Moreover, after application, most fungal spores do not usually persist at high levels for long enough to continue the infection process. This means that, like chemical herbicides, mycoherbicides often need to be reapplied. Although many potential mycoherbicides have been identified, success has been limited using this approach.

Table 1.1. Registered mycoherbicides and their target weeds (Charudattan and Dinoor, 2000; Landcare Research, 2005; Te Beest and Templeton, 1985).

Mycoherbicide	Active ingredient (fungus)	Target weed	
		Common name	Scientific name
BioMal®	*Colletotrichum gloeosporioides* f. sp. *malvae*	Round-leaved mallow	*Malva pusilla*
Collego®	*Colletotrichum gloeosporioides* f. sp. *aeschynomene*	Northern joint vetch	*Aeschynomene virginica*
DeVine®	*Phytophthora palmivora*	Stranglevine or Milkweed vine	*Morrenia odorata*
Dr BioSedge®	*Puccinia canaliculata*	Yellow nutsedge	*Cyperus esculentus*
ECOclear®	*Chondrostereum purpureum*	Black cherry and Blackberries	*Prunus serotina* and *Rubus* spp.
LuBao 2®	*Colletotrichum gloeosporioides* f. sp. *cuscutae*	Dodder	*Cuscuta* spp.
Myco-Tech™ paste	*Chondrostereum purpureum*	Deciduous tree spp.	-
Smolder	*Alternaria destruens*	Dodder spp.	
Stumpout®	*Cylindrobasidium leave*	Black wattle and Golden wattle	*Acacia mearnsii* and *A. pycnantha*

1.3.2 Classical biological control

Classical biological control involves the importation and release of co-evolved, exotic biological agents from the centre of origin of the target weed with the aim of creating a natural epidemic in a weed population. The agents are first quarantined until positively identified, certified free from companion species, parasitoids, and symptoms of diseases. They are also tested on plant materials not available in their country of origin to ensure their specificity. Once permission to release the agents from quarantine is granted, they are mass reared and distributed or released.

One of the most famous examples of successful biological control of weed is that of prickly pear (*Opuntia stricta* Haworth) with a moth, *Cactoblastis cactorum* Berg (Dodd, 1940). "Secondary parasites" (fungi and bacteria) were credited with aiding the insect in causing the final death of the weed (Dodd, 1940). At the time the moth was released in 1926, 24 million ha were infested with the weed. Thirteen years after the introduction of the moth, the infestation of prickly pear in Queensland was reduced by more than 99% (Landcare Research, 1996). The control of water-hyacinth (*Eichhornia crassipes*) in Australia (Hatcher and Paul, 2001) and Uganda (Ogwang and Molo, 2004) are classic examples. The weevils *Neochetina bruchi* and *N. eichhorniae* reduced plant density of water-hyacinth along Lake Victoria and Lake Kyoga by 80% (Ogwang and Molo, 2004).

1.3.3 Advantages and disadvantages of biological control

Charudattan (2001) presented several justifications for biological control with pathogens. These included the phasing out of several older herbicides, public resistance towards genetically altered food crops (e.g. herbicide-tolerant transgenic crops), increasing problems with herbicide-resistant weeds, government-instituted mandates for reducing chemical pesticide usage and consumer preference for non-chemical alternatives in food production.

A successful biological control programme reduces or in some cases, removes the need for conventional (often chemical) methods of control for a weed species that is growing prolifically in the absence of its natural pests and pathogens. The benefit-to-

cost ratio of successful control can be very high, especially when earlier successes in one country form the basis for repeating the introductions elsewhere (Waterhouse, 1998). Crawley (1989) observed that while the overall success rate for biological weed control may be relatively low, the successes are permanent and highly cost effective. Furthermore, biological weed control is often target specific and, therefore, potentially less harmful to non-target plant species in the ecosystem. Biological control agents remove weeds gradually, allowing favourable plant species to replace them without exposing large areas of land to erosion and limiting invasion by other undesirable species. Moreover, the agents pose minimal or no health risk to handlers.

Disadvantages of biological control are that the level of control may vary from place to place because of differences in climate, soil type, vegetation, management practices etc. The weed is not usually eliminated but kept in equilibrium with the population of the biocontrol agents and there could be non-target effects. In addition, classical biological control is often perceived to be too slow and the development process is also time-consuming (Charudattan and Dinoor, 2000). These issues may act as major constraints to the widespread implementation of this control strategy.

1.4 Pathogens for gorse control

1.4.1. Fungal pathogens associated with gorse

In an attempt to look for fungal pathogens for biological control of gorse, a survey across New Zealand of fungi associated with diseased stem and leaf tissues of gorse and broom was conducted between 1991 and 1993 (Johnston *et al*., 1995; Johnston and Parkes, 1994). Isolations were made from gorse collected at 115 sites throughout the country. Table 1.2 is the list of fungi isolated or collected from gorse in New Zealand.

Table 1.2. Fungi isolated from gorse in New Zealand (Dingley, 1969; Hughes, 1966; Johnston *et al*., 1995; Johnston and Parkes, 1994; McKenzie and Johnston, 1999; Pennycook, 1989).

