Biological Control of *Pyrenophora teres*

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Master of Science (M.Sc.)

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Declaration

I hereby declare that the work herein, submitted for the degree of Masters of Agricultural Science in Letterkenny Institute of Technology, is the result of my own investigation, except where reference is made to published literature. I also certify that the material submitted in this thesis has not been previously submitted for any other qualification.

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Abstract

Pyrenophora teres the causal organism of barley net blotch disease is responsible for substantial loss in crop yield with yield losses as high as 40% under favourable conditions. Current control measures are achieved using an integrated approach, combining chemical control, the use of resistant cultivars and crop hygiene practices. In this study several bacterial isolates from soils around Ireland were screened for their potential to act as biological control agents against this pathogen. *In vitro* and *in vivo* tests identified four bacterial isolates, namely; *Pseudomonas fluorescens* strain MKB100 and MKB156, *Pseudomonas* sp. MKB158 and MKB194 having the potential to reduce disease development compared to control test plants. There was a significant positive correlation between the effects of *P. teres* alone, or in combination with *Ps. fluorescens* strain MKB100 or MKB156, *Pseudomonas* sp. MKB158 or *Pseudomonas* sp. MKB194 on net blotch disease development on seedling and detached leaf assays of barley cultivars Lux and Tavern (r = 0.996 and 0.932, P <0.01, P < 0.05 respectively). Under glasshouse conditions, *Ps. fluorescens* MKB100 and MKB156 bacterial isolates reduced disease development in barley seedlings by 70 and 69%, respectively when compared to control plants. *Pseudomonas fluorescens* strains MKB100 and MKB156 significantly inhibited the development of disease symptoms in the barley cultivar Lux under field conditions, with *Ps. fluorescens* MKB156 reducing the percentage diseased leaf area by 54% compared to *P. teres* inoculated control plants. Irrespective of bacterial isolate, bacterial application pre-fungal inoculation was generally more effective in reducing the AUDPC for net blotch disease than was application post-*P. teres* inoculation. Future success of these potential biological control agents will depend on their ability to consistently suppress net blotch under field conditions and on their ability to remain viable if integrated into a disease control program.

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List of Abbreviations

ANOVA	Analysis of variance
approx.	approximately
AUDPC	Area under the disease progress Curve
°C	Degrees Celsius
$cm^{(2)}$	Centimetre (squared)
cv.	Cultivar
dpi	days post inoculation
g	Gram
GS	Growth Stage
h	Hour
%	Percent
km	Kilometre
LB	Luria-Bertani broth
m^2	Metre squared
ml	Millilitre
ml^{-1}	Per millilitre
$ml\ l^{-1}$	Millilitre per litre
ng	nanogram(s)
ND	No data
NPK	Nitrogen: Potassium: Phosphorus
NUV	Near ultra violet
p.	Page
PDA	potato dextrose agar
RH	Relative humidity
SWD	Sterile distilled water
sec	second
SEM	Standard error of mean
spp.	Species
temp	temperature
µl	Micro litre
µm	Micro meter
$v\ v^{-1}$	volume per volume
wt.	Weight
$w\ v^{-1}$	weight per volume

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Chapter 1: Literature Review

1.0 General introduction

Net blotch is a damaging foliar disease of barley caused by the fungal pathogen *Pyrenophora teres* Drechsl. [anamorph *Drechslera teres* (Sacc) Shoemaker] and is responsible for losses in grain yield and quality (Smedegaard-Petersen, 1974). Two formae speciales of the pathogen are known to exist, the net form *Pyrenophora teres* f. sp. *teres*, and the spot form *Pyrenophora teres* f. sp. *maculata*; these differ distinctly in the symptoms they produce on the host leaves (Smedegaard-Petersen, 1971). The net form initially produces dark brown net-like lesions (Steffenson and Webster, 1992) and the spot type produces dark brown elliptical lesions usually surrounded by a chlorotic zone (Smedegaard-Petersen, 1971; Van den Berg and Rossnagel, 1991). Net blotch disease has increased in importance in recent years, mainly due to the barley cultivars currently grown. None contain resistance to net blotch (Wilcoxson *et al.*, 1992). *Pyrenophora teres* has a worldwide distribution and can be found wherever barley is grown; the net form is more common than the spot form (Khan, 1987; Steffenson *et al.*, 1991) however the spot form appears to be more prevalent in Denmark (Smedegaard-Petersen, 1971) and France (Arabi *et al.*, 1992).

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1.1 Nomenclature and morphological characteristics of *P. teres*

Pyrenophora teres was originally classified as a member of the genus *Helminthosporium* by Link in 1803 (Kwasna, 1995). The genus *Helminthosporium* has been divided into 6 genera including 3 anamorphic genera (*Bipolaris*, *Drechslera*, and *Exserohilum*) and 3 teleomorphic genera (*Cochliobolus*, *Pyrenophora*, and *Setosphaeria*) (Kachlicki, 1995). Six species of *Drechslera* have been described as

pathogens of barley (Wallwork *et al.*, 1995). The specific name *Drechslera teres* (Sacc.) Shoem. was created by Shoemaker (1959). Mycelia give rise to spores known as conidia (asexual structures) (Anon, 1981). *Drechslera teres* is classified in the subdivision Deuteromycotina as a member of the class Hyphomycetes (Agrios, 1988). The sexual stage of *D. teres* was described in 1923 by Drechsler and he named it *Pyrenophora teres* (Drechs.) (Smedegaard-Petersen, 1972). The ascigerous stage consists of small black fruiting bodies known as perithecia which bear the ascospores (Smedegaard-Petersen, 1971). Perithecia of the spot-form are produced more frequently than perithecia of the net-form on ascospore-inoculated barley seedlings (Smedegaard-Petersen, 1972).

Much confusion has arisen regarding the identification of *P. teres* and no single feature can be used to distinguish *P. teres* from related species (Shipton *et al.*, 1973). Smedegaard-Petersen (1971) discovered that two types of symptoms were produced by *P. teres* on barley, i.e. net- and spot-like symptoms. He respectively named these as *Pyrenophora teres* (Drechs) f. sp. *teres* and *Pyrenophora teres* (Drechs) f. sp. *maculata* (Smedg). Due to the fact that they could be readily crossed, they are considered as different types of the same species instead of two different species (Smedegaard-Petersen, 1971). *Pyrenophora japonica* which produces oval necrotic lesions is often mistaken for *P. teres* f. sp. *maculata* and has many characteristics in common with it, but this pathogen appears to be limited to Japan and is thought to have evolved from *P. teres* (Kenneth, 1962). Both species are parasites of cultivated barley but differ in conidial characteristics and certain host plant reactions (Scott, 1991). In comparison with *P. teres*, conidia of *P. japonica* are longer, lack the inflated basal cell and regularly have secondary conidiophores (Scott, 1991).

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Isolates of *Pyrenophora* can vary in colony morphology and sporulating characteristics, especially after successive subculturing on V 8 juice agar (McDonald, 1967). Mycelium is brown to black or grey in colour and white tufts of aerial mycelium are produced in some species when cultured on rich media such as potato glucose agar (Smedegaard-Petersen, 1971). Club-like mycelial tufts are commonly produced by both the net and spot forms of *P. teres* when mycelium comes into contact with the wall of the Petri dish (Smedegaard-Petersen, 1971). Continuous subculturing on artificial media results in a decline in isolate pathogenicity (Piening, 1963). Culturing *P. teres* on potato dextrose agar results in the secretion of orange pigments (known as anthroquinones) by the fungus into the medium (Kenneth, 1962). The pigments produced vary in colour depending on the medium, the isolate, and the conditions under which the isolates are grown (Kenneth, 1962). As with other *Drechslera* species, conidial production is diurnal. Conidiophore formation requires UV light between 310 and 355nm and conidia form in the dark or in the absence of wavelengths between 310-495nm (Onesirosan & Banttari, 1969). Sporulation appears to be more abundant on lima bean agar than on potato glucose agar (Smedegaard-Petersen, 1971).

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Perithecia, asci and ascospores of the net and spot forms are morphologically identical (Smedegaard-Petersen, 1971). Conidia of the net and spot form vary in size, however this depends on the environmental conditions under which they are produced (Smedegaard-Petersen, 1971). Conidiophores are light brown and arise singly or in small groups of two to three; often the basal cell is swollen and conidiophores vary in length from 57 to 202 μm with 1-6 septa in the net form and 68 to 291 μm with 2-8 septa in the spot form (Smedegaard-Petersen, 1971). Conidiophores can arise from between epidermal cells and stomata of infected plant tissue (Deadman, 1988;

Deadman and Cooke, 1991). Each conidiophore produces several conidia, which are straight, cylindrical and rounded at the ends (Anon, 1981). They are light yellowish brown in colour and net and spot form conidia typically measure 52-138 x 13-16 µm and 62-138 x 13-18 µm, respectively (Smedegaard-Petersen, 1971). Conidial chain formation is frequent on agar media incubated in damp chambers where up to 7 conidia in a row have been observed; a lower frequency of chain formation was observed under field conditions (Kenneth, 1962).

Pycnidia are flask-shaped, thin walled, light yellow to brown structures and can vary in size from 35-176 µm in diameter (Kenneth, 1962; Smedegaard-Petersen, 1972). Pycnidia were readily produced in culture on poor nutrient media, but Kenneth (1962) did not succeed in germinating pycnidia-borne pycnidiospores on poor nutrient media. Unlike conidia, pycnidia are produced without specific light/dark requirements (Kenneth, 1962).

Perithecia are flask shaped and vary in size from 35-176 µm (Smedegaard-Petersen, 1971). Asci are club-shaped and contain one to eight ascospores, which are yellow to brown in colour and possess three transverse septa and one longitudinal septum (Smedegaard-Petersen, 1971). Asci and ascospores measure 161-260 x 31-44 µm and 42-61 x 16-25 µm, respectively (Smedegaard-Petersen, 1971). The fruiting bodies have been found on straw, on dead diseased leaves, on seeds and in culture (Kenneth, 1962).

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1.2 Geographic distribution and economic importance

Net blotch occurs sporadically in most barley growing areas of the world, which makes it difficult to assess its overall economic importance (Murray *et al.*, 1998). Both the net and the spot form of the disease occur in barley production areas with

cool moist climatic conditions (Suganda *et al.*, 1993). The net form has a worldwide distribution and can be found in most European countries, including the UK (Locke *et al.*, 1981; Jordan and Allen, 1984), France (Toubia-Rahme *et al.*, 1994), Finland (Robinson and Jalli, 1999), Ireland (Deadman and Cooke, 1987). Both forms are found in Australia (Wallwork *et al.*, 1995), Canada (Tekauz and Buchannon, 1977), and the USA (Shipton *et al.*, 1973). The spot form is comparatively rare and was only classified as a distinct disease in the late 1960's in Denmark (Smedegaard-Petersen, 1971). While the net form appears to be more prevalent worldwide the spot form appears to be more prevalent in Denmark, with 71% of the sites sampled in 1969 being infected with *P. teres* f. sp. *maculata,* compared to 65% being infected with *P. teres* f. sp. *teres* (Smedegaard-Petersen, 1971). The distribution of the two forms may be dependent on region; in Western Canada the spot form accounted for only 8% of the disease in 1974, compared to 46% of the disease in the Eastern region (Tekauz and Buchannon, 1977).

Kenneth (1962) anticipated that *P. teres* originated in Israel and the Middle East where barley (*Hordeum vulgare* L.) was one of the first domesticated cereals, most likely originating in the Fertile Crescent area of the Near East. The extreme variability of the fungus and the appearance of all 3 reproductive stages of the fungus (conidial, pycnidial and pseudothecial) in Israel indicate that this was the centre of origin of the pathogen.

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The most serious effect of net blotch disease is a reduction in the quality of barley grain. Net blotch reduces grain carbohydrate content thereby reducing brewing quality (Kamul and Naguib, 1957) and yield (Smedegaard-Petersen, 1974). Shipton (1966) reported a yield loss of 17% and a reduction in bushel weight, thousand-grain weight and grain size in net blotch-infected plots when compared to crops that were

controlled by regular fungicide sprays. Jordan (1981) found that the greatest yield loss occurred when infection occurred at GS 30 (Zadoks *et al.*, 1974), before the end of tillering, causing a reduction in grain number, thousand-grain weight and an overall yield decrease of 19%. When infection occurred later (GS 45), yield was only reduced by 2.8%. These results agree with those of Rintelen (1969) who reported that with spring barley, yield losses of 20% resulted when plants were infected before tillering and losses of 10% resulted from when infection occurred between tillering and flowering. A reduction in leaf area, plant height and total weight result from seed infection (Wallwork *et al.*, 1995) and sowing infected seed enhances the losses associated with subsequent *P. teres* infections (Smedegaard-Petersen, 1974). An increase in the severity of the disease occurred in the 1970's due to the adoption of more intensive farming practices (a decrease in crop rotation) and a change in the barley cultivars being grown (Smedegaard-Petersen, 1974). *Pyrenophora teres* has a narrow host range. It infects both wild and cultivated barley, but some studies showed that artificial inoculation of other members of the genera *Poaceae* with *P. teres* resulted in net blotch disease symptoms (Singh, 1956). Such plants may act as alternative hosts and sources of inoculum.

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1.3 Symptoms

Net blotch obtains its name from the net-like symptoms produced by the net form (*P. teres*. f. sp. *teres*) on the host plant (Smedegaard-Petersen, 1971). Symptoms associated with the net form of the disease are most common on leaves but can also occur on leaf sheaths, flowers, and grain (Smedegaard-Petersen, 1971). Primary infection from seed can be easily distinguished from secondary infection (from air- or splash-dispersed conidia or ascospores) (Jordan, 1981). Symptoms resulting from

primary seed infection appear as water-soaked stripes extending up the first seedling leaf or a pale grayish lesion in the center of the lamina (Jordan, 1981). Symptoms of the net form caused by secondary aerial infection appear as minute spots or streaks, which grow to form longitudinal and transverse brown streaks on the leaves (with vertical and horizontal lines within the lesions), producing a characteristic net-like pattern with a zone of chlorosis surrounding the lesion (Smedegaard-Petersen, 1971; Jordan, 1981; Van den Berg and Rossnagel, 1991) (Figure 1.1A). Resistant reactions result in smaller and fewer lesions developing (Keeling and Banttari, 1975).

Spot type symptoms produced by *P. teres* f. sp. *maculata* on barley include dark brown elliptical necrotic spots with chlorotic margins of varying width on the leaf blade (Smedegaard-Petersen, 1971) (Fig. 1.1B). This is followed by extension of the chlorosis over the entire leaf. Chlorosis is thought to contribute more to the disease severity than do the number of lesions (Smedegaard-Petersen, 1971; Scott, 1995). Disease resistant reactions manifest as necrotic lesions lacking chlorotic margins (Smedegaard-Petersen, 1971). In resistant interactions the plant continues normal growth and suffers little damage (Smedegaard-Petersen, 1971).

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1.3.1 Role of phytotoxins in net blotch disease development

Both *P. teres.* f. sp. *teres* and *P. teres* f. sp. *maculata* produce substances that are toxic to plants (i.e. phytotoxins) (Smedegaard-Petersen, 1977). Three toxins, toxin A (L,L-N-(2-amino-2carboxyethyl)aspartic acid), toxin B (anhydroaspergillomarasmine A) and toxin C (aspergillomarasmine A) have been isolated from culture filtrates of both forms of *P. teres* (Weiergang *et al.*, 2002b). Toxins A and B have been detected in infected barley leaves (Smedegaard-Petersen, 1977). Toxin A and B have similar physical and chemical properties. Both are readily soluble in water and 0.5N HCL, are

heat-stable and dialysable (Smedegaard-Petersen, 1977). Bach *et al.* (1979) transformed toxin C into the less toxic toxin B by treatment with trifluoracetic acid. Toxin C also converts to toxin B in modified Fries media at low pH values due to a ring closure of toxin C (Friis *et al.*, 1991).

When applied to detached leaves, toxin A caused dark yellow, chlorotic symptoms but little necrosis, toxin B is only slightly toxic and didn't cause any necrotic symptom development and toxin C caused distinct necrotic symptoms and zones of light yellow chlorosis (Weiergang *et al.*, 2002a). However they do not induce the well-defined net or spot lesions (Weiergang *et al.*, 2002a). Toxins affect the surrounding healthy tissue by increasing the cellular respiration level (Mandahar & Garg, 1978). The form of the isolate of *P. teres* (net or spot type) was not correlated with the type or amount of toxins A, B and C produced (Weiergang *et al.*, 2002b). Barley cvs most susceptible to infection by *P. teres* are most sensitive to the toxins produced by the fungus, suggesting that these toxins may be used to select resistant barley lines for a breeding programme (Weiergang *et al.*, 2002a). The use of these toxins in *in-vitro* selection of insensitive cell lines and the regeneration of plants from these cells may also aid the development of new net blotch-resistant barley cvs (Chawla and Wenzel, 1987; Furusawa *et al.*, 1988; Rines and Luke, 1985).

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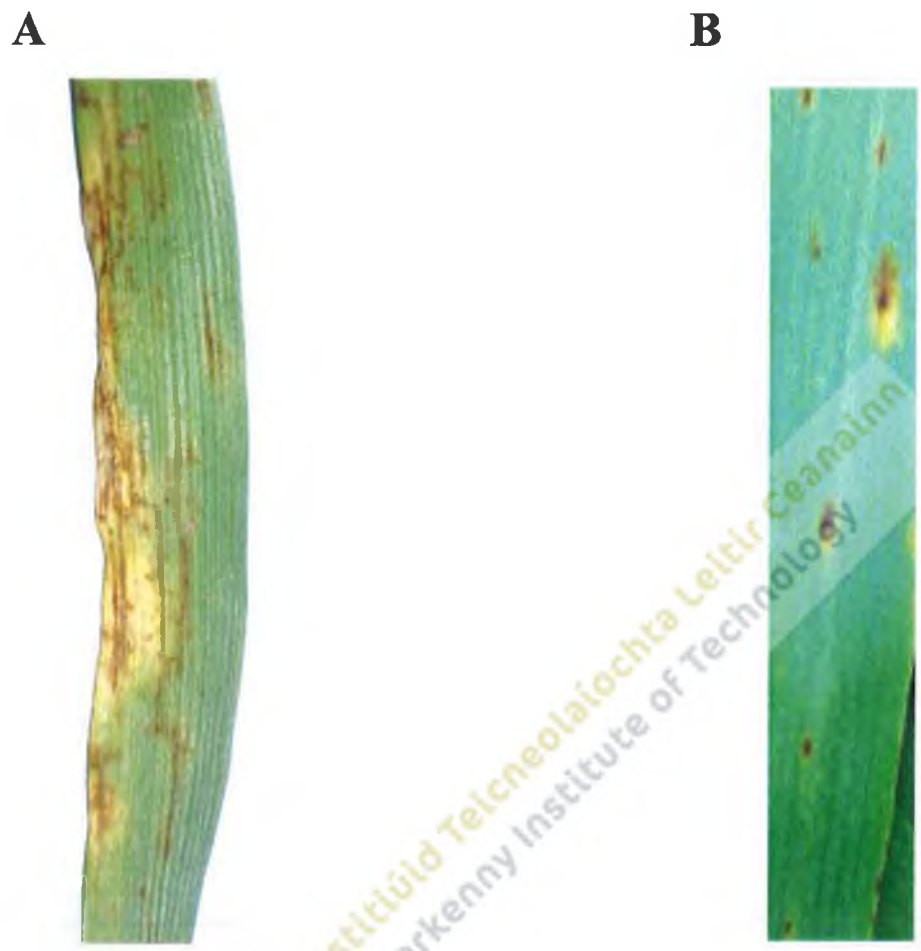

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Figure 1.1 Symptoms associated with the net form (A) and the spot form (B) of net blotch disease of barley (cultivar Lux) caused by *Pyrenophora teres* f. sp. *teres* and *Pyrenophora teres* f. sp. *maculata,* respectively

1.4 Epidemiology of the disease

Net blotch is a polycyclic disease: the disease cycle includes one sexual generation where ascospores are produced from the fruiting bodies (pseudothecia) and several asexual generations where conidia are produced (Murray *et al.*, 1998). Although ascospores are thought to initiate primary infection, the asexual stage is more important in the establishment and spread of the disease (Murray *et al.*, 1998). Figure 1.2 illustrates the net blotch disease cycle.

