BIOLOGICAL CONTROL OF CHOCOLATE SPOT AND RUST ON FABA BEANS

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DECLARATION

The contents of this thesis are the sole work of the author and have not been previously submitted for the award of a degree to any other university.

Andrew J Jackson

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SUMMARY

The potential of bacterial and fungal isolates from root and soil samples of faba bean and other crops, was assessed for biological control of *Botrytis fabae* (chocolate spot) and rust (*Uromyces viciae-fabae*) through *in vitro* and *in vivo* screening.

In total, 690 bacterial and fungal isolates were obtained and screened with *B. fabae* mycelium in a preliminary screen on agar plates. Potential antagonism was exhibited by approximately 30% of these isolates, as indicated by at least one of the following visual symptoms: a brown discoloration of mycelium or media, a zone of inhibition of mycelial growth, a zone free of sclerotial formation or contact inhibition between isolates. Detached leaf studies rejected a further 20% of the isolates due to their pathogenicity towards the leaves. Further replicated antagonism tests *in vitro* allowed more detailed studies, where the percentage inhibition in radial growth of *B. fabae* and the zone of inhibition were recorded. Some isolates which exhibited potential antagonism in the initial preliminary screen did not do so in this test. Bacterial isolate LL1.F23, identified as *Pseudomonas fluorescens*, exhibited the largest inhibition zone (11.2 mm), inducing malformations and severe browning of mycelium at the culture edge. Overall, fungi produced smaller inhibition zones than bacterial isolates.

Germination tests with *B. fabae* conidia showed that isolate LL1.F23 significantly reduced the germination, length of germ-tubes produced and appressorium formation relative to the control. Other isolates reduced germination significantly but to a lesser degree. Some fungal isolates inhibited mycelial

growth of *B. fabae* through the production of volatile and non-volatile antibiotics. However, due to the production of secondary colonies of antagonists, the results were variable and inconsistent. Spore germination tests on *U. viciae-fabae* in the presence of antagonists showed that uredospore germination was reduced by up to 90%.

Following *in vitro* screening, 35 isolates (15 fungal and 20 bacterial isolates) were chosen for application to bean plants in the glasshouse. Against chocolate spot, the fungal isolate AP1.S20 (*Penicillium chrysogenum* Thom.) showed the greatest potential, reducing disease levels by up to 80%. Fungal isolate AG1.F4 (*Geomyces pannorus* (Link) Singler & J.W. Carmich.) and bacterial isolate P1.S13B, reduced chocolate spot infection to 19% and 12% of the control respectively, when applied simultaneously with *B. fabae* conidia. The percentage leaf area covered with rust pustules was reduced by 78% following simultaneous application of isolate AP2.R16 with rust uredospores. The most consistent reductions in chocolate spot and rust were obtained with simultaneous inoculation of the antagonist and the pathogen. It was found with rust that the greater the concentration of antagonist inoculum, the greater the reduction of rust on the leaf surface. A suspension of 1 x 10^8 spores ml^{-1} of isolate MX1.L34 (*Penicillium brevicompactum* Dierckx.) reduced chocolate spot symptoms to 8.5% of the control, with significant disease control attained at concentrations as low as 1 x 10^4 spores ml^{-1}.

Cell-free culture filtrates of four fungal isolates, one of each of the fungal species identified, when bioassayed with *B. fabae* conidia, exhibited antifungal activity reducing both

germination and germ-tube growth significantly. Studies were initiated to isolate these inhibitory agent(s) through partial purification of the culture filtrates. Cell-free filtrates of isolate AP1.S20 (*P. chrysogenum*) were extracted with four solvents: ethyl acetate, chloroform, hexane and petroleum ether, and screened *in vitro* for antifungal activity against *B. fabae* conidia. The ethyl acetate fraction of isolate AP1.S20 gave the largest inhibition of spore germination (95.7%), while the petroleum ether fraction significantly reduced germ-tube extension to 19% of the control. Thin layer chromatography of these solvent extracts displayed up to 15 discrete fractions from each solvent sample. On application of these to tlc plates, the germination of *B. fabae* conidia was inhibited at one discrete spot from each solvent extract, each with the same Rf value. Preliminary results using detached leaves showed that both the culture filtrate and a spore suspension of isolate AP1.S20 significantly reduced lesion development and lesion spread.

1.

1. INTRODUCTION AND REVIEW OF LITERATURE

Severe yield losses can be caused by aggressive attacks of *Botrytis fabae* (chocolate spot), with fungicides only giving partial disease control due to the widespread occurrence of resistance to fungicides. Rust (*Uromyces viciae-fabae*) appears late in the growing season but is of increasing importance in the United Kingdom causing significant yield losses of up to 20% (Lapwood, McEwan & Yeoman, 1984).

With the increasing awareness of the problems and expense of conventional methods of disease control, including fungicides and breeding programmes, biological control of plant pathogenic fungi has many attractions. Aerial plant surfaces, commonly know as the phyllosphere or the phylloplane, provide a habitat for a diverse and complex range of epiphytic micro-organisms, many of which influence the growth of pathogens.

1.1 Vicia faba L.: Domestication and production

Broad bean (*Vicia faba* L.) belongs to the order *Leguminosae* and is an important grain legume of the North temperate zone. The bean is a papilionaceous flowering plant of the genus *Vicia* and the species *faba*. This crop is grown world-wide on approximately 3.6 million ha, with a total annual production of about 4 million tonnes (Elliott & Whittington, 1979). It is a traditional crop in Europe used as: (i) field beans (*V. faba minor* and *equina*) originating from West-Asiatic areas for animal feeding or, (ii) as broad beans (*V. faba major*) originating from the Mediterranean area and which are used for direct human

consumption (Austin *et al.*, 1986; Chapman, 1983). In an attempt to standardise English usage the term 'faba bean' has been widely adopted to denote the whole species (Bond *et al.*, 1985; Bond, 1976).

In Western Europe, the use of fresh or preserved *V. faba* is confined to restricted areas and large seeded varieties (Bond *et al.*, 1985), with the United Kingdom the third largest producer in Europe (Hebblethwaite, 1984). Unripe seeds, cooked or raw are used for human consumption as a good source of protein, vitamins and a moderate source of minerals.

Faba beans have been grown in the U.K. since pre-Roman times, but after peak production in the 1860s and 1870s, the area of faba beans declined progressively (Heath, 1987). Reasons for this decline include: the agricultural depression, increased labour costs and the importation of cheaper protein. There has been renewed interest in the crop due to rising costs of protein rich foods and a desire for EEC countries, since 1973, to be self-sufficient in protein rich grains (Bond *et al.*, 1985; Heath, 1987). This is encouraged by a subsidy to cultivate grain legumes, and although guaranteed prices have been lost, they still retain a fixed support (Campredon, 1993). At present grain legumes account for 7.5% of the protein consumption in the EEC, or 4.54 million tonnes (Rexen & Hardy, 1993). The calculation of subsidies has been transferred from volume to hectares, reducing the race to increase yield (Campredon, 1993), allowing increased interest in cultivars with respect to disease resistance as well as quantity and application of N_2 fertilisers. The International Centre for Agricultural Research in the Dry Areas (ICARDA) was established

in 1976, and one of the main crops studied is faba beans.

For future research activities, two objectives have been outlined for the EEC. Firstly, increasing the protein derived from grain legumes and secondly, to explore the possibilities of other uses for grain legumes, not necessarily in terms of protein (Rexen & Hardy, 1993).

Faba beans can be sown in either the autumn or spring, but the majority are autumn-sown (winter beans). Winter beans are long established as an important component of cereal rotations, particularly on heavy textured soils in the English Midlands (Heath, 1987). Faba beans have a low input requirement and command a minimum price. Disadvantages of growing winter beans include a notorious reputation for low and variable yields, coupled with the risk of a late harvest of tall, diseased, and lodged crops of uneven maturity. Spring beans have a slightly higher protein content, but are lower yielding, more sensitive to drought and aphid attack, and of later maturity and harvest date than winter beans (Heath, 1987). Faba beans are used as a rotational 'break' crop in continuous cereal growing systems, preventing a build up of soil borne cereal diseases, supplying nitrogen and improving the soil structure (Bond *et al.*, 1985).

V. faba plants are distinctly annual with strong hollow erect stems bearing basal branches arising from leaf axils. Alternate pinnate leaves comprise between 2 and 6 entire oral leaflets each up to 8 cm long. Plant height varies with environmental conditions from 50-200 cm. Traditional cultivars produce between 2 and 10 typical papilionaceous flowers in each auxiliary raceme starting at between the 5th and 10th node (Bond *et al.*, 1985). In most soils, Legumes produce the so called

nitrogen root nodules via the symbiotic interaction of the host plant with root infectious bacteria of the genus *Rhizobium* (Bond *et al.*, 1985).

1.2 Principal limitations to production and yield

1.2.1 YIELD

Faba beans are a relatively inexpensive crop in terms of input costs compared to cereals, but the variability in yield of faba beans is higher than that of cereals, as the crop is more subject to the effects of adverse environments, especially drought, excessive rainfall and to pests and diseases (Austin *et al.*, 1986).

Yield variability in beans has long been attributed to various factors, including its indeterminate growth habit associated with excessive vegetative growth in wet seasons, and excessive reproductive losses (Heath, 1987). Breeders are currently developing genotypes with a determinate growth habit which are shorter and more resistant to lodging than conventional types.

1.2.2 DISEASES AND PESTS

Severe yield losses can be caused by aggressive attacks of *B. fabae* (Chocolate spot) on winter beans, with husbandry methods and fungicides giving only partial control (Hebblethwaite, 1984). Rust (*U. viciae-fabae*) appears later in the growing season but is of increasing importance in the United Kingdom causing significant yield losses (20%) (Lapwood *et al.*, 1984).

As a necrotroph, *B. fabae* is capable of a saprophytic existence and relatively rapid growth on a wide variety of substrates. It is opportunistic and has enzymes which help it to compete for space and nutrients on the leaf surface (Barnett & Binder, 1973). *U. viciae-fabae* is biotrophic and has a close association with the host, which furnishes continuous supplies of nutrients (Barnett & Binder, 1973).

Ascochyta fabae (leaf and pod spot) is seed transmitted, but in the U.K. freedom for infection is an important aspect of seed certification (Bond *et al.*, 1985). It can only be partially controlled by the currently available techniques such as chemical and cultural methods (Tivoli, 1993). Foot rot (*Fusarium*) limits production mainly on soils of poor fertility or structure (Bond *et al.*, 1985).

Aphid damage can be controlled with insecticides when attacks are forecast, and the weed parasite *Orobanche crenata* can be devastating but chemical control is often not economic unless the crop has potential for large yields (Austin, Dickinson & Goodfellow, 1977).

1.2.3 BREEDING

Breeding programmes to improve faba beans have only been conducted on a serious basis for the last 5-10 years, even though genetic variation existed (Hebblethwaite, 1984) (see Sections 1.3.1.2 and 1.4.1.2).

The driving force in the evolution of *V. faba* has been the high levels of natural cross pollination, with heterosis giving advantages to heterozygotes, and as a consequence, leading to

frequent exchanges of genes among and within populations (Bond *et al.*, 1985; Bond, 1993).

Agricultural research is becoming more and more specialised, often requiring synergy between different disciplines (Tivoli, 1993). In the past the breeder would devise and implement a programme himself for breeding pathogen resistance, but now he needs to work with a pathologist who provides essential information on the pathogen to integrate this with genetic and other control methods (Tivoli, 1993).

1.3 Chocolate spot (Botrytis fabae and Botrytis cinerea)

This disease is compound and is caused by both *B. fabae* and *B. cinerea*, although Harrison (1988) found evidence to suggest that while both species can cause chocolate spot in the field, *B. fabae* is the more important pathogen because it is more aggressive than *B. cinerea*. Both species of *Botrytis* belong to the class, Imperfecti fungi (Deuteromycetes) and the order, Moniliales (Agrios, 1978). The effectiveness of this aggressive phase of the disease can be devastating on autumn sown crops of winter beans in North-western Europe (Bond *et al.*, 1985), and is regarded as one of the major causes of yield instability. *B. fabae* is a facultative parasite, unable to mobilise assimilates and attacks only senescing tissue (Williams, 1975).

Symptoms include reddish brown to chocolate coloured spots appearing on leaves and pods and brown streaks on stems (Plate 1a). With an aggressive infection the spots coalesce, with large dark lesions appearing causing defoliation, eventually

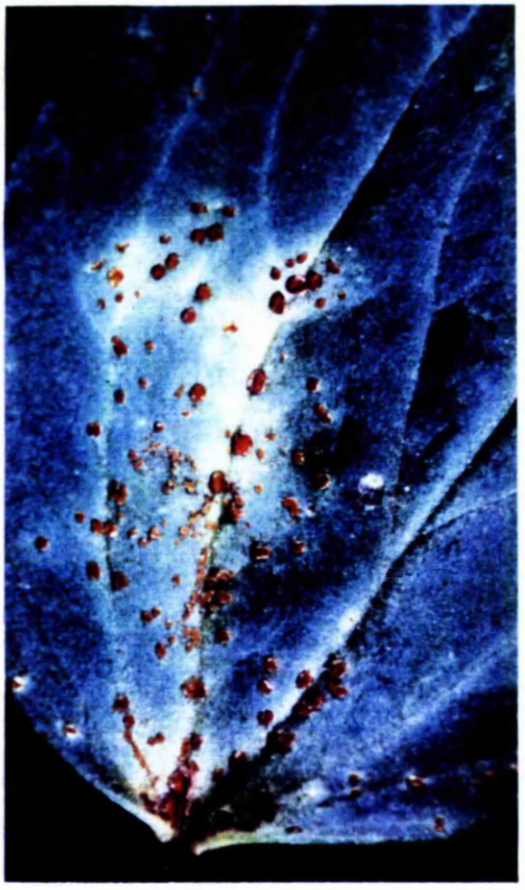
Plate 1: Visual symptoms of foliar diseases of faba beans.

(a) Reddish brown to chocolate coloured spots on bean leaves caused by *B. fabae*.

(b) Rust pustules of *U. viciae-fabae*, bearing reproductive uredospores, characteristically orange-brown in colour.

(a)

(b)



killing the entire shoot system (Harrison, 1988). Dead tissue gradually turning brown with characteristic chocolate coloured spots due to the fungus converting colourless tryosin to brown melanin (Butler & Jones, 1961).

Infected plants suffer a loss of leaf area, although the plants may maintain a relative growth rate comparable to healthy plants. From calculations of the Net Assimilation Rate (NAR), a measure of photosynthetic efficiency, Williams (1975) showed that diseased plants were 30-40% more productive than control plants, probably because the remaining healthy leaves on infected plants compensated for the reduction in leaf area (Williams, 1975). Bainbridge *et al.*, (1985) found that chocolate spot reduced yields when severe epidemics developed, either early in the season or during flowering. The level of disease at pod set and shortly after, is the most critical factor in determining loss of yield (Williams, 1978).

Infection is mainly by conidia liberated from conidiophores produced in lesions or decaying vegetation. Chocolate spot is more prevalent in cool, moist or wet weather, on heavy, poorly drained soils, in sheltered sites and where potassium and phosphorus levels are low (Anon, 1984).

B. fabae conidia germinate on leaves to produce appressoria on germ-tubes before penetration of the cuticle. Unless infection is heavy the invading fungus enters no further than the epidermis, below the point of penetration (Butler & Jones, 1961). In aggressive infections, there is a heavier deposit of conidia on leaves and hyphae invade the intercellular spaces of mesophyll tissue. Infection may extend from one leaf surface to the other causing cell walls to expand before

completely collapsing.

B. fabae has the specific ability to kill host cells soon after penetration, allowing it to become established and reducing any active resistance mechanisms. The importance of killing large numbers of host cells and suppressing host resistance is indicated by the effects of inoculum concentrations on fungal development (Mansfield, 1980; Mansfield & Hutson, 1980; Rossall, Mansfield & Hutson, 1979).

Growth of *B. fabae* within its host plant is entirely necrotrophic (Mansfield & Hutson, 1980). The development of resistant reactions to non-pathogenic species, like *B. cinerea*, is associated with less penetration and the death of far fewer plant cells during the early stages of infection compared to that caused by *B. fabae* around the site of penetration.