Isolates identified directly on gorse	**Isolates obtained in culture**
Amylostereum sacratum (= basidiomycete macrofungus)	*Alternaria* sp.
Arcyria ferruginea (myxomycete)	*Alternaria alternata*
Bjerkandera fumosa (macrofungus)	*Ascochyta* sp.
Byssomerulius corium (macro)	*Botryosphaeria lutea*
Coniophora sp.	*Chaetomium* sp.
Coniothyrium sphaerospermum	*Chondrostereum purpureum* (macrofungus)
Corticium utriculicum (macro)	*Cladosporium cladosporioides*
Dacrymyces lacrymalis (macro)	*Colletotrichum* sp.
Daldinia bakeri (macro)	*Cosmospora aurantiicola* (= *Fusarium lavarum*)
Daldinia childiae (macro)	*Epicoccum purpurascens* (= *E. nigrum*)
Diaporthe eres	*Fusarium graminum*
Dichomitus leucoplacus (macro)	*Geotrichum* sp.
Eutypella stellulata	*Gibberella avenacea* (= *F. avenaceum*)
Flagellospora sp.	*Gibberella baccata* (= *F. lateritium*)
Gibberella cyanogena (= *F. sulphureum*)	*Gibberella pulicaris* (= *F. sambucinum*)
Gibberella tumida (= *F. tumidum*)	*Marielliottia biseptata* (= *Dreschlera*)
Lachnum virgineum	*Microsphaeropsis* spp.
Metacapnodium fraserae (sooty mold)	*Pestalotiopsis* sp.
Monilia aurea	*Phomopsis* spp.
Nectria ochroleuca	*Pleiochaeta setosa*
Nodulisporium sp.	*Pleospora herbarum*
Periconia minutissima	*Septoria* sp.
Phaeosolenia densa (macro)	*Septoria slaptonensis*
Phoma sp.	
Phoma ulicis	
Physarum cinereum (myxomycete)	
Pleospora tarda (= *Stemphylium*)	
Polyporus arcularius (macro)	
Polyporus squamosus (macro)	
Proliferodiscus dingleyae (macro)	
Radulum sp. (macro)	
Rhinocladiella sp.	
Sphaerobolus stellatus (macro)	
Stereum hirsutum (macro)	
Podoscypha pergamena (macro)	
Tomentella ferruginea (macro)	
Trichocladium basicola (= *Thielaviopsis basicola*)	
Tritirachium sp.	
Ulocladium sp.	

Among the fungal pathogens commonly found on gorse, *F. tumidum* has the greatest potential for development as a mycoherbicide (Johnston and Parkes, 1994). Inoculation of young gorse plants with *F. tumidum* resulted in high mortality under favourable conditions (Fröhlich and Gianotti, 2000; Morin *et al*., 1998). However, since natural infection was low, development of a mycoherbicide was considered a more suitable approach to control the weed. Another fungal pathogen, *Chondrostereum purpureum* (Fr) Pouzar causes silver leaf disease of gorse. This fungus is also a wound pathogen of pipfruit trees, raspberries, roses, willows and eucalypts (Dye, 1972; Gadgil and Bowden, 1982). A study by Bourdôt *et al*. (2006) found no evidence of synergism between these two fungi. This present study only assessed the potential of *F. tumidum* for gorse control since *C. purpureum* has a wide host range including economic and native plants (Bishop, 1978).

1.4.2 The genus *Fusarium*

Fusarium is a form genus in the Hyphomycetes (subdivision Deuteromycotina) that produces macroconidia, microconidia, and chlamydospores. All species form macroconidia with a foot-shaped basal cell (that may not be distinct for some species) (Gams and Nirenberg, 1989). Sclerotia may be produced but they are not important taxonomically. The key taxonomic criteria are the shape and size of the macroconidia, the presence or absence and the shape of microconidia and chlamydospores, and the structure of conidiophores (Windels, 1992). Moreover, colony morphology, pigmentation, and growth rates on agar are useful secondary characters for identification. In the genus *Fusarium*, over 90 species are recognised, depending upon the taxonomic system used (Windels, 1992). *Fusarium* is the anamorph (asexual state) of *Gibberella.* Some species cause disease in humans and animals (Nelson *et al.*, 1990), and some species produce mycotoxins such as fumonisins, trichothecenes type A (T-2 toxin), trichothecenes type B (nivalenol) and zearalenone (Bergers *et al*., 1985; Kroschel and Elzein, 2004; Moss, 2002; Mule *et al*., 1997).

1.4.3 *Fusarium tumidum*

Fusarium tumidum is a foliar, non-systemic pathogen that causes lesions on leaves, spines, and stems of gorse as well as tip dieback (Broadhurst and Johnston, 1994). In New Zealand, it was first isolated from lupin (*Lupinus* spp.) in 1976 by G.F. Laundon and identified by Gerlach and Nirenberg (1982). This fungus has been found to commonly infect gorse and broom and to form a *Gibberella* teleomorph in nature (Broadhurst and Johnston, 1994).

F. tumidum produces macroconidia which are thick walled and distinctly septate, curved, fusiform and somewhat irregular in shape (Fig. 1.3). The dorsal surface is strongly curved while the ventral surface is less curved and are relatively large measuring 28-122 x 6-12.5µm (Broadhurst and Johnston, 1994). Perithecial initials develop in some cultures, but do not mature unless compatible isolates are crossed (Brayford, 1997). The sexual stage of *F. tumidum* is *Gibberella tumida.* Under favourable condition, the conidia germinate within 24 h (Fig. 1.4) and can grow well on most agar media across a wide range of pH. The conidia are the active ingredient for mycoherbicide preparation (Fröhlich and Gianotti, 2000).