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1.4.1 Source, production and characteristics of inoculum

Both the net and spot form of the disease arise from infected seed and seedling leaves which become infected with net blotch on emergence from seed (Jordan, 1981). Mycelium in the caryopsis grows and infects the developing coleoptile which then carries the disease above ground as it emerges. Ascospores, conidia and pycnidia may form on the surface of infected grain (Kenneth, 1962). Ascospores and conidia spread from infected residues via wind or water and these in turn multiply on the growing foliage (Anon, 1981) (Figure 1.2). Conidia that are produced on the surface of necrotic lesions of the net form may cause secondary infection lesions on the developing foliage (Jordan, 1981). Fructifications generally develop from the necrotic leaf area (Shipton *et al.*, 1973). Conidia chains arise from between epidermal cells (usually containing 2-3 conidia per chain) (Kenneth, 1962). Conidia may even be produced on lesions that develop on resistant cvs (Keeling, 1967; Shipton *et al.*, 1973). The amount of chlorosis resulting from the spot form is a more important indicator of the disease severity (Smedegaard-Petersen, 1971) and conidia of the spot form were not found on necrotic lesions but were present on chlorotic tissue (Van den Berg & Rossnagel, 1991).

The process of sporulation in *P. teres* consists of conidiophore production and formation of conidia, and the optimum temperature for conidial production was 21 °C in culture (Onesirosan and Banttari, 1969). *In vitro* experiments revealed that conidia were produced within 24 h after previous conidia had been brushed off the conidiophore; also if conidiophores had been broken they regenerated themselves anew (Kenneth, 1962). Conidia developed within 7 days on leaves which had been removed from a diseased crop and incubated on moistened filter paper at 18 °C (Jordan, 1981). Shaw (1986) found that under dry conditions, the time to conidial production on barley leaves ranged from 25 days at 10 °C to 11 days at 20 °C, while under wet conditions, this time was reduced by 20% at all temperatures. Size of conidia is influenced by the substrate they are produced on; longer conidia with a larger number of septae developed on leaf as compared to on straw surfaces (Smedegaard-Petersen, 1972). Conidia have the ability to germinate from an early age regardless of colour, size or septation, thereby making it difficult to speak of mature conidia (Kenneth, 1962). Under field conditions conidia require air currents for separation from conidiophores (Kenneth, 1964) and germination has not been observed among conidia still attached to the conidiophore (Kenneth, 1962).

Perithecia are formed on straw after heavy rainfall and asci and ascospores develop during the late autumn and the following spring and summer, depending on climatic conditions (Smedegaard-Petersen, 1972) (Figure 1.2). Perithecia are most frequently located on the median part of the straw and occasionally on the stubble, highlighting the importance of removing the straw after harvest (Smedegaard-Petersen, 1972). The presence of perithecia is associated with autumn attacks on growing crops and on volunteer plants (Smedegaard-Petersen, 1972). Perithecia of *P. teres* produce long, thick, tapering dark setae (Kenneth, 1964) which may be absent

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on older perithecia (Smedegaard-Petersen, 1972). The production of perithecia in culture has been reported, however successful production of ascospores in culture is not common (Smedegaard-Petersen, 1972). McDonald (1963) was successful in producing ascospores on sorghum seed embedded in Sach's nutrient agar by mixed suspensions of 2 compatible single-spore isolates. A 1:1 segregation ratio of mating genes was obtained when ascospore progeny from perithecia produced in culture were backcrossed to parental isolates verifying that *P. teres* is heterothallic. McDonald (1963) found that perithecia and ascospores developed at 10-15 °C but not at 20 or 25 °C (in both light and darkness) and needed between 1 and 6 months to mature. Smedegaard-Petersen (1971) found mature perithecia that yielded ascospore progeny were produced when monoconidial isolates of the net type and the spot type were crossed, and the progeny segregating into net and spot type symptoms when inoculated on barley. Under damp conditions conidia are often produced on the surface of immature perithecia, either on short conidiophores on the setae or on conidiophores between the setae and these can give rise to secondary infections (Smedegaard-Petersen, 1972), even when ascocarps are infertile (Shipton *et al.*, 1973).

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Pycnidia have been reported by a number of authors and have been found on natural substrates and also in culture (Kenneth, 1962; McDonald, 1963; Smedegaard-Petersen, 1972), particularly on poor nutrient media such as tap-water media and Sach's agar (Kenneth, 1962) and they are successfully produced over a wide temperature range (10-30 °C) in culture. But there is little evidence of the ability of pycnidia-borne pycnospores to germinate normally (Kenneth, 1962) and the function of the pycnidial stage is unknown (Kenneth, 1962; Smedegaard-Petersen, 1972). Kenneth (1962) postulated that the pycnidial body is a spermagonium and the

pycnospores are spermatia. Smedegaard-Petersen (1972) successfully germinated pycnospores on potato dextrose agar however infection experiments using pycnospores to infect barley seedlings were unsuccessful (Smedegaard-Petersen, 1972; Jordan, 1981).

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1.4.2 Dispersal

Conidia are released by strong air currents whereas ascospores are forcibly discharged (Shipton *et al.*, 1973). Deadman and Cooke (1991) studied the factors responsible for net form conidial dispersal and found that higher numbers of conidia were caught using a Burkard high throughput jet spore trap in a wind tunnel, when wind blew over dry infected leaves, as compared to when mist was blown over infected leaves. Deadman and Cooke (1989) suggested the decrease in conidial numbers with increasing spore trap height was due to the downward run-off of leaf water containing conidia. Jordan (1981) also found that that few conidia were present above the crop canopy on rain-free days and none during rainfall. Martin and Clough (1984) found that airborne release of conidia was significant only when the canopy was dry or nearly dry, with maximum release occurring 2-5 days (depending on temperature) after a $\geq$ 16 h period of leaf wetness. Jordan (1981) found that the maximum release of net-form conidia occurred under the following conditions: continuous rainfall (21.3mm) for 370 min, followed by 10 h of sunshine and a maximum temperature of 22 °C. They speculated that there was a greater availability of spores on the lower older leaves. But conidia are capable of being sufficiently dispersed by air currents to infect the upper leaves and heads (Jordan, 1981). With regard to the distances conidia travel, it was found that there was a decline in the numbers caught with increasing distance from the source (Deadman and Cooke, 1989), and so infection is thought to

be more likely from within the crop than from neighbouring fields. Van den Berg & Rossnagel (1990) found that similar trends for conidial dispersal were observed for net and spot–form isolates, with peak spore release episodes between 1200 and 1800 h and 100% germination at 6 h post inoculation (at the optimum temperature).

The rate at which perithecia mature and ascospores are released is highly dependent on time and moisture (Kenneth, 1964). Perithecia are formed on the trash and stubble from the previous season, and over winter there until conditions are suitable in the spring (Figure 1.2). Then the perithecia break open and release asci and ascospores that infect the crop (Arnst *et al.*, 1978; Parry, 1990). Ascospores developed within an ascus after 48 days when diseased leaves were taken from a field crop in 1979 and incubated on moistened filter paper at 18 °C (Jordan, 1981). Under wet conditions mature perithecia broke apart and ascospores were forcibly discharged into the surrounding water (Jordan, 1981). Water droplets were held by the perithecial setae and asci were discharged into the air. Smedegaard-Petersen (1972) found that release of ascospores from the ascus was dependent on rain splash, however Jordan (1981) caught ascospores in still air at lateral distances of up to 14 mm from a perithecium.

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1.4.3 Colonisation of the host

Free water and temperature are important factors influencing the ability of conidia to germinate and leaf infection to occur (Shipton *et al.*, 1973). Van den Berg and Rossnagel (1990) found that the optimal temperature for conidial germination differed between the net and the spot form; 25 °C for the net form and 20-25 °C for the spot form. A leaf wetness period of at least 4.5 h is required for infection (Singh, 1963c) and Van den Berg and Rossnagel (1990) found that 100% germination was achieved

within 6 h post conidial inoculation at the optimum temperature. However the net form required a longer leaf wetness period than the spot form in order for infection to occur and the minimum leaf wetness period decreased with increasing temperatures for both forms (Van den Berg and Rossnagel, 1990). The germination rate of *P. teres* is age-dependent: Shaw (1986) found that when diseased leaves were stored in a bag in the dark at 4 °C the germination rate of conidia decreased by 20% in 5 months and more rapidly thereafter. However the fungus has survived for longer on infected stubble (Shipton *et al.*, 1973).

Conidial spores germinate most frequently from end cells (Smedegaard-Petersen, 1971; Van Caseele and Grumbles, 1977) producing germtubes, which grow to various lengths before producing appressoria (Van Caseele and Grumbles (1977). Appressoria are formed which are club-shaped and infection pegs form beneath the appressorium (Van Caseele and Grumbles, 1977). Entry into the host occurs by penetration into epidermal cells rather than through stomata (Van Caseele and Grumbles, 1977) which is followed by the development of a primary vesicle. Following penetration of the epidermal cell colonising hyphae developed intercellularly in the mesophyll tissue causing disorganisation of the internal membrane of cells and cell collapse (Keon and Hargreaves, 1983). The first visible symptoms of disease are evident on barley leaves two days post-inoculation (Smedegaard-Petersen, 1977a; Keon and Hargreaves, 1983). Net blotch disease results in an increase in plant respiration (Smedegaard-Petersen, 1977b; Smedegaard-Petersen, 1980; Mandahar and Garg, 1978). This may be due to the toxins produced by the pathogen which affect surrounding cells away from necrotic lesions (Mandahar and Garg, 1978). Stimulation of the Emden Myerhof Pathway, the tricarboxylic acid cycle and the hexose monophosphate pathway occurs together with the

uncoupling of phosphorylation from oxidation in these pathways and these events may be responsible for the increase in respiration in net blotch-affected barley leaf tissue. The effect of net blotch disease on plant respiration in infected leaf tissue is cultivar dependent. A temporary increase in the level of respiration was observed in resistant cultivars infected with *P. teres* f. *teres*, whereas in susceptible plants the increase in respiration occurred at a slower rate and continued until the beginning of necrosis (Smedegaard-Petersen, 1980).

Ascospores may germinate within a few hours (Drechsler, 1923) and infect the plant in as little as 5 h as long as free moisture is available. When the perithecia nears maturity it loses its flat hemispherical shape and develops a short cylindrical beak (Kenneth, 1962). Under wet conditions, mature perithecia break apart and release asci which release the ascospores over a short distance. The ascospores contained in the ascus are not separated but contained in a gelatinous sheath and dispersal is dependent on water splash. *In vitro*, ascospores germinated readily in the presence of water when groups of crushed asci from mature perithecia were placed on leaves of barley seedlings and disease symptoms were visible after 2-3 days (Smedegaard-Petersen, 1972). Jordan (1981) observed germinated ascospores *in vitro* and penetration of the leaves by germ tubes, which can form from any or all cells of an ascospore (Steffenson, 1997) however little information is available regarding ascospore colonisation of the host.

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1.4.4 Factors affecting symptom development, dispersal and epidemiology

Pyrenophora teres can cause infection at any stage in the development of the plant and the age, nutritional status and genotype of the host plant are important factors in

determining the resulting level of disease symptoms (Khan and Boyd, 1969a). There have been conflicting reports regarding the effect plant age has on the disease caused by *P. teres*. Piening (1968) found an increased amount of antifungal compounds in older leaves while Khan and Boyd (1969a) and Tekauz (1986) found an increase in resistance with plant age. However, using young isolates of high infectivity, Singh (1963b) found an increased susceptibility to net blotch in older as compared to younger plants. The flag leaf has been found to be most resistant to disease, regardless of the age of the plant (Singh, 1956; Tekauz, 1986). The nutritional condition of the host is also an important factor affecting infection (Shipton *et al.*, 1973). High amounts of nitrogen and phosphorus increased susceptibility to net blotch infection (Singh, 1956). Deficiencies in other mineral elements such as calcium, iron, magnesium, potash and sulphur resulted in reduced infection (Singh, 1963d). Following inoculation, a 48 h reduction in light intensity (under conditions of high humidity) has been correlated with an increase in the number of net blotch lesions per unit of leaf and a breakdown in host resistance (Khan and Boyd, 1969a). The relationship between isolate age and infectivity requires further investigation. Khan and Boyd (1969a) found that younger isolates of the pathogen (10 day old) produced a greater proportion of immature and non-infective conidia than older cultures, therefore reducing the level of infection on barley seedlings under glasshouse conditions.

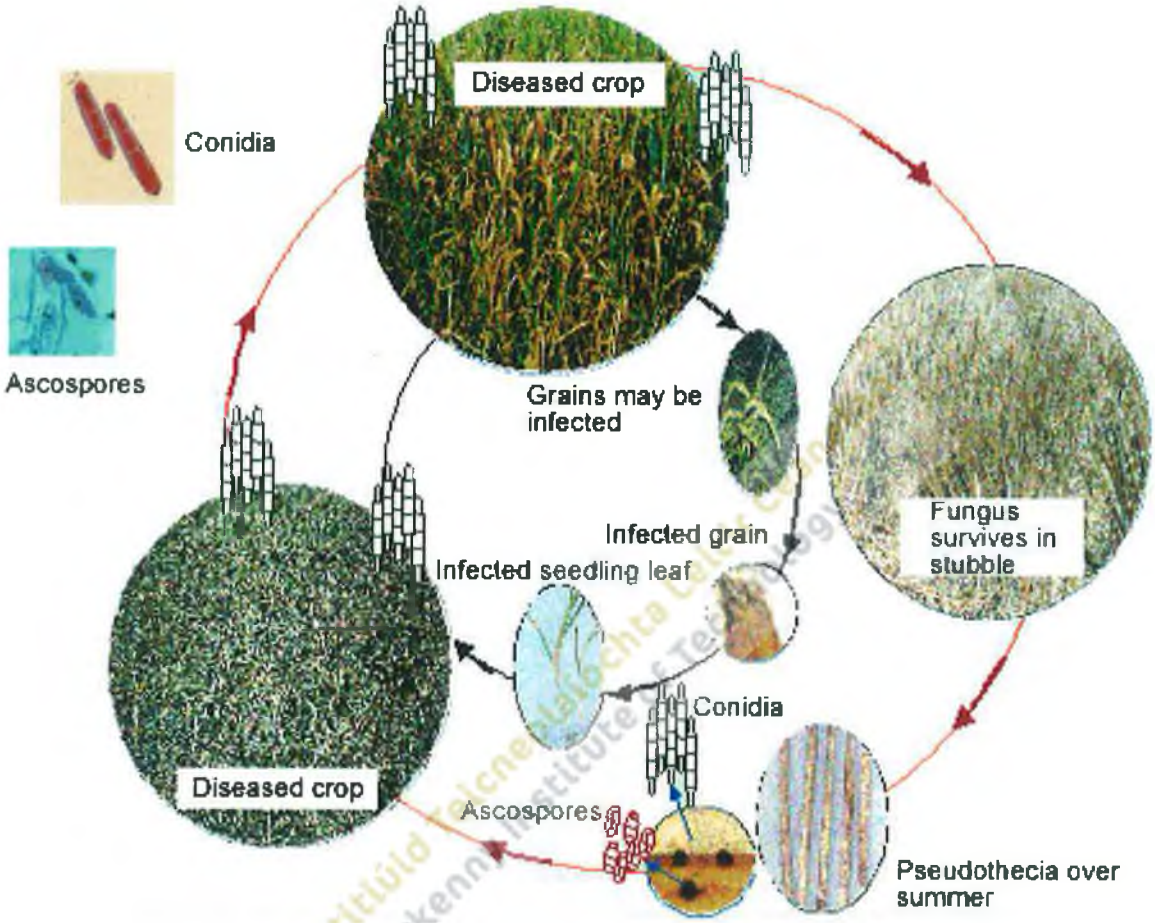

Figure 1.2 Net blotch disease cycle. After: Platz (2004)

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1.4.5 Virulence of *Pyrenophora teres* populations

The virulence of *P. teres* populations is influenced by their genetic makeup, toxin profiles, the reproductive state of the pathogen, environmental factors and host genotypes. Variable virulence is often detected with populations of sexually reproductive, genetically variable pathogens such as *P. teres* (Peever & Milgroom, 1994; Peltonen *et al.*, 1996). High genetic variability has even been detected within *P. teres* populations isolated from the same plant (Peltonen *et al.*, 1996). *In vitro*, isolates of net and spot forms that produce low levels of toxins A and B are less virulent and cause less disease lesions, whereas more virulent isolates produce higher toxin levels and induce more damaging symptoms such as watersoaking and chlorosis (Smedegaard-Petersen, 1977). Virulence may also vary from region to region and from year to year (Tekauz, 1990; Steffensen & Webster, 1992). The introduction of new barley cultivars in Western Australia brought about a change in the virulence of *P. teres* with 73% of net type isolates demonstrating avirulent reactions on previously susceptible cultivars Beecher and Atlas, while moderately resistant cultivars Stirling and O' Connor had become susceptible about a decade after their release in Western Australia (Khan, 1982). Gupta and Loughman (2001) compared the variation in virulence of net type isolates collected between 1975-1985 and 1995-1996 in Western Australia on a set of 47 barley cultivars under glasshouse conditions and found that the virulence has remained stable for the last 19 years. This is thought to be due to a lack of selection pressure for pathogen virulence because highly resistant cultivars have not been grown in this region. Spot type isolates were also examined (1995-1996) and variation in virulence was found on cultivar Herta, however this variation has been reported for the first time from this region and will be used in the development of resistant varieties. Virulence is also influenced by the reproductive

state of the pathogen (Peever and Milgroom, 1994; Peltonen *et al.*, 1996) as the occurrence of the sexual state allows recombination between pathotypes (Cromey and Parkes, 2003).

Pon (1949) first reported the variation in virulence in net type isolates in the United States. *P. teres* is characterised by a large genetic variability leading to the formation of numerous physiologic races (Graner *et al.*, 1996). Khan and Boyd (1969b) identified three distinct races WA-1, WA-2 and WA-3 in Australia, which could be differentiated on two barley cultivars CI1179 and CI7584. Steffenson and Webster (1992) identified 13 pathotypes from 91 isolates collected in California which could be differentiated on 22 barley genotypes. 15 pathotypes were identified from a collection of 23 net type isolates and 4 pathotypes were identified from a collection of 8 spot type isolates on 25 differential barley genotypes (Wu *et al.*, 2003). Net and spot isolates used were from 12 different barley growing regions of the world. Variation among pathotypes is more common among net type isolates than for spot type isolates (Wallwork *et al.*, 1995; Wu *et al.*, 2003). Distinct pathotypes of *P. teres* f. *teres* have been reported from many production areas including Australia (Khan and Boyd, 1969b), Europe (Smedegaard-Petersen, 1971) and North America (Singh, 1962; Steffenson and Webster, 1992). Variation in virulence has also been detected in *P. teres* f. *maculata* (Tekauz, 1990; Tekauz and Mills, 1974; Wu *et al.*, 2003). Breeding for resistance in a region requires knowledge of these pathotypes for both forms of *P. teres*.

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In culture, isolate virulence decreases after repeated subculturing (Kwasna, 1995). Subculturing *P. teres* repeatedly on media containing amino acids maintains the pathogens ability to sporulate (Piening, 1963) and a temperature of 21 °C and 12 h light/12 h dark light regime are essential for *in vitro* conidial production (Onesirosan

& Banttari, 1969). Characteristics of *P. teres* isolates are better maintained when conidia and ascospores are used in subculturing whereas mutation is more frequent when mycelium is sub-cultured (McDonald, 1967; Shipton *et al.*, 1973). Isolates should be regularly passed through a susceptible host in order to maintain virulence.

1.5 Control

Net blotch disease and its control have received more attention in recent years due to the increasing popularity of barley, the use of more intensive management practices (Gupta and Loughman, 2001), earlier sowing of crops (Locke, 1982), the use of short rotations (Gupta and Loughman, 2001), the emergence of resistance to fungicides among populations of *P. teres* (Doyle, 2005) and the EU directive prohibiting the use of mercury-based seed dressing in 1991 (Rennie and Cockerell, 1993). An integrated method of control, combining good crop hygiene practices, the use of resistant cvs and chemical control (both as seed dressing and foliar applications), is currently the most effective net blotch disease management strategy.

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1.5.1 Cultural control

Cultural control involves the manipulation of cultural practices to avert pathogen-associated damage. Cultural practices that help suppress disease development include the use of certified seed, appropriate cropping and conventional tillage practices and the use of crop rotation.