Cell wall degrading enzymes are the principle cause of cell death during the development of chocolate spot (Harrison, 1983c). Secretion of polygalacturonase, hemicellulases and proteases by fungi accompany infection in some fungal pathogens. However, it is not quite clear how important these enzymes are in penetration of *V. faba* leaves by *B. fabae* (Urbanek, Kuzniak & Malolepsza, 1987). In the peripheral zone of lesions on beans containing only traces of *B fabae* mycelium, galactanase, protease, polygalacturonase and arabinase were all increased in activity. This paralleled the intensive secretions of these enzymes by the accumulated mycelium in the central zone, indicating that their role is not limited to supplying the pathogen with nutritive substances (Urbanek *et al.*, 1987).

These enzymes probably diffuse out from the infection

site, penetrating host tissue ahead of the pathogen's mycelium, destroying host defences and making the spread of the disease possible. β-1,3-glucanase, which has been identified at the infection site, may either be produced by the pathogen as a tool of attack or by the host plant as a defensive agent (Urbanek *et al.*, 1987).

The age of a leaf at the time of infection has a profound effect on the pathogenicity of *B. fabae* e.g. older leaves develop more lesions than younger ones, but exhibit less chlorosis. Established lesions on young leaves of intact plants at high humidities increased in size at half the rate of those on old leaves (Helibronn & Harrison, 1989).

B. fabae requires about 4 conidia, compared with 400 *B. cinerea* spores, for infection on *V. faba* leaves (Baker & Cook, 1974). As the age of spores increases, their ability to infect leaves decreases due to a decline in nutrient reserves. Infections of *B. fabae* decrease faster at 22^{o}C than 10^{o}C and also faster at low humidities than high (Buxton, Last & Nour, 1957). Indeed, lesion growth is highly dependent on humidity and temperature (Harrison, 1980), but their is no evidence of an interaction between them. Harrison also found that light intensity and presence of a water film did not affect the rate of lesion growth. *B. fabae* conidia are predominantly dispersed by wind (Harrison & Lowe, 1984).

At present there is no satisfactory means of controlling the disease by targeting the overwintering fungi (Harrison, 1979). *B. fabae* overwinters either as sclerotia embedded in dead bean stems or as hyphae living saprophytically and/or as resting mycelium in the soil and necrotic crop debris.

1.3.1 DISEASE CONTROL - TRADITIONAL

Several strategies can be employed to control chocolate spot, including crop hygiene and breeding for disease resistance, but fungicide application is the most effective and widely used (Harrison, 1988).

1.3.1.1 Fungicide application

Wilson (1937) and Kosogorova (1963) found that Bordeaux mixture gave good control of chocolate spot. More recently, foliar application of organic fungicides, especially benomyl, has been effective against the disease (McEwen *et al.*, 1981). Although benomyl is widely used to control chocolate spot, strains of *B. cinerea* resistant to it, and other MBC fungicides, are becoming very common (Harrison, 1988; Parry, 1993). Resistance can manifest itself in one of two ways, either through a complete loss of disease control, or through a gradual loss of effectiveness of the fungicide over several years (Parry, 1993). The fungicide used may still give some disease control, but not as effective as it used to be. Fungicides with more than one mode of action and which thus affect several metabolic processes, reduce the chance of resistant strains of the pathogens arising.

A range of systemic compounds are used to control chocolate spot, including: benodamil, carbendazim, fenpropimorph, prochloraz, propiconazole, thibendazol, thiophanate-methyl and tridemorph (Ivens, 1993; Bainbridge *et al.*, 1985; Elliott & Whittington, 1980). Jones (1993) recommends using MBC fungicides in tank mixes with chlorothalonil. Bennett & Lane (1992) tested captan, thiram,

carbendazim and benomyl *in vitro* against *B. fabae* where they all limited pathogen growth, but unfortunately, trials *in vivo* were not conclusive.

The timing of fungicide application to achieve good disease control is important (Bainbridge *et al.*, 1985). Due to seasonal variation in weather affecting the development of chocolate spot, Gladders, Ellerton & Bowerman, (1991) emphasised that a flexible approach to fungicide application is required to control chocolate spot before it develops into a severe infection. Dobson & Giltrap (1991) found a 2-3 spray programme starting at early flowering gave acceptable control. Chlorothalonil mixed with benomyl or mycolozolin (Gladders *et al.*, 1991) showed promise for maintaining and improving chocolate spot control. Alternating the application of fungicides with biological control agents to control *B. cinerea* has been suggested as a means of reducing the selection pressure towards resistance to common fungicides (Elad & Cohen, 1991; Elad & Zimand, 1991).

1.3.1.2 Cultivars - Breeding resistance

Genetic resistance to chocolate spot of faba beans is not good in current commercial cultivars (Hanounik, Jellis & Hussein, 1990). Various authors have reported the inability to find absolute resistance to *B. fabae* (Elliott & Whittington, 1978; 1979; Bond *et al.*, 1985).

The lack of a high degree of resistance is largely due to the inability of *V. faba* to cross with related but resistant species, such as *V. narbonensis* (Lawes, Bond & Poulsen, 1983). Until a method to transfer this resistance is achieved, breeding

efforts are aimed at slowing down the development of the disease (Harrison, 1988). ICARDA have produced an accession showing consistent resistance to *B. fabae* [reference number ILB 438/938 (=BPL710/1179)] which originated from Ecuador (Bond 1990, personal communication). This line is now being crossed with the U.K. adapted varieties of winter beans.

Inhibitory effects of covicine, vicine and L-dopa on *in vitro* growth of *B. cinerea* might also occur *in vivo* (Harrison, 1988), so breeding for high levels of these compounds might confer resistance to *B. fabae*. No attempts to breed for resistance to *B. cinerea* have been reported (Harrison, 1988). The extrapolation of screening tests based on measuring resistance to infection, leaf expansion or sporulation carried out in the laboratory and the glasshouse, often relate poorly to disease resistance of plants grown in the field (Harrison, 1988).

The possibility of inducing mutations with improved disease resistance has been examined by Abdel-Hal & Kamel (1974), using gamma rays, but no firm indication of any useful degree of resistance has been found.

1.3.1.3 Crop hygiene

Crop burning after harvest, rotation of crops to reduce inoculum levels, ploughing in plants after harvest and destroying volunteer weeds and seedlings can all help to control chocolate spot, reducing the carry over of diseased material (Harrison, 1988).

1.3.1.4 Summary

Accurate weather forecasting and understanding of conidial dispersal/survival, infections, lesion expansion and sporulation in relation to microclimate, and changes in disease susceptibility as the season progresses, may allow potential disease severity to be predicted (Harrison, 1988). This would allow fungicides to be applied before any high risk period. It is unlikely that bean cultivars with complete resistance to attacks by *B. cinerea* and *B. fabae* will be developed in the near future, so the use of varieties with a moderate degree of resistance and possibly the use of micro-organisms antagonistic to *Botrytis* could substantially reduce levels of chocolate spot (Harrison, 1988), provided good crop hygiene and reliable disease forecasting are possible. Bond, (1990, personal communication) emphasised that varietal resistance could play a large part in biological control of chocolate spot.

1.3.2 BOTRYTIS CONIDIA

Quantitative studies have been carried out to compare different *Botrytis* species (Mansfield & Hutson, 1980). Here germination, formation, growth and death of infection hyphae were noted at each inoculation site. Differences between pathogenic and non-pathogenic species were much greater in terms of successful penetrations and formation of infection hyphae than in germination of conidia. *B. fabae* was found to penetrate quickly into bean leaves, causing rapidly spreading lesions at all inoculation sites after 4 days. Mansfield & Hutson (1980) found the effect of inoculum concentration was much more pronounced with *B. fabae* than *B. cinerea*, finding that *B. fabae* infection

hyphae grew at almost a linear rate within the epidermis of bean leaves.

The percentage of *B. cinerea* conidia which will germinate in water decreases sharply with the age of cultures (Chou & Preece, 1968). With increasing age of conidia, partial loss of endogenous substrates occur through leaching (leading to loss of water-soluble components) and respiration (Blakeman, 1971). Pollen reduced the inoculum threshold needed for infection, restoring germination and infectivity of moribund conidia, changing non-aggressive lesions to aggressive, and increasing the severity of a *Botrytis* attack (Chou & Preece, 1968).

Last (1960) found that conidia of *B. fabae* lost their infectivity with increasing age, such that 40 day old conidia, had lost their ability to infect, while still able to germinate satisfactorily. Such aged conidia can be made infective when suspended in a nutrient solution such as orange juice or 4.5% sucrose (Last, 1960; Deverall & Wood, 1961). *B. fabae* conidia were found to germinate after 45 days (Last, 1960), whereas after 14 days Chou & Preece (1968) recorded a germination of just 2% for *B. cinerea* conidia. Last (1960) showed that conidia of *B. fabae* lost their ability to infect quicker than they lost their ability to germinate showing clearly that there is a greater energy requirement for infection than germination.

Exogenous nutrient materials present on the foliage can have a marked effect on increasing growth and subsequent infection by facultative parasites (Blakeman, 1971). Brown (1922) found that natural exudates or added nutrients enhanced infection of *B. cinerea* on petals of *Cerus* and *Gloxinia*, as well as on leaves of broad bean. Experiments with *B. cinerea* on

Rust (Uromyces vicae-fabae)

Subdivision:	Basidiomycotina
Class:	Teliomycetidae (Hemibasidiomycetes)
Order:	Uredinales
Genus:	Uromyces
Species:	viciae-fabae

capsules of different varieties of castor bean showed that the susceptibility of varieties was dependent on the amount of sugar in leachates (Orellana & Thomas, 1962).

The higher rate of germination and growth rate of *B. fabae* compared to *B. cinerea* (Purkayastha & Deverall, 1965) could well be related to the fact that *B. fabae* conidia are on average five times larger than those of *B. cinerea*, containing higher levels of food reserves.

Conidial production by *B. fabae in vitro* is stimulated by irradiation; near uv irradiation at wavelengths of 375-400 nm induced most sporulation (Harrison, 1981). The optimum osmotic potential of the culture medium for the formation of conidia was found to be about -27 bars, although mycelial growth was greater at even lower osmotic potentials (Harrison & Helibronn, 1988). Abundant conidial production occurred when the fungus was grown in media over a range of pH values, but conidia were not produced in continuous darkness or in temperatures greater than 24.5^{o}C (Harrison, 1984b). Leach & Moore, (1966) found that production of conidia by *B. fabae* on agar was promoted strongly by high salt or sucrose concentrations and so they considered the effect to be due to low osmotic potential.

1.4 Rust (Uromyces viciae-fabae)

Rust fungi are members of the subdivision Basidiomycotina, family *Pucciniaceae* and the order Uredinales. *Uromyces viciae-fabae* belongs, together with the *Ustilaginaes*, to the *Teliomycetidae*, a subclass of the *Basidiomycetes*, and are distinguished by the appearance of the basidium on the

teliospore (Grove, 1913; Scott & Chakravorty, 1982).

U. viciae-fabae is reported on faba beans and many other leguminous species and occurs in almost every part of the world (Wilson & Henderson, 1966). Bean rust is autoecious and is an obligate parasite attacking only living tissue of hosts, exhibiting a high degree of specialisation (Shaw, 1963).

Rust is a major disease of faba beans in the Middle East and in North Africa where epidemics cause yield losses as high as 20% (Rashid & Bernier, 1984). Losses up to 45% occurred when susceptible cultivars were used in Australia (Rashid & Bernier, 1991). These authors reported yield losses up to 68% on susceptible lines, but using modern resistant lines, damage did not exceed 10%. During a series of field experiments at Rothamsted (Lester, 1982; cited in Lapwood *et al.*, 1984) on spring beans, rust had become prevalent, attacking the crop during August and leading to almost complete defoliation by late August/early September.

Colonisation of the host involves the formation of intracellular haustoria and intercellular mycelium which penetrates the host plant tissues (Brand, 1985).

Uredospores and basidiospores of rust fungi typically penetrate and infect their host plants in different ways (Littlefield & Heath, 1979). Germ-tubes of dikaryotic uredospores normally form appressoria over stomata and penetrate with an infection peg. Once in the substomatal chamber, the fungus differentiates a vesicle from which one or more elongated infection hyphae arise. After the formation of the haustorial mother cells, intracellular haustoria develop in host cells

(Freytag *et al.*, 1988). In contrast, mono- or binucleate basidiospores of *U. viciae-fabae* penetrate into epidermal cells directly via appressoria, which generally occurs close to the anticlinal walls of the epidermis. After penetration of the cuticle and the cell wall, typically, a vesicle develops within the epidermal cell, below the cuticle, without disturbing the host plasmalemma. This vesicle elongates into a primary hyphae that may branch and grow into adjacent epidermal and palisade cells or exit from the epidermal cell into the intercellular space (Freytag *et al.*, 1988).

The host plant reaction to rust infection is affected by environmental conditions including light and temperature, influencing the physiological responses of the host (Shaw, 1963) and the formation of infection structures (Emge, 1958).

After infection, whitish spots soon appear on the leaves and stems bearing reproductive spores, uredospores and teleutospores (Butler & Jones, 1961), with uredospores the more common form found on bean leaves (Buczacki & Harris, 1981).

Uredospores of rust fungi are airborne and germinate using their own reserves, requiring no exogenous nutrients (Allen, 1965). Uredosori, which bear uredospores develop on both sides of leaves as well as on the stems and the petioles (Butler & Jones, 1961). They are round to ovate, spiny with 3 or 4 germ pores and measure 20 to 30 μm by 18 to 26 μm. Teleutospores occur on leaves, but mainly on the stems and are dark brown, almost black in colour (Butler & Jones, 1961).

No symptoms of the disease are macroscopic until about 4-6 days after inoculation where the fungus has formed a

subepidermal stroma, which are small white slightly raised spots on the leaf undersurface, before erupting through the epidermis (Plate 1b) to release mature uredospores (Dixon, 1981). These uredospores are differentiated and are characteristically orange-brown in colour (Brand, 1985).

This timing of uredospore maturation and release depends on environmental conditions and the nutritional status of the host (Shaw, 1963), although, added nutrients have no stimulatory effect on germination (Caltrider, Ramachandran & Gottlieb, 1963). Establishment of rust colonies following inoculation of plants with uredospores depends on the properties of the host plant surface, conferred by the epidermal cells (Maheshwari, Allen & Hildebrandt, 1967). They concluded that the efficacy of surface stimulus appears to be determined to a large extent by chemical composition of this surface. A high atmospheric humidity is required for spread, with the optimum temperature for germination of uredospores ranging from 16^{o}C to 22.5^{o}C (Butler & Jones, 1961).

U. viciae-fabae may attack younger active tissue utilising assimilates, depressing photosynthesis and raising respiration mainly affecting activity during pod filling stage (Williams, 1978). Defoliation also can follow a severe attack (Buczacki & Harris, 1981). Pustules act as foci for the accumulation of many metabolites and the fungus derives nutrients by alteration of the direction of the normal phloem transport.

The larger the pustule size then the lower the frequency of pustules formed on a leaf, due to competition between individual pustules for essential substances (Shaw, 1963). Primary sori are 1-2 mm in diameter with secondary sori

developing outside the infection site as a ring (Dixon, 1981). Leaf longevity decreases as the intensity of infection increases (Yarwood, 1961). A common symptom of infection by biotrophic fungi is the visible 'green islands' which form around uredosori on the leaf surface.

Rusts are less subject to competition from epiphytes but are commonly attacked by antagonists once pustules have ruptured for spore release (Baker & Cook, 1974). Rusts are less susceptible to antagonism during prepenetration compared to necrotrophic pathogens like *Botrytis*, which are nutrient dependent at this stage of development (Fokkema, 1976).

Uredospore germination is affected by a crude inhibition involving a volatile substance (Allen, 1955) and requires water to avert this self inhibition. Good germination is obtained in the presence of free water and oxygen (Shaw, 1964).

The critical period of pathogenesis is the time during which the pathogen becomes established in the host (Inman, 1962) i.e. the time elapsing between inoculation and symptom development.

1.4.1 DISEASE CONTROL - TRADITIONAL

A range of chemicals have been and are being used to control rust on faba beans.

1.4.1.1 Fungicide application

Recent studies in England (Lapwood *et al.*, 1984) reported a 16% increase in kernel weight and a 17% increase in grain yield from

fungicide protected faba bean plots, compared to unprotected plots under natural rust infection.