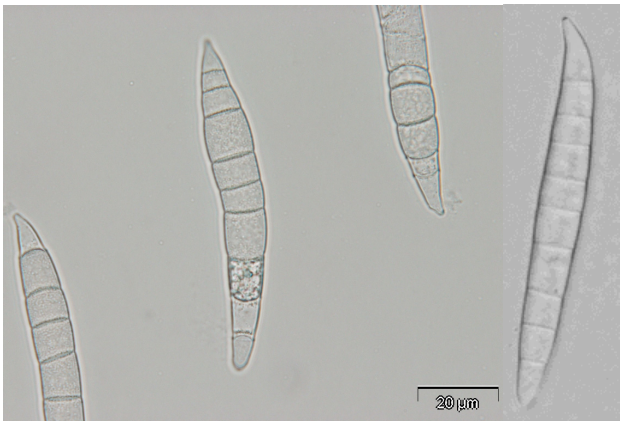

Figure 1.3. Macroconidia of *Fusarium tumidum* (520 µm^2).

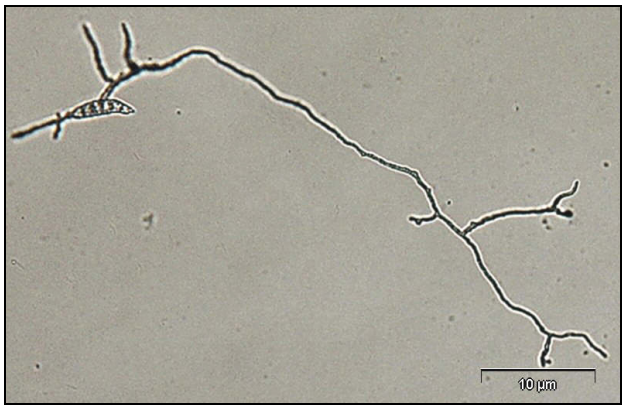

Figure 1.4. Germinating conidium and growing hyphae of *Fusarium tumidum*.

The most important constraint for infection by *F. tumidum* is available water during the initial phases of the infection process (Fröhlich and Gianotti, 2000). Studies by Morin *et al.* (1998; 1995) have shown that *F. tumidum* is capable of killing gorse only when exposed to a moist environment during the initial infection phases. When gorse plants were exposed to dew period immediately after inoculation, the mortality rate was higher than when exposure of inoculated plants to dew was delayed. Delaying exposure to dew by 48 h reduced disease severity, whilst *F. tumidum* killed all young gorse exposed to a 24-h dew period immediately after inoculation. Additionally, exposure to long dew period causes more infection than short dew period. Dry weight of gorse was reduced significantly as the dew period was increased from 12 h to 48 h after inoculation (Morin *et al.,* 1998). The minimum dew period required for infection also increases with age of the plant. For example, a 24-h dew period was needed to infect 8-wk-old seedlings while twice that period was required to infect 12-wk-old plants (Morin *et al.,* 1995).

F. tumidum is able to infect gorse plants within a wide range of temperature (12-27^{o}C), and has been reported to infect the weed at temperatures as low as 5^{o}C (Morin *et al.*, 1998). However, infection increases with temperature during the initial phase with 22^{o}C reported as the optimum temperature for maximum reduction in gorse plant dry weight (Morin *et al.*, 1998).

Disease incidence in gorse generally increases with an increase in concentration of *F. tumidum* conidia (Fröhlich *et al*., 2000; Morin *et al*., 1995) and the volume of

application (Fröhlich *et al*., 2000). Both Fröhlich *et al*. (2000) and Morin *et al*. (1995) recommended 1 x 10^6 conidia/mL as effective against gorse and found it sufficient to kill 95% of 6-wk-old gorse (Morin *et al*., 1998). Application rates beyond and below this level have been found to be uneconomical and ineffective, respectively (Fröhlich *et al*., 2000). Furthermore, suspensions containing more than 1 x 10^6 conidia/mL are viscous, due to the large size of the conidia and may be difficult to apply with commonly used agricultural spray nozzles.

The ability of *F. tumidum* to kill gorse decreases as the age of the plant increases. For example, the dry weight of 4 and 8-wk-old plants infected by the fungus was reduced by 89 and 73%, respectively. In contrast, the dry weight of older plants (12 and 16 wk old) was reduced by only 55% (Morin *et al.*, 1998). In another study, *F. tumidum* spores in invert emulsion failed to kill 72 wk old gorse despite causing more than 75% tissue damage (Fröhlich and Gianotti, 2000). Bourdôt *et al*. (2006) reported a 45% reduction in the density of regenerative gorse shoots by the pathogen.

F. tumidum spores are thought to germinate and infect plants through the stomata (P. Johnston, pers. comm., 2003). There is however, no report on this in the literature.

Risk assessment

Host range tests of *F. tumidum* on 20 non-target plant species closely related to gorse (within the family Fabaceae) or which are of commercial importance in New Zealand have shown that tree lucerne, (*Chamaecytisus palmensis*) and tree lupin (*Lupinus arboreus*) were most severely affected by the pathogen (Fröhlich and Gianotti, 2000). These plant species are among the closest relatives of gorse and broom. The fungus however, had less effect on more distantly related legumes that are native in New Zealand, or are used as economic crops or as cover crops in plantation forests (Barton *et al*., 2003). The tests also showed that susceptibility to infection decreased with increasing age of the plants. Moreover, plants with woody stem were more resistant than those with succulent stem. Pine species (*Pinus radiata*) of any age were not significantly affected (Fröhlich and Gianotti, 2000).