Use of certified clean seed is vital as seed-borne inoculum has the ability to contaminate straw on which it will produce abundant inoculum the following year (Piening, 1968). It is well-established that seed-borne inoculum is a source of primary inoculum for both forms of the pathogen; conidia, perithecia and sclerotia of *P. teres*

can be found on the surface of the seeds (Jordan, 1981). Although barley straw is a major source of inoculum, the seeds contribute to the introduction of the pathogen into plots which were previously free from disease (Youcef-Benkada *et al.*, 1994) highlighting the importance of using clean certified seed.

Cropping practices such as sowing date, application of nitrogen, the rogueing of diseased plants and the use of conventional tillage all affect net blotch disease development. Early sowing means a longer growing season with increased exposure to carry-over inoculum. In field experiments conducted in 1982/1983 and 1983/1984 in Centre and Lancaster counties, Pennsylvania, USA, the greatest and least net blotch disease severities generally occurred in the earliest and latest winter barley plantings, respectively; later planting resulting in an increase in yield, thousand grain weight and seed number per head when compared with earlier planting (Delserone and Cole, 1987). But, in the Centre country trial conducted in 1982/1983, planting date had no influence on disease severity; the authors speculated that this may have been due to the warm and humid spring weather allowing for more cycles of secondary infection in this trial, which in turn masked any apparent effect of planting date on disease severity. Application of nitrogen fertilizer ensures high yield and quality; however it may favour disease development. Locke *et al.* (1981) reported a direct positive correlation between the level of nitrogen applied and the resulting level of net blotch disease on barley. Nitrogen application results in increased relative humidity within the crop canopy which favours the development of disease (Jordan and Hutcheon, 1999).

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The incidence of net blotch has increased in recent years due partly to retention of stubble and minimum tillage (Jordan and Allen, 1984). This leads to a build-up of inoculum that is present on straw debris from previous crops (Jordan,

1981; Jordan and Allen, 1984). *Pyrenophora teres* perithecia produced on straw can retain its infection potential for up to 9 months (Piening, 1967). Sclerotioid organs of *D. teres* can survive for 2 years in the soil and can develop into perithecia if fertilised by spermatia, or into sclerotia if fertilisation is unsuccessful (Ali-Haimoud *et al.*, 1993). Indeed, sowing crops some distance from areas that were previously sown with barley is advisable to prevent wind dispersal of ascospores or conidia (Shipton *et al.*, 1973). Removal of inoculum may be carried out in a number of ways; deep ploughing (to a depth of 20cm), shallow cultivations (to a depth of 15cm), burning, etc. In an experiment that analysed the influence of straw disposal and cultivation methods on the incidence and severity of net blotch, more conidia were found to be present in plots with standing stubble or chopped straw, compared to those plots where the straw had been burnt (Jordan and Allen, 1984). Direct-drilled barley plants were diseased with *P. teres* in a shorter period of time, in comparison to plants sown in ploughed areas (Jordan and Allen, 1984). Piening (1968) found that 42% net blotch infection resulted from a plot where the straw and stubble were lightly diced, compared to only 8% infection where the stubble had been ploughed under. Ascospores of the pathogen were responsible for half the net blotch lesions produced on volunteer barley plants (Piening, 1968). In the past, burning was an important and effective method for the eradication of inoculum sources, however it is not environmentally acceptable as it may be detrimental to nesting birds, cause smoke pollution and removes organic matter from the soil.

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Crop rotation can help minimize plant disease potential by reducing populations of disease organisms surviving in the soil or on crop residues. Although crop rotation reduces the risk of many cereal diseases, it does not eliminate them. Crop rotation is essential for the control of this disease because mono-cropping

encourages build-up of pathogen populations (Pusey, 1996). But crop rotation is generally more effective for soil-borne than for aerial plant diseases such as net blotch (Apple, 1977). Crop rotation also benefits the soil by maintaining a balance of nutrients and improving soil structure (Pusey, 1996). Partial control may be achieved by using rotations of different barley cvs, provided the cvs. have resistance to different races of the pathogen (Campbell, 1989).

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1.5.2 Resistant cultivars

The use of resistant barley cvs is the most effective method of net blotch disease control (Shipton *et al.*, 1973). Due to the influence of plant growth stage, plant genotype, isolate of the pathogen and the environment on disease resistance, plant disease resistance genes are difficult to map and to isolate (Molnar *et al.*, 2000). An increase in the expression of resistance genes is often observed in adult plants (Jonsson *et al.*, 1998). Most cvs. react differently to *P. teres* at different growth stages, with most cvs. displaying susceptible reactions at the seedling stage and more resistant reactions at the heading stage to the net (Tekauz, 1986) and the spot form of the pathogen (Scott, 1992). However, the susceptibility of 2 barley cvs. (Heartland barley and the resistant line CI 5791) to net blotch caused by 2 spot form isolates increased with increasing plant age (Tekauz, 1986). This enhanced resistance at the adult plant stage may be attributed to genes conferring seedling resistance still functioning to protect adult plants from the fungus, or additional genes conferring adult plant resistance may be present in high frequencies in the barley germplasm tested (Graner *et al.*, 1996). Stefffenson *et al.* (1996) found different QTL that function at different developmental stages with a higher number of QTL's identified at the adult plant stage and only one QTL was in common for adult and seedling plant

resistance. Loci from the same general chromosome region contribute to resistance at both stages of plant development (Steffenson *et al.*, 1996). Jonsson *et al.* (1998) found a significant correlation in disease resistance of barley seedlings inoculated at the one- and two-leaf stages and disease reactions on the fourth and flag leaves when plants were grown in a growth chamber as well as disease levels recorded on the three uppermost leaves in a field experiment. Previously Buchannon and McDonald (1965) and Arabi *et al.* (1990) found a high correlation between seedling resistance to *P. teres* and resistance at the adult plant stage.

Schaller and Wiebe (1952) reported that varieties of barley from the Manchuria region of China were more resistant to net blotch than those from Abyssinia, Switzerland, Norway, Sweden and China. Later, Buchannon and McDonald (1965) found Ethiopian varieties to be most resistant. As the Middle East is reported to be the centre of origin to the genus *Hordeum,* it is not surprising that these areas contain varieties that are resistant (Kenneth, 1962). Highly resistant cultivars such as Clipper lack lesions or produce lesions much reduced in size to net blotch isolates (Scott, 1991) whereas the cultivar Dampier may be regarded as highly susceptible as it shows no resistance against the disease (Khan, 1987). Gupta and Loughman (2001) assessed the current virulence of *P. teres* (net form) on barley in Western Australia and found CI 9214 represents a universally resistant line while Dampier, Stirling and Prior represent universally susceptible lines. Wu *et al.* (2003) found that barley genotypes of Rojo (from the USA), Coast (USA), CIho (9819 (Ethiopia), Ciho (Ukraine), CIho 7584 (USA), CIho 5822 (Ukraine), ND B112 (USA), and FR 926-77 (USA) were resistant to all the net and spot type isolates used in the study which were from 12 barley growing regions of the world representing a diverse collection of isolates. Use of net blotch disease resistant cultivars reduces the

severity of the disease during the growing season but also reduces the amount of initial inoculum available to cause disease the following season (Murray *et al.*, 1998).

The genetics of barley resistance to net blotch disease is complex, with numerous genes affecting resistance. Several major resistance genes (*R* genes) have been identified in barley that confer high levels of resistance against *P. teres* (Graner *et al.*, 1996; Khan and Boyd 1982; Steffenson 1997) as have some genes that confer partial resistance against the pathogen (Arabi *et al.*, 1990; Douglas and Gordon, 1985; Harrabi *et al.*, 1993; Steffenson and Webster, 1992b). Major resistance genes include *Rpt1* located on chromosome 3, *Rpt2* on chromosome 5 and *Rpt3* on chromosome 2 (Søgaard and von Wettstein-Knowles, 1987). Net blotch was evaluated at the seedling stage in the glasshouse and at the adult plant stages in the field to determine the number and chromosomal location of quantitative trait loci controlling resistance to *P. teres* f. *teres* isolate ND89-19 on a cv. Steptoe × Morex cross, quantitative trait loci (QTL) located on chromosomes 4 and 6 were found to confer seedling resistance and seven QTL positioned on all chromosomes, except chromosome 5, conferred adult plant resistance to the disease (Steffenson *et al.*, 1996). Spaner *et al.* (1998) identified QTL on chromosomes 1, 3, 4, 6, and 7 of a Harrington × TR306 cross that conferred adult plant resistance to natural *P. teres* infections, but none co-segregated with those reported for adult plant resistance by Steffenson *et al.* (1996). The chromosome 3 and 4 QTL detected by Spaner *et al.* (1998) correspond to QTL for heading date, and the authors proposed that these QTL may reflect escape from disease through differential maturation. In an experiment where five barley genotypes which varied in resistance to *P. teres* and 10 F_1 progeny were inoculated with the pathogen in controlled conditions; all analyses showed that average effects of allelles were of much greater importance than dominance in controlling resistance (Douglas and Gordon, 1985).

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Genotypes with partial resistance endure a moderate level of disease during epidemics which may not be acceptable to growers who expect significant reductions in yield with moderate levels of disease (Steffenson *et al.*, 1991). Potential yield loss caused by *P. teres* f. *teres* was investigated on two barley genotypes Kombar (susceptible cultivar) and UC 603 (cultivar which possesses incomplete resistance to *P. teres* f. *teres*) over a two year period when net blotch incidence was high (Steffenson *et al.*, 1991). Although infection responses for both cultivars were high (determined by large lesion types) in growth chamber and field studies, the incomplete resistance of UC 603 effectively reduced yield loss due to *P. teres* with yield losses of 3.2-5.3% in contract to Kombar which showed yield losses of 31-35%. Results of this study show that satisfactory yields can still be obtained with genotypes possessing incomplete resistance in the presence of moderate amounts of disease.

A significant variation in pathogenicity of *P. teres* among French and Syrian isolates was found in a study conducted by Arabi *et al.* (2003). Cultivars CI-5791, 79-SIO-10 and Arrivate which were considered in the past as resistant (Arabi *et al.*, 1992; Jonsson *et al.*, 1999; Williams *et al.*, 1999) exhibited susceptible reactions when inoculated with French isolates R5 and S5 suggesting that these isolates may be considered as a physiological race of the pathogen (Arabi *et al.*, 2003). Since pathotype varies from region to region and virulence varies from pathotype to pathotype (Steffenson and Webster, 1992) results identifying resistance may not be applicable to another region which contains different isolates.

Variability in response of cultivars to isolates of *P. teres* may arise from differences in environmental conditions. The barley genotype greatly influences the effect of environmental conditions on net blotch disease development. The effect of factors studied in the reaction of barley to net blotch is highly dependent on the

variety of barley used in the study; high post-inoculation temperatures (36 °C) resulted in breakdown of resistance to net form net blotch in two Ethiopian and four Manchurian cultivars to an isolate of *P. teres* under glasshouse conditions (Khan and Boyd, 1969a). However high preinoculation temperatures or high light intensity during a 48 h high humidity incubation period enhanced resistance of these cultivars.

The breeding of net blotch disease resistant barley cvs is time consuming and costly. Therefore, the development of molecular tools that could identify the presence of resistance genes in germplasm early on in the preliminary selection stage and that could track the inheritance of these genes in subsequent crossing progeny would be extremely beneficial for breeders. It would also facilitate the pyramiding of resistance into new populations (Choo *et al.*, 1994). The cloning and characterisation of the resistance genes would enable the production of disease resistant barley by genetic modification (GM) technology.

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1.5.3 Chemical control

Chemical control, both as seed dressing and foliar application, is an important component of an integrated control strategy for net blotch disease. The introduction of the EU directive (1991) prohibiting the use of mercury based products saw an increase in the incidence of net blotch disease in Britain (Rennie and Cockerell, 1993). The main group of fungicides used against net form net blotch belong to the triazole (Locke, 2000) and strobilurins groups (Whitehead, 2004). The curative triazole fungicides inhibit the C14-demethylase enzyme, thus inhibiting the production of sterols essential for the maintenance and integrity of fungal membranes (Gisi *et al.*, 2000). In a cereal fungicide trial conducted by Syngenta 2003 (Kimpton, 2004), the

strobilurin azoxystrobin was more effective than the tested triazole propiconazole for controlling net form net blotch disease on barley cv. Harrington when applied as a single application under field conditions (0.43 t/ha increase vs. 0.17 t/ha increase compared to untreated control) (Kimpton, 2004). Strobilurins disrupt electron transport in fungal mitochondria; they bind to the Qo site (the outer, quinone oxidizing pocket) of the cytochrome bc1 enzyme complex (complex III), thus blocking electron transfer in the respiration pathway and leading to energy deficiency due to a lack of ATP and ultimately this causes cell death (Becker *et al.*, 1981). Second generation strobilurins such as Acanto (picoxystrobin) have curative as well as preventative properties; this curative activity is more effective against net blotch (form *teres*) (Doyle, 2002). A field trial comparing disease control programmes on spring barley cultivar Lux was conducted at Kildalton, Co. Kilkenny in 2001 where net form net blotch was severe. A two spray programme (GS 31 and GS 39) including the strobilurins Amistar and Opera gave the highest yields (> two-fold increase) and reduced net blotch disease severity by 94%, when compared to unsprayed plots (Dunne, 2001). The highest reduction in disease severity using a single application was 66% (compared to untreated controls) and this was achieved using the strobilurin Opera at GS 39.

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A decline in the sensitivity of propiconazole and prochloraz was found in net blotch isolates tested in England between 1989-1996 (HGCA Project Report No. 132) (Locke, 1996). Sensitivity testing was conducted whereby isolates were subcultured on potato dextrose agar that was either unamended or contained propiconazole and prochloraz, and the percentage growth rate of the cultures on plates containing fungicide was compared to that on unamended agar. Up until 1996 control of net blotch was largely dependent on the DMI fungicides, however other chemistries such

as azoxystrobin (strobilurin) and cyprodinil (aniliopyrimidine) have become available in controlling the disease (Locke, 2000). Further testing of these fungicides to net blotch isolates in 1999 revealed that the mean sensitivity of these isolates were similar to results obtained three years previously indicating that the shift in sensitivity that had occurred between 1989 – 1996 had stopped (Locke, 2000). Sensitivity to the anilinopyrimidine fungicide cyprodinil was tested in 1999 and it was found that isolates of *P. teres* were very sensitive to this fungicide indicating that if use of this chemical increases, a shift in the sensitivity of the *P. teres* population in the UK may be expected (Locke, 2000). Locke (2000) examined if any resistance to the strobilurin fungicide azoxystrobin was present in UK *P. teres* populations in 1999, using a spore germination assay testing 176 isolates (obtained from 22 winter barley crops in England) and no resistance was detected. In the last few years (2002 – 2005), partial resistance to strobilurins has been detected among Belgian, French, UK and Irish net form isolates of *P. teres* (Doyle, 2005). *Pyrenophora teres* resistance to strobilurins has been associated with a change from phenylalanine to leucine at position 129 (F129L) of the cytochrome b gene which prevents the strobilurin binding, reducing their effectiveness (Kim *et al.*, 2003). While this mutation has rendered isolates partially resistant to strobilurins, the G134A cytochrome b gene mutation detected in *Mycosphaerella graminis* and *Blumeria graminis* f. sp. *tritici* and *hordei* isolates confers major rather than partial resistance to strobilurins (Gisi *et al.*, 2002). The G134A mutation has not yet been detected in strobiliurin-resistant *P. teres* isolates (Doyle, 2005).

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Environmental conditions and timing of application greatly influence fungicide efficacy. The aim of fungicide application is to maximise the green leaf area of the top three leaves during grain filling; in barley it is the 2nd and 3rd leaves (flag 1

and flag 2) that are most important (Weppler and Hollaway, 2004). In Ireland, it is recommended that fungicides are applied as a T1 spray at GS31 and a (T2) spray at GS 37-49 (as crops frequently show signs of disease such as net blotch prior to first node), using triazoles or a triazole-strobilurin mixture (Dunne, 2002).

The use of recommended fungicide rates is important as the application of low rate doses can increase the likelihood of fungicide resistance emerging in *P. teres* population. Also, the use of a mixture of fungicides (of different modes of action) to control net blotch disease is desirable, as it reduces the risk of fungicide resistance emerging in *P. teres* populations and the usage of fungicide mixtures can reduce the number of fungicide applications required throughout the growing season (Whitehead, 2004).

Under field conditions an application of herbicides (diquat and paraquat) were effective in decreasing *P. teres* conidial production because it destroyed infected green leaf tissue, thereby reducing inoculum production potential and removing potential sites for re-infection (Jordan and Allen, 1984). Toubia-Rahme *et al.* (1994) showed that, under controlled environment conditions, a pre-inoculation treatment of barley leaves with glyphosphate completely inhibited sclerotioid formation by both the net and spot form of the pathogen; paraquat also had an inhibitory effect on sclerotioid formation by both forms, but was not as effective as the glyphosphate treatment. When applied post-inoculation, glyphosphate and paraquat reduced the number of sclerotioid structures produced by both the net and spot forms of *P. teres,* but was more effective against the spot form. It would be interesting to see if similar results could be achieved under field conditions.

Present-day control of net blotch disease of barley relies on the use of seed-dressing and foliar formulations of fungicides, with current chemical groupings

available for control of net blotch including strobilurins, triazoles, benzimidazole, chlorothalonil, morpholine, chlorophenyl, anilinopyrimidine, guanidine, carboxamide and dithiocarbamates (Whitehead, 2004). A mixture of a strobilurin and a triazole is the recommended treatment in Ireland against net blotch; using a mixture is important in order to avoid resistance developing to the strobilurin chemicals (Doyle, 2005).

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1.5.4 Biological control

Biological control or 'biocontrol' is defined as a strategy for reducing disease incidence or severity by direct or indirect manipulation of microorganisms (Maloy, 1993). Cook and Baker (1983) described it as "*the reduction of inoculum density or disease-producing activities of a pathogen or parasite in its active or dormant state, by one or more organisms, accomplished naturally or through manipulation of the environment, host, or antagonist, or by mass introduction of one or more antagonists*". Although biocontrol offers a positive alternative to chemical pesticides, the overall contribution of biocontrol represents about 1% of agricultural chemical sales (Lidert, 2001). There are currently 13 bacteria and 12 fungi registered with the US Environmental Protection Agency which can be sold as biocontrol agents against plant disease (Fravel, 2005).

While several researchers have investigated the potential of bacteria and fungi to control cereal diseases, presently, *Pseudomonas chlororaphis* strain MA 342 marketed as Cedomon (BioAgri AB, Stockholm, Sweden) is the only biocontrol agent commercially available for the control of net blotch disease of barley (Copping, 2004). This bacterium is formulated as a seed treatment containing 1 X 10^6 colony forming units (cfu) per gram. *Pseudomonas chlororaphis* MA 342 competes with pathogens for nutrients and space, encourages the plant's natural defence system,

promotes the development of roots and shoots (Copping, 2004) and suppresses fungal growth by producing the antifungal compound 2,3-deepoxy-2,3-didehydrorhizoxin (Hökeberg, 1998). This product is commercially available in the United States (and is registered with the US Environmental Protection Agency) and is available in Sweden, Norway, Finland and Austria (BioAgri, Sweden). *Pseudomonas chlororaphis* has been included in Annex I of Directive 91/414/EEC since March 2004 (Anon, 2004).

It is anticipated that more biological agents will be registered for the control of net blotch disease, especially for use in the growing organic cereal production tillage sector. An important criterion for the selection of such agents will be to ensure that they are adapted to the climate and soil in which they are to be used (Leyns *et al.*, 1990; de Bruyne *et al.*, 1991; Hökeberg *et al.*, 1997). The mechanisms of pathogen suppression by bacteria include the production of antimicrobial substances, induced resistance, competition between the biocontrol microorganism for nutrients and plant surface area (Weller, 1988; Pedersen *et al.*, 1999) and competition for iron through the production of siderophores (Whipps, 2001; Baaker *et al.*, 1993). These mechanisms need not be exclusive, and it is desirable that any new biocontrol agents developed for the control of net blotch disease possess as many of these attributes as possible. Also any biological control agent should be formulated so that it is has a long and stable shelf life, is easy to apply and is active once applied and multiplies on the plant surface.