Chemical control involved foliar sprays which can be useful to halt the early build up of disease. Yeoman, Lapwood & McEwan, (1987) found that from a range of materials used in agriculture, maneb and mancozeb gave the best control of rust on spring-sown field beans. They found that disease levels and yield were not significantly different when a second spray was given in the same season. Recommended chemicals to use include: fenpropimorph, triadimefon, propineb, maneb or mancozeb (Ivens, 1993). Control of *U. viciae-fabae* on lentils was studied using a tank mixture of triadimefon + propineb (Sepulveda & Mario Alvarez, 1989) applied pre-bloom, full bloom and early pod maturity. Disease control and yield were significantly better in plots treated either pre-bloom or full bloom, together with improved seed size.

1.4.1.2 Cultivars - Breeding resistance

Specific resistance to single pustule rust isolates can be found in many faba bean accessions (Rashid & Bernier, 1986b), and several genes for resistance have been identified in inbred lines. Any population of *U. viciae-fabae* is composed of numerous races, so cultivars with specific single genes for resistance are expected to give only short lived control. Some faba bean accessions have the ability to retard rust development, known as slow rusting or partial resistance (Rashid & Bernier, 1986a). This type of resistance is governed by a single dominant gene and is race specific. Unfortunately, this is only useful in the presence of one or more components of

resistance, and in some slow rusting populations tolerance to rust has varied from year to year (Rashid & Bernier, 1991). This could be the result of adaptability of genotypes to environmental variation between years and to heterogeneity of the population, comprising of a few plants that are either susceptible or resistant to specific isolates, as well as plants characterised by slow rusting.

Faba beans are very herterogenous and this lack of crop uniformity is noted by the reaction of cultivars to both rust and chocolate spot (Rashid & Bernier, 1984), and is due to the partially outcrossing nature of the crop. When accessions were tested with a mixture of isolates of rust, there was considerable variability in reaction. A few accessions contains several plants exhibiting resistant to highly resistant reactions.

1.4.1.3 Crop hygiene

The fungus overwinters primarily as uredospores or resting mycelium on living plant material provided by volunteer beans, old green bean debris and autumn sown crops. The elimination of such volunteers and contaminated debris will reduce inoculum levels.

1.4.2 RUST UREDOSPORES

Unlike most saprophytic fungi, rust uredospores germinate erratically and inconsistently (Allen, 1955), and germination is influenced by the presence of an endogenous inhibitor. The activity of the crude inhibitor involves a volatile substance

(at room temperature) which has reduced effect on aeration. Putative inhibitors include the following organic compounds: 2,4-dinitrophenol, 2-methyl-L,4,-naphthoquinone and coumarin (Allen, 1955), as well as phenolic derivatives of benzoic and cinnanic acids and indole acetic acid (Shaw, 1964). The production of self inhibitors may be caused either by the metabolic breakdown of materials or by their release from a bound form in the mature spores (Bell & Daly, 1962). As water is needed for the release of inhibitors, oxygen is required for inhibitor production with lipid metabolism predominant during germination. These inhibitors (or similar compounds) could be useful for fungistatic control of rust diseases (Bell & Daly, 1962).

Prolonged floating is required before the inhibitor can be easily washed out of spores to prevent the concentration of inhibitor rising again (Allen, 1955). The presence of this inhibitor in uredospores accounts for the erratic germination of untreated uredospores. Uredospores germination *in vitro* gradually reduced as the concentration of spores was increased above 2×10^4 spores ml^{-1} indicating the operation of self inhibitors.

Uredospores have a low water content of 10-15% and swell before producing a germ-tube (Shaw, 1964). Good germination takes place in the presence of free water and oxygen. Uredospore metabolism is supported solely at the expense of endogenous reserves, chiefly lipids (Shaw, 1963), as adding nutrients *in vitro* had no stimulatory effect on germination (Caltrider *et al.*, 1963). During germination the nonsponifiable lipids, fatty acids and the protein all increased

(Caltrider *et al.*, 1963).

Host reaction to rust is affected by light and temperature, which influence the physiological response of the host (Shaw, 1963). These conditions also affect the differentiation of infection structures of the fungus. Final emergence of the germ-tube is subject to direct effects of the environment upon the mobilisation of spore reserves (Allen, 1955). The viability of uredospores at the time of landing on leaf surfaces varies considerably according to rust species, the previous history of the spores and the density of the spore deposit (Manners, 1981).

The principle stimulant in rust uredospores has been identified as the volatile compound n-nonanal (Manners, 1981), which was isolated from air when passed over uredospores. The biological importance of such stimulants and inhibitors is uncertain, but it seems clear that they must influence the germination or rust uredospores on leaves (Manners, 1981). Certain volatile substances thought to occur in intercellular spaces have been found to stimulate differentiation of appressoria.

On leaves, germ-tubes of rust uredospores tend to grow at right angles to the long axis of the leaves. This pattern is disrupted if the cultivar is resistant. Tropisms controlling the direction of germ-tube length are positive hydrotropism, negative thigmotropism and dia-thigmotropism.

Teliospores of *U. viciae-fabae*, which are not constitutionally dormant, do not require light as a signal for initiating the germination process (Freytag *et al.*, 1988).

Basidiospore germlings derived from *Uromyces* spp. differentiate *in vitro* and *in vivo*, although differentiation is slower in axenic conditions and infection structures are not intracellular, e.g. on artificial membranes spores take 3 days to form primary hyphae compared to 24 hours on host tissue (Freytag *et al.*, 1988). The surface consistency of the substrate is critical for basidiospores of *Uromyces* spp. to differentiate (Freytag *et al.*, 1988), where there was a great increase in the numbers of vesicles and primary hyphae formed. These authors found that glass clearly induces appressorium formation but not vesicles, because the physiochemical surface of glass is characteristically inhibitory to post appressorial fungal growth. Uredospores contrast to basidiospores, since they respond to topographical features by developing appressoria and further infection structures.

1.5 Biological control

With the increasing awareness of the problem and expense of conventional methods of disease control, including fungicides and breeding programmes, biological control of pathogenic fungi has many attractions (Skidmore, 1976).

The concept of Biological control allows the incidence of disease either to be eliminated or its presence reduced by the introduction of one or more antagonistic organisms (control agents). Antagonism commonly means a relationship between organisms in which one organism, the antagonist, creates adverse circumstances for the other one which is to be controlled. It refers to the result of an interaction without giving any indications as to the possible underlying mechanism (Fokkema,

1976). Therefore, biological control is aimed at modifying the population biology of the host/parasite/antagonist system (Lynch & Ebben, 1986). Most approaches involving bio-control have been directed at suppressing disease induced by soil-borne pathogens, due primarily to the more stable and less extreme environment of the rhizosphere compared to the atmosphere (Lynch & Ebben, 1986).

A reduction in saprophytic flora sensitive to fungicides can result in an increase in disease outbreaks by disrupting the biological balance in the phyllosphere (Fokkema, 1976; Blakeman & Fokkema, 1982). This phenomenon is explained as a retardation of naturally occurring antagonism, since the fungicide sensitive, non-target saprophyte population has been reduced (Blakeman & Fokkema, 1982). These saprophytes would normally reduce the nutrients available for pathogen growth. The occurrence of benomyl-resistant *B. cinerea* on sprayed cyclamen plants may be a consequence of the reduction of antagonistic *Penicillium* spp. (Van Dommelen & Bollen, 1973; cited in Blakeman & Fokkema, 1982). Also pesticide spray programmes in apple orchards have been shown to have a substantial quantitative and qualitative effect on non-target epiphytic micro-organisms (Andrews & Kenerley, 1978).

Aerial plant surfaces provide a habitat for epiphytic micro-organisms, many of which influence the growth of pathogens (Blakeman, 1993; Blakeman & Fokkema, 1982) especially water availability and sunlight. The surface of aerial shoots support a bacterial flora made up predominantly of a few characteristic genera (Crosse, 1971), making this habitat distinctive in comparison with the rhizosphere. The surface of aerial plant

parts, known as the phyllosphere or phylloplane, has a microflora which is characterised by its diversity and complexity (Elad, 1990). Rapid environmental changes in the phyllosphere can occur, varying the size of any bacterial or fungal population. The residual surviving inoculum is normally sufficient to give rise to large cell numbers when favourable conditions for multiplication occur (Sztejnberg & Blakeman, 1973). The biological components of the phyllosphere are influenced by a number of localised microclimates (Elad, 1990) due to environmental conditions, morphological features, nutritional levels, cultivation methods, crop protection treatments and leaf surface chemicals, supporting a diverse range of saprophytes and pathogens. In devising ways of enhancing the antagonistic effects of micro-organisms against plant pathogens, it is necessary to understand the nature of this specialised microhabitat (Blakeman & Fokkema, 1982).

There are few examples of successful applications of biological control in the phyllosphere compared to the rhizosphere. These reasons include:- (i) the availability of cheap, effective foliar fungicides and their ease of application to the foliage, (ii) direct competition of saprophytes with applied chemicals, (iii) conditions on the canopy of plants do not favour introduced organisms, (iv) other factors which affect the suitable niche for growth of the biocontrol agent (Baker & Cook, 1974).

Most attempts at biological control have involved the application of the antagonistic micro-organisms to the plant, with success depending on the ability of the antagonist to multiply and colonise the plants (Blakeman & Fokkema, 1982). It

is possible to assist growth through manipulation of the localised phyllosphere environment, e.g. application of nutrient solutions, or increasing the humidity.

There is an increasing awareness of the negative side effects of fungicides on the ecosystem, and this interest may result in biological control and fungicidal treatments being considered in terms of the most effective means of disease control in relation to ecological damage which may result from their use (Fokkema, 1976; Mendgen, Schiewe & Falconi, 1992). Fungicide application can result in changes, reducing or eliminating antagonistic flora (Baker & Cook, 1974). Fungicide application has also been reported to affect the biological balance, often leading to an increase in disease levels (Blakeman & Fokkema, 1982). There is also an increasingly strict legislative and environmental climate raising costs and the timescale of development of new fungicides (Elad & Zimand, 1991). Many agrochemical and biotechnology companies are investing in the research and development of disease bio-control, with the intention of incorporating biological control into integrated crop management programs.

Saprophytes have the ability to act as nutrient sinks reducing nutrient levels available to stimulate necrotrophic pathogens. The nutrients on the plant surface are derived from various sources including chemical components from leaf tissues, exogenous substances, exudates, by products, and even pollutants (Elad, 1990). Some saprophytes also have the ability to control pathogens through the production of secondary metabolites.

Biological control agents will not persist and be active or effective in the phyllosphere unless they are adapted to the

plant environment, and can compete effectively with other micro-organisms (Elad, 1990). Often, attempts at biological control fail because of the lack of persistence of the antagonist on or within the host, and so introduction of naturally occurring resident micro-organisms is preferred to organisms from other habitats. Success depends on a thorough understanding of the micro-environment of the host surface and detailed knowledge of the growth and development of the pathogen and antagonist (Elad, 1990). It is important to understand the mechanism of antagonism to optimise the efficacy of the bio-control agent (Blakeman & Fokkema, 1982).

1.5.1 EXUDATES

Exudation occurs through the cuticle via ectodesmata or microcapillaries and on drying may leave a residue on the leaf surface or in the surface wax, allowing the properties of the surface wax to influence the exudation process (Baker & Cook, 1974). Exudation of nutrients through hydathodes is also common. These exuded drops may be blown or shaken off the leaf, evaporate, or be retracted when the humidity falls (Baker & Cook, 1974), but they markedly affect the development of fungi on the leaf surface. Some leakage occurs through lenticels to the mesophyll tissue of leaves.

Omar, Salem & El-Gantiny, (1989) found that the physiological status of faba bean leaves changed with plant age, and increases seen in the density of micro-organisms reflected variations in type and amount of nutrients available from the leaves at different ages.

Leaf pretreatments with chemicals or a period in darkness (24 h) can alter the permeability of bean leaves and susceptibility of leaves attached by conidia of *B. fabae* (Sol, 1968; 1969). Higher quantities of leachable sugars and amino acids were detected after pretreatments with decenylsuccinic acid, after which there was an observed increase in spore germination and in infection (Blakeman, 1971).

The nature and amount of exudates reaching the leaf surface can be altered by fertiliser application which in turn affects the chemical composition of plants (Blakeman, 1971). Higher levels of sugar and amino acids were present on broad bean leaves when roots were supplied with nitrogen in the form of ammonium as opposed to nitrate (Sol, 1968; 1969), resulting in increased spore germination and infection. Any treatment which raises the nutrient status of leaves (e.g. increasing fertility or increasing light intensity), resulted in higher levels of exudation (Blakeman, 1971). Blakeman also noted that treatments causing an increase in permeability will almost certainly result in enhanced exudation of small molecular weight nutrients, chiefly sugars and amino acids. Depending on plant species a change in permeability could also result in increased exudation of antifungal compounds. The rate of exudation probably depends on the size and shape of molecules. Plant leachates also contain traces of antimicrobial substances such as phenolic compounds, organic acids or alkaloids (Turkey, 1971) but are seldom present in high enough concentrations in infection droplets to prevent germination or growth of pathogens (Blakeman, 1993).

1.5.2 MICRO-ORGANISMS

Air currents disseminate both transient and resident epiphytes to aerial plant surfaces. The transients remain inactive or die whereas the residents may multiply (Baker & Cook, 1974). A restricted characteristic flora is maintained on aerial plant parts.

1.5.2.1 Resident flora

In the phyllosphere there is a characteristic flora, where micro-organisms that multiply on healthy plant surfaces without affecting the plant are termed residents (Baker & Cook, 1974). They are generally non-specific and occur on a range of plants, but a few are quite specific. As leaves mature micro-organism numbers generally increase due to the presence of nutrients from leakage through ectodesmata, pollen, flower parts and other debris on the leaf surface. Leaves parasitised by rusts, powdery mildews, foliar nematodes, or mites, or having a mechanical injury, are apt to have higher populations of resident epiphytes than are healthy leaves (Baker & Cook, 1974). Studies on micro-organisms on bean leaves (Omar *et al.*, 1989) have shown that in the bean leaf phyllosphere bacteria greatly outnumber fungi and that these micro-organisms colonise the areas over and around the midrib and veins on the leaf surface.

1.5.2.2 Transient flora

Transient or casual micro-organisms on aerial parts settle there from the air but do not multiply, except perhaps in foreign debris on the leaf surface (Baker & Cook, 1974). Normal accumulation of pollen apparently promotes antagonistic

microflora that inhibits pathogens, but pollen applied with the pathogen may provide a food base for infection, and in the absence of inhibitory micro-organisms, cause severe disease levels (Baker & Cook, 1974). Other than plant age or nutrient availability, the season affects the micro-organism density (Omar *et al.*, 1989).

1.5.2.3 Succession of micro-organisms

Plant pathogens are normally transient on aerial parts before penetration of the host. During this brief period they are exposed to interactions with the resident epiphytes, including competition for nutrients and exposure to antibiotics and/or enzymes. There is also interaction and succession among the epiphytes themselves (Baker & Cook, 1974). Bacteria may be the first to colonise rapidly growing tissue (Blakeman, 1993), and yeasts and other fungi become more plentiful as the host matures through the season and becomes moribund (Leben, 1965).

Bacteria, yeasts and yeast like organisms normally outnumber hyphomycetes. Bacteria present on aerial plant surfaces are predominantly gram-negative (Blakeman, 1993; Blakeman & Brodie, 1976) and are predominantly pigmented forms unlike those isolated from soil (Baker & Cook, 1974). Bacteria in the phyllosphere belong to the following genera:- *Erwinia*, *Pseudomonas*, *Flavobacterium* and *Xanthomonas* (Blakeman & Brodie, 1976). *Lactobacillus*, *Corynebacterium* and *Bacillus* are also regularly isolated. Actinomycetes are rarely found on leaves and stems although they are common in soil (Baker & Cook, 1974).

Bacterial pathogens occur widely as epiphytes on non-diseased plants, whereas most fungal pathogens are less common

or absent as a non-parasitic phase on healthy plants (Blakeman & Brodie, 1976). This could reflect the fact that the majority of fungal pathogens are not well adapted for survival in the mycelial state on aerial plant surfaces. However, fungi do exist as saprophytes in the soil or in dead tissue (Blakeman & Brodie, 1976).