F. tumidum produces type A trichothecenes (neosolaniol and T-2 toxin) (Altomare *et al*., 1995; Mule *et al*., 1997; Morin *et al*., 2000). Trichothecenes are a family of tetracyclic sesquiterpenoid substances produced by several species of *Fusarium*. Being phytotoxins, they can produce symptoms such as chlorosis, necrosis and wilting (McClean, 1996). They inhibit the enzyme peptidyl transferase and therefore protein synthesis (McCormick, 2003) and have been implicated in some human diseases (Beardall and Miller, 1994; Cheeke, 1998). Reports indicate that trichothecenes play a role in the pathogenesis of *Fusarium* spp. (Proctor *et al*., 1995; Zonno and Vurro, 2002). However, Morin *et al*. (2000) found no correlation between levels of T-2 tetraol produced and pathogenicity of different *F. tumidum* isolates. The isolate of *F. tumidum* used for this study, produces relatively low levels of trichothecenes (Morin *et al*., 2000) and, therefore, is likely to be safer compared with other *F. tumidum* isolates.

There have been problems with the commercial formulation of *F. tumidum.* Field application of the pathogen formulated in water and three invert emulsions failed to induce severe disease epidemics (Fröhlich *et al*., 2000). Moreover, due to the large populations of gorse growing at places difficult to reach, spraying is often considered not feasible. These constraints have necessitated the need for an alternative method for delivery of the pathogen. Using gorse-associated insect species as vectors of *F. tumidum* is a novel strategy of mycoherbicide delivery.

1.5 Insects for gorse control

Several insect species have been released for classical biological control of gorse in New Zealand. Two of these: Gorse seed weevil *Apion ulicis* Förster, (Coleoptera: Apionidae) and Gorse pod moth *Cydia ulicetana* Denis and Schiffermüller (Lepidoptera: Tortricidae) feed on seeds while the remaining insects feed on foliage. The foliage feeding insects include Gorse spider mite *Tetranychus lintearius* Dufour, Gorse thrips *Sericothrips staphylinus* Haliday (Thysanoptera: Thripidae), Gorse soft shoot moth *Agonopterix ulicetella* (Stainton), Gorse colonial hard shoot moth *Pempelia genistella*, and Gorse stem miner *Anisoplaca ptyoptera*. *Anisoplaca ptyoptera* is a native insect and has never been considered for the biocontrol of gorse in New Zealand because it attacks the rare native brooms. The light brown apple moth

Epiphyas postvittana Walker (Lepidoptera: Tortricidae) has been accidentally introduced into the country through imports and is abundant on gorse and broom throughout the country. Four of these insects *(A. ulicis, C. ulicetana, E. postvittana* and *S. staphylinus*) were chosen for this study based on their establishment in this country and availability.

1.5.1 Gorse seed weevil *(Apion ulicis)*

Apion ulicis was released in Nelson and Alexandra in February 1931 with the aim of destroying gorse seeds. It is now widespread and common in both North and South Islands (Kuschel, 1972). The adult weevil is about 1.8-2.5 mm long (Fig. 1.5). It is grey, pear shaped and can be found throughout the year, but is more common in spring and early summer. The female weevil oviposits through a hole in the side of an immature gorse pod that has not been previously attacked by other females (Hoddle, 1991). Several eggs and larvae (up to 30) can be found in a single pod (Hill *et al*., 1991). The eggs take about a month to hatch into white larvae with brown heads, which feed on the seeds for about 6-8 wk and then pupate for about a month. The new adults emerge from the pods and hibernate on gorse during winter. There is only one generation each year, and the adults live for 12 months.

A. ulicis can reduce the number of viable seeds considerably. In a study by Sixtus *et al*. (2003), only 11% of gorse seeds damaged by the weevil remained viable. Gorse sets a significant amount of seed in autumn and winter and since the weevil is reproductively active only during spring, the autumn-winter seeds escape predation (Cowley, 1983). Consequently, only 36% of the annual seed crop is destroyed (Cowley, 1983) although the weevils infect 60-90% of the spring seeds (Hill *et al.*, 1993).

1.5.2 Gorse pod moth *(Cydia ulicetana)*

Cydia ulicetana was first imported from England by the Department of Scientific and Industrial Research (DSIR) in 1989, mass reared and released throughout New Zealand in 1992. The adults are 5-8 mm long, and pale brown in colour (Fig. 1.5). The female moths are bigger than the males and they lay shiny white eggs. The eggs hatch 2 wk after laying, into white larvae (caterpillars) with black heads, which chew their way

into the pods and feed on the seeds. Each larva destroys at least two seedpods during its development, consuming all seed present inside each pod. The larvae consume *A. ulicis* larvae encountered in pods although, less than 20% of pods contain both species (Hill, 1982). The larvae feed for about 1 month and then leave the pods to pupate on the gorse stems. The new adult moth emerges after about 2 wk, thus, one generation from egg to adult takes about 2 months. *C. ulicetana* has two generations each year and in conjunction with the seed weevil, removes up to 100% of the spring seed and 15% of the autumn seed crop of gorse. Assessment at one site showed that the two seed-feeding insects: *A. ulicis* and *C. ulicetana* together destroyed about 50% of the annual seed crop (Hill *et al*., 2000). Non-target plants attacked by *C. ulicetana* are broom, Russell lupin, tree lupin and lotus (Landcare Research, 2004).