Ali-Haimoud *et al.* (1993) observed that mycelial suspensions and culture filtrates of several fungal isolates (strains of *Trichoderma koningii*, *T. viride*, *T. pseudokoningii* and of two unidentified fungi) and of the actinomycete *Micromonospora* spp. significantly inhibited the *in vitro* formation of sclerotioid organs on *P. teres* var. *teres*- and var. *maculata*-inoculated barley straw. Sclerotioid

organs are important survival and reproductive structures (these frutifications can form perithecia if fertilized or sclerotia if not and they can remain viable in soil for up to 2 years) (Ali-Haimoud *et al.*, 1993). Both the mycelium and culture filtrate of *T. viride* and *T. pseudokoningii* also significantly inhibited sclerotioid organ germination on barley straw, whether applied pre- or post- *P. teres* inoculation. The spot form of the pathogen was generally more sensitive to the culture filtrates than was the net form. However, Amundsson and Hökeberg (1984) found that several known antagonists, including *Trichoderma* spp., *Serratia* spp. and various strains of *Pseudomonas fluorescens*, were not effective against *P. teres*. More recently, Hökeberg *et al.* (1997) found that *Pseudomonas chlororaphis* strain MA 342 seed treatment resulted in a > 98 % reduction in the incidence of *P. teres*-infected plants derived from pathogen-inoculated seed grown under field conditions. The disease control and yield increases resulting from this bacterial seed treatment were similar to those achieved by treating seed with the fungicide Panoctine Plus 400 (guazatine and imazalil). This bacterium also suppressed common bunt of wheat (caused by *Tilletia caries*) under field conditions, had a shelf life of up to six weeks and freezing did not influence its biocontrol efficacy.

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1.6 Objectives of research

This research examined the ability of bacteria isolated from Irish cereals, cereal rhizospheres and cereal weeds to inhibit net blotch disease development both in glasshouse studies and in a preliminary field trial. Furthermore, the mode by which potential biocontrol agents suppressed this disease was assessed.

Chapter 2: Materials and Methods

2.1 Fungal and bacterial inoculum

Mycelium of the highly aggressive isolate *Pyrenophora teres* var. *teres* strain N45 (isolated from Irish barley in 1999) was subcultured from –70 °C stocks onto potato dextrose agar (PDA; Oxoid, UK) and incubated at 20 °C for 7 days under near-UV light. Conidia were scraped from the surface of the agar mat into 0.2% v v^{-1} Tween 20. This suspension was filtered through a double layer of J-cloth and the inoculum concentration was adjusted to 2 X 10^4 spores ml^{-1} 0.2 % Tween 20.

Bacterial isolates, originating from Irish cereal plant, cereal rhizosphere soil samples and from weeds growing in cereal fields (collected in 2002), were previously identified by both morphological and 16S rRNA sequence analyses (Khan *et al.*, 2006) (Table 2.1). Bacteria were subcultured from –70 °C stocks onto nutrient agar (Oxoid, UK), incubated at 25 °C for 3 days, and subcultured into 100 ml of Luria Bertani (LB) broth (Luria and Burrows, 1957) (100 ml) and incubated at 25 °C, 180 rpm for 24 h. The optical density (OD) of the resulting inoculum was adjusted to $OD_{0.3}$ with SDW.

2.2 Plant material

The barley used in this study included the winter cvs. Siberia and Calcutta and the spring cvs Lux, Fractal, Tavern and Prestige (kindly supplied by Goldcrop, Seedtech and Powerseeds, Cork, Ireland). Cultivars Siberia and Lux are rated as susceptible to moderately susceptible to net blotch, and cultivars Calcutta, Fractal, Tavern as moderately resistant to resistant, based on the 2002 – 2004 Department of Agriculture, Food and Rural Development (DAF) Cereal Variety and Evaluation Programme

(ratings = 4, 5, 6, 6 and 7, respectively on a scale of 0 – 9, 9 being highly resistant) (DAFRD, 2002; DAF 2003, 2004). Prestige was not evaluated in the DAFRD trials, but was rated as moderately susceptible (comparable to cv. Lux) in the Danish Annual Report of the National Field Trials 2003 (Pedersen, 2004).

2.3 Seedling trials

Seedling trials were conducted to screen bacteria for their biocontrol potential, to further test the efficacy of bacteria that appeared promising in the screen, to determine the relationship between time of bacterial application (relative to fungal inoculation) and biocontrol efficacy, and to determine if select bacteria induced systemic resistance to net blotch disease.

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Screening trials: In an initial screening trial, ninety-nine bacteria (Table 2.1) were screened for their potential to control net blotch disease under glasshouse conditions. Seeds of the spring cv. Lux were germinated in the dark at 20 °C on moist Whatman No. 1 filter paper (Whatman International Ltd., England) for 48 h. Germlings were planted in 12 cm diameter pots containing John Innes Compost No.2 (3 seeds per pot) and grown in the glasshouse with a 16/8 h light/dark regime. At growth stage (GS) 13 (Zadoks *et al.*, 1974), the whole plant was inoculated until runoff (approx. 10ml) with either a bacterial isolate $OD_{0.3}$ or LB broth (control plants) using a hand-held sprayer. Leaves that received bacterial or LB treatment were then sprayed 24 h later with a *P. teres* var. *teres* strain N45 conidial suspension (2×10^4 spores ml^{-1} 0.2 % Tween 20) or with 0.2 % Tween 20. Plants were covered with polythene bags to increase humidity and enhance spore germination. Bags were removed 24 h post-inoculation. Three plants (in one pot) received each treatment combination. The bags were replaced for

an additional 24 h. Visual disease symptoms were assessed 5, 7 and 10 days post-*P. teres* inoculation using the numerical scale devised by Tekauz (1985) (scale = 0 – 10, 10 being very susceptible) and these disease scores were used to calculate the area under the disease progress curve (AUDPC) (Shanner and Finney, 1977). The screening trial was conducted once.

Further seedling trials were then conducted to assess the biocontrol efficacy of those bacteria showing possible net blotch disease suppression in the initial screening trial, i.e. unidentified isolates MKB52, MKB141 and MKB66, *Bacillus megaterium* strain MBK135, *Pseudomonas* sp. MKB158, *Pseudomonas* sp. MKB194 and *Ps. fluorescens* strains MKB100 and MKB156. These seedling trials were conducted as described above for the initial screening trial, except that two barley cvs. (Lux and Tavern) were used, 6 plants per cv. (2 plants per pot) were subjected to each treatment combination and this experiment was conducted twice.

Timing trials: Seedling trials were conducted to determine the effect of the time of application of *Ps. fluorescens* strains MKB100 and MKB156, and *Pseudomonas* sp. MKB194 on net blotch disease development. These trials were conducted as described above except that bacterial and LB broth treatments were performed at either 6, 4 and 2 days pre- or 2, 4 and 6 days post-*P. teres* or 0.2% Tween 20 treatment and the visual disease scores 4, 6, 8 and 10 days post-fungal inoculation were used to calculate AUDPC

2.4 Dual culture plate tests

The ability of four bacteria (*Ps. fluorescens* strains MKB100 and MKB156, and *Pseudomonas* sp. MKB158, and *Pseudomonas* sp. MKB194) to directly inhibit the growth of *P. teres* var. *teres* was investigated using dual culture plate tests. These tests

were performed using a modification of the method described by Pedersen *et al.* (1999). Each PDA plate was inoculated with a mycelial plug (0.5 cm diameter) from a 14-day-old PDA culture of *P. teres* var. *teres* strain N45 and 1 µl of either bacterial suspension $OD_{0.3}$ or LB broth (control treatments) was inoculated 4 cm away from the *P. teres* plug. Plates were incubated at 20 °C in darkness. Fungal colony diameter was measured after 4, 5 and 6 days and used to determine the growth rate (mm day^{-1}) of *P. teres* on dual culture plates, relative to that of the pathogen grown in the presence of LB broth. This experiment was conducted twice and each time included three replica PDA culture plates per treatment combination.

2.5 Systemic resistance trials

The ability of *Ps. fluorescens* strains MKB156 and MKB100, and *Pseudomonas* sp. MKB194 to induce systemic seedling resistance to net blotch disease was investigated under contained environment conditions. Seeds were surface-sterilised as described by Fischer (2003) and germinated on filter paper, as described above. Sterile John Innes Compost No. 2 was amended with bacterial inoculum or LB broth (15 ml per 150 g soil) and placed in 7 cm diameter pots. After 24 h, the germlings were planted in these pots (2 per pot). At GS 13, the above ground plant tissue was inoculated with *P. teres* var. *teres* strain N45 or 0.2% Tween 20, as described above. The plants were grown in a climate-controlled growth room at 12 h day/12 h night temperatures of 20/12 °C with a 12-h light period (700 µmol m^{-2} s^{-1}) and constant humidity of 85%. Disease was assessed as described above for the screening trial. Six plants (2 per pot) received each treatment combination and the experiment was conducted twice.

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2.6 Detached leaf assay

The biocontrol efficacy of unidentified isolates MKB23 and MKB44, *Bacillus sphaericus* strain MBK232, *Pseudomonas* sp. MKB158, *Pseudomonas* sp. MKB194, *Ps. fluorescens* strains MKB100 and MKB156 and *Pseudomonas tolaasii* MKB286 against net blotch disease on barley cvs Lux, Tavern, Siberia, Calcutta, Fractal and Prestige was assessed using a detached leaf assay. Seedlings of barley were grown as described above for seedling trials. At GS 13, leaf segments (3.5 cm) from the 2nd leaves were placed in 9 cm diameter Petri dishes on 0.5 % wv^{-1} water agar (technical agar No. 3, Oxoid, UK) amended with 0.05 g l^{-1} kinetin (Sigma, UK) (3 leaf segments per plate, adaxial surface uppermost). A 10 µl droplet of either *P. teres* inoculum (2 x 10^4 spores ml^{-1} 0.2 % Tween 20) or 0.2% Tween 20 was placed on the centre of the leaf segments. When the droplet had evaporated (2 h post-inoculation), a 10 µl droplet of either bacterial inoculum $OD_{0.3}$ or LB broth was applied to the same position in the centre of the leaf segments. Six leaf segments (3 per plate) were subjected to each treatment combination. Plates were incubated at 20 °C in a controlled environment with a 12 h white light (700 µmol m^{-2} s^{-1})/12 h dark cycle. Disease was assessed as the area of the disease lesion at 3, 4 and 5 days post-inoculation (dpi) and these values were used to calculate the AUDPC (Shaner and Finney, 1977). This experiment was conducted twice.

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2.7 Field trial

The spring barley cv. Lux was grown in 18 x 1 m^2 field plots [separated by 1m that incorporated an oat (cv. Freddy) guard row] in Thornfield, UCD in 2005. Eight grams of barley seed was applied to each plot, as was 4 g of NPK 10-10-20 (Agrifert, Switzerland) fertiliser at the time of planting. The field trial was enclosed in netting to

prevent seed loss due to birds. Chemical treatments were as follows: GS 20, herbicides CMPP (600g mecoprop-p l^{-1}, 1.5 l Ha^{-1}) (BASF, Ireland) and Oxytril (200g bromoxynil and 200g bioxynil l^{-1}, 1.5 l Ha^{-1}) (Bayer Crop Science, UK);.GS 25, the aphicide Decis Quick® (25 g deltramethrin l^{-1}, 0.25 l Ha^{-1}) (Bayer Crop Science, UK); GS28, Corbell (750g fenpropimorph l^{-1}, 1 l Ha^{-1}) (BASF, Ireland) to control mildew. At GS39 the first and second leaves of 10 plants per plot were sprayed to runoff (approx. 10 ml) with either bacterial inoculum $OD_{0.3}$ or LB broth, using a hand-held sprayer. Plots were then covered with clear polythene bags and after 48 h, the bags were removed and the aforementioned leaves were then sprayed to runoff with either *P. teres* var. *teres* strain N45 inoculum (2×10^4 spores ml^{-1} 0.2 % Tween 20) or 0.2 % Tween 20. The polythene bags were replaced for 24 h. All inoculations took place under wind-free conditions and when the temperature was < 18 °C. The treatments were administered in a randomised plot design, with 3 replicate plots per treatment combination. At GS 60 a further 10 plants per plot were sprayed until runoff with either bacterial inoculum or LB broth, covered in polythene bags and 24 h later sprayed with the fungal inoculum or 0.2% Tween 20. The polythene bags were replaced for 24 h. Disease was assessed as the area of the disease lesion at 5, 7 and 10 dpi and these values were used to calculate the AUDPC. This experiment was conducted once.

2.8 Statistical analysis

Normal data distribution was confirmed for the seedling experiment, timing trials, systemic resistance trial, field experiment, and the dual culture plate test using the Ryan Joiner test (Ryan *et al.*, 1985) and the Johnston transformation test (Johnson, 1995) was used to normalise the distribution of the data from the detached leaf assay. These tests were performed in Minitab (Minitab release 13.32©, 2000 Minitab Inc.,

USA). Analysis of variance (ANOVA) of all the above mentioned tests (incorporating Tukey's pairwise comparison at the 5% level of significance) was performed using SPSS (SPSS release 11.0.1©, SPSS Inc., USA). The Pearsons Product Moment Correlation between AUDPC values from the detached leaf assay and the seedling test, and between the seedling test and the field trial data were performed using SPSS (SPSS release 11.0.1©, SPSS Inc., USA).

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Table 2.1 Code and origin of bacterial isolates

Isolate code	Identity[a]	*Origin*	
		Host tissue[b]	**Location**
MKB 135	*Bacillus megaterium*	Wheat rhizosphere	Castledermot, Co. Kildare
MKB 221	*B. mojavensis*	Wheat rhizosphere	Claregalway, Co. Galway
MKB 15	*B. mycoides*	Barley rhizosphere	Castlefin, Co. Donegal
MKB 23	*B. mycoides*	Barley soil	Castlefin, Co. Donegal
MKB 45	*B. mycoides*	Barley rhizosphere	Castlefin, Co. Donegal
MKB 73	*B. mycoides*	Barley rhizosphere	Claregalway, Co. Galway
MKB 44	*B. sphaericus*	Barley soil	Castlefin, Co. Donegal
MKB 232	*B. sphaericus*	Barley soil	Piltown, Co. Tipperary
MKB 41	*Exiguobacterium* sp.	Barley rhizosphere	Castlefin, Co. Donegal
MKB 49	*Exiguobacterium* sp.	Barley soil	Castlefin, Co. Donegal
MKB 150	*Earthworm bacterium*	Barley soil	Claregalway, Co. Galway
MKB 29	*Pantoea agglomerans*	Barley soil	Castlefin, Co. Donegal
MKB 158	*Pseudomonas* sp.	Oat rhizosphere	Castlefin, Co. Donegal
MKB 194	*Pseudomonas* sp.	Wheat leaf	Ardee, Co Louth
MKB 90	Ps. fluorescens	Oat chaff	Castlefin, Co. Donegal
MKB 100	*Ps. fluorescens*	Oat rhizosphere	Castlefin, Co. Donegal
MKB 133	*Ps. fluorescens*	Wheat rhizosphere	Castledermot, Co. Kildare
MKB 156	*Ps. fluorescens*	Barley rhizosphere	Claregalway, Co. Galway
MKB 286	*Ps. tolaasii*	Barley leaf	Piltown, Co. Tipperary
MKB 2	ND	Barley leaf	Castlefin, Co. Donegal
MKB 4	ND	Barley leaf	Castlefin, Co. Donegal
MKB 6	ND	Barley leaf	Castlefin, Co. Donegal
MKB 8	ND	Barley chaff	Castlefin, Co. Donegal
MKB 9	ND	Barley chaff	Castlefin, Co. Donegal
MKB 12	ND	Barley grain	Castlefin, Co. Donegal
MKB 17	ND	Barley rhizosphere	Castlefin, Co. Donegal
MKB 20	ND	Barley grain	Castlefin, Co. Donegal
MKB 22	ND	Barley leaf	Castlefin, Co. Donegal
MKB 23	ND	Barley soil	Castlefin, Co. Donegal
MKB 25	ND	Barley grain	Castlefin, Co. Donegal
MKB 26	ND	Barley grain	Castlefin, Co. Donegal
MKB 27	ND	Barley rhizosphere	Castlefin, Co. Donegal
MKB 30	ND	Barley leaf	Castlefin, Co. Donegal
MKB 31	ND	Barley leaf	Castlefin, Co. Donegal
MKB32	ND	Barley leaf	Castlefin, Co. Donegal
MKB 36	ND	Barley rhizosphere	Castlefin, Co. Donegal
MKB 37	ND	Barley grain	Castlefin, Co. Donegal
MKB 43	ND	Barley rhizosphere	Castlefin, Co. Donegal
MKB 46	ND	Barley rhizosphere	Castlefin, Co. Donegal
MKB 47	ND	Barley rhizosphere	Castlefin, Co. Donegal
MKB 48	ND	Barley rhizosphere	Castlefin, Co. Donegal
MKB 50	ND	Barley soil	Castlefin, Co. Donegal

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Table 2.1 Continued...

Isolate code	Identity[a]	*Origin*	
		Host tissue[b]	Location
MKB 52	ND	Barley rhizosphere	Castlefin, Co. Donegal
MKB 53	ND	Barley soil	Castlefin, Co. Donegal
MKB 54	ND	Oat leaf	Castlefin, Co. Donegal
MKB 56	ND	Oat leaf	Castlefin, Co. Donegal
MKB 57	ND	Oat leaf	Castlefin, Co. Donegal
MKB 58	ND	Oat grain	Castlefin, Co. Donegal
MKB 59	ND	Oat grain	Castlefin, Co. Donegal
MKB 60	ND	Oat grain	Castlefin, Co. Donegal
MKB 61	ND	Oat leaf	Castlefin, Co. Donegal
MKB 62	ND	Oat chaff	Castlefin, Co. Donegal
MKB 63	ND	Oat chaff	Castlefin, Co. Donegal
MKB 64	ND	Oat grain	Castlefin, Co. Donegal
MKB 65	ND	Oat grain	Castlefin, Co. Donegal
MKB 66	*ND*	Oat rhizosphere	Castlefin, Co. Donegal
MKB 67	ND	Oat leaf	Castlefin, Co. Donegal
MKB 68	ND	Oat grain	Castlefin, Co. Donegal
MKB 69	ND	Oat grain	Castlefin, Co. Donegal
MKB 70	ND	Oat rhizosphere	Castlefin, Co. Donegal
MKB 72	ND	Oat rhizosphere	Castlefin, Co. Donegal
MKB 74	ND	Barley soil	Claregalway, Co. Galway
MKB 75	ND	Barley leaf	Claregalway, Co. Galway
MKB 76	ND	Barley leaf	Claregalway, Co. Galway
MKB 77	ND	Barley leaf	Claregalway, Co. Galway
MKB 78	ND	Barley rhizosphere	Claregalway, Co. Galway
MKB 79	ND	Barley rhizosphere	Claregalway, Co. Galway
MKB 81	ND	Barley leaf	Claregalway, Co. Galway
MKB 89	ND	Oat chaff	Claregalway, Co. Galway
MKB 101	ND	Wheat leaf	Castledermot, Co. Kildare
MKB 104	ND	Wheat rhizosphere	Castledermot, Co. Kildare
MKB 105	ND	Wheat leaf	Castledermot, Co. Kildare
MKB 106	ND	Wheat leaf	Castledermot, Co. Kildare
MKB 108	ND	Wheat leaf	Castledermot, Co. Kildare
MKB 111	ND	Barley weed leaf	Castlefin, Co. Donegal
MKB 112	ND	Barley weed leaf	Castlefin, Co. Donegal
MKB 120	ND	Oat grain	Castlefin, Co. Donegal
MKB 121	ND	Oat chaff	Castlefin, Co. Donegal
MKB 124	ND	Barley rhizosphere	Claregalway, Co. Galway
MKB 125	ND	Barley weed leaf	Castlefin, Co. Donegal
MKB 138	ND	Barley weed leaf	Castlefin, Co. Donegal
MKB 140	ND	Oat chaff	Castlefin, Co. Donegal

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Table 2.1 Continued...