1.5.3 ANTAGONISM

Plant pathogens invade aerial parts of the host through unbroken surfaces, natural openings, wounds or senescent or dead tissues, and when in the epiphytic phase of growth before infection of plants, they are most vulnerable to biological control. Specialised fungal parasites require fewer spores to infect than do less specialised pathogens. Rusts can achieve 10-50% infection from single uredospores, whereas *B. fabae* will require 4 conidia and *B. cinerea* more than 100 times this number to achieve 10% effectiveness (Baker & Cook, 1974).

A convenient way to classify epiphytic/pathogen interaction is according to the probable mode of action involved. Generally, 3 main types of antagonism are recognised: antibiosis, hyperparasitism and nutrient competition, but mechanical obstruction, pH alteration, stimulation of host defence mechanisms and induced resistance are also important. These methods of antagonism are not mutually exclusive (Fravel, 1988).

1.5.3.1 Antibiotic production

Antibiotics are organic compounds of low molecular weight

produced by microbes, either of a volatile or non-volatile nature, and which confer a selective advantage to microbes in competition for nutrient and space in their ecological niche (Fravel, 1988). Production of antibiotics active against fungi by leaf surface bacteria has not been widely reported (Blakeman & Brodie, 1976).

The occurrence of inhibition zones between antagonists and a pathogen on agar is commonly considered to be the result of the production of antibiotics (Fokkema, 1976), but could be the result of staling products (Blakeman & Fokkema, 1982). Where antibiotic production has been identified as the main cause of antagonism by a saprophyte, a cell-free culture filtrate or a semipurified antibiotic preparation may be used as opposed to live inoculum (Blakeman & Fokkema, 1982), especially if the antagonist is a poor coloniser.

Production of antibiotics has been shown to be associated with pathogenicity in that culture filtrates from pathogenic isolates reproduced disease symptoms in the host, where loss of pathogenicity by an isolate was accompanied by a failure to produce the antibiotic (Sinden & Devay, 1967). Of course, the ability to produce an antibiotic in culture does not necessarily mean that the antibiotic is produced *in vivo* and also that it may not necessarily be responsible for the disease protection properties of a particular bacterium (Blakeman & Brodie, 1976).

Antibiosis is not an exclusive role on bio-control, but it often acts in concert with competition and/or parasitism (Fravel, 1988).

1.5.3.2 Hyperparasitism

Hyperparasitism covers a multitude of different interactions including: minor to major morphological disturbances, overgrowth of hyphae of one fungus by another, penetration and direct parasitism via haustorial production and lysis of one hypha by another (Blakeman & Fokkema, 1982; Skidmore, 1976). Bacteria in soil have frequently been reported to lyse fungal spores, germ-tubes and hyphae, probably via the action of chitinolytic enzymes which degrade hyphal walls (Blakeman & Brodie, 1976).

In necrotrophic interactions the parasitic fungus derives nutrients from dead fungus host cells (usually killed by a parasite). The response of susceptible species varies from cessation of growth and vacuole formation to deformation of hyphal tips and ultimately cell rupture (Skidmore, 1976). Necrotrophic hyperparasitic interactions have been used to control pathogens on leaf surfaces.

Biotrophic hyperparasitism occurs when a parasite secures nutrients from living cells of the fungus host through haustorial formation without killing the host (Skidmore, 1976).

1.5.3.3 Nutrient competition

For some foliar pathogens (like *Botrytis*), exogenous nutrients are essential for infection. Low nutrient levels are associated with water films on leaf surfaces, because bacteria and yeasts are able to take up nutrients from these dilute solutions more rapidly and in greater quantity than germ-tubes of fungal pathogens and therefore exogenous nutrients are reduced (Blakeman & Fokkema, 1982). The greater the amount of hyphal

growth and development of appressoria by the pathogen on the leaf surface prior to penetration, the greater will be the demand for nutrients (Blakeman, 1971; Baker & Cook, 1974). Most fungal dispersed spores are partially or wholly nutrient dependent for their germination. This ensures that their germination does not occur in a location lacking nutrients and other materials essential for continued growth (Baker & Cook, 1974; Clark & Loebeer, 1976). Therefore germ-tubes are subject to competition by residential epiphytes.

On newly expanded green leaves the nutrient supply is limited and saprophytic growth is poor. As leaves age the nutrients on the leaf surface increase (Turkey, 1971), which coincides with the extension of the phyllosphere fungal population. Addition of nutrients, either sugars or amino acids, to spores *in vitro*, removes inhibitions attributable to nutrient impoverishment (Skidmore, 1976). In mixed inoculation experiments, isolates of resident phyllosphere fungi moderated the stimulatory effect of pollen on pathogen development by reducing mycelial growth and causing less necrosis (Skidmore, 1976; Fokkema, 1973). In a non-enriched phyllosphere, nutrients leached from the underlying leaf tissue may determine the growth and development of the pathogen (Fokkema, 1976).

The source of substrates for growth of fungal pathogens on the surface of leaves originates either from materials formed within the leaf or from materials deposited on the leaf (Blakeman, 1971). This includes:- aerosols produced by other plants, metabolic by-products from other microbes, chemical constituents of host wax and metabolites from cells (Emmett & Parberry, 1974). Bacteria on the leaf surface are able to

convert the form of substances, for example, carbohydrate (sugar) to glucose and fructose, resulting in the formation of substances which restrict or even prevent the growth of pathogenic fungi. Cell walls can be lysed by the production of chitinolytic enzymes or other lytic compounds.

Leaf saprophytes undoubtedly act as scavengers, reducing the nutrients available for pathogens on the leaf surface (Baker & Cook, 1974). The effectiveness with which bacteria remove nutrients from dilute solutions on plant surfaces is associated with the development of extracellular polysaccharides (Paton, 1960). The formation of polysaccharides from simple sugars creates a concentration gradient which attracts solutions to the polymer.

Sztejnberg & Blakeman (1973) postulated that the failure of conidia to germinate was due to uptake of nutrients into the polysaccharide sheath of adjacent bacteria, leading to an increasingly steep diffusion gradient from within to outside the conidium. Endogenous supplies of soluble nutrients might then be depleted from the conidia.

The effectiveness of nutrient competition depends on the need by pathogen spores and germ-tubes for nutrients required for their growth (Fokkema, 1976). Micro-organisms also compete for oxygen and space to grow on the leaf surface. Antagonists are usually saprophytes, since they are more tolerant of environmental extremes and have a wider distribution than many pathogens.

1.5.3.4 Mechanical obstruction

Fungi may extensively colonise a substrate to the exclusion of other fungi, including pathogens which arrive later. Fokkema (1978) considered that the extensive growth of *Cladosporium herbarium* on pollen rich rye leaves could prevent pathogen spores from reaching leaf surfaces.

1.5.3.5 pH alteration

An increase in pH may be a universal inhibitory mechanism, decreasing pectinase activity and so interfering with cell wall degrading enzymes, especially in wounded tissues (Skidmore, 1976). Newhook, (1951a; 1951b) found that when saprophytic bacteria and fungi had colonised wounds of lettuce crops, the pH was raised to 7.8-8.4, whereas in lesions of, *Botrytis* the pH reached a maximum of 6.8-7.2. The inhibition of growth could only be partially explained through the production of antibiotics, suggesting that pH could be a more universal inhibitory mechanism reducing pectinase production in wounded tissue (Fokkema, 1976).

Rasanayagam & Jeffries (1992) found that ectomycorrhizal fungi inhibited *Pythium ultimum*, causing swelling and hyphal bursting in pH levels below pH 3. This inhibition of growth initally indicated antibiotic metabolites, but similar symptoms were induced by acid alone, through acidification of the medium, rather than being due to pH dependent antibiotic production. Local acidification of any substrate is not necessarily due to the production of specific antifungal agents (Rasanayagam & Jeffries 1992). It could be caused by secretion of acidic compounds by the fungus, or the secretion of anions from the

medium, resulting in an increase in H^+ ion concentration in the vicinity.

Bhatt & Vaughan (1963) studying *Cladosporium*, which is well documented as a successful antagonist, found that it produced very few inhibitory substances on agar but the pH of the medium was raised to 8.0. Production of acid by epiphytic bacteria can make conditions unfavourable for pathogen growth. For example, *Erwinia herbicola in vitro* was shown to lower the pH to levels which inhibit fireblight (Riggle & Klos, 1972). *E. herbicola* utilised all the organic nitrogen present to reduce the pH to levels which inhibited the pathogen, *E. amylovora*, which would only grow on such media after suitable pH adjustment and the addition of peptone.

1.5.3.6 Induction of phytoalexins

Phytoalexins are chemicals produced by the host plant in response to infection toxic to bacteria and fungi (Campbell, 1989). Phylloplane fungi are able to induce phytoalexin or similar compounds in undamaged leaves which is considered a possible method of antagonism (Skidmore, 1976). Prior, or simultaneous treatment of the host plant with cell-free extracts of heat killed bacterial cells can also protect plants, inducing a protective response in the host tissues (Blakeman & Fokkema, 1982).

Epiphytic bacteria need entry into the plant to confer protection and so large populations of bacteria on plant surfaces greatly increases the chance of disease protection because there is a greater likelihood of sufficient bacteria entering natural openings (Blakeman & Brodie, 1976; Baker &

Cook, 1974). It is considered unlikely that epiphytic bacteria confer any direct disease protection through stimulation of phytoalexin production in plants, where phytoalexins are characteristically associated with necrotic tissue (Keen & Kennedy, 1974).

B. fabae has much greater growth within the epidermis of bean plants compared to *B. cinerea*, which could suggest that *B. fabae* is specially adapted for rapid penetration into its host (Mansfield & Hutson, 1980). It can penetrate, killing epidermal cells and produce infection before there is any active resistance response from the invaded plants. In *V. faba*, wyerone derivatives (phytoalexins) have been shown to accumulate within epidermal tissue inoculated with *B. cinerea* or with low concentrations of *B. fabae*, between 7 and 8 hours after inoculation (Mansfield, 1980). *B. fabae* appears able to metabolise wyerone acid, which is the predominant phytoalexin produced in response to infection (Mansfield, 1980; Mansfield & Hutson, 1980), while *B. cinerea* cannot.

1.5.3.7 Induced resistance

There are indications that phyllosphere organisms, potential plant pathogens and saprophytes can all reduce the development of parasites by direct antagonism but are also able to stimulate host plant resistance to infection. However, there is a high specificity required in the inducing agent. Few studies have been attempted on a field scale due to the problems of applying living bacteria or fungi (Schonbeck & Dehne, 1986).

Culture filtrates of saprophytic bacteria and fungi can induce resistance against the bean rust fungus, without any

direct antagonistic effect on the pathogen (Schonbeck, Dehne & Bednt, 1980). The inducers often have to be applied before infection to initiate the appropriate defence system (Mendgen *et al.*, 1992). Recently, Murray & Walters (1992) showed that inocultation of the lower leaves of broad beans with rust leads to the development of systemic induced resistance to rust infection in the upper leaves.

There has been some commercial success with a few biological control agents. Ethanolic and aqueous extracts (tea) are effective against cucumber mildew (*Sphaeroptheca fuliginea* and *Erysiphe cechoracearum*) if used protectively and applied regularly (Mendgen *et al.*, 1992). 'Mycosan' contains aluminium sulphate, wettable sulphur and other constituents such as horsetail extract, yeast and silica, whereas 'Ulmasud' is a refined clay mineral consisting of silica, aluminium oxide and titanium dioxide (Mendgen *et al.*, 1992). Both 'Mycosan' and 'Ulmasud' are capable at controlling powdery and downy mildews, acting directly on the fungus. Others like clay minerals or silica may impede recognition of the plant surfaces which some fungi need for infection or they may stimulate the plant defences (Mendgen *et al.*, 1992). None of these compounds offer replacements for conventional fungicides, but have a role in 'alternative' agriculture.

1.5.3.8 Fungistatic substances

In addition to nutrients, leaves may exude fungistatic substances causing inhibition of germination of spores or restriction of their germ-tube growth (Blakeman, 1971). Phenols are the most widely known fungistatic substances

produced by leaves.

Apart from fungistatic substances formed within the plant cells and exuded on to the leaf surface, there is increasing evidence of the role of constituents of cuticular waxes in limitation of fungal growth on leaf surfaces (Blakeman, 1971). It is difficult to assess antifungal substances known to occur in cuticular wax and their role in disease resistance because of the physical effect of wax on the deposition of inoculum on the leaf surface (Blakeman, 1993).

1.5.4 BIOLOGICAL CONTROL OF BOTRYTIS

There are a few reports of investigations of biological control of chocolate spot on faba beans, with most literature relating to *B. cinerea*. In the most recent study on *B. fabae*, Bennett & Lane (1992) integrated fungicide application *in vitro* with the antagonist *Trichoderma viride*. *T. viride* alone greatly suppressed *B. fabae* growth and the addition of benomyl or carbendazim stopped pathogen growth completely. However, these differences were less marked on subsequent *in vivo* trials. These authors thought that systemic fungicides could provide better control, and that the use of *Trichoderma* would help prevent the build up of resistance to fungicides. Morris & Lane (1990) observed great variation among the *Trichoderma* strains they examined, finding that *B. cinerea* conidial germination was greatly affected by nutrition, but that *B. fabae* was inhibited by antibiotics produced in culture filtrates of *Trichoderma* in both *in vitro* and *in vivo* trials. Culture filtrates provided a degree of control of new outbreaks of *B. fabae*, but they concluded that preventative preinoculation with the mycoparasite

offered better control (Morris & Lane, 1990).

Other studies of inhibition of Chocolate spot in the phyllosphere (Hanounik & Hasanain, 1986) showed a strong inhibitory effect (measuring spore germination and germ-tube elongation) *in vitro* from both leaf washings and diffusates collected from resistant cultivars compared to those from a susceptible one. Verdie & Le Normand, (1984) isolated two prominent bacteria, *Pseudomonas fluorescens* and *Erwinia herbicola*, which suppressed *B. fabae in vitro*. Two fungi which inhibited *B. fabae* growth *in vitro* were *Nigrospora* spp. and *Chaetomium* spp. (Omar *et al.*, 1979). Omar also found that bacteria with white, brown or cream pigments (rather than those with red, yellow or orange pigments) reduced *B. fabae* growth, together with two unidentified actinomycete species which showed antagonism against the pathogen.

Gliocladium roseum and *Penicillium* spp. were found to be suppressive to *B. cinerea* in a full range of tests in the laboratory (Peng & Sutton, 1991), growth room and the glasshouse. Interestingly, Peng, Sutton & Kevan, (1992) spread inoculum of the antagonistic micro-organism, *G. roseum*, on a colony of honey bees using a dispenser at the hive entrance/exit. Field density of the microbe was more consistent using this method of spread rather than a regular inoculum spray at weekly intervals.

Elad, Zimand & Chet, (1989) screened non-pathogenic micro-organisms for their ability to inhibit grey mould (*B. cinerea*) on bean and other crops. *Trichoderma* isolates were relatively more efficient than others, but they found that the effectiveness of a specific isolate was affected by the host

involved. When *Trichoderma* spp. were applied to vineyards, there was a significant reduction in disease incidence to 64-68% of the control, compared to 72-76% reduction with *Trichoderma* + Folpan and only a 45-46% reduction when the fungicide Ronilan (Vinclozolin) was tested.

T. harzianum has been applied under commercial conditions (Elad & Cohen, 1991), both in vineyards and in glasshouse crops to control *B. cinerea*. When applied to the vineyards as a wettable powder formulation it resulted in similar levels of disease control to the fungicides applied. In glasshouse experiments, where tomato plants were sprayed either with *T. harzianum* alone or alternated with the fungicide iprodione, both types of treatment gave levels of control of grey mould similar to a wide range of commercially used fungicides. Similar results were obtained by Elad & Zimand, (1991), also using *T. harzianum* as a biological agent applied either on its own, as a tank mix with diethofencarb + carbendazim or iprodione, or applied in alternation with the same chemical. Alternating bio-control agents with a fungicide is also effective in reducing populations of *B. cinerea*, and has potential to reduce pressure towards resistance to common fungicides and to reduce pesticide residues in treated agricultural products (Elad & Cohen, 1991; Elad & Zimand, 1991).