1.5.3 Light brown apple moth (*Epiphyas postvittana)*

Epiphyas postvittana is native to Australia and has been introduced accidentally to New Zealand. The males have a forewing length of 6-10 mm with a light brown area at the base distinguishable from a much darker, red-brown area at the tip (Fig. 1.5). Females (7-13 mm) are bigger than the males. The larvae cause damage to gorse foliage and pods (Thomas, 1984) and have been shown to transmit spores of *Botrytis cinerea* on grapes (Bailey *et al*., 1997). As the moth is cool-adapted, its pest status is most severe in the cooler regions of New Zealand. *E. postvittana* is polyphagous and is known to feed on over 250 host species in New Zealand. Host plants include gorse, apples, pears, grapes, citrus varieties, kiwifruit, lucerne and lupin.

1.5.4 Gorse thrips *(Sericothrips staphylinus)*

Sericothrips staphylinus is a short, robust thrips that appears black except for distinctive white rudimentary wing pads and a layer of shiny adpressed hairs on the abdomen (Fig. 1.5). It was introduced from England in 1991 and has established readily. A second population of thrips, originating from Europe and imported from Hawaii, was released in 2001. Females are longer (1.02-1.17 mm) than males (0.74-0.87 mm) and lay white to pale yellow eggs which hatch after an average of 20 days (Hill *et al*., 2001). The larvae pass through two larval stages, a prepupal stage and a pupal stage before the adult stage. The mean developmental time from eggs to adult at

19°C is 42 days with life-time fecundity averaging 76.2 eggs (Hill *et al.*, 2001). *S. staphylinus* is normally brachypterous although winged adults occur occasionally; hence, its spread is very slow. It sucks the watery sap from gorse shoots, stunting shoot growth and reducing both flowering and seeding. Davies *et al.* (2005) reported 57% reduction of gorse shoot dry weight by *S. staphylinus* attack. Studies indicate that thrips prefer young new foliage and are capable of significant damage to gorse regrowth and seedlings (Fowler and Griffin, 1995). Hill *et al.* (2001) observed heavy damage to potted gorse under laboratory conditions with visible damage present at release sites in the field. This damage was caused by larval and adult gorse thrips feeding on the mesophyll tissue.

Despite the important role played by phytophagous insects in controlling gorse, the use of insects alone is not sufficient to completely control the weed (Hill *et al*., 2000; Syrett *et al.,* 1999). The interaction between these insect species and *F. tumidum* will be studied in this project.

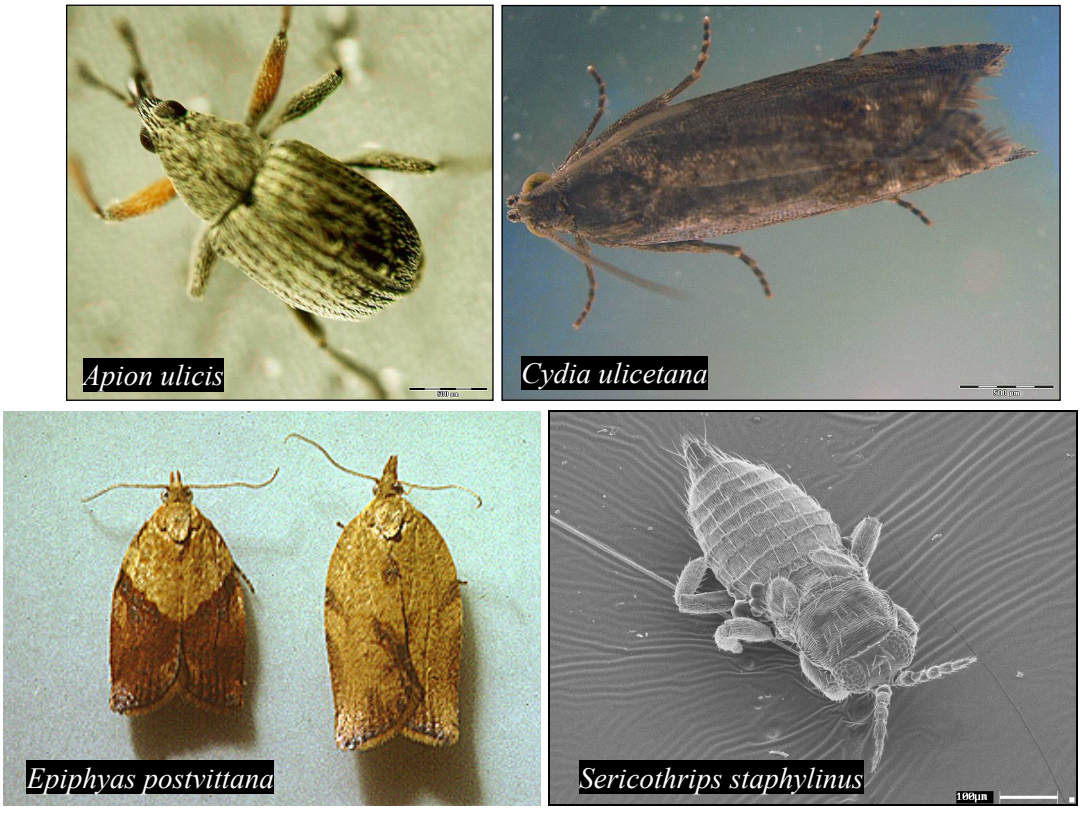

Figure 1.5. Four gorse-associated insect species. *Apion ulicis,* Bar = 500 µm; *Cydia ulicetana,* Bar = 500 µm; *Epiphyas postvittana,* male: left (photograph courtesy of Hortnet); *Sericothrips staphylinus,* Bar = 100 µm.