Isolate code	Identity[a]	*Origin*	
		Host tissue[b]	Location
MKB 141	ND	Oat rhizosphere	Castlefin, Co. Donegal
MKB 143	ND	Oat grain	Castlefin, Co. Donegal
MKB 153	ND	Oat grain	Castlefin, Co. Donegal
MKB 164	ND	Oat grain	Castlefin, Co. Donegal
MKB 177	ND	Wheat leaf	Castledermot, Co. Kildare
MKB 181	ND	Wheat leaf	Castledermot, Co. Kildare
MKB 190	ND	Wheat leaf	Piltown, Co. Tipperary
MKB 193	ND	Wheat leaf	Ardee, Co Louth
MKB 209	ND	Barley weed	Ardee, Co Louth
MKB 210	ND	Barley weed	Piltown, Co. Tipperary
MKB 230	ND	Barley weed	Claregalway, Co. Galway
MKB 232	ND	Wheat leaf	Piltown, Co. Tipperary
MKB 244	*ND*	Wheat leaf	Piltown, Co. Tipperary
MKB 249	ND	Barley rhizosphere	Claregalway, Co. Galway
MKB 258	ND	Barley rhizosphere	Claregalway, Co. Galway
MKB 271	ND	Wheat leaf	Ardee, Co Louth
MKB 281	ND	Wheat leaf	Piltown, Co. Tipperary

[a]Positive identification through analysis of 16s region of ribosomal DNA by Khan *et al.* (unpub. data), ND = not determined

[b]Bacteria were isolated from either the surface of cereal leaves or grain, from cereal rhizosphere soils, or from weeds growing in a barley field.

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Chapter 3: Results

3.1 *In vivo* effect of bacteria on net blotch disease of barley seedlings

A preliminary screen was conducted to determine if any of 99 bacterial isolates (Table 2.1) showed potential for suppressing net blotch symptom development caused by *P. teres* var. *teres* (strain N45) on barley cv. Lux seedlings under glasshouse conditions. Figure 3.1 illustrates the typical net blotch symptoms observed on diseased plants. Based on the results (detailed in Appendix 1.1), eight bacterial isolates were chosen for further study on the basis of (i) their ability to inhibit net blotch disease, (ii) their identity, and (iii) their ability to inhibit septoria tritici blotch and seedling blight of wheat caused by *Mycosphaerella graminicola* and *Fusarium* spp., respectively (Kildea, S., Khan, Fisher, S., Egan, D. and Doohan, F.M., unpubl. data; Khan *et al.*, 2006) (see Appendix 1.2 for a more in-depth explanation of why isolates were chosen for further study). These eight bacteria were unidentified strains MKB52, MKB141 and MKB66, *Ps. fluorescens* strains MKB100 and MKB156, *Pseudomonas* sp. strain MKB158, *Pseudomonas* sp. strain MKB194 and *Bacillus megaterium* strain MKB135.

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Figure 3.1 Typical net blotch symptoms on a seedling leaf of barley cultivar Lux 7 days post-inoculation with *Pyrenophora teres* var. *teres* strain N45

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The ability of these eight bacteria to suppress net blotch disease of seedlings of two barley cvs. (Lux and Tavern) was further investigated (see Appendix 1.2 for statistical analysis). All bacterial isolates reduced net blotch disease development on both cvs (Figure 3.2); however, unidentified bacterial isolates MKB52, MKB66 and MKB141 did not significantly reduce disease levels, relative to the disease levels on leaves treated with *P. teres* and LB ($P > 0.05$). There was a significant correlation between the results obtained for cv. Lux and Tavern ($r = 0.978$; $P < 0.01$) (Appendix 1.2.3). Control plants treated with 0.2% Tween 20 + LB broth, or 0.2 % Tween 20 + bacterial culture exhibited no visual disease symptoms and appeared healthy.

Pseudomonas fluorescens strains MKB100 and MKB156, *Pseudomonas* sp. MKB158 and *Pseudomonas* sp. MKB194 all significantly reduced the net blotch disease symptoms on both barley cvs by > 46 % ($P < 0.05$), relative to the disease levels on *P. teres*-inoculated, LB-treated control plants (Figure 3.2). *Pseudomonas fluorescens* strains MKB100 and MKB156 were the most efficient biocontrol agents; they reduced the net blotch AUDPC values on both cvs by at least 64%, relative to *P. teres*-inoculated, LB-treated control plants (Figure 3.2). No bacteria had a detrimental effect on plant health, as determined by visual examination of negative control plants treated with bacterial inoculum and 0.2 % Tween 20.

3.2 Effect of timing of application on the biocontrol efficacy of bacterial isolates

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This experiment was conducted to determine at what time of application, relative to *P. teres* inoculation, were *Ps. fluorescens* strain MKB100 and MKB156, and *Pseudomonas* sp. MKB194 most effective in controlling net blotch disease on barley cv. Lux. These bacterial isolates were chosen for this study as they were most

effective in controlling *P. teres* symptoms in the seedling experiment and bacteria were applied -6 to + 6 days relative to *P. teres* inoculation.

Irrespective of bacterial isolate, bacterial application pre-fungal inoculation was generally more effective in reducing the AUDPC for net blotch disease than was application post-*P. teres* inoculation (Figure 3.3; see Appendix 1.3 for statistical analysis). The lowest disease was observed on plants treated with *Ps. fluorescens* MKB100 2 days pre *P. teres* inoculation (AUDPC = 57% of that recorded for *P. teres*-inoculated, LB-treated plants). But this value was not significantly lower than those AUDPC values obtained for plants treated with either, *Ps. fluorescens* MKB156 2 and 6 days pre-fungal inoculation or *Pseudomonas* sp. strain MKB194 6 days pre-fungal inoculation; these treatments resulted in a significant 47 – 53% reduction in the AUDPC values for net blotch disease ($P < 0.05$). When the results of this experiment are compared to the results obtained in the seedling experiment detailed in Section 3.1 where bacteria were applied 1 day-pre fungal inoculation, it appears that bacteria performed better when applied 1 rather than 6, 4 or 2 days pre-*P. teres* inoculation (>71 vs. > 37 % reduction in AUDPC, respectively, relative to *P. teres*-inoculated, LB treated plants). As with the previous experiments, no bacteria had a detrimental effect on plant health, as determined by visual examination of negative control plants treated with bacterial inoculum and 0.2% Tween 20.

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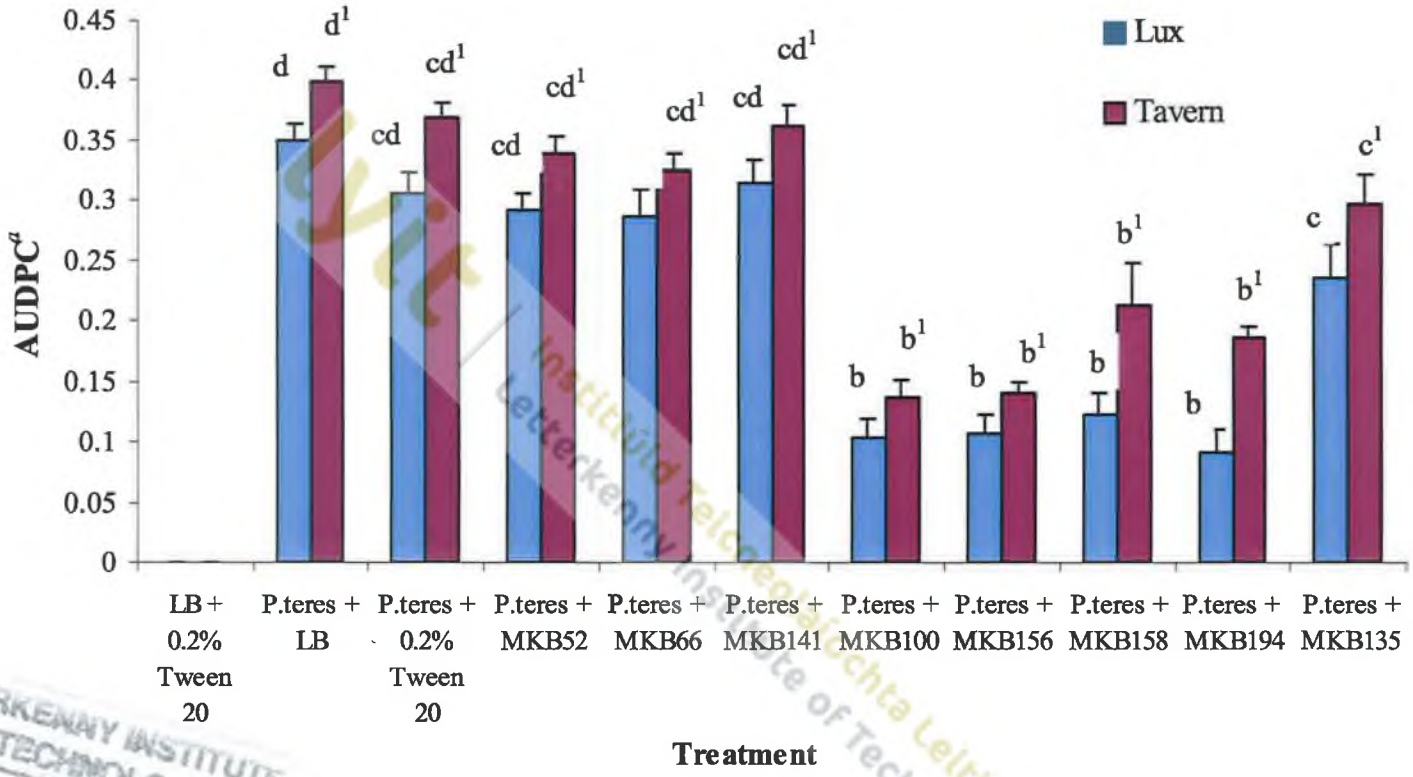

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Figure 3.2 Effect of bacterial treatments on net blotch of barley seedlings on cultivars Lux and Tavern. At growth stage 13 (Zadoks et al., 1974) the whole plant was inoculated with either a bacterial isolate or 0.2% Tween 20 or Luria Bertani broth (LB) followed 24 h later with *P. teres* var *teres* strain N45 inoculum. Codes: *P. teres* and Luria Bertani broth (LB) *Pyrenophora teres*; MKB52, MKB141, MKB66, unidentified bacterial isolates; MKB135, *Bacillus megaterium*; MKB158 and MKB194, *Pseudomonas* sp.; MKB100 and MKB156, *Ps. fluorescens*. Disease was scored 5, 7 and 10 days post-inoculation and used to calculate the area under the disease progress curve (AUDPC[a]). Bars represent SEM. For any given treatment, values followed by the same letter are not significantly different from each other ($P = 0.05$).

3.3 Antagonism of bacteria towards *P. teres*

Dual culture plate tests were performed in order to determine if *Ps. fluorescens* strains MKB100 and MKB156, *Pseudomonas* sp. MKB 158 and *Pseudomonas* sp. MKB194 that significantly inhibited net blotch disease development in the seedling blight tests (see Section 3.1) had a direct inhibitory effect on the *in vitro* growth of *P. teres* on PDA. None significantly retarded the radial growth of *P. teres*, relative to the growth of the pathogen in the absence of bacteria (Statistical analysis of results: Appendix 1.4; Figure 3.4).

3.4 Suppression of *P. teres* by induced systemic resistance

Pseudomonas fluorescens strains MKB100 and MKB156, and *Pseudomonas* sp. MKB194 were three of the bacteria that caused the greatest reduction in net blotch disease of barley cvs Lux and Tavern when applied 1 day pre-fungal inoculation, but were not directly antagonistic to the pathogen (see Sections 3.1, 3.2 & 3.3); further seedling blight tests were performed to determine if these bacteria induced systemic barley resistance to net blotch. To test this, we compared net blotch disease development on *P. teres*-inoculated plants grown in bacterium- or LB broth-amended soil (i.e. the bacterium was spatially separated from the pathogen). Control plants grown in soil amended with either bacterial cultures or LB broth, and treated with 0.2% Tween 20 exhibited no disease symptoms and appeared healthy. Growth of plants in *Ps. fluorescens* MKB156 and *Pseudomonas* sp. MKB194 amended soil resulted in a significant reduction in net blotch disease development on *P.* teres-inoculated plant leaves (AUDPC scores = 42 and 36% of the scores attributed to control plants grown in LB-amended soil and inoculated with *P. teres*) (Figure 3.5; *P*

< 0.05; See Appendix 1.5 for statistical analysis). But the disease scores on *P. teres*-inoculated plants grown in soil amended with *Ps. fluorescens* MKB100 were not significantly different from those *P. teres*-inoculated plants grown in LB amended soil (AUDPC = 0.28 and 0.34, respectively).

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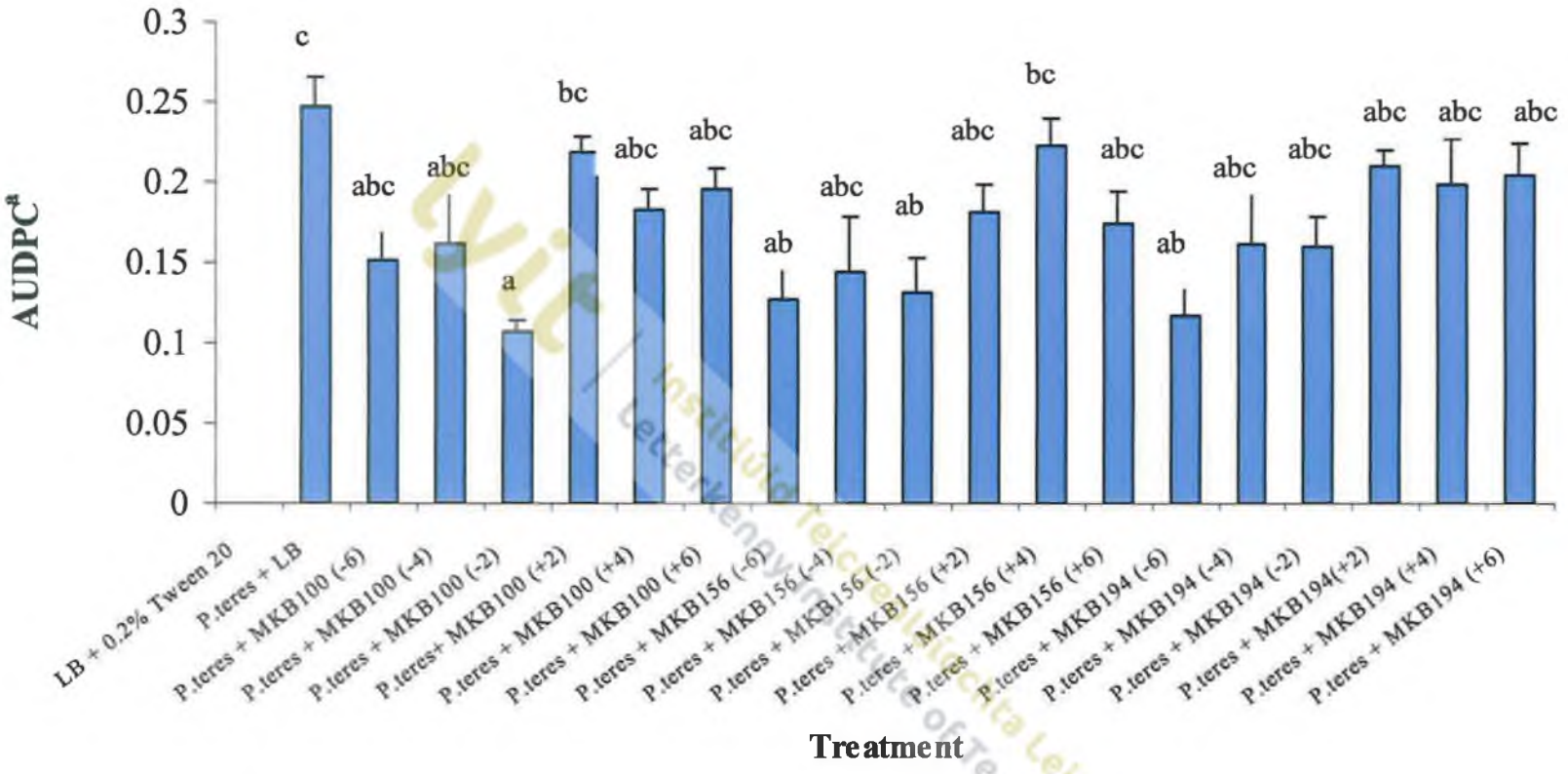

Figure 3.3 Effect of timing of bacterial application, relative to *Pyrenophora teres* leaf inoculation, on the development of net blotch disease on barley cultivars. Plants were inoculated with bacterium or treated with Luria Bertani (LB) broth 6, 4 or 2 days pre-, or 2, 4 or 6 days post-fungal inoculation at growth stage 13. Disease was scored 4, 6, 8 and 10 days post-fungal inoculation and these values were used to calculate the area under the disease progress curve (AUDPC[a]). Bacterial codes: *Pseudomonas fluorescens* MKB100 and MKB156, *Pseudomonas* sp. MKB194. Numbers in parentheses represent the number of days pre- (-) or post- (+) fungal inoculation that the bacterium was applied. Bars represent SEM. For any given treatment, values followed by the same letter are not significantly different from each other ($P = 0.05$).

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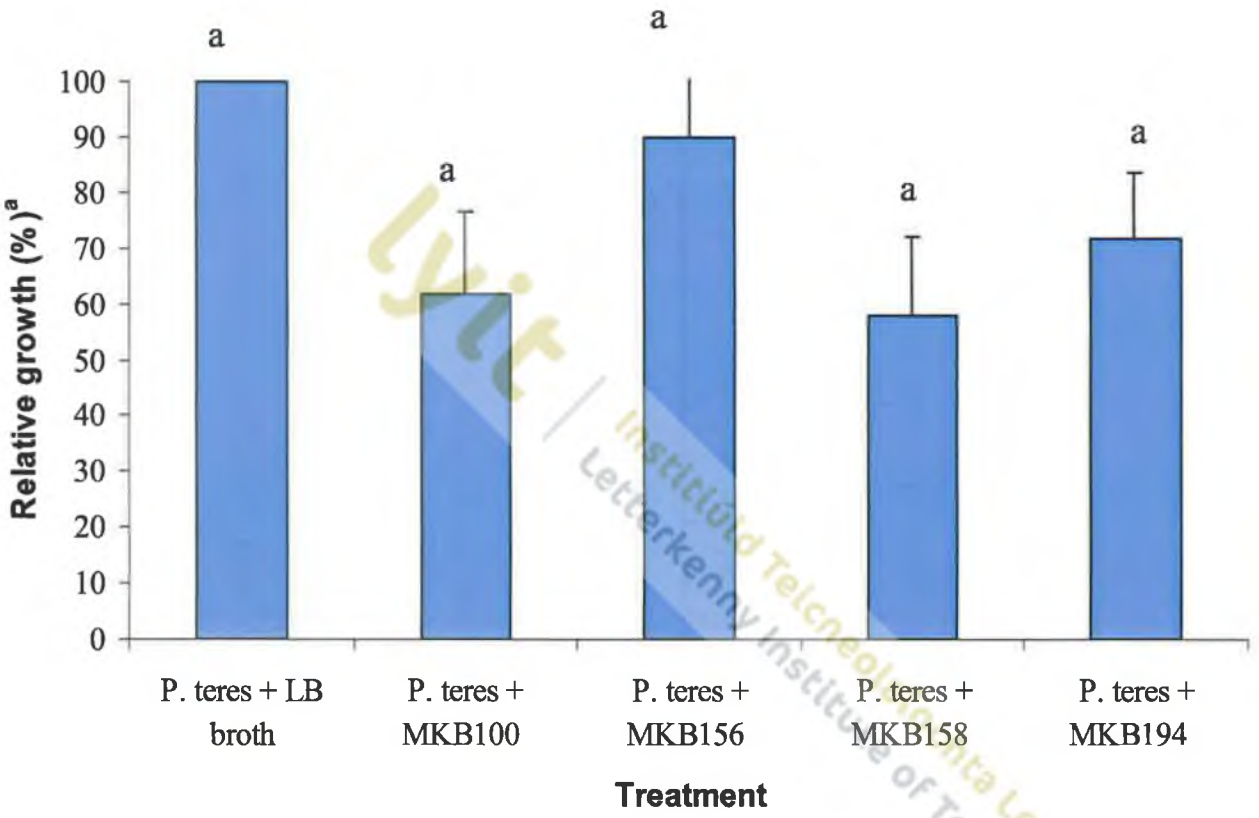

Figure 3.4 Effect of bacteria on the radial growth of *P. teres* in dual culture plate test. Plates were inoculated with a mycelial plug of *P. teres* var *teres* strain N45 and, at a distance of 4 cm, with a loopful of either bacterial suspension or Luria Bertani (LB) broth. Fungal colony diameter was measured 4, 5 and 6 days post-inoculation and results were used to calculate the %[a] growth of the fungus on plates co-inoculated with bacteria, relative to the growth on plates treated with *P. teres* and LB broth. Bacterial codes: MKB100 and MKB156, *Pseudomonas fluorescens*; MKB194, *Pseudomonas* sp. Bars represent SEM. For any given treatment, values followed by the same letter are not significantly different from each other ($P = 0.05$).