Strains of *Bacillus* spp., *Pseudomonas* spp. and *Chromobacterium* spp. isolated from lettuce leaves were all shown to be antagonistic to *B. cinerea in vitro* (Newhook, 1951a). Newhook (1951b) showed that saprophytic fungi isolated from lettuce leaves prevented *B. cinerea* attack under controlled conditions; they included species of *Fusarium*, *Phoma*,

Trichoderma, *Penicillium* and to a lesser extent *Cladosporium*. The saprophytes needed a period of establishment with high humidity or free water and temperatures between 15°C and 25°C. He found that either the pH was raised, inhibiting the growth of *B. cinerea* through a reduction in the activity of pectinase enzymes, or antibiotics were produced.

Wood (1951) controlled *B. cinerea* on lettuce by spraying with species of *Bacillus*, *Penicillium* and *Trichoderma*, but found that control in field conditions was more difficult due to the death of inoculum in dry conditions, removal of spores from foliage during heavy rain and low temperatures. Bhatt & Vaughan (1963) isolated saprophytic fungi from strawberry fruits which antagonised *B. cinerea* in culture. *Cladosporium herbarum*, *Aureobasidium pullulans* and *Dendrophoma obscurans* all failed to prevent germination, but inhibited germ-tube growth. *C. herbarium* increased the pH on the strawberry fruits to above 6.0, and inhibited the activity of pectic enzymes of *B. cinerea*. All the antagonists limited the growth of *B. cinerea* by producing antifungal metabolites, colonising infection sites, like dead or senescing flower parts, thus preventing the pathogen from entering fruit.

Epiphytic bacteria were found to multiply in water droplets placed on chrysanthemum leaves containing *B. cinerea* conidia (Blakeman & Frazer, 1971). This suggests that the multiplication of bacteria was partially stimulated by nutrients leaked from the conidia, since the greater the number of bacteria, the higher the proportion of conidia which failed to germinate. A similar study on beetroot leaves (Blakeman, 1972) found that leakage of nutrients from leaves, and conidia of *B.*

cinerea, affected the numbers of bacteria and the extent to which conidial germination was inhibited. As the beetroot plants aged there was an increased leakage of amino acids and carbohydrates from the leaves, which selectively stimulated a *Pseudomonas* isolate. This isolate inhibited spore germination to a greater extent on the leaves of older plants.

Brodie & Blakeman (1975) found while using radiolabelled media, that there was substantial leakage from conidia into a suspension of water, of up to 20%. In the absence of bacteria, the conidia were able to reabsorb a proportion of these leachates on their germination and early growth of their germ-tubes. There is likely to be an adverse effect on germination of conidia if substances initially leaked by them which are needed for germination but cannot be reabsorbed after their removal by bacteria (Bristow & Lockwood, 1972).

The importance of nutrients in stimulation of *B. cinerea* and, the utilisation of exogenous substances by conidia and bacteria was studied by Brodie & Blakeman (1975). Using glutamine as the sole exogenous nutrient, the bacteria were more effective in removing glutamine from solution than conidia, despite the fact that the total conidial volume used was approximately ten times that of the bacteria. Nearly all the glutamine taken up by the bacteria was removed in the first 3 h of the experiment, whilst uptake by conidia occurred at a steady rate over a 24 h period (Brodie & Blakeman, 1975). The effectiveness with which bacteria utilised glutamine would suggest that competition for exogenous nutrients might adversely affect germination of conidia on plant surfaces.

On leaf surfaces there are a number of different amino

acids and simple sugars (Blakeman, 1972) which could alter the pattern of competition observed with glutamine alone. *In vitro* experiments using algal protein hydrolysate which contains most of the amino acids on leaves, showed that the uptake of amino acids by bacteria was still much more rapid than by conidia (Brodie, 1975) (80% after 5 h compared with 10% after a similar time by conidia). Thus bacteria deplete most of the amino acids before *B. cinerea* conidia can obtain significant quantities. In solution containing amino acids and glucose, the decrease in germination of conidia with increasing numbers of bacteria was directly proportional to uptake of amino acids (Blakeman & Brodie, 1976).

At concentrations of amino acids equivalent to those found on leaf surfaces, bacteria effectively removed them from solution, as recorded using ^{14}C-labelled compounds (Blakeman & Frazer, 1971). With higher concentrations of amino acids, a greater proportion of label remained in solution, which may explain the failure of bacteria to inhibit germination of conidia at higher nutrient levels (Blakeman & Frazer, 1971). *B. cinerea* conidia concentrations of at least 5×10^5 conidia ml^{-1} were required to remove 70% of ^{14}C in 24 h from an amino acid mixture. When higher numbers of conidia were used, there was no increase in uptake of the label and germination decreased due to self inhibition (Brodie, 1975).

Interesting, Mansfield & Deverall (1974) suggested that local differences in bacterial populations over broad bean leaves is a possible explanation for the apparent random distribution of *B. cinerea* inoculation sites that failed to develop lesions.

1.5.5 BIOLOGICAL CONTROL OF RUST

Germination and germ-tube growth of rust fungi can be adversely affected by a large number of leaf surface micro-organisms (Manners, 1981). These effects have been demonstrated both *in vitro* and on leaf surfaces, but in some cases the inhibitory effect operating *in vitro* did not operate *in vivo*. Germination of uredospores in the presence of micro-organisms can be reduced by as much as 90-99% (Manners, 1981). Dramatic reductions like this suggest that successful biological control is possible.

Filtrates of *Aspergillus clavatus* cultures lysed uredospore germ-tubes, greatly reducing germination of *Puccinia recondita, P. graminis* f.sp. *tritici* and *P. coronata* (Morgan, 1963). Morgan (1963) also showed that the active substance which *Bacillus pumilus* produced in culture filtrates was inhibitory to germ-tube development, lysing the fungus both before and after autoclaving. The effectiveness of the autoclaved culture filtrate of *B. pumilus* in controlling the rust shows that substances other than or as well as enzymes were involved in lysis of the fungi (Morgan, 1963). Pustule formation was reduced to 10-25% of the controls on the leaf surface.

Kapooria & Sinha (1969) found that all of the species screened (24 in total) in their experiments reduced germination of *Puccinia penniseti* in hanging drop experiments. Applying *Fusarium oxysporum, Chaetomium globosum* and *Aspergillus japonicus* simultaneously with rust spores to leaves, reduced pustule number by 50-90%. The greater the proportion of antagonistic spores, the larger the reduction in pustule number.

Mishra & Tewari (1976) found that phylloplane saprophytic fungi inhibited uredospore germination of *P. graminis tritici in vitro* in hanging drop experiments and germination tests using culture filtrates from different isolates, especially when the proportion of rust uredospores in the mixture was small. They used the most active species *in vitro* for trials on the leaf surface. Those giving greatest inhibition were *Penicillium notatum, Myrothecium roridum, Cladosporium herbarum* and *Nigrospora sphaerica*.

Docherty & Preece (1978) isolated *Bacillus cereus* which was normally associated with uredospores of *Puccinia allii* on leeks. The bacterium completely inhibited germination of uredospores on agar, and when a suspension containing 10^9 cells ml^{-1} was sprayed onto leaves, infection was reduced to 0.4-7% that of the controls. Concentrations of *B. cereus* suspensions of 10^7 cells ml^{-1} and below showed no significant control.

Kamyshko *et al.*, (1975) found that 11 species of actinomycetes which produced antibiotics suppressed uredospore germination in *P. graminis* and *P. recondita*. Various *Pseudomonas* isolates tested *in vivo* against leaf rust, *P. recondita* f.sp. *tritici* were found to reduce symptom expression.

Fungal hyperparasites of rusts, *Darluca filum, Tuberculina vinosa* and *Verticillium lecanii*, and their potential for biological control, have been discussed by Kranz (1981). *D. filum* enters the host via cracks in the epidermis caused by the eruption of the fruitification of the rust fungi, whereas *V. lecanii* infects open uredosori, while *T. vinosa* predominantly colonises pycnia and aecia (Kranz, 1981). *D. filum* gave best control when inoculated 3 days after application of *Puccinia*

recondita uredospore suspension, allowing abundant infection of the rust (Swendsrud & Calpouzos, 1972). Kranz (1981) concluded that timing of inoculation was essential for infection. For their successful use as biological control agents or as part of an integrated control system, more information is required on growth of these hyperparasites.

Parker & Blakeman (1984a) assessed the microflora associated with uredospores of *U. viciae-fabae* and their effects on germination of the rust. Germination of uredospores on glass showed no lysis of germ tubes (Parker & Blakeman, 1984a), but lysis was observed on leaves, indicating that constituents of the microflora were able to attack the cell walls of uredospores. They found that the presence of uredospores encouraged the development of bacterial populations and reduced yeast colonies, possibly due to leachates emanating from uredospores or from leaf parts ruptured by pustules. Increased numbers of micro-organisms have been shown to be present on rust infected plum, poplar and wheat leaves (McKenzie & Hudson, 1976) compared to unifected leaves.

Rust fungi infect directly through the epidermis or through stomata and usually do not require exogenous nutrients for germination. Biotrophic fungi, like rusts, are less subject to competition from epiphytes than are most fungi during infection, as they are not subject to nutrient competition (Fokkema, 1976). However, obligate parasites are commonly subject to attack by antagonists once the pustules have ruptured from spore release (Baker & Cook, 1974).

One of the most critical stages in the life cycle of rusts is the germination and growth of germ-tubes of uredospores on

the leaf surfaces of the host leaf, before forming an appressorium over a stoma and finally penetrating into the leaf (Parker & Blakeman, 1984b). Leakage of nutrients from uredospores and leaves has been shown to influence germination, growth of germ-tubes and infection by uredospores of *U. viciae-fabae* (Parker & Blakeman, 1984b). Increased germination where uredospores from young and old pustules were washed may have been due to a loss of self inhibitors (Marte, 1971). Uredospores also contain self stimulators of germination. Leaf leachates are known to contain a wide variety of compounds (Turkey, 1971) and so this stimulation of growth of germ-tubes of *U. viciae-fabae* uredospores in broad bean leachates could have been caused by nutrients or stimulators (Parker & Blakeman, 1984b).

Pollen on leaves stimulates germination, growth of germ-tubes and lesion formation of many saprophytes and necrotrophic pathogens. Growth of germ-tubes of *U. viciae-fabae* uredospores was reduced by pollen diffusates, caused by concentrations of nutrients released from pollen grains which were too high (Parker, 1982). Many exogenously applied sugars or amino acids caused a stimulation of uredospore germination at low concentrations. High concentrations inhibited germination, indicating that uredospores are adapted to germinate in low concentrations of nutrients found in leaf leachates (Parker & Blakeman, 1984b). During washing of uredospores, loss of endogenous nutrients indicated an association between reduced growth of germ-tubes and subsequent infection. It is likely, therefore, that exogenous nutrients may also influence infection, particularly by old and leached uredospores. Reduced growth of germ-tubes and infection shown by washed

uredospores is presumed to be due to loss of endogenous nutrients. Significant quantities of amino acids have been found in leachates of uredospores of *U. viciae-fabae* (Parker & Blakeman, 1984a). On leaves, the rapid consumption of leached endogenous nutrients by saprophytes would prevent their reabsorption by spores, affecting germination and influencing rust development. Of course, rust uredospores require little or no exogenous nutrients for germination, making nutrient competition an unlikely mechanism of spore inhibition (Fokkema, 1981).

Parker & Blakeman (1984b) studied the extent to which nutrient relationships affect the behaviour of uredospores on leaf surfaces. Washing of young uredospores usually increased their germination, while reducing germ-tube growth, whereas washing stimulated germ-tube growth but not germination of older uredospores. Antagonism could involve not only a reduction in germination of uredospores but also inhibition of germ-tube length and growth (Parker & Blakeman, 1984a).

1.6 Objectives

1. To produce a protocol for the isolation of bacteria and fungi from a wide range of plant and soil samples.

2. To subsequently screen these isolates, both *in vitro* and *in vivo*, for antagonism towards two foliar pathogens of *Vicia faba* (i) Chocolate spot (*Botrytis fabae*) and (ii) *Uromyces viciae-fabae* (rust).

3. Identification of the most antagonistic organisms from these screens for further studies.

4. To determine the mode of action of these antagonists.

5. To further screen these selected isolates for the production of antifungal compounds direct from culture filtrates.

2.

2. GENERAL MATERIALS AND METHODS

2.1 Growth and maintenance of bean plants before inoculation

Seeds of faba bean (*V. faba*) cv. Threefold White were sown in 5 inch pots (4 per pot) containing Levington M3 potting compost (Fisons). The trays were placed in a ventilated glasshouse under natural daylight supplemented to a 16 h photoperiod with 400 W mercury vapour lamps. The maximum daylight temperature was 24°C, falling to a minimum of 9°C at night.

2.2 Maintenance of Uromyces viciae-fabae

Bean rust, *U. viciae-fabae* was maintained on stock bean plants grown under glasshouse conditions listed above, (Section 2.1). Plants with 3 to 4 sets of leaves were inoculated with a uredospore suspension. This suspension was obtained by lightly brushing the surface of infected leaves with a soft camel-hair brush and placing it in a small volume of distilled water. The resultant suspension was painted on the upper surfaces of the leaves. Inoculated plants were covered with a clear polythene bag for 48 h to maintain a high humidity necessary for spore germination. Infected plants were maintained under these conditions until required. Approximately every 14 days after inoculation spores were collected, suspended in distilled water as above and painted onto infected plants.

2.2.1 GROWTH AND INOCULATION OF PLANT MATERIAL

Eighteen day old plants grown as above and with three sets of

leaves were sprayed to run off with a spore suspension of 25 mg uredospores to 100 ml distilled water, using an aerosol spray unit (Fisons). Tween 80 was added to the spore suspension, as a surfactant, to obtain a dilution of 0.01% (v/v).

2.3 Maintenance of Botrytis fabae

One strain of the pathogen *B. fabae*, obtained from Dr J.G. Harrison (Scottish Crop Research Institute), was used in all experiments. This pathogen was maintained on potato dextrose agar (PDA, Oxoid Ltd.). Approximately every 14 days a section of mycelium was removed from the advancing edge of the culture and subcultured onto fresh PDA plates and incubated at 20°C.

2.3.1 SPORULATION OF BOTRYTIS FABAE

B. fabae cultures sporulated *in vitro* under the conditions described by Harrison (1978). Maximum conidia production occurred on Medium X (Last & Hamley, 1956) with the addition of 10% sucrose. After this medium was inoculated onto the plates they were incubated at 20°C under near uv radiation.

An alternative, and also a more successful method for *B. fabae* conidial production was used in this study, published by Leach & Moore (1966), using a high concentration of inorganic salts. About 200 g of unmacerated bean leaf material was autoclaved for 20 mins at 120°C (1 kg cm^{-2}) in 1 litre distilled water, after which 20 g agar, 20 g sodium nitrate and 160 g sucrose were added to the mixture.

2.3.2 GROWTH AND INOCULATION OF PLANT MATERIAL

Plants with three sets of leaves were sprayed with a *B. fabae* conidia solution adjusted to 5 x 10^5 conidia ml^{-1} using an Improved Neubauer Haemocytometer.

2.4 Maintenance of fungal and bacterial cultures

Bacteria were maintained on nutrient agar (NA) using the streak plate method to obtain isolated colonies. Fungi were subcultured every 14 days on PDA medium. Some samples were also plated onto nutrient agar containing penicillium-G-potassium (100 mg ml^{-1}) and streptomycin sulphate (100 mg ml^{-1}) to suppress bacterial growth. All cultures were routinely incubated at 20°C ± 1°C, but for longer term storage they were kept in a cold room at 4°C ± 1°C.

2.5 Statistical analysis

Data were analysed using the statistical software package Minitab, release 7.2 Standard version (Minitab Inc. 1989) or Genstat 5 (released, 1990), to calculate means and standard error of the mean, using one way analysis of variance and covariance. Confidence intervals were calculated using the student t-test.

In tables and figures throughout this thesis significant differences are shown as follows:- $P<0.05$ = *, $P<0.01$ = **, and $P<0.001$ = ***.

3.

3. IN VITRO SCREENING OF BACTERIAL AND FUNGAL ISOLATES FOR ANTAGONISM TO BOTRYTIS FABAE AND UROMYCES VICIAE-FABAE

3.1 Introduction

This chapter reports on an examination of the *in vitro* screening of micro-organisms antagonistic to the foliar pathogens *Botrytis fabae* (Chocolate spot) and Rust (*Uromyces viciae-fabae*) on faba beans (*Vicia faba* L.).