1.6 Insect-Pathogen interactions

Fungi and insects form the major groups of agents used in biological control of weeds. While some studies have shown positive interactions between these organisms, others have reported negative interactions. Hatcher (1995) provided a range of examples demonstrating varying types of insect-pathogen interactions. These include synergism (where the effect of the interaction is greater than the sum of the individual effects), additive (where the effect of the interaction is equivalent to that obtained from adding the damage from insect and fungus alone) and inhibitory (interaction causes a reduction in a plant variable significantly less than that caused by the weaker of the two agents alone). Equivalent interaction causes a reduction in a plant variable equivalent to the damage obtained from either insect or fungus alone (usually the agent causing the greater damage).

1.6.1 Positive effect of insects on plant pathogenic fungi

Insects may facilitate fungal infection by two main actions: first by acting as vectors for spore transmission (Bradbury, 1998; Morrison *et al.*, 1998; Suckling *et al.*, 1999) and secondly, by providing wound sites for fungal entry (Friedli and Bacher, 2001 a, b; Kluth *et al*., 2001). Insect's attack on weeds may also create a suitable environment for the growth and development of fungi (Hatcher, 1995; Klein and Auld, 1996).

1.6.1.1 Vectoring of pathogens

Several insects have been shown to disseminate pathogens from infected plants to healthy ones thereby helping to spread diseases. *Thrips obscuratus* Crawford treated with spores of a fungicide-resistant strain of *Monilinia fructicola* Wint. infected the flowers and fruit of peaches in the field by dispersing the spores (Ellis, 1993). Dissemination of *Fusarium oxysporum* f. sp. *radicis-lycopersici* and sorghum ergot *Claviceps africana* from diseased plants to healthy plants by adult fungus gnat and corn earthworm moths *Helicoverpa zea*, respectively have been reported (Gillespie and Menzies, 1993; Prom *et al.,* 2003). In an experiment on transmission of *Puccinia carduorum* by three coleopteran insect species; *Cassida rubiginosa*, Müller, *Trichosirocalus horridus* (Panzer) and *Rhinocyllus conicus* Froelich, Kok and Abad (1994) confirmed spore dispersal by these insects. Most of the urediniospores were

observed on setae of legs of all the insects. The study also found that the insects could carry large numbers (> 20 per leg) of rust spores and were not adversely affected by the fungus. In an experiment to control *Botrytis* fruit rot (caused by *B. cinerea*) in strawberry fields, Kovach *et al.* (2000) showed that bumble bees and honey bees could successfully disseminate *Trichoderma harzianum* spores to control the disease. Other examples of the vectoring of pathogenic fungi by insects include reports by Guadelupe *et al*. (1999) and Paine *et al*. (1997). However, studies by Hill *et al.* (2003) showed low transmission of the fungal pathogen, *Phoma clematidina* by inoculated fly, *Phytomyza vitalbae* Kaltenbach (Diptera: Agromyzidae) to infect the old man's beard *Clematis vitalba*. Only 10% of leaflets of the weed developed *P. clematidina* infection caused by flies sprayed with conidial suspension of the pathogen.

Transmission of propagules on the cuticle is more important in fungal dispersal by insects than through the gut. This is due to the fact that, passage through the gut causes damage to propagules and reduces their viability. In addition, the abundance and diversity of fungal propagules is greater on the cuticle than in the faeces (Ingham, 1992; Williams *et al*., 1998). Consequently, this study focused on the external microflora of four insect species in a bid to determine their potential to vector *F. tumidum* to infect gorse plants.

1.6.1.2 Provision of wound sites for pathogens

Insects cause various degrees of physical damage to plant tissues through feeding, oviposition and seeking shelter. Extensive damage is caused by the larvae which feed on the foliage and seed. Studies have shown that damaged plant tissues are generally more susceptible to fungal infection than healthy ones as they provide easy access for fungal penetration (Dillard and Cobb, 1995; de Nooij, 1988). For instance, the injured tissue of cabbage caused by both artificial damage and insect feeding (Lepidoptera larvae) was colonised by *Sclerotinia sclerotiorum* (Dillard and Cobb, 1995). The weevil *Ceutorhynchidius troglodytes* provided an entry wound for the pathogenic fungus *Phomopsis subordinaria* in the plant tissue of *Plantago lanceolata*. The weevils were indispensable for the infection to occur, with no infection occurring in the absence of the weevil (de Nooij, 1988). It appears that insect damage enhances

susceptibility of weeds to infection not only by providing entry for the pathogen but, by increasing stress on the weed (Leath and Byers, 1977; Moellenbeck *et al.,* 1992).