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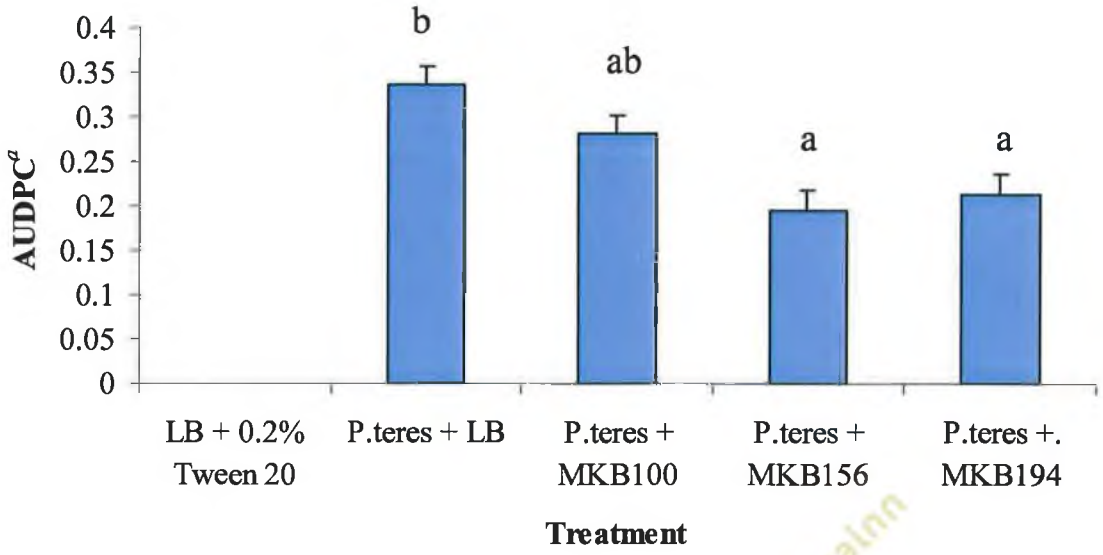

Figure 3.5 Effect of bacteria on the systemic resistance response of barley (cultivar Lux) to net blotch disease. Plants were grown in either Luria bertani (LB) broth- or bacterium-amended soil. At growth stage 13, the above-ground green tissue was spray-inoculated with conidia of *Pyrenophora teres* var. *teres* strain N45. Disease was scored 5, 7, and 10 days post-fungal inoculation and these values were used to calculate the area under the disease progress curve (AUDPC[a]). Bacterial codes: MKB100 and MKB156, *Pseudomonas fluorescens*; MKB194, *Pseudomonas* sp. Bars represent SEM. For any given treatment, values followed by the same letter are not significantly different from each other ($P = 0.05$).

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3.5 Detached leaf assay

Detached leaf experiments were conducted in order to determine (a) if the effect of bacteria on net blotch disease development on cvs Lux and Tavern was similar on detached leaves and growing plant leaves, and (b) the effect of barley cultivar on the efficacy of select biocontrol agents. Eight bacteria were chosen for detached leaf tests. *Pseudomonas flourescens* strain MKB100 and MKB156, *Pseudomonas* sp. MKB158 and *Pseudomonas* sp. MKB194 were chosen because they gave good control in the initial screening and subsequent seedling blight tests (> 70 % reduction in diseased leaf area in the screening test and > 46 % reduction in AUDPC in the subsequent seedling tests, relative to *P. teres*-inoculated, LB-treated plants; see Appendix 1.1 and 1.2 and Section 3.1). Unidentified isolates MKB23 and MKB44, *Bacillus sphaericus* MBK232 and *Pseudomonas tolaasii* MKB286 were chosen to include more bacterial species, and, in the initial screen test (Section 3.1 and Appendix 1.1) they reduced diseased leaf area by < 70 % (43, 65, 57 and 47 % reduction, respectively, relative to the disease leaf area observed on *P. teres*-inoculated, LB-treated leaves).

All eight bacteria significantly reduced net blotch disease development on detached leaves of cvs. Lux and Tavern (Figure 3.6). *Pseudomonas* sp. MKB194 was the most effective bacterial isolate tested, reducing the net blotch AUDPC on cvs Lux and Tavern to < 4 % of the score attributed to *P. teres*-inoculated, LB-treated detached leaves (Figures 3.6 & 3.7). There was a significant positive correlation between the effect of *P. teres* alone, or in combination with *Pseudomonas flourescens* strain MKB100 or MKB156, *Pseudomonas* sp. MKB158 or *Pseudomonas* sp. MKB194 on net blotch disease development on seedling and detached leaves of cv. Lux and Tavern (r = 0.996 and 0.932, $P < 0.01$, $P < 0.05$ respectively) (Appendix 1.6.7). *Ps. tolaasii* MKB286 and *B. sphaericus* MKB232 were both as effective as *Ps.*

fluorescens strains MKB100 and MKB156, or *Pseudomonas* sp. MKB158 in reducing the net blotch AUDPC on detached leaves (Figure 3.6; $P > 0.05$), and, as stated above, the former two bacteria did not perform well in the initial seedling screening trial and were not selected as potential biocontrol agents.

Barley cv. did not influence the biocontrol efficacy of *Ps. fluorescens* strains MKB100 and MKB156, and *Pseudomonas* sp. MKB194 in detached leaf tests, as treatment with these three bacteria significantly reduced the net blotch AUDPC on leaves of all six cvs tested to less than 73 % of the AUDPC values obtained for *P. teres*-inoculated, LB-treated leaves (Table 3.1; $P < 0.05$) (see Appendix 1.6 for statistical analysis of the results). However, the biocontrol efficacy of *Pseudomonas* sp. MKB158 in detached leaf tests was influenced by cultivar. For five of the six cvs. used, this bacterium reduced net blotch AUDPC by > 76 %, but for cv. Prestige, it only reduced AUDPC by 69%, relative to the AUDPC for *P. teres*-inoculated, LB treated leaves. The biocontrol efficacy of unidentified isolates MKB23 and MKB44, *Bacillus sphaericus* MBK232 and *Pseudomonas tolaasii* MKB286 against net blotch disease of detached leaves was also influenced by cultivar (Table 3.1).

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3.6 Effect of bacteria on the *in vivo* development of net blotch on adult barley plants

A preliminary experiment was conducted to assess the ability of *Ps. fluorescens* strains MKB100 and MKB156, to control net blotch disease of barley cv. Lux under field conditions. Based on the results of earlier experiments (see Sections 3.1 and 3.2), bacteria were applied 1 day pre-fungal inoculation and, within the same field trial, the efficacy of bacteria against net blotch on seedlings (GS 39) and on adult plants (GS 60) was compared. Unfortunately, no disease developed on plants inoculated with *P.*

teres at GS 39, irrespective of whether they had been pre-treated with LB broth (positive control) or bacteria, and therefore such a comparison could not be made. Neither bacterial isolate affected the health of either seedling or adult plants, as observed on negative controls treated with bacterium and 0.2 % Tween 20.

In contrast to what happened on seedlings, symptoms developed as early as 3 days post-*P. teres* inoculation of plants at GS 60. Statistical analyses of the data are presented in Appendix 1.7. *Pseudomonas fluorescens* strains MKB100 and MKB156 both significantly inhibited the development of disease symptoms, relative to *P. teres* inoculated, LB-treated control plants ($P < 0.05$) (Figure 3.8). *Pseudomonas fluorescens* strain MKB156 caused the greatest suppression of net blotch development on adult plants (Figures 2.8 & 2.9; AUDPC = 53% of the score attributed to positive control *P. teres*-inoculated, LB-treated plants). But the efficacy of this bacteria in controlling net blotch was not significantly better than that of *Ps. fluorescens* MKB100 ($P < 0.05$) that reduced net blotch AUDPC to 44% of that observed on positive control plants ($P < 0.05$). There was a significant correlation between the results obtained from the seedlings experiment and the field experiment ($r = 0.990$; $P < 0.05$) (Appendix 1.7.1).

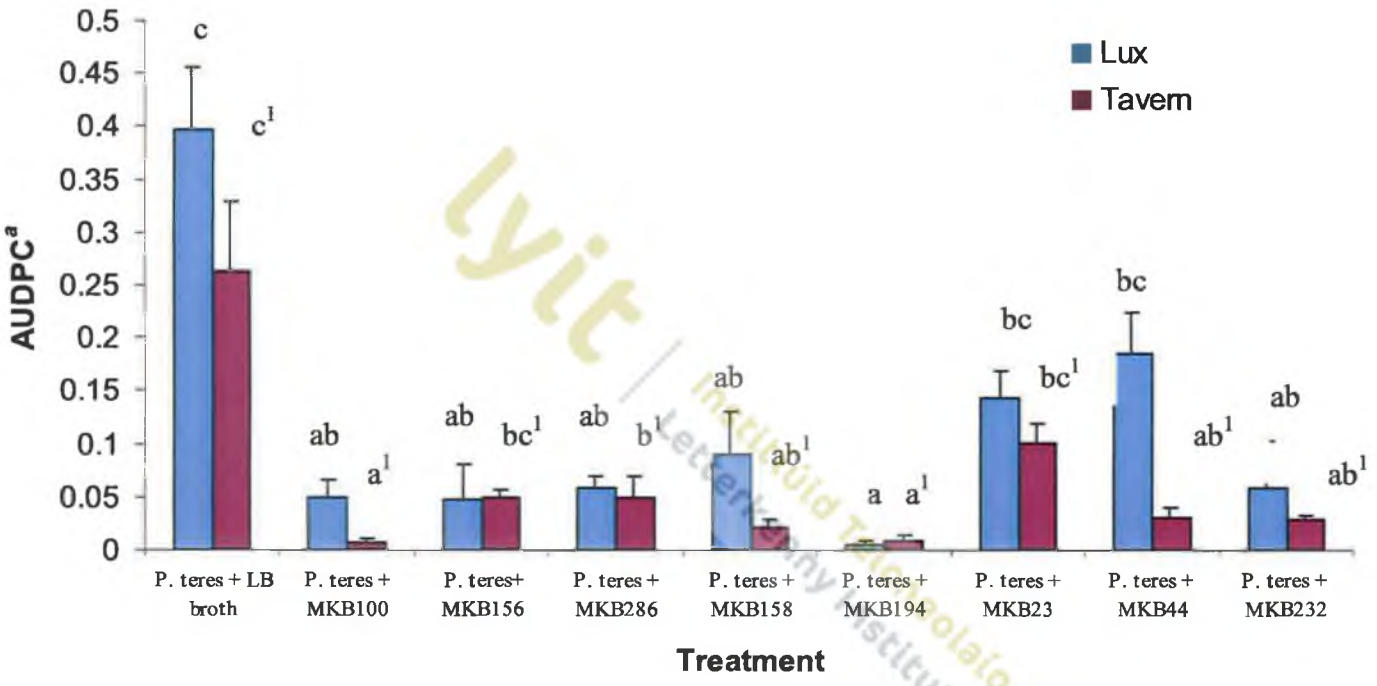

Figure 3.6 Effect of bacterial treatments on net blotch disease development on detached barley (cultivars Lux and Tavern) leaves. Leaves were inoculated with conidia of *Pyrenophora teres* var. *teres* strain N45 and either treated with Luria bertani (LB) broth or bacterial inoculum. The size of the disease lesion at 3, 4 and 5 days post-inoculation was determined and used to calculate the area under the disease progress curve (AUDPC[a]). Bacterial codes: MKB100 and MKB156, *Pseudomonas fluorescens*; MKB286, *Pseudomonas tolaasii*; MKB158 and MKB194, *Pseudomonas* sp.; MKB23, *Bacillus mycoides*; MKB44 and MKB232, *Bacillus sphaericus*. Bars represent SEM. For any given treatment, values followed by the same letter are not significantly different from each other ($P = 0.05$).

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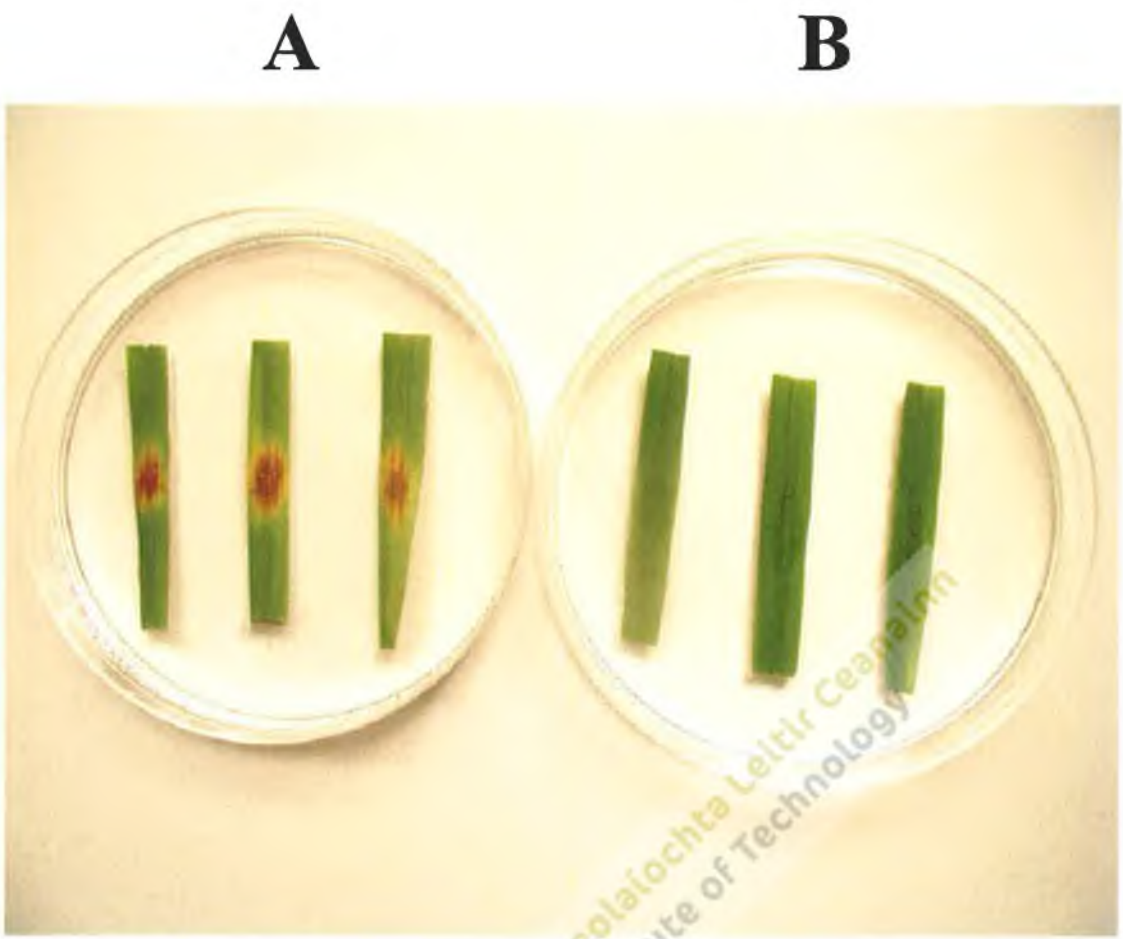

Figure 3.7 Effect of *Pseudomonas* sp. MKB194 on net blotch disease development on detached barley (cultivar Lux) leaves. Leaves were inoculated with conidia of *Pyrenophora teres* var. *teres* strain N45 and either co-inoculated with Luria bertani (LB) broth (**A**) or treated with *Pseudomonas* sp. MKB194 (**B**). Plates were photographed 7 days post-inoculations

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Table 3.1 Effect of barley cultivars on the efficacy of biocontrol agents in controlling net blotch disease development on detached leaves

Treatment[a]	Barley cultivar AUDPC value[b]					
	Lux	Tavern	Fractal	Prestige	Siberia	Calcutta
Pyrenophora teres + Luria Bertani broth	0.398^{c}	0.263^{c}	0.335^{d}	0.413^{d}	0.210^{d}	0.278^{b}
P. teres + *Ps. fluorescens* MKB100	0.050^{ab}	0.008^{a}	0.021^{ab}	0.022^{ab}	0.007^{ab}	0.009^{a}
P. teres + *Ps. fluorescens* MKB156	0.048^{ab}	0.050^{bc}	0.090^{abc}	0.091^{abcd}	0.035^{bc}	0.068^{ab}
P. teres + *Pseudomonas* sp. MKB158	0.089^{ab}	0.023^{ab}	0.079^{abc}	0.125^{bcd}	0.019^{abc}	0.049^{ab}
P. teres + *Pseudomonas* sp. MKB194	0.005^{a}	0.01^{a}	0.013^{a}	0.015^{a}	0.006^{a}	0.011^{a}
P. teres + *B. mycoides* MKB23	0.143^{bc}	0.101^{bc}	0.038^{abc}	0.187^{cd}	0.076^{cd}	0.105^{b}
P. teres + *B. sphaericus* MKB44	0.186^{bc}	0.031^{ab}	0.120^{abc}	0.042^{abc}	0.026^{abc}	0.053^{ab}
P. teres + *B. sphaericus* MKB232	0.059^{ab}	0.029^{ab}	0.172^{cd}	0.124^{bcd}	0.051^{cd}	0.067^{ab}
P. teres + *Ps. tolaasii* MKB286	0.060^{ab}	0.049^{b}	0.130^{bcd}	0.118^{bcd}	0.02^{abc}	0.039^{ab}

[a]Detached leaf segments were inoculated with conidia of *Pyrenophora teres* var. *teres* strain N45 and either treated with Luria bertani (LB) broth or co-inoculated with bacterium. Plates were photographed 7 days post-inoculation. [b]The size of the disease lesion at 3, 4 and 5 days post-inoculation was determined and used to calculate the area under the disease progress curve (AUDPC). For any given treatment, values followed by the same letter are not significantly different from each other ($P = 0.05$).

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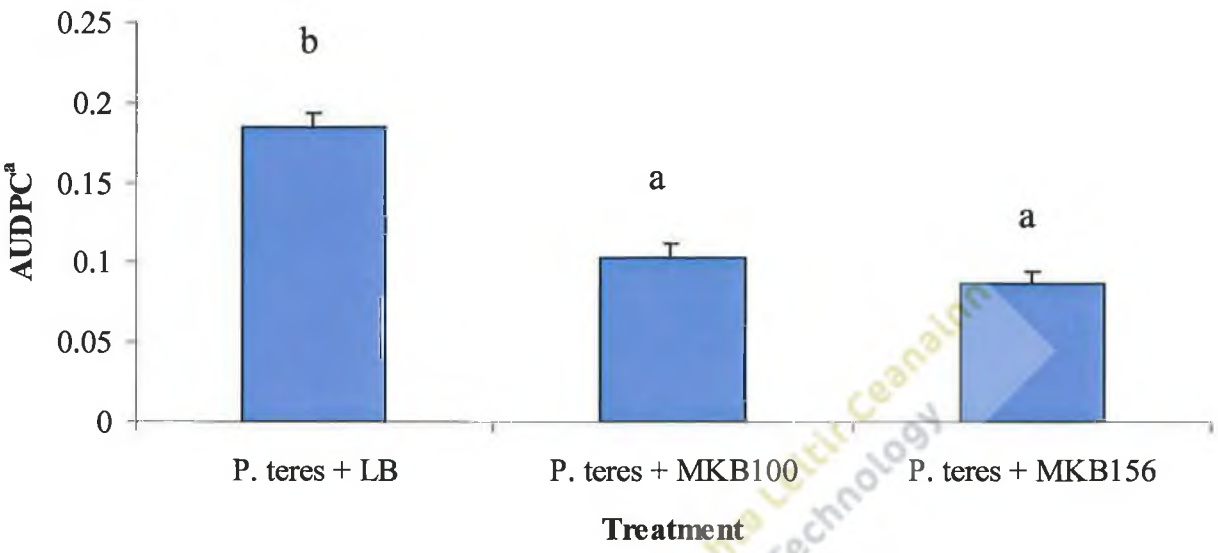

Figure 3.8 Effect of bacteria on net blotch disease of adult barley plants. At growth stage 60, barley plants (cultivar Lux) were treated with Luria Bertani broth or were inoculated with bacterium; 24 h later plants were treated with 0.2% Tween 20 or inoculated with conidia of *Pyrenophora teres* var. *teres* strain N45. Disease was scored 5, 7 and 10 days post-*P. teres* inoculation and the values were used to calculate the AUDPC[a]. Bacterial codes: MKB100 and MKB156, *Pseudomonas fluorescens.* Bars represent SEM. For any given treatment, values followed by the same letter are not significantly different from each other ($P = 0.05$).