Recent studies of inhibition of Chocolate spot *in vitro* (Hanounik & Hasanain, 1986) show strong inhibitory effects of both leaf washings and diffusates from resistant cultivars, compared to those from a susceptible one. Indeed, antagonistic bacteria have been isolated which suppressed both *B. cinerea* and *B. fabae in vitro* (Verdie & Le Normand, 1984), while the isolate *Trichoderma viride* greatly reduced *B. fabae* growth *in vitro* (Morris & Lane, 1990). Faba beans can also produce phytoalexins and other induced inhibitors under the stimulation of antagonistic epiphytic micro-organisms (Arase, Fujita & Kondo, 1990).

Both volatile and non-volatile antibiotics have been identified in previous studies (Morris & Lane, 1990), where they altered the growth of *B. fabae* mycelium and sclerotial development. Individual isolates of antagonists vary in their ability to produce antibiotics, in their level of activity and therefore in their potential to control pathogen development. Micro-organisms isolated from various sources, have therefore been shown to be effective in controlling both *B. fabae* and *B. cinerea in vitro* (Section 1.5.4).

Screening strategies are essentially the same whether applied to aerial, subterranean or aquatic habitats (Andrews, 1985). All screening methods are based on the evidence that the candidate organism either interferes with the pathogen or reduces disease development, with microbes being isolated in initial screening studies. Interference of growth implies some form of disruption or inhibition and can be evaluated in the laboratory on agar media or by microscopic examination. Reduction in disease can be measured by rating disease severity such as lesion area, pustule number or plant biomass.

Conditions for the isolation of micro-organisms are often on a general growth medium for fungi and bacteria, with selection based on nutritional characteristics or the presence of an antibiotic agent (Andrews, 1985). Another option is to apply mixed populations of micro-organisms to agar plates seeded with a pathogen to select those microbes which have antagonistic activity, judged on the production of inhibition zones. Such an approach combines both isolation and screening.

Some researchers prefer to screen micro-organisms with certain traits, such as good growth, stability and sporulation in culture or use a particular species or genus that is known to display antagonism. However, selective approaches should not be used unless there is evidence that antagonistic activity is restricted to a specific group of microbes (Andrews, 1985). The agar plate test is one of the most commonly used screening methods for antagonists. Agar plate methods can provide clues to their presumptive mode of antagonism and also the system to be used for further studies.

Any *in vitro* system needs to be designed appropriately to

allow successful screening (Jackson, Whipps & Lynch, 1991b). In this thesis a multivariate primary screening system is used to identify potential antagonists and assess this potential, and initial studies are performed to identify their individual modes of action.

3.2 Materials and methods

3.2.1 ISOLATION PROCEDURES

Potential antagonists were isolated from soil and foliage samples obtained from faba bean and other crops throughout Scotland (Table 3.1).

Soil samples of 10 g each were mixed with 90 ml sterile distilled water and shaken thoroughly to place the microflora into suspension (Tautorus & Townsley, 1987). Leaf samples (about 20 cm^2) were shaken vigorously in jars containing 50 ml sterile distilled water for 1.5 h (Fokkema & Lorbeer, 1974). From each suspension, a ten-fold dilution series in sterile distilled water was prepared, down to 10^{-7} dilution. Samples of 0.1 ml from each dilution were spread onto nutrient agar plates (Tautorus & Townsley, 1987) and incubated for 5 days at 20°C.

3.2.2 ANTAGONISM TO BOTRYTIS FABAE

3.2.2.1 Preliminary screen

Preliminary tests for antagonism to *B. fabae* were unreplicated and used dual culturing in sterile petri dishes (Newhook, 1951a). Three streaks of bacterial isolates were inoculated near the periphery of a plate (Plate 2a), with *B. fabae*

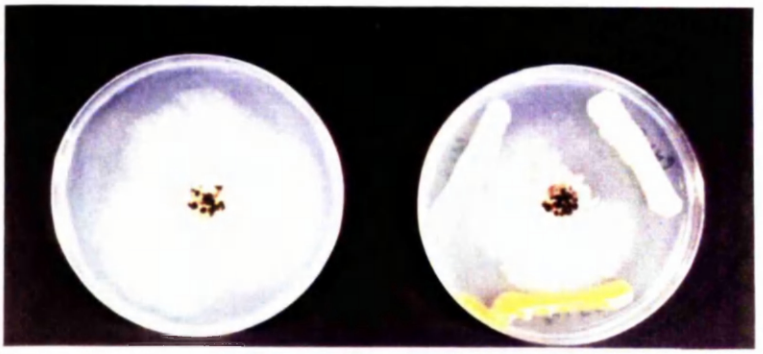
Plate 2: In vitro screening of bacterial and fungal isolates against B. fabae.

(a) Layout of preliminary *in vitro* screen of bacterial isolates for antagonism towards *B. fabae*.

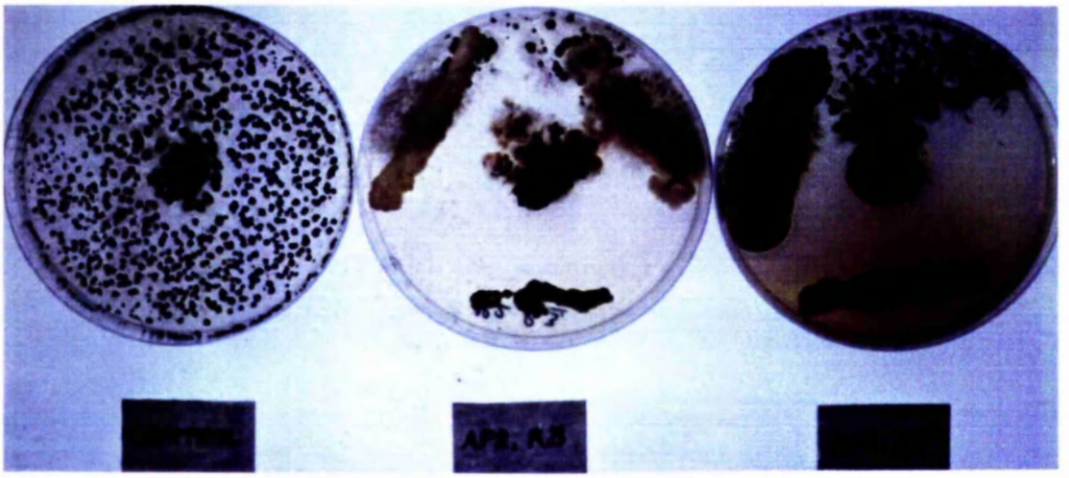
(b) Visual characteristics, bacterial: large clear zones of *B. fabae* growth with isolates AP2.R3 and AP2.R8 inhibiting both mycelium and sclerotia formation; brown coloration of *B. fabae* mycelium and also the PDA medium.

(c) Visual characteristics, fungal: browning of *B. fabae* mycelium with all of the isolates, especially T3.F19. Small zones free of pathogen mycelium are noticable, with inhibition of sclerotial formation near the point of interaction.

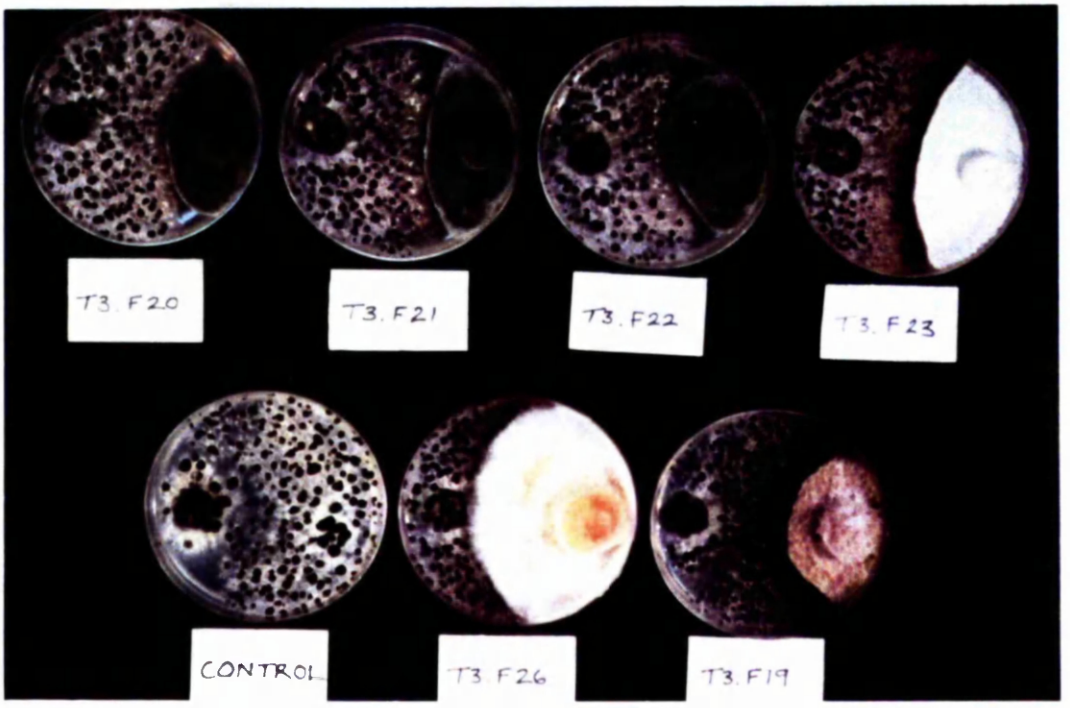
(a)



(b)



(c)



inoculated in the centre as a 4 mm diameter agar plug taken from the edge of an actively growing culture of *B. fabae*. Fungal isolates and *B. fabae* were placed at opposite sides of a 60 mm petri dish. Visual assessments of any interactions were noted.

3.2.2.2 Detached leaf tests

Isolates showing antagonism to *B. fabae in vitro* were screened for their pathogenicity to detached leaves of broad bean (cv. Threefold White). Mature leaves, detached from healthy plants grown under glasshouse conditions (Section 2.1), were surface sterilised in 0.5% sodium hypochlorite solution for 2 minutes (Hanounik & Maliha, 1984), washed twice in sterile distilled water (10 min each) and dried on sterile filter paper.

Sterile distilled water (2.5 ml) was added to 90 mm Petri dishes, each containing 2 pieces of filter paper (Whatman number 1). Two sterile leaves supported on a glass rod to avoid direct contact with the wet filter paper, were placed in each dish. Three 10 µl spots of sterile water were placed on the left-hand side of each leaf (6 per plate), with the right-hand side acting as a control. To each spot a loopful of bacterial cells or fungal spores were added (Fig. 3.1). Control plates tested sterile distilled water alone or with *B. fabae* conidia. There were two replicate plates of each treatment. Dishes were sealed and placed in a growth cabinet at 20°C. Tissue damage was assessed at 2-day intervals, and it was noted whether isolates were pathogenic or non-pathogenic to the bean leaves.

Table 3.1
Sources of field samples screened to isolate fungi and bacteria.

Code	Crop	Cultivar	Sample contents	Isolated
MB1.F	Bean	Maris Bead	necrotic foliage	9.10.90
MB1.S	Bean	Maris Bead	soil sample	9.10.90
MB2.F	Bean	Maris Bead	necrotic foliage	9.10.90
MB2.S	Bean	Maris Bead	soil sample	9.10.90
T1.F	Bean	Troy	necrotic foliage	9.10.90
T1.S	Bean	Troy	soil sample	9.10.90
Z1.S	Bean	mixed	soil sample	9.10.90
MX1.F	Bean	mixed	foliage/stem	9.10.90
MX1.L	Bean	mixed	necrotic foliage	9.10.90
MX2.F	Bean	mixed	foliage/stem	9.10.90
MX2.L	Bean	mixed	necrotic foliage	9.10.90
A1.R	Bean	Alfred	root material	11.10.90
A1.NS	Bean	Alfred	necrotic stem/pod	11.10.90
A1.GS	Bean	Alfred	green stem/pod	11.10.90
A1.L	Bean	Alfred	foliage	11.10.90
TFW1.GS	Bean	Triplefold White	green stem/pod	11.10.90
T3.F	Bean	Troy	necrotic foliage	16.10.90
T4.F	Bean	Troy	necrotic foliage	16.10.90
LL1.F	Bean	Local Landrace	foliage	16.10.90
C1.F	conifer	-	foliage	26.10.90
AP1.F	Bean	Aquadulce	foliage (plot)	26.10.90
AP1.S	Bean	Aquadulce	soil sample (plot)	26.10.90
AP2.R	Bean	Aquadulce	root sample (plot)	1.10.90
AG1.R	Bean	Aquadulce	root (glasshouse)	26.10.90
AG1.F	Bean	Aquadulce	foliage(glasshouse)	26.10.90
BL1.F	beech	-	foliage	26.10.90
PN1.F	pine needles	-	foliage	1.10.90
DL1.F	leaf litter	-	decaying foliage	1.10.90
BE1.F	Bean	Bunyards Exhibition	foliage	1.10.90
P1.S	fallow plot		soil sample	1.10.90

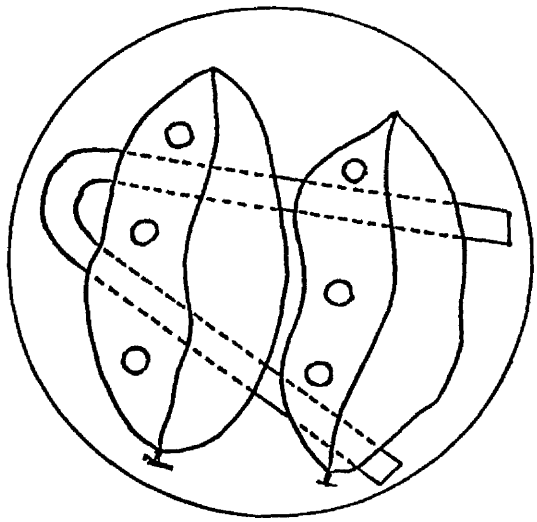
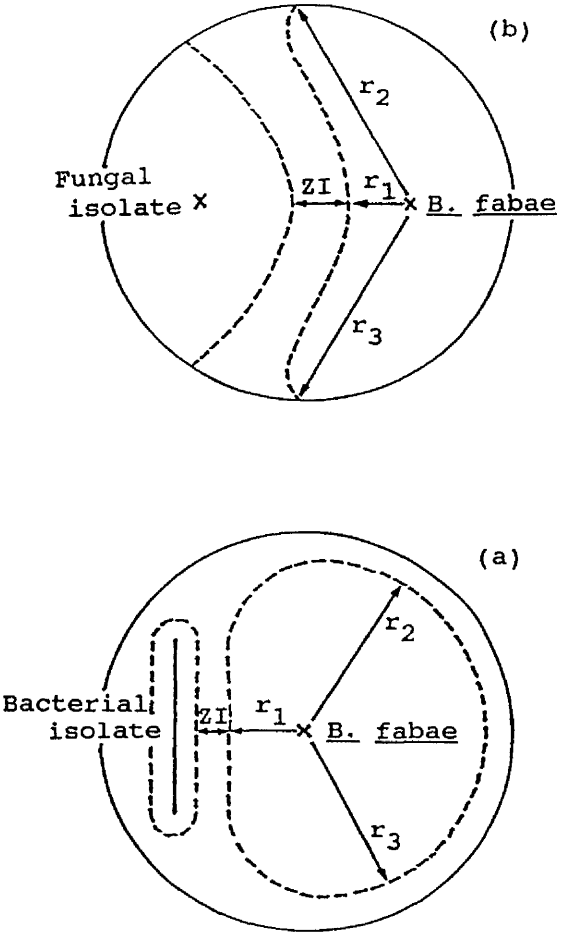


Fig. 3.1
Layout of detached bean leaves in 90 mm diameter petri dishes, to test the pathogenicity of bacterial and fungal isolates towards *V. faba* leaves. The bean leaves were spotted on the left-hand side of the leaf.