1.6.1.3 Provision of suitable environment for pathogens

Insect attack on weeds may create a suitable environment for the growth and development of pathogens. For example, Klein and Auld (1996) observed that *Colletotrichum orbiculare*, had enhanced infection of Bathurst burr (*Xanthium spinosum*), after wounding as a result of ruptured plant cells releasing moisture and possibly, nutrients, which facilitated the growth of the fungus. Insect damage could provide similar moisture and release nutrient promoting fungal infection. The mealybug *Rastrococus invadens* Williams, deposits honey dew providing nutrients and results in the development and growth of *Metacapnodium fraserae* covering leaves of fruit trees and ornamental plants (Agounké *et al*., 1980). A similar interaction exists between the whitefly and *M. fraserae*. The nymphs and the adult whiteflies feed on the cell sap and excrete the excess sugar as honey on the leaves. The fungus grows on the honeydew deposits and forms sooty mold on the leaves and fruits (Hatcher, 1995) thereby reducing the leaf photosynthetic capacity (Murray and Walters, 1992) and plant growth.

1.6.2 Effect of plant pathogenic fungi on insects

Plant pathogenic fungi can affect insects either directly or indirectly. In direct effects, the insect is affected by fungus-produced toxins. Indirect effects include those whereby the fungus alters host-plant quality for the insect (Hatcher, 1995).

Some insects prefer infected plant leaves to healthy ones. For instance, the spotted cucumber beetles, *Diabrotica undecimpunctata,* preferred feeding on leaf discs from cucumber with necrotic lesions caused by *Cladosporium cucumerinum* infection, rather than healthy leaves (Moran, 1998). In another study, adults of the weevil *Apion onopordi* emerging from *Puccinia punctiformis* infected stems were significantly larger than those developing in healthy stems (Friedli and Bacher, 2001a, b). Scriber and Slansky (1981) noted that when confronted with a nutritionally inferior food, phytophagous insects often eat more of it in order to compensate for its poor quality.

Gastrophysa viridula feeding on *Uromyces rumicis*-infected *Rumex crispus* consumed up to 2.5 times as much plant material as those feeding on healthy *R. crispus* (Hatcher *et al.*, 1994). This increased food consumption can have a long-term effect on the target weed by reducing biomass production.

1.6.3 Negative insect-pathogen interactions

Studies have shown that most insects do not serve as vectors for plant pathogens. For example, 94% of 53 species of insects reported as pests of bean were found to be non-vectors of plant pathogens (Vega *et al.*, 1995). Some insects may vector fungal pathogens but only for a short period (i.e. less than 24 h) (Hill *et al*., 2003).

The main negative effect of insects on pathogenic fungi is caused by mycophagy, the consumption of pathogen tissues (i.e. mycelia, spores or exudates) by insects (Hatcher and Paul, 2000; Padgett *et al.,* 1994). Fungal tissue contains 75-95% water. By dry weight, the nitrogen content of fungal mycelia generally ranges from 1 to 7%; carbohydrate and protein contents are 50 and 20-40%, respectively (Hatcher, 1995). Fungal tissue is also a good source of B vitamins, choline and sterols, and is free of condensed tannins (Martin, 1979). These features make mycophagy an attractive proposition.

Toxic metabolites of some fungi, can affect insects feeding on them or feeding on infected plant tissues. For example, *Heliothis virescens* (Lepidoptera) had reduced larval weight, inhibited pupation and increased larval development time by feeding on plants infected with *Fusarium moniliforme*, *F. oxysporum* and *Alternaria alternata* (Abbas and Mulrooney, 1994). In another study, Kruess (2002) showed that the chrysomelid beetle *Cassida rubiginosa*, consumed more, developed faster, survived better and was larger when fed on leaves from healthy *Cirsium arvense,* rather than leaves from plants infected by *Phoma destructiva.* This may explain the avoidance of certain fungal-infected tissue by insects (Wilson *et al.,* 2000).

Any negative interaction between the proposed insects and *F. tumidum* needs to be determined to improve the efficiency of the smart auto-inoculation system to control gorse.

1.6.4 Smart auto-inoculation systems

Smart auto-inoculation systems are a promising complementary strategy to be used in combination with mycoherbicides. In this approach, insects are used as vectors to transmit pathogens to target weeds. The approach is made feasible by using auto-inoculation systems baited with pheromones or kairomones to attract insects to the inocula. The insects will carry some of the pathogen inocula on their body (cuticle) and deposit them on the target weed upon visit to infect the weed (Fig. 1.6). Moreover, male insects may transmit spores on their cuticle to female insects during mating. This will increase the chances of spreading the spores among the weed population. Under favourable environmental conditions, the spores will germinate and infect the weed. This 'lure-load-infect' concept proposed in this research is a new area of research in biological control of weeds.