Figure 3.9 Effect of *Pseudomonas fluorescens* strain MKB156 on net blotch disease of adult barley plants. At growth stage 60, barley plants (cultivar Lux) were treated with bacterium (**A**) or were inoculated with Luria Bertani broth (**B**); 24 h later plants (**A** and **B**) were inoculated with conidia of *Pyrenophora teres* var. *teres* strain N45. Plants were photographed 12 days post-*P. teres* inoculation

4 Discussion

The current study investigates the potential of several bacterial isolates to act as biological control agent(s) for *Pyrenophora teres,* the causal agent of barley net blotch. From initial screening through to more detailed seedling trials, *Ps. fluorescens* strains MKB100 and MKB156 and *Pseudomonas* sp. strain MKB194 gave excellent *in vivo* net blotch disease control under conditions of high disease pressure, reducing the level of disease by 68%, 67% and 64% respectively, relative to that of the *P. teres* – LB inoculated control leaves. Unfortunately an attempt to analyse field performance of *Ps. fluorescens* MKB100 and MKB156 against net blotch of seedlings failed due to the prevalence of unfavourable environmental conditions for disease development post-*P. teres* inoculation. An adult plant biocontrol test conducted within the same field trial showed that *Ps. fluorescens* MKB100 and MKB156 reduced the level of disease caused by *P. teres* on barley plants by 44 and 52%, respectively. The ability of these bacterial isolates to suppress net blotch disease of barley under field conditions and in response to high disease pressure is promising. However due to time limitations this field trial was only conducted once and would need to be repeated in order to determine if the effects are consistent. More extensive field testing programmes should investigate the performance of bacteria against both seedling and adult plant net blotch disease.

Johnsson *et al.* (1998) found that 94% control in the number of infected plants per m^2 was achieved when seed was inoculated with *Pseudomonas chlororaphis* MA 342 and 98% control using the fungicide Panoctine Plus when compared to untreated naturally infected seed in spring barley field experiments conducted in Sweden over a six year period (1991-1996). In the current work the bacteria selected for adult barley tests were *Ps. fluorescens* MKB100 and MKB156 which reduced the level of disease

caused by *P. teres* by 44 and 52% respectively. However this experiment was conducted on plants that were inoculated with bacterial inoculum, followed 24 h later with conidial inoculum of *P. teres* at GS60 and this experiment was conducted once due to time constraints. Parameters assessed in the current experiment involved scoring the disease symptoms on individual plants using a scale devised by Tekauz (1985) at 5, 7 and 10 days post inoculation while Johnsson *et al.* (1998) assessed the number of infected plants at the 3-4 leaf stage. *Pseudomonas chlororaphis* MA 342 was as effective as conventional fungicides in controlling net blotch and has been consistent in its biocontrol efficacy under field conditions in Sweden since 1991 (Johnsson *et al.*, 1998; Tombolini *et al.*, 1999). The colonisation pattern of MA 342 appears to be similar to the colonisation pattern of *P. teres* and a close physical relationship between them may be required for biocontrol (Tombolini *et al.*, 1999). Seed treatment can be advantageous because as biocontrol agents grow and proliferate, they can colonise and protect newly formed plant parts to which they were not initially applied (Harman, 1990). Testing the efficacy of the *Pseudomonas* bacteria identified in this study as seed treatment, may enhance the level of control achieved in controlling *P. teres*.

All three bacterial isolates that showed promise for control of net blotch disease of seedlings *in vivo* were fluorescent Pseudomonads- with two that also showed promise for controlling net blotch of adult plants under field conditions. Many biocontrol bacteria are Pseudomonads (Ligon *et al.*, 2000). *Pseudomonas chlororaphis* MA 342 is effective against a range of cereal seed-borne diseases including *Pyrenophora teres* (net blotch), *D. graminea* (leaf stripe), *D. avenae* (oat leaf stripe), *Tilletia caries* (common bunt in wheat), *Ustilago avenae* (loose smut in oats), *U. hordeii* (covered smut), and has some effect against *Microdochium nivale*

and *Stagonospora nodorum* (Hökeberg *et al.*, 1997; Johnsson *et al.*, 1998). *Pseudomonas fluorescens* 2-79RN$_{10}$ is an effective biocontrol agent of take-all disease of wheat caused by the fungal pathogen *Gaeumannomyces graminis* (Weller, 1988; Bull *et al.*, 1991), *Pseudomonas fluorescens* strain WCS417r is effective in reducing root rot of *Arabidopsis* caused by *Fusarium oxysporum* and leaf blight of *Arabidopsis* caused by *Pseudomonas syringae* (Pieterse *et al.*, 1996).

Potential modes of action of biocontrol agents include direct inhibition of pathogen growth, production and secretion of antimicrobial compounds, competition with the pathogen for nutrients and plant surface area, and/or induction of host systemic resistance to the pathogen (Whipps, 1997; Pieterse *et al.*, 2001). From the dual culture experiment, it appears that neither *Ps. fluorescens* strains MKB100 nor MKB156, nor *Pseudomonas* sp. strain MKB194 directly inhibit the growth of *P. teres*. Pedersen *et al.* (1999) found that, in dual culture, strains of *Pseudomonas fluorescens*, *Pseudomonas chlororaphis* and *Pseudomonas involutus* reduced the mycelial growth of *Fusarium moniliforme*, *F. oxysporum* and *Rhizoctonia solani* on potato dextrose agar. Eight *Pseudomonas fluorescens* isolates inhibited the growth of *R. solani* when tested using the dual culture method (Kazempour, 2004). It is not known whether *Pseudomonas chlororaphis* strain MA 342 inhibits *P. teres* in dual culture as this experiment was not conducted (Hökeberg *et al.*, 1997).

In the current experiment we did not test whether these Pseudomonads secreted antifungal metabolites, but Khan *et al.* (2006) showed that culture filtrate of *Ps. fluorescens strains* MKB100 and MKB156 (produced by these bacteria in co-culture with *Fusarium* isolates) showed no antifungal activity towards *Fusarium* species. On the basis of the dual culture tests and the work of Khan *et al.* (unpubl. data) it appears unlikely that these bacteria produce and secrete antifungal compounds

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in *in vitro* culture conditions. Further experimentation is required to confirm that such compounds, particularly antifungal volatiles, are not produced by these bacteria, especially since several metabolites with antifungal properties are known to be produced by other strains of *Ps. fluorescens* and other Pseudomonads (Thomashow and Weller, 1996; Ligon *et al.*, 2000). Also, it may be that such compounds are produced *in vivo* but not *in vitro* or are only produced under particular *in vitro* conditions. *Pseudomonas chloraphis* MA 342 suppresses fungal growth by producing the antifungal compound 2,3-diepoxy-2,3-didehydrorhizoxin and the biocontrol efficacy of mutants which have lost their ability to produce this compound is compromised (Hökeberg, 1998). The colonisation pattern of this bacterium is similar to that of the pathogen; both are present on sites around the embryo and in the glume of barley seeds, and 2,3-deepoxy-2,3-didehydrorhizon is present in the glume (Tombolini *et al.,* 1999). Take-all is suppressed by a fluorescent Pseudomonad that produces the antibiotic phenazin-1-carboxylic acid in the rhizosphere of wheat (Thomashow *et al.,* 1990). Phenazines are broad spectrum antibiotic metabolites which enhance microbial competitiveness and the pathogenic potential of the organisms that synthesise them, and phenazine-1-carboxylic acid is the end product of phenazine biosynthesis in *Ps. fluorescens* 2-79 (Ahuja *et al.*, 2004). Phenazines uncouple oxidative phosphorylation in target organisms and generate toxic intercellular oxygen species (Turner and Messenger, 1986). Pyrrolnitrin is a natural product produced by some *Pseudomonas* spp. and is active against *Rhizoctonia* spp. and *Fusarium* spp. (Ligon *et al.*, 2000). Pyrrolnitrin inhibits reduced nicotinamide adenine dinucleotide (NADH) and succinate oxidase in fungal mitochondria by blocking electron transport in the flavine region (Arima *et al.*, 1964; Tripathi and

Gottlieb, 1969). Pyrrolnitrin provided the chemical model for the development of the commercial fungicide fludioxonil (Ligon *et al.*, 2000).

The growth rate of *Ps. fluorescens* strains MKB100 and MKB156, and of *Pseudomonas* sp. MKB194 on detached barley leaves did not exceed that of other Pseudomonads that showed no biocontrol efficacy against *P. teres* (results not shown). Therefore it seems unlikely that competition with *P. teres* for space and nutrients is their primary mode of action. Under field conditions Johnsson *et al.* (1993) found that early establishment of populations exceeding 10^5 CFU per blossom of *Ps. fluorescens* Pf A-506 and *Ps. agglomerans* Eh C9-1 on pear blossoms suppressed establishment and growth of fireblight caused by *Erwinia amylovora*, thereby decreasing disease incidence by excluding the pathogen from infection sites. *Pseudomonas chlororaphis* is a vigorous rhizosphere-inhabiting bacterium that rapidly colonises the roots of treated plants outcompeting phytopathogenic species for nutrients (Copping, 2004). The colonisation pattern of this bacterium thus appears to be involved in the biological control of *P. teres*.

The results of seedling experiments (in which bacteria and *P. teres* were spatially separated) showed that the induction of host systemic resistance was responsible for at least part of the biocontrol efficacy of *Ps. fluorescens* strain MKB156 and *Pseudomonas* sp. MKB194. Induced systemic resistance (ISR) is a plant-mediated mechanism where host plant defences are stimulated and plants are protected systemically (Hammerschmidt, 1999). ISR has been demonstrated in a number of plants where the inducing bacteria and the challenging pathogen are kept spatially separated (Van Loon *et al.*, 1998; Elad, 2000). Results of research in which *Ps. fluorescens* strain WCS417r and either *F. oxysporum* or *Ps. syringae* were spatially separated demonstrated that the former induced systemic resistance against

root rot and leaf blight of Arabidopsis caused respectively by the latter two organisms (Pieterse *et al.*, 1996; Pieterse *et al.*, 2002). Resistance to these pathogens is induced independently of SA and PR accumulation before infection but requires an intact response of the plant hormones jasmonic acid and ethylene (Pieterse *et al.*, 1998). *Pseudomonas putida* strain RE8 suppresses Fusarium wilt of radish by induced systemic resistance (De Boer *et al.*, 2003) and induced systemic resistance against *Peronospora parasitica* in *Arabidopsis thaliana* has been demonstrated in response to root inoculation with *Pseudomonas fluorescens* (Lavicoli *et al.*, 2003). ISR involves colonisation of the rhizosphere with plant growth promoting rhizobacteria and the activation of latent resistance mechanisms, mediated at least in some cases, by a jasmonate/ethylene sensitive pathway (Hammerschmidt, 1999). Because many defence genes are activated, pathogens are unlikely to develop resistance to these defences (Kuc, 2001). It would be interesting to determine what signalling pathways and defence mechanisms are involved in *Ps. fluorescens* strain MKB156- and *Pseudomonas* sp. MKB194-induced systemic resistance to net blotch disease. The identification of genes that are differentially expressed when infected with a fungal pathogen and bacteria may enable us to establish the biocontrol agents' mode of action, what signalling pathways mediate ISR, and would enable us to assess the contribution of host genotypes to induction (Kahmann and Basse, 2001). Khan *et al.*, (2006) found that the presence of *Pseudomonas* sp. strain MKB158 in soil activated ISR in aerial wheat and barley tissue, resulting in reduced severity of Fusarium seedling blight of these cereals. Microarray analysis showed that, by 24 h post-*Fusarium* inoculation of barley stems, 27 genes were more highly expressed in the stem tissue of plants grown in soil amended with *Pseudomonas* sp. MKB158, as compared to those grown in soil amended with LB broth. By analysis of these genes,

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Khan *et al.*, (2006) has identified four probable pathways involved in rhizobacteria-mediated induced systemic resistance, three of which are auxin-regulated and one of which may be regulated by abscisic acid.

Evaluating the usefulness of a detached leaf test for the screening of bacterial populations for potential biocontrol agents against net blotch disease was carried out. But bacteria performed well as biocontrol agents in the detached leaf test that did not perform well in the initial seedling screening experiment. Because the initial screening experiment was only conducted once, care must be taken in drawing conclusions from this as regards the usefulness of the detached leaf test. There was a significant positive correlation between the effect of *P. teres* alone, or in combination with *Ps. fluorescens* strain MKB100 or MKB156, *Pseudomonas* sp. MKB158 or *Pseudomonas* sp. MKB194 on net blotch disease development in replicated seedling detached leaf tests conducted using cvs Lux and Tavern. Sharma (1984) found that the reaction of twenty-one barley cultivars to inoculation with *P. teres* was highly significant when correlated with the degree of leaf damage on whole plants grown outdoors. However, no correlation was found between greenhouse results and those obtained with either detached leaf or field experiments on the reaction of six cultivars to *P. teres* infection (Deadman and Cooke, 1986)

The detached leaf test also highlighted that at least on leaf segments if not on whole plants, the efficacy of some bacteria as biocontrol agents is host genotype-dependent. Barley cultivars did not influence the biocontrol efficacy of *Ps. fluorescens* strains MKB100 and MKB156, and *Pseudomonas* sp. MKB194 against net blotch disease in detached leaf tests. Experiments need to be conducted to ensure that this hold true on whole plants. Other research has reported that the efficacy of biocontrol agents can be host cultivar-dependent. Ryan *et al.* (2004) found that the

efficacy of *Streptomyces* spp. as biocontrol agents against Fusarium dry rot disease was moderately affected by the potato cultivar used. But Schisler *et al.* (2002) found that the efficacy of *Bacillus* spp. and *Cryptococcus* spp. as antagonists in controlling *Fusarium* head blight disease was not wheat cultivar-dependent.

More intensive field trials should further investigate the effect of timing of application, environmental conditions and formulation on the biocontrol efficacy of *Ps. fluorescens* strains MKB100 and MKB156 and *Pseudomonas* sp. strain MKB194 against net blotch disease of barley. Such trials should assess the effect of biocontrol agents on disease incidence, severity and plant yield. In the current work, irrespective of bacterial isolate, bacterial application pre-fungal inoculation was generally more effective in reducing the AUDPC for net blotch disease than was application post-*P. teres* inoculation. Khan *et al.* (2001) found that timing of application of *Bacillus* and *Cryptococcus* antagonists relative to time of *F. graminearum* inoculation of wheat heads (+ or – 4 h, or immediately before or after pathogen inoculation) did not influence their efficacy in reducing Fusarium head blight disease symptom severity. However, the ability of *Bacillus* strain AS 43.4 to reduce yield (1000 grain weight) loss due to *F. graminearum* was influenced by time of bacterial application: while application of this bacterium at – 4 h pre-*F. graminearum* inoculation, or immediately before or after pathogen inoculation restored yield to 85%, application 4 h post-*F. graminearum* reduced yield by 5.5 % of that obtained for negative control plants. Environmental conditions and formulation will also influence how biocontrol bacteria perform and persist on plants and this should be considered when designing field experiments.

The method used to apply bacteria and the amount applied affects their distribution and pattern of colonisation and consequently affects the efficacy of

biocontrol agents. In this research the bacteria were applied to seedlings as both a foliar application and as a soil amendment and to adult plants in the former manner. Comparison of seedling tests results suggests that soil amendment was not as effective as foliar application in controlling net blotch. But, while similar concentrations of bacterial cells were applied to the leaf and to the soil rhizosphere, it may be necessary to use higher concentrations of bacteria in the latter case to achieve the same bacteria cell /plant cell contact ratio which undoubtedly will influence bacterial biocontrol efficacy. Identifying the mechanism of biocontrol employed by the bacteria helps determine the most effective method of application. The fact that *Ps. fluorescens* MKB156 and *Pseudomonas* sp. MKB194 induce ISR against net blotch is exciting, as these may be useful as seed dressings for inhibiting the development of net blotch disease from seed-borne inoculum.

Formulation influences the possible methods by which biocontrol bacteria can be applied, and also greatly influences the efficacy and stability of biocontrol agents. Formulation stability and shelf life are important determinants regarding the market potential of a biocontrol agent. Pseudomonads are fast growing, easy to culture, and they utilise a range of metabolisable organic compounds (Whipps, 2001). Barley seeds dressed with the commercial net blotch biocontrol agent can be stored dry for at least two years without losing the disease-suppressing effect exhibited by the bacterial treatment under field conditions (Johnsson *et al.*, 1998). Seed dressing involving the application of *Pseudomonas chlororaphis* MA342 involved growth of the bacterial culture in Tryptic Soy Broth (Difco Ltd.) on a shaker for 2 days and 300 ml of this bacterial broth was applied per kg of seed which resulted in approximately 10^7 cfu per seed; the seeds were then spread out and dried for >12 h (Johnsson *et al.*, 1998). The commercially available biocontrol agent Blighban A506® is based on *Pseudomonas*

fluorescens A506 which is distributed as a pellet formulation comprised of freeze dried cells of A506 which suppress disease through pre-emptive exclusion of the pathogen (Wilson and Lindow, 1993). Best control with this product is achieved when the product is sprayed onto newly opened flowers prior to the arrival of the pathogen. Bacterial isolates identified in this study should be applied using each of the above mentioned formulations in order to test the influence of formulation on biocontrol efficacy.

Climatic conditions at the time of application will influence the efficacy of bacteria in controlling net blotch disease of barley. Field trials and *in vitro* experiments should be conducted in order to determine if the biocontrol efficacy of *Ps. fluorescens* strains MKB100 and MKB156 and *Pseudomonas* sp. strain MKB194 is consistent under the range of environmental conditions that prevail in the regions and at the time of year which they are likely to be used. As these three bacteria were isolated from an oat rhizosphere, a barley rhizophere and a wheat leaf, they should be adapted to cereals and cereal rhizospheres. Also, they were isolated in Ireland and should be adapted to temperate climates. The performance of MA 342 was tested against *P. teres* under various climatic conditions cropped to cereals in Sweden with soil types varying from clay soils to sandy soils and variations in temperatures and levels of precipitation. Results found that the effects of MA 342 were consistent under different climatic conditions from the South of Sweden to the North (Johnsson *et al.*, 1998).

Using a combination of bacteria may lead to an increased suppression of disease, as demonstrated by De Boer *et al.* (2003) who found that a combination of *Pseudomonas putida* strains WCS58 and RE8 was 20 % more effective in reducing the % diseased plants caused by Fusarium wilt of radish compared to a single strain

treatment. De Boer *et al.* (2003) attributed the enhanced biocontrol activity of the bacterial combination to be the result of pseudobactin-mediated competition for iron combined with induced systemic resistance. It has been reported that a synergistic effect may result from integrating the usage of chemical fungicides and biocontrol agents; if compatible, the combination should give a higher level and a broader range of disease control (Elad *et al.,* 1980; Schroth and Hancock, 1981; Chakravarty *et al.*, 1990). Future experimentation should determine the compatibility between *Pseudomonas fluorescens* strains MKB100 and MKB156 and *Pseudomonas* sp. strain MKB194, and between each of these and triazoles and strobilurins. Using fungicides in combination with biocontrol agent(s) would reduce both the quantity of chemical pesticides being used and the risk of resistance developing to the chemical pesticide (Fryod, 1997).

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5 Conclusion

Net blotch cause by *Pyrenophora teres* is one of the most economically devastating diseases of cultivated barley (Weiland *et al.*, 1999) with yield losses as high as 40% in seasons or production areas which favour the pathogen (Shipton *et al.*, 1973). Current control is achieved using an integrated approach, combining chemical control, the use of resistant cultivars and crop hygiene practices. Due to the adverse effects chemical fungicides have on the environment and the threat of resistance to these fungicides, there is an increasing demand for biological control. Biological control of cereal diseases by bacteria or their secondary metabolites has previously been reported (Weller, 1988; Levy *et al.*, 1989; Bull *et al.,* 1991; Hökeberg *et al.*, 1997; Khan *et al.*, 2001; Johansson *et al.*, 2003). In the current study bacterial isolates, which were found in close proximity to cereals (Khan *et al.*, unpubl. data) were examined to investigate their potential to control net blotch disease of barley.