3.2.2.3 Replicated antagonism

The fungal and bacterial isolates which damaged detached leaves least and were antagonistic to *B. fabae* in the initial *in vitro* tests, were selected for examination in more detail. One 40 mm long streak of each bacterial isolate was made on a 90 mm PDA plate (Fig. 3.2a) with an agar plug of *B. fabae* inoculated in the centre (Fokkema, 1973). Fungal isolates and *B. fabae* were placed at opposite sides of a 90 mm petri dish (Royse & Ries, 1978) (Fig. 3.2b). Plates were incubated at 20^{o}C with 5 replicates of each treatment.



Inhibition of radial growth (%) was calculated as:

$$100\left[\left(\frac{(r2+r3)}{2}-r1\right)\Big/\frac{(r2+r3)}{2}\right]$$

Fig. 3.2

Diagrams showing the layout of petri dishes when inoculated to measure antagonistic properties of (a) bacterial and (b) fungal isolates to *B. fabae* [Fokkema (1973), Royse & Ries (1978)]. ZI = zone of inhibition

Two parameters of inhibition were calculated: (i) the width of the inhibition zone and (ii) the % inhibition of radial growth of *B. fabae* (Fokkema, 1973), which is calculated from three measurements of radial growth (Fig. 3.2). Measurements were taken 2,4,8,12, and 16 days after inoculation. The statistical package Genstat 5 was used to carryout analysis of variance and covariance on the resulting data.

3.2.2.4 Antibiotic production

Non-volatile antibiotic production was estimated by placing a 5 mm inoculum disc of antagonist centrally on PDA plates that was covered with sterile cellophane, 50 μm thick (Dennis & Webster, 1971a) and placed in a growth cabinet at 20^{o}C. After 7 days growth, the antagonist and cellophane were removed and a 5 mm disc of the pathogen (*B. fabae*) was placed centrally on the agar and its growth rate recorded over the following 5 days (Jackson *et al.*, 1991b). To determine if complete inhibition was due to the production of a fungistatic or fungitoxic antibiotic the pathogen inoculum disc could be transferred to fresh PDA medium and examined for fresh growth (Jackson *et el.*, 1991b). Five replicates were used in all these experiments.

The production of volatile antibiotics was studied using two petri dishes sealed together. The uppermost plate was inoculated centrally with *B. fabae*, and the lower one simultaneously with the test antagonist (Jackson *et al.*, 1991b; Dennis & Webster, 1971b). The inhibition of growth (%) was calculated by comparing the growth of *B. fabae* in the presence of a test antagonist, to the growth of control cultures.

3.2.2.5 Spore germination tests

A suspension of spores of antagonistic fungal isolates, at a concentration of 2 x 10^6 spores ml^{-1}, were prepared from 14 day old agar plate cultures, by washing the cultures with 5 ml sterile distilled water containing 0.01% Tween 80 and agitated with a glass rod (Andrews, Berbee & Nordheim, 1983). The resulting solution was filtered through 4 layers of muslin to remove any mycelial debris. Bacteria were grown on nutrient agar slants, and when 7 days old, and the cells were suspended in sterile distilled water and adjusted to 2 x 10^9 cells ml^{-1}. Conidia of *B. fabae* were collected from sporulating cultures (Section 2.3.1) and a suspension of 5 x 10^5 conidia ml^{-1} was prepared. All solutions were counted using a Improved Neubauer haemocytometer.

Studies on the germination of *B. fabae* conidia, in the presence of either fungal or bacterial antagonists, were carried out on glass slides. Two 20 µl drops were placed 2.5 cm apart on each slide. The final concentration on the slides was 2.5 x 10^5 *B. fabae* conidia ml^{-1}, 1.0 x 10^6 spores ml^{-1} for fungal isolates and 1.0 x 10^9 cells ml^{-1} for bacterial isolates. The slides were placed in transparent boxes (4 slides per box), lined with moistened filter paper (Whatman number 1), on a glass rod frame and incubated in a growth cabinet at 20°C, with 6 replicate slides per treatment.

Treatments:-

control	*B. fabae* conidia alone
fungal isolates:-	MB2.F45
	AP1.S20
	AP2.R16
	AG1.F4

bacterial isolates:- MB2.S5B
LL1.F23

After 12 h and 36 h incubation the samples were stained with lactophenol cotton-blue to allow easier examination of the fungus (Shipton & Brown, 1962). The following assessments were made from non-contacting conidia in randomly chosen fields of view:-

(1) germination (100 conidia)
(2) average germ-tube length (20 germinated conidia)
(3) appressorium formation (100 germinated conidia)

A spore with a germ-tube of any length was considered to have germinated (Purkayastha & Deverall, 1965).

3.2.3 ANTAGONISM TO UROMYCES VICIAE-FABAE

3.2.3.1 Spore germination bioassay

In vitro tests on germination of *U. viciae-fabae* uredospores were carried out in the presence of bacterial cells and fungal spores. Rust uredospores were harvested and collected as in Section 2.2. Around 50 mg of uredospores were floated on 100 ml sterile distilled water as a monosporic layer at $4^{o}C$ for 16 h to release self inhibitors produced by the spores (Wynn, 1976). They were then washed for 2-3 minutes with sterile distilled water and suspended in sterile distilled water containing a surfactant (0.01% Tween 80) to a concentration of 5×10^{4} uredospores ml^{-1} using an Improved Neubauer haemocytometer. 170 μl of this solution was transferred to wells of a microtitre plate with a capacity of 400 μl. These wells already contained 170 μl suspensions of either potential bacterial or fungal

antagonists (Baker *et al.*, 1983) and were incubated overnight at $16^{o}C$, in the dark.

A suspension of fungal spores of concentration 5×10^{5} spores ml^{-1} was prepared from a plate culture by washing with distilled water, loosening spores with a glass rod and filtering through four layers of muslin, thus removing any mycelial debris (Docherty & Preece, 1978). Bacteria were grown on nutrient agar slants and their cells suspended in sterile distilled water and adjusted to a concentration of 2×10^{9} cells ml^{-1}. This gave final concentrations of 25000 rust uredospores ml^{-1}, fungal suspensions of 2.5×10^{5} spores ml^{-1} and bacterial suspensions of 1×10^{9} cells ml^{-1} in the microtitre plate wells.

After 16 h the suspensions in the microtitre wells were stained with lactophenol cotton-blue to dye the uredospore germ-tubes allowing easy visual assessment and to stop further germ-tube formation and growth before examination (Bell & Daly, 1962). Samples were placed on microscope slides and examined at x100 magnification. One hundred uredospores were counted for each of the ten replicate wells per treatment. Germination has occurred when the germ-tube was equal to or greater than the diameter of the spore (Bell & Daly, 1962).

3.2.4 INFECTION AND LEAF STAINING STUDIES

Preliminary studies were performed on the effects of antagonists on infection of detached bean leaves by *B. fabae* and *U. viciae fabae*.

3.2.4.1 *Uromyces viciae-fabae*

Here a technique for clearing and staining wheat stem rust was followed (Shipton & Brown, 1962). Mature bean leaves were infected with rust uredospores and grown under glasshouse conditions as described in Section 2.2.1. Detached bean leaves infected with rust and between 2 and 7 days from inoculation, were cut into areas of approximately 15 x 15 mm and immediately immersed in 10-15 ml of alcoholic lactophenol cotton-blue (1 part lactophenol to 2 parts 95% alcohol). Lactophenol cotton-blue was prepared as follows [lactophenol, 100 ml (phenol 20 ml, lactic acid 20 ml, glycerine 40 ml, distilled water 20 ml); glacial acetic acid, 4 ml; 1% aqueous aniline blue, 3 ml].

The solution containing the leaf sections was brought to boiling and simmered for 1 minute. After the leaves sank, the solution was again brought to boiling for about 30 seconds. The leaves remained in the stain for approximately 48 h at room temperature and then were removed, rinsed in water and placed in chloral hydrate (5 g chloral hydrate to 2 ml water) for 30-50 minutes to clear the leaves before mounting on a microscope slide in 50% glycerine.

3.2.4.2 *Botrytis fabae*

Leaf discs about 10 mm in diameter were cut from mature healthy bean leaves using a cork borer, which were then floated in sterile distilled water at 20°C in petri dishes. From a conidial suspension (1×10^5 ml^{-1}) a 20μl sample containing approximately 2000 conidia was applied to the disc surface. Detached leaves were also infected through spraying with a suspension of *B. fabae* conidia of similar concentration.

Fungal antagonists AP1.S20, AP2.R16 and AG1.F4 were applied to the leaf discs or detached leaves from a suspension of 1 x 10^6 spores ml^{-1}, either prior to, simultaneously with or after the inoculation of the pathogen.

After periods varying from 12 h to 96 h, samples were cleared in either 75% lactic acid for 48 h at 40°C (Erb, Gallegly & Leach, 1973), chloral hydrate (200% w/v) or methanol. After clearing, these samples were stained in lactophenol cotton-blue solution for 2-4 days. Some samples were placed back into methanol for 2-4 h to clear excess stain before examination.

These samples were mounted in 50% glycerine or Glycerine-gelatin mounting medium for fixing fungal preparations on slides. The mounting media has the following composition [gelatin 7 g, distilled water 42 ml, glycerol 50 ml and phenol 1 g]. The boiled mixture is a dense, gelatinous substance, but is clear in the cooled state. When used, such fixed preparations are preserved for long periods (Kiraly *et al.*, 1974). Slides can be sealed with a nail varnish solution before storage in the dark.

3.3 Results

3.3.1 ISOLATION

In total, 690 bacterial and fungal isolates were obtained, including some duplicates. Isolation was usually easiest from series dilutions of 10^{-5}, 10^{-6} and 10^{-7} of the original solutions. The addition of antibiotics to suppress bacterial growth was very effective, allowing easier isolation of fungi.

3.3.2 ANTAGONISM TO BOTRYTIS FABAE

In the preliminary screen, the following characteristics were taken to indicate potential antagonism: a brown discoloration of mycelium (either antagonist and/or fungal isolate) and/or media, a zone of inhibition of mycelial growth, a zone free of sclerotial formation with mycelial growth or contact inhibition i.e. no overlapping growth (Plate 2b and 2c).

Of all the isolates examined, 205 (30%) showed evidence of antagonism to *B. fabae* in dual culture, but the proportion was much larger among fungi (63%) than bacteria (21%) (Table 3.2). The majority of isolates showing antagonism *in vitro* were isolated from soil or foliage samples collected from faba bean crops. Of the total number of selected bacteria 88%, and 72% of the total of selected fungal isolates, were isolated from samples collected from faba beans. The remaining 12% and 27% of the 205 isolates selected were collected from samples from other crop types, and comprised bacteria and fungi respectively (Table 3.3).

3.3.2.1 Detached leaf tests

Out of the 205 isolates selected, 39 were shown to cause necrosis of detached leaves and were rejected from the screening programme (Table 3.2). Isolate P1.S18 (from soil sample-fallow field) showed a inhibition zone of 17 mm in the preliminary screen but was later found to be pathogenic on bean leaves and so was rejected at this stage of the screening programme. Other isolates gave results which were variable and were thus also retained for further antagonism tests. *B. fabae* conidia in the

Table 3.2
Results of *in vitro* screening tests.

	Total No. tested	No. selected in initial screen (%)	No. rejected after detached leaf tests (%)	Number remaining
Bacteria	566	120 (21)	13 (13)	107
Fungi	124	85 (63)	26 (30)	59
Total	690	205 (30)	39 (19)	166

Table 3.3
Origin of soil and foliage samples utilised for screening for antagonistic micro-organisms.

	Total No. selected	Sample origin[a]		Crop type[a]	
		Foliage (%)	Soil (%)	Bean (%)	Other (%)
Bacteria	120	67 (55)	53 (63)	106 (88)	14 (12)
Fungi	85	54 (45)	31 (37)	62 (72)	23 (27)
Total	205	121 (59)	84 (41)	168 (82)	37 (18)

a figures in parentheses are the percentages of total number of isolates selected

control cultures caused necrotic lesions after 1-2 days, which later covered the whole leaf before sporulating (Plate 3).

On inoculation of the antagonist onto the leaf surface using a metal inoculation loop, there was a risk of physical surface damage, allowing the formations of lesions and allowing antagonists to enter into epidermal cells of the leaves.

3.3.2.2 Replicated antagonism

In all, 45 bacterial and 18 fungal isolates were selected from the 107 bacterial and 59 fungal isolates (Tables 3.4 & 3.5) which showed some antagonism in the preliminary screen and passed the detached leaf test. The replicated tests for antagonism revealed little variation in the radial growth rate of *B. fabae* among control cultures, although differences in colour and sclerotial formation were noticable. The analyses of variance showed significant differences ($P<0.01$) between different isolates of bacteria in measurements of inhibition of radial growth (%) of *B. fabae* on all recording days except the first from inoculation. On the other hand, significant differences between fungal isolates ($P<0.05$) were apparent only 12 and 16 days after inoculation. This is likely to be the result of the different types of growth shown by bacteria and fungi. Bacterial colonies were slow to spread across the agar surface, whereas many fungal isolates grew rapidly and sporulated over the entire plate surface.

However, measuring radial growth of *B. fabae* alone does not take account of variation in growth rates among the different bacterial and fungal isolates being screened for antagonism (Fokkema, 1973). Any isolate showing an inhibition

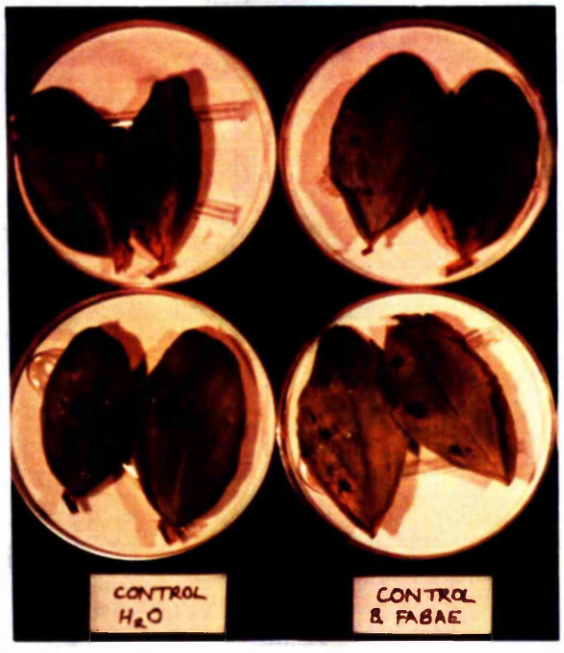
Plate 3: Detached leaf tests.

(a) Controls: leaves were either inoculated with water or a suspension of *B. fabae* conidia. Necrotic lesions at all of the 6 inoculation sites can be observed.

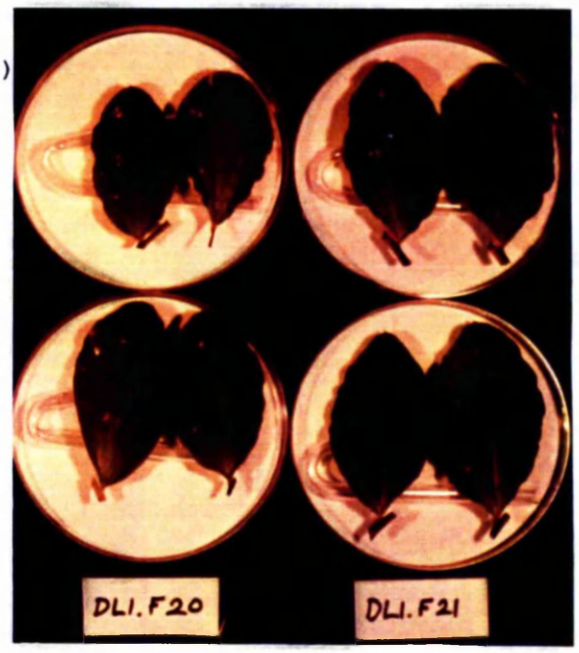
(b) Both isolates show little or no damage to the bean leaves. One small individual lesion with isolate DL1.F21 was concluded to be the result of the method of inoculation, through damage to the leaf surface.

(c) Two fungal isolates rejected from the *in vitro* screening programme due to pathogenicity towards bean leaves, especially the isolate T3.F23.