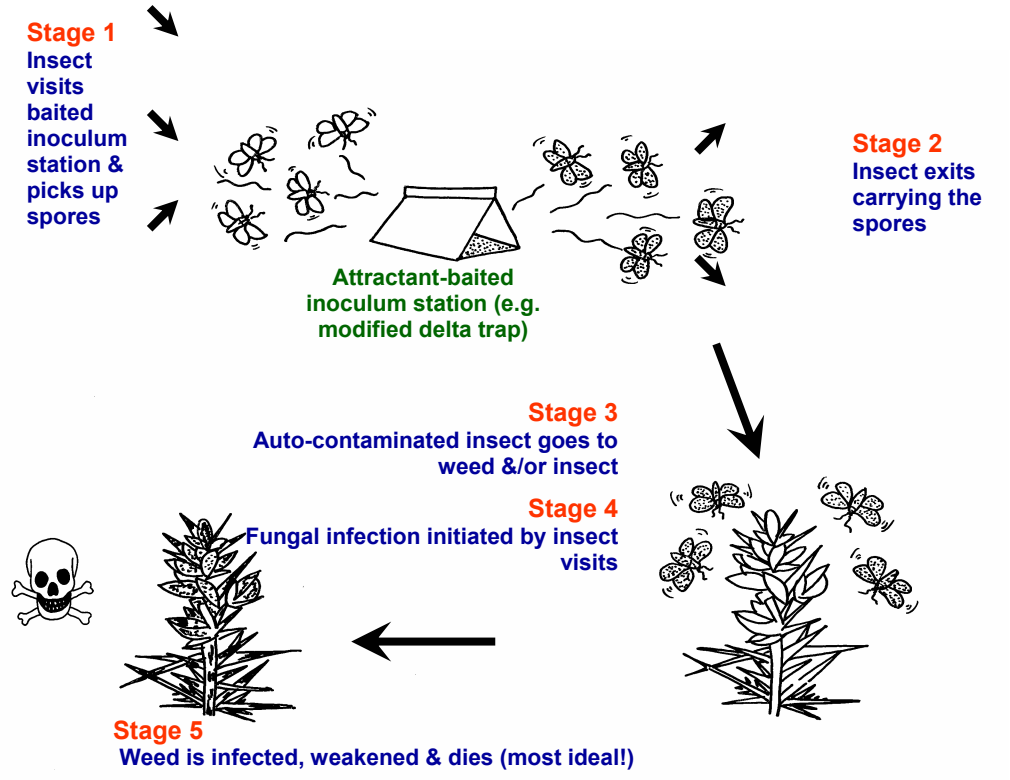

Figure 1.6. Proof of concept: Lure-load-infect. (Diagram courtesy of Dr A.K.W. Hee).

1.7 Natural surface microflora on insects

Insects host numerous bacterial and fungal populations on their cuticle and in their gut with interactions ranging from pathogenesis to symbiosis. In order to use insects as vectors of *F. tumidum* in a biocontrol programme, it is important to select insect species that do not naturally carry microflora that might inhibit the efficacy of the mycoherbicide. To assess the potential of the insect species chosen for this study to vector *F. tumidum* conidia, the natural surface microflora of the insects need to be determined. A detailed review of insect microflora is presented in Chapter Two.

1.8 Microbial identification

Until recently, microbial identification has been based on morphological characters such as spore shape and arrangement of mycelium. However, this method can be time-consuming and often requires a specialist. Moreover, results can be confusing with certain species. Of late, molecular techniques such as random amplification of polymorphic DNA (RAPD) (Paavanen-Huhtala *et al.,* 1999), restriction fragment length polymorphism of Polymerase Chain Reaction amplified rDNA (RFLP-PCR) and DNA sequence of gene fragments (Steenkamp *et al.*, 1999) have provided valuable additional characters that enable the distinction of fungi (Cubeta *et al*., 1991; Chen, 1992).

A growing number of studies have also reported the use of 16S rRNA sequencing for the identification of bacteria and their phylogenetic relationships, associated with insects (Moran *et al*., 2003; Peloquin and Greenberg, 2003). For example, the endosymbionts of mealybugs (Munson *et al.*, 1991), whiteflies (Clark *et al*., 1992), weevils (Campbell *et al*., 1992), ants (Haiwen *et al*., 2005), aphids (Haynes *et al*., 2003), and wasps (Breeuwer *et al.*, 1992) have been determined. Using PCR-RFLP and sequence of bacterial 16S rDNA gene, 10 different bacteria from the red imported fire ant (*Solenopsis invicta*), nine *Leuconostoc* species and acetic acid bacteria have been identified (Haiwen *et al*., 2005; Jang *et al*., 2003; Ruiz *et al*., 2000).

Another method for assessing the functional diversity of bacterial communities is the Biolog system. The Biolog MicroPlate tests the ability of bacterial isolates or of

microbial communities to utilise or oxidise a pre-selected panel of different carbon sources. The test yields a characteristic pattern of purple wells, which constitutes a "Metabolic Fingerprint" of the capacities of the inoculated organisms (Bochner, 1989). Molecular techniques can identify individual bacteria to the species level whereas the Biolog system is more suitable for characterisation of bacterial microbial communities. Consequently, molecular techniques were used for this study.

1.9 Overall aim of the Ph.D.

The overall aim of this study was to test the hypothesis that insects can vector *F. tumidum* to infect gorse. The objective was to investigate the feasibility of using insects as vectors of *F. tumidum* to gorse and for the fungus to cause sufficient infection to significantly weaken or kill the weed. This has been sub-divided into the following specific objectives:

- To determine the external microflora of four potential insect vectors.
- To determine the factors influencing pathogenicity of *F. tumidum* on gorse.
- To determine the effect of *F. tumidum* on gorse seedling emergence and growth.
- To determine the ability of the four insect species to vector *F. tumidum* spores.
- To assess the combined effect of both *F. tumidum* and the insect species with the greatest potential to vector *F. tumidum* and to provide information on the type of interaction between the two biocontrol agents.

1.10 Thesis format

This thesis comprises six chapters; four of which are experimental chapters. Each experimental chapter comprises a summary, introduction, materials and methods, results, discussion and reference sections and is presented as a manuscript for publication. This has resulted in some repetition although this has been kept to a minimum.

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