Bacterial isolates *Pseudomonas fluorescens* MKB100 and MKB156 significantly reduced net blotch disease on seedlings in the glasshouse, reducing disease by 70 and 69% respectively relative to *P. teres* – Luria Bertani broth treated control plants. The level of success of these bacterial isolates on adult barley plants in the field study, was reduced to 44 and 53% respectively. The reduction in the level of control by the bacterial isolates, from the seedling results in the glasshouse to the adult plants in the field highlights the problem associated with lack of consistency. This reduction in efficacy may be due to environmental conditions, as application in the field meant direct exposure to varying climatic conditions. Future work should investigate application of this bacterium as a seed treatment where an increased level of control may be achieved in the soil. Also, application as seed formulations means that microorganisms are subjected to conditions where extremely conducive

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environments prevail (Warrior *et al.*, 2000). The environment surrounding the above ground portion of plants is very different from that in the soil (Pusey, 1996). Maintaining viability is fundamental to formulating a biocontrol agent (Pusey, 1996) and in the current study bacteria were grown in Luria Bertani broth and applied as a foliar application. However storage and survival of the bacteria in LB over long periods may not be possible and alternative methods may have to be sought.

Results from the seedling experiment where the pathogen and the bacteria were kept spatially separated showed that induced systemic resistance is involved in the biocontrol activity of *Ps. fluorescens* MKB156 and *Pseudomonas* sp. MKB194. Future work on the molecular mechanisms that mediate the biological control interaction need to be conducted so as to understand the exact mode of action elucidated by the bacterium. Microarray analysis identifies genes that have been up- or down-regulated that play a role in disease pathways. Microarray analysis may give a better understanding of the molecular phenomena underlying the systemic resistance towards net blotch disease of barley induced by the potential biocontrol agents. Studies of genes expressed differentially at different timepoints post inoculation could provide valuable information on the involvement of these bacteria in the control of the fungus.

Future success of *Pseudomonas fluorescens* MKB156 will depend on its ability to consistently suppress net blotch under field conditions and on its ability to remain viable if integrated into a disease control program.

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Appendix 1: Effect of bacteria on net blotch disease of barley

Appendix 1.1 Preliminary analysis of the effect of bacteria on net blotch disease of barley seedlings

Treatment [a]	Relative diseased leaf area (%)[b]
P. teres + MKB52	8.7
P. teres + *Pseudomonas* sp. MKB194	9.7
P. teres + MKB8	11.3
P. teres + *Pantoea agglomerans* MKB29	15.5
P. teres + *Pseudomonas* sp.MKB158	16.3
P. teres + *Ps. fluorescens* MKB100	18.9
P. teres + MKB66	20
P. teres + MKB54	20.3
P. teres + MKB140	20.3
P. teres + *Bacillus megaterium* MKB135	22.4
P. teres + *Bacillus mycoides* MKB15	23.2
P. teres + MKB141	23.4
P. teres + *Ps. fluorescens* MKB133	23.4
P. teres + MKB67	25.3
P. teres + MKB108	25.5
P. teres + MKB150	26.9
P. teres + MKB65	27.4
P. teres + MKB121	28.9
P. teres + MKB143	28.9
P. teres + *Ps. fluorescens* MKB156	28.9
P. teres + MKB258	29.7
P. teres + MKB164	30
P. teres + MKB124	32.4
P. teres + MKB104	32.4
P. teres + MKB138	32.6
P. teres + MKB20	34.5
P. teres + *B. sphaericus* MKB44	34.7
P. teres + MKB62	35
P. teres + MKB26	35.5
P. teres + MKB57	37.1
P. teres + MKB89	38.2
P. teres + MKB74	38.4
P. teres + *Exiguobacterium* sp. MKB41	38.4
P. teres + MKB79	39.5

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Appendix 1.1 continued....

Treatment [a]	Relative diseased leaf area (%)[b]
P. teres + MKB181	39.5
P. teres + MKB190	39.7
P. teres + MKB68	40
P. teres + MKB43	41
P. teres + MKB153	41
P. teres + MKB12	41.6
P. teres + MKB6	42.6
P. teres + MKB112	42.9
P. teres + MKB210	42.9
P. teres + MKB232	43
P. teres + MKB50	43.1
P. teres + MKB281	43.1
P. teres + MKB32	43.9
P. teres + MKB77	43.9
P. teres + MKB53	44.2
P. teres + MKB58	44.2
P. teres + MKB48	46
P. teres + MKB69	48.1
P. teres + MKB249	48.7
P. teres + MKB60	48.9
P. teres + MKB105	48.9
P. teres + MKB72	49.2
P. teres + *B. mycoides* MKB73	49.7
P. teres + MKB78	50.3
P. teres + *Exiguobacterium* MKB49	50.8
P. teres + MKB111	51
P. teres + MKB177	51
P. teres + MKB17	51.3
P. teres + MKB125	51.3
P. teres + MKB9	51.8
P. teres + MKB244	51.8
P. teres + *B. mycoides* MKB45	52.1
P. teres + MKB70	52.1
P. teres + MKB22	52.4
P. teres + MKB230	52.6
P. teres + MKB4	52.9
P. teres + MKB30	52.9
P. teres + MKB25	53.2
P. teres + MKB23	53.2
P. teres + *Ps fluorescens* MKB90	53.2
P. teres + *Ps. tolaasii* MKB286	53.2
P. teres + MKB209	53.9

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Appendix 1.1 **continued....**

Treatment [a]	Relative diseased leaf area (%)[b]
P. teres + MKB120	54.2
P. teres + MKB101	55
P. teres + MKB63	55.8
P. teres + *B. mycoides* MKB23	57.1
P. teres + MKB81	57.1
P. teres + MKB59	57.6
P. teres + MKB64	58.1
P. teres + MKB27	58.4
P. teres + MKB61	58.4
P. teres + MKB56	58.9
P. teres + MKB2	61.3
P. teres + MKB75	61.6
P. teres + MKB31	62.1
P. teres + MKB47	62.4
P. teres + MKB271	63.2
P. teres + MKB37	63.4
P. teres + MKB36	65
P. teres + MKB106	66.8
P. teres + MKB193	71.6
P. teres + MKB46	73.7
P. teres + *B. sphaericus* MKB232	73.9
P. teres + MKB221	78.1
P. teres + MKB76	81.4
P. teres + 0.2% Tween 20	84.5
P. teres + LB	100

[a]The whole plant was treated with *P. teres* or 0.2 % Tween 20 at GS 13 and with bacteria or Luria Bertani broth (Luria and Burrows, 1957) 1 days pre-fungal inoculation.

[b]Disease was assessed at 5, 7 and 10 days post-fungal inoculation; these values were used to calculate the area under the disease progress curve (AUDPC) (Shanner and Finney, 1977) and results are expressed as the AUDPC values of samples, relative to those of *P. teres*-inoculated, LB-treated plants.

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Appendix 1.2 Effect of bacterial treatments on net blotch development on cultivars Lux and Tavern seedlings under glasshouse conditions

Unidentified strains MKB52, MKB141 and MKB66 were chosen as, of the 80 unidentified bacteria tested, they caused greatest inhibition of symptom development (≥ 78%, relative to that on LB-treated *P. teres*-inoculated leaves). Of the 4 *Ps. fluorescens* isolates tested, those selected included strains MKB100 and MKB156. *Ps. fluorescens* strain MKB100 caused greatest suppression of symptom development (≥ 81%) and MKB100 also controlled *Fusarium culmorum* seedling blight disease of wheat and barley by >83 % under controlled environment conditions (Khan *et. al.*, unpubl. data). Although *Ps. fluorescens* strain MKB156 only reduced net blotch disease by 45%, it was chosen for further study because it had a direct inhibitory effect on the growth of *F. culmorum*, *F. graminearum* and *F. poae* in dual culture plate tests (Khan *et. al.*, unpubl. data). *Pseudomonas* sp. MKB194, *Pseudomonas* sp. MKB158 and *Bacillus megaterium* strain MKB135 were selected for further study because they reduced net blotch disease by > 69% and, under controlled environment conditions, *Pseudomonas* sp. MKB158 reduced the severity of *F. culmorum* seedling blight of wheat and barley by > 74% (Khan *et al.*, unpubl. data) and, under both controlled environment and field conditions, *Bacillus megaterium* strain MKB135 reduced the severity of septoria tritici blotch disease of wheat symptoms by > 59%.

Appendix 1.2 continued…

Data: Normalised AUDPC values
Test: One-way ANOVA incorporating Tukeys Pairwise comparison test at the 5% level of significance

* 1 = SDW, 2 = *Pyrenophora teres* + LB, 3 = *P. teres* + MKB52, 4= *P. teres* + MKB141, 5 = *P. teres* + MKB66, 6 = *P. teres* + *B. megaterium* MKB135, 7 = *P. teres* + *Pseudomonas* sp. MKB158, 8 = *P. teres* + *Ps. fluorescens* MKB100, 9 = *P. teres* + *Ps. fluorescens* MKB156, 10 = *P. teres* + *Pseudomonas* sp. MKB194, 11 = *P. teres* + 0.2% Tween 20.

1.2.1 One-way ANOVA: homogeneous subsets for cv Lux

			Subset for alpha = .05			
	* Bacteria	N	1	2	3	4
Tukey HSD[a]	1.00	6	.0000			
	10.00	6		.0913		
	8.00	6		.1031		
	9.00	6		.1069		
	7.00	6		.1231		
	6.00	6			.2363	
	5.00	6			.2863	.2863
	3.00	6			.2919	.2919
	11.00	6			.3063	.3063
	4.00	6			.3142	.3142
	2.00	6				.3498
	Sig.		1.000	.975	.115	.349

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 6.000.

1.2.2 One-way ANOVA: homogeneous subsets for cv Tavern

			Subset for alpha = .05			
	* Bacteria	N	1	2	3	4
Tukey HSD[a]	1.00	6	.0000			
	8.00	6		.1380		
	9.00	6		.1399		
	10.00	6		.1860		
	7.00	6		.2133		
	6.00	6			.2970	
	5.00	6			.3253	.3253
	3.00	6			.3390	.3390
	4.00	6			.3613	.3613
	11.00	6			.3694	.3694
	2.00	6				.3983
	Sig.		1.000	.099	.131	.124

Means for groups in homogeneous subsets are displayed.
a. Uses Harmonic Mean Sample Size = 6.000.

Appendix 1.2.3 Correlation of results from seedling experiment between cultivars Lux and Tavern

Test: Pearsons Product Moment Correlation

Correlations

		VAR00001	VAR00002
Lux	Pearson Correlation	1	.978**
	Sig. (2-tailed)	.	.000
	N	11	11
Tavern	Pearson Correlation	.978**	1
	Sig. (2-tailed)	.000	.
	N	11	11

**. Correlation is significant at the 0.01 level (2-tailed).

Appendix 1.3 Effect of timing of bacterial application on net blotch development on cv. Lux

* 1 = *P. teres* + Luria Bertani broth (LB), 2 = *P. teres* + *Pseudomonas fluorescens* strain MKB100 6 days pre-fungal inoculation, 3 = 4 days pre-fungal inoculation, 4 = 2 days pre-fungal inoculation, 5 = 2 days post fungal inoculation, 6 = 4 days post fungal inoculation, 7 = 6 days post fungal inoculation, 8 = *P. teres* + *Pseudomonas fluorescens* strain MKB156 6 days pre-fungal inoculation, 9 = 4 days pre-fungal inoculation, 10 = 2 days pre-fungal inoculation, 11 = 2 days post fungal inoculation, 12 = 4 days post fungal inoculation, 13 = 6 days post fungal inoculation, 14 = *P. teres* + *Pseudomonas* sp. strain MKB194 6 days pre-fungal inoculation, 15 = 4 days pre-fungal inoculation, 16 = 2 days pre-fungal inoculation, 17 = 2 days post fungal inoculation, 18 = 4 days post fungal inoculation and 19 = 6 days post fungal inoculation

1.3.1

Data: Normalised AUDPC values

Test: One-way ANOVA incorporating Tukeys Pairwise comparison test at the 5% level of significance

	* Treatment + Time	N	Subset for alpha = .05		
			1	2	3
Tukey HSD [a]	4.00	6	.1072		
	14.00	6	.1169	.1169	
	8.00	6	.1275	.1275	
	10.00	6	.1314	.1314	
	9.00	6	.1450	.1450	.1450
	2.00	6	.1514	.1514	.1514
	16.00	6	.1597	.1597	.1597
	15.00	6	.1614	.1614	.1614
	3.00	6	.1617	.1617	.1617
	13.00	6	.1742	.1742	.1742
	11.00	6	.1811	.1811	.1811
	6.00	6	.1831	.1831	.1831
	7.00	6	.1958	.1958	.1958
	18.00	6	.1989	.1989	.1989
	19.00	6	.2036	.2036	.2036
	17.00	6	.2097	.2097	.2097
	5.00	6		.2186	.2186
	12.00	6		.2225	.2225
	1.00	6			.2478
	Sig.		.084	.063	.081

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 6.000.

Appendix 1.4 % Growth inhibition of *P. teres* by bacterial treatments in dual culture plate tests

Data: % Growth inhibition values
Test: One-way ANOVA incorporating Tukeys Pairwise comparison test at the 5% level of significance

	* Treatment	N	Subset for alpha = .05
			1
Tukey HSD[a]	P. teres + MKB158	3	57.8947
	P. teres + MKB100	3	61.5630
	P. teres + MKB194	3	71.7703
	P. teres + MKB156	3	89.7927
	P. teres + LB	3	100.0000
	Sig.		.309

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Appendix 1.5 Disease symptom development on barley leaves (cv. Lux) grown in soil amended with bacterial treatments and leaves inoculated with *P. teres* var *teres*.

Data: Normalised AUDPC values
Test: One-way ANOVA incorporating Tukeys Pairwise comparison test at the 5% level of significance

	* Treatment	N	Subset for alpha = .05	
			1	2
Tukey HSD[a]	P. teres + MKB156	6	.1960	
	P. teres + MKB194	6	.2136	
	P. teres + MKB100	6	.2807	.2807
	P. teres + LB	6		.3357
	Sig.		.107	.419

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 6.000.

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Appendix 1.6 Effect of bacterial isolates on net blotch development on detached leaves.

Data: Transformed AUDPC values (diseased leaf area)

Test: One-way ANOVA incorporating Tukeys Pairwise comparison test at the 5% level of significance

* 1 = *P. teres* + LB, 2 = *P. teres* + *B. mycoides* MKB23, 3 = *P. teres* + *Bacillus sphaericus* MKB44, 4 = *P. teres* + *Ps. fluorescens* MKB100, 5 = *P. teres* + *Ps. fluorescens* MKB156, 6 = *P. teres* + *Pseudomonas* sp. MKB158, 7 = *P. teres* +. *Pseudomonas* sp. MKB194, 8 = *P. teres* + *Bacillus sphaericus* MKB232, 9 = *P. teres* + *Pseudomonas tolaasii* MKB286.

1.6.1 One-way ANOVA: homogeneous subsets for cv. Calcutta

	* Treatment	N	Subset for alpha = .05	
			1	2
Tukey HSD[a]	7.00	3	-1.3800	
	4.00	3	-1.1400	
	6.00	3	-.2167	-.2167
	9.00	3	-.2133	-.2133
	3.00	3	.0167	.0167
	8.00	3	.0867	.0867
	5.00	3	.2033	.2033
	2.00	3		.6667
	1.00	3		1.4367
	Sig.		.092	.071

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

1.6.2 One-way ANOVA: homogeneous subsets for cv. Fractal

	* Treatment	N	Subset for alpha = .05			
			1	2	3	4
Tukey HSD[a]	7.00	3	-1.1176			
	4.00	3	-.9715	-.9715		
	2.00	3	-.6001	-.6001	-.6001	
	6.00	3	-.1777	-.1777	-.1777	
	5.00	3	.0291	.0291	.0291	
	3.00	3	.3332	.3332	.3332	.3332
	9.00	3		.4284	.4284	.4284
	8.00	3			.6349	.6349
	1.00	3				1.6841
	Sig.		.058	.072	.148	.090

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

1.6.3 One-way ANOVA: homogeneous subsets for cv. Tavern

	* Treatment	N	Subset for alpha = .05		
			1	2	3
Tukey HSD[a]	4.00	3	-1.2632		
	7.00	3	-1.1982		
	6.00	3	-.4019	-.4019	
	3.00	3	-.1346	-.1346	
	8.00	3	-.1055	-.1055	
	9.00	3		.2128	
	5.00	3		.3656	.3656
	2.00	3		.9368	.9368
	1.00	3			1.6923
	Sig.		.133	.056	.059

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

1.6.4 One-way ANOVA: homogeneous subsets for cv. Prestige

	* Treatment	N	Subset for alpha = .05			
			1	2	3	4
Tukey HSD[a]	7.00	3	-1.4968			
	4.00	3	-.9740	-.9740		
	3.00	3	-.6513	-.6513	-.6513	
	5.00	3	.0381	.0381	.0381	.0381
	6.00	3		.1306	.1306	.1306
	8.00	3		.1968	.1968	.1968
	9.00	3		.2385	.2385	.2385
	2.00	3			.6256	.6256
	1.00	3				1.5682
	Sig.		.055	.205	.161	.056

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

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1.6.5 One-way ANOVA: homogeneous subsets for cv. Siberia

	* Treatment	N	Subset for alpha = .05			
			1	2	3	4
Tukey HSD[a]	7.00	3	-1.6276			
	4.00	3	-1.2764	-1.2764		
	6.00	3	-.4707	-.4707	-.4707	
	9.00	3	-.3140	-.3140	-.3140	
	3.00	3	-.1369	-.1369	-.1369	
	5.00	3		.1526	.1526	
	8.00	3			.4852	.4852
	2.00	3			.8696	.8696
	1.00	3				1.7874
	Sig.		.073	.094	.135	.156

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

1.6.6 One-way ANOVA: homogeneous subsets for cv. Lux

	* Treatment	N	Subset for alpha = .05		
			1	2	3
Tukey HSD[a]	7.00	3	-1.3407		
	5.00	3	-.5045	-.5045	
	8.00	3	-.3678	-.3678	
	4.00	3	-.2494	-.2494	
	9.00	3	-.0706	-.0706	
	6.00	3	-.0221	-.0221	
	2.00	3		.5316	.5316
	3.00	3		.7416	.7416
	1.00	3			1.7506
	Sig.		.194	.248	.271

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

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1.6.7 Correlation between detached leaf results and seedling experiment on cvs. Lux and Tavern respectively. Treatments included in correlation: *P. teres* + LB, *P. teres* + *Ps. fluorescens* MKB100, *P. teres* + *Ps. fluorescens* MKB156, *P.teres* + *Pseudomonas* sp. MKB158 and *P. teres* + *Pseudomonas* sp. MKB194

Test: Pearsons Product Moment Correlation

Correlations

		VAR00001	VAR00002
VAR00001	Pearson Correlation	1	.996**
	Sig. (2-tailed)	.	.000
	N	5	5
VAR00002	Pearson Correlation	.996**	1
	Sig. (2-tailed)	.000	.
	N	5	5

**. Correlation is significant at the 0.01 level (2-tailed).

Correlations

		VAR00003	VAR00004
VAR00003	Pearson Correlation	1	.932*
	Sig. (2-tailed)	.	.021
	N	5	5
VAR00004	Pearson Correlation	.932*	1
	Sig. (2-tailed)	.021	.
	N	5	5

*. Correlation is significant at the 0.05 level (2-tailed).

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Appendix 1.7 Effect of bacterial isolates on net blotch development on adult plants

Data: Normalised AUDPC values (diseased leaf area)

Test: One-way ANOVA incorporating Tukeys Pairwise comparison test at the 5% level of significance

	* Treatment	N	Subset for alpha = .05	
			1	2
Tukey HSD[a]	P. teres + MKB156	30	.0872	
	P. teres + MKB100	30	.1035	
	P. teres + LB	30		.1845
	Sig.		.332	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 30.000.

1.7.1 Correlation between results from seedling experiment and field experiment on cv. Lux. Treatments included in correlation: *P. teres* + LB, *P. teres* + *Ps. fluorescens* MKB156 and *P. teres* + *Ps. fluorescens* MKB100

Test: Pearsons Product Moment Correlation

Correlations

		VAR00001	VAR00002
VAR00001	Pearson Correlation	1	.990*
	Sig. (1-tailed)	.	.046
	N	3	3
VAR00002	Pearson Correlation	.990*	1
	Sig. (1-tailed)	.046	.
	N	3	3

*. Correlation is significant at the 0.05 level (1-tailed).

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