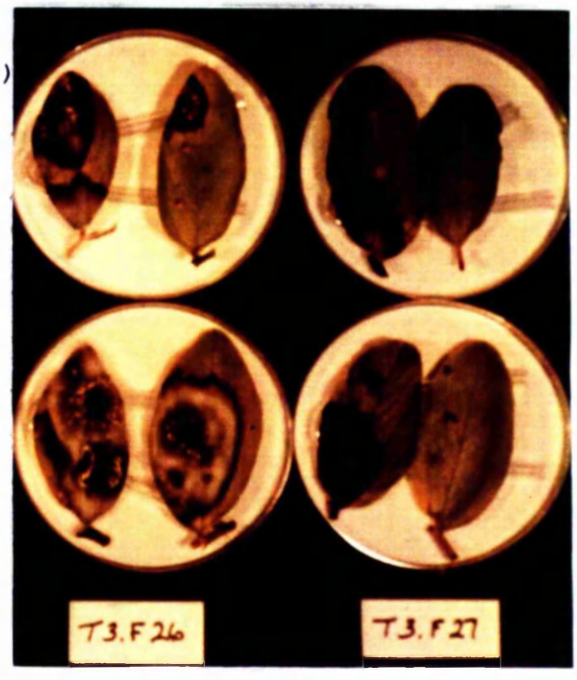
(a)



(b)



(c)



zone of zero or greater, together with significant inhibition ($P<0.05$) of radial growth of *B. fabae*, was taken to show antagonistic properties. Of the 45 bacteria and 18 fungal isolates selected, 19 and 14 of these isolates respectively, showed inhibition zones with significant reductions in radial growth (Tables 3.4 & 3.5).

Large variations between isolates were seen in the mean values for both of the parameters measured (Fig. 3.3), with a high correlation (coefficient 0.793) between inhibition zones and the percentage inhibition of radial growth using bacterial isolates. Such a correlation was not seen with the fungal isolates (-0.273).

With some isolates, a negative inhibition zone was recorded when the pathogen overran the test isolate with no significant inhibition of radial growth of the pathogen (Fig. 3.4a). Eight isolates (all bacterial) failed to establish on the agar plate (Fig. 3.4b), and as a result further experiments were carried out establishing these isolates on the agar plates before inoculation with *B. fabae* mycelium. These isolates were grown for 2-6 days to allow significant colony growth before carrying out antagonism tests. Of these isolates, only one, DL1.F3 (after 6 days establishment before *B. fabae* inoculation) showed a significant reduction in radial growth (62.4%) and a large inhibition zone of 10 mm on agar. Isolate P1.S22 exhibited a zone free of sclerotial development (20-30 mm) next to the bacterial streak, but with no inhibition zone of mycelium.

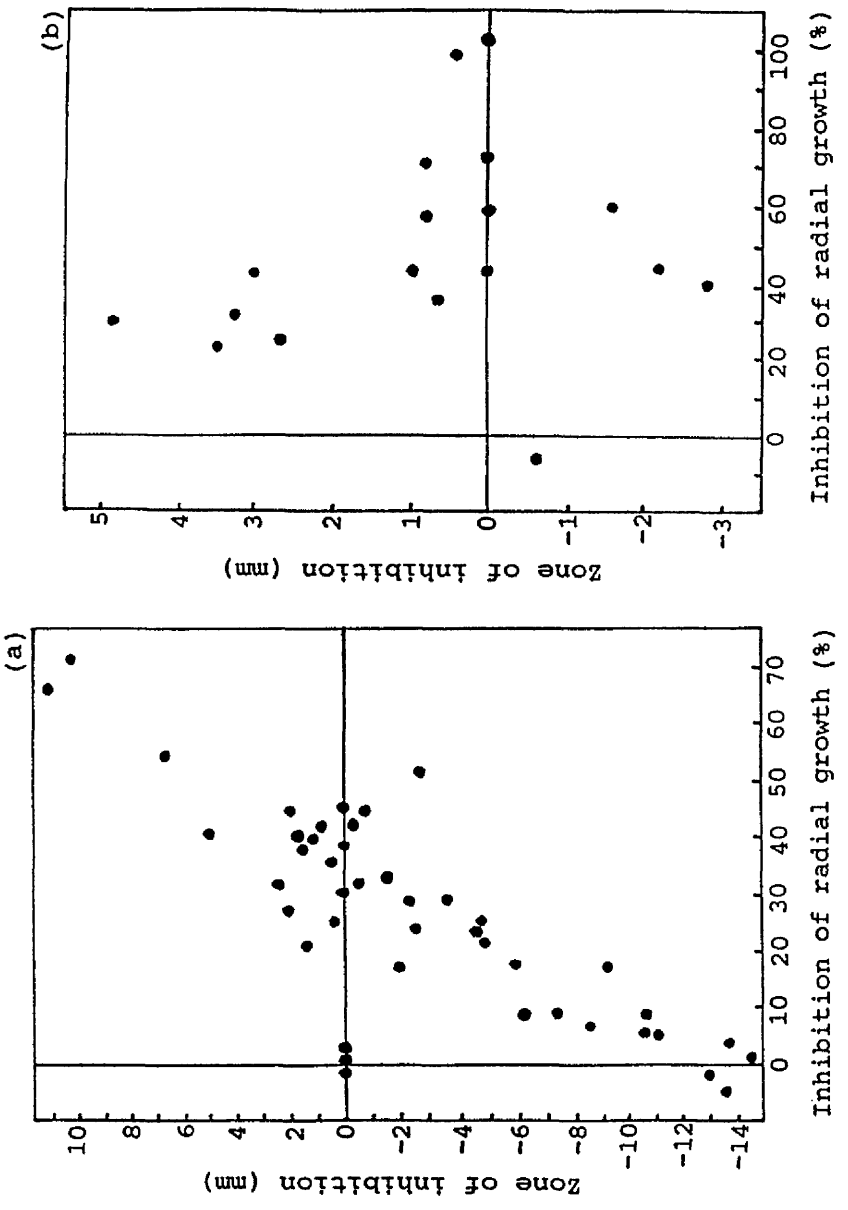


Fig. 3.3
Relationships between the size of the inhibition zones (mm) and the inhibition of radial growth (%) of *B. fabae in vitro*, induced by different (a) bacterial and (b) fungal isolates.

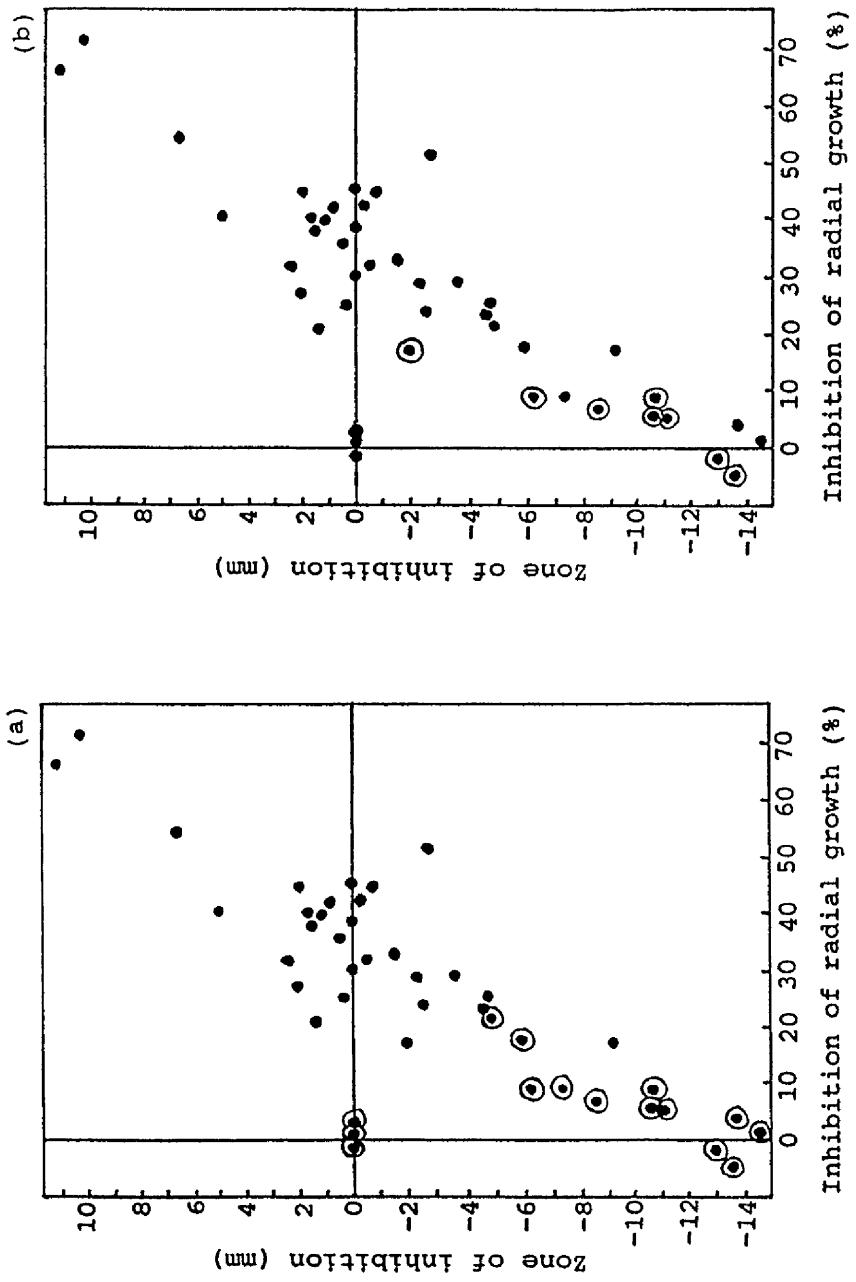


Fig. 3.4

Relationships between the size of the inhibition zones (mm) and the inhibition of radial growth (%) of *B. fabae* showing (a) bacterial isolates with no significant inhibition of radial growth (circled), and (b) bacterial isolates that were slow to establish *in vitro* in the replicated agar screen (circled).

Table 3.4
Results of screening bacterial isolates for antagonism to *B. fabae in vitro*.

Isolate	% inhibition of radial growth[a]		Zone of inhibition (mm)[b]		Browning of medium[c]	Browning of mycelium[d]	Inoculum[e]	Sclerotial formation[f]
MB1.S 15	29.45	*	-2.2	-	-	-	-	
MB2.F 28	55.04	*	**6.8**	+++	+	+	-	
MB2.F 33	25.68	*	-4.4	-	-	-	-	+
MB2.S 4	2.99	ns	-13.8	-	-	-	-	
MB2.S 5B	72.37	*	**10.2**	++++	+	-	-	
MB2.S 6	0.84	ns	-14.4	-	-	-	-	
MB2.S 7	41.26	*	**5.0**	++	+	+	-	-
MB2.S 8	19.70	*	-2.0	-	+	+	+	
MB2.S 16	24.83	*	-2.4	-	-	+	-	
MB2.S 19	45.05	*	**0.0**	+	-	+	-	+
MB2.S 20	40.42	*	**0.8**	+	+	+	-	
T1.S 1	30.67	*	**0.0**	+	+	+	-	-
T1.S 5B	23.58	*	-4.4	-	+	-	-	-
T1.S 11A	25.44	*	**0.4**	+	-	-	-	
T1.S 14	31.94	*	**2.4**	+	-	-	-	-
Z1.S 11A	-5.01	ns	-13.6	-	-	-	+	
Z1.S 14	9.63	ns	-10.4	-	+	+	+	+
MX1.F 2	36.95	*	**0.2**	+	-	-	-	
MX1.F 40	43.68	*	-0.4	-	-	+	-	
MX1.L 23	52.41	*	-2.6	-	-	+	-	+
MX2.F 5	4.28	ns	-10.6	-	+	+	+	
A1.R 10	21.44	ns	-4.8	-	-	+	-	+
A1.NS 5	9.7	ns	-7.5	-	+	+	-	-
A1.GS 10	28.98	*	-3.6	-	-	-	-	+
A1.GS 12	32.50	*	-0.4	-	-	+	-	+
A1.L 2	38.73	*	**1.4**	+	+	+	-	-
TFW.GS 2	38.42	*	**0.0**	+	-	+	-	-
T3.F35	-1.96	ns	-13.0	-	-	-	+	
LL1.F 16	8.71	ns	-8.5	-	-	-	+	+
LL1.F 21	0.5	ns	**0.0**	+				
LL1.F 23	66.07	*	**11.2**	++++	-	+	-	-
AP1.S 6	18.19	ns	-5.6	-	-	+	-	+
AP1.S 16	33.07	*	-1.6	-	+	+	-	+
AP2.R 8	22.68	*	**1.5**	+	-	-	-	-
AP2.R 12A	45.23	*	**2.0**	+	+	+	-	-
AP2.R 13A	39.22	*	**1.8**	+	+	+	-	-
AG1.R 2	0.4	ns	**0.0**	+	-	+	+	+
AG1.R 3	40.10	*	**1.0**	+				
AP2.R 3	-1.1	ns	**0.0**	+				
DL1.F 3	9.53	ns	-6.0	-	+	-	+	-
P1.S 5	17.31	*	-9.2	-	+	+	-	+
P1.S 6	43.96	*	-0.2	-	+	+	-	-
P1.S 13B	27.49	*	**2.0**	+	+	+	-	-
P1.S 18	16.36	ns	-4.2	-				
P1.S 22	4.22	ns	-11.0	-	+	-	+	+

(See notes at Table 3.5)

Table 3.5
Results of screening fungal isolates for antagonism to *B. fabae in vitro*.

Isolate	% inhibition of radial growth[a]		Zone of inhibition (mm)[b]		Browning of medium[c]	Browning of mycelium[d]	Sclerotial formation[e]
MB2.F 45	36.3	*	**0.6**	+	-	-	
MB2.S 52	40.0	*	-2.8	-	-	+	+
Z1.S 22	44.9	*	**0.0**	+	-	+	+
MX1.F 62	58.9	*	**0.0**	+	-	+	
MX1.L 32	99.5	*	**0.4**	+	-	+	
MX1.L 34	100.0	*	**0.0**	+	-	+	
AP1.S 19	-0.6	ns	-0.6	-	-	+	
AP1.S 20	58.6	*	**0.8**	+	-	+	
AP2.R 15	29.6	ns	**3.4**	++	+	+	+
AP2.R 16	37.7	ns	**1.0**	+			
AP2.R 18	60.0	*	-1.6	-	+	+	
AP2.R 19	73.5	*	**0.0**	+	-	+	
AG1.R 4	70.6	*	**0.8**	+	+	+	
AG1.R 5	23.4	ns	**2.8**	+	-	+	
AG1.R 8	42.8	*	**3.0**	++	+	+	
AG1.R 13	25.4	ns	**3.4**	++			
AG1.F 4	31.2	*	**4.8**	++	+	+	+
DL1.F 19	44.6	*	-2.2	-	-	+	+

Notes:

a * significant from control ($P<0.05$), ns not significant

b inhibition zones (modified from Renwick *et al.*, 1991)

-	no inhibition zone
+	inhibition zone 0 - 3 mm
++	inhibition zone 3 - 6 mm
+++	inhibition zone 6 - 9 mm
++++	inhibition zone > 9 mm

c + browning, - no browning

d + browning, - no browning

e + sclerotia enhanced, - sclerotia inhibited

Fig. 3.5
Labelled bacterial isolates screened for antagonism to *B. fabae*.

Isolate	Number
MB1.S 15	1
MB2.F 28	2
MB2.F 33	3
MB2.S 4	4
MB2.S 5B	5
MB2.S 6	6
MB2.S 7	7
MB2.S 8	8
MB2.S 16	9
MB2.S 19	10
MB2.S 20	11
T1.S 1	12
T1.S 5B	13
T1.S 11A	14
T1.S 14	15
Z1.S 11A	16
Z1.S 14	17
MX1.F 2	18
MX1.F 40	19
MX1.L 23	20
MX2.F 5	21
A1.R 10	22
A1.NS 5	23
A1.GS 10	24
A1.GS 12	25
A1.L 2	26
TFW.GS 2	27
T3.F35	28
LL1.F 16	29
LL1.F 21	30
LL1.F 23	31
AP1.S 6	32
AP1.S 16	33
AP2.R 8	34
AP2.R 12A	35
AP2.R 13A	36
AG1.R 2	37
AG1.R 3	38
AP2.R 3	39
DL1.F 3	40
P1.S 5	41
P1.S 6	42
P1.S 13B	43
P1.S 18	44
P1.S 22	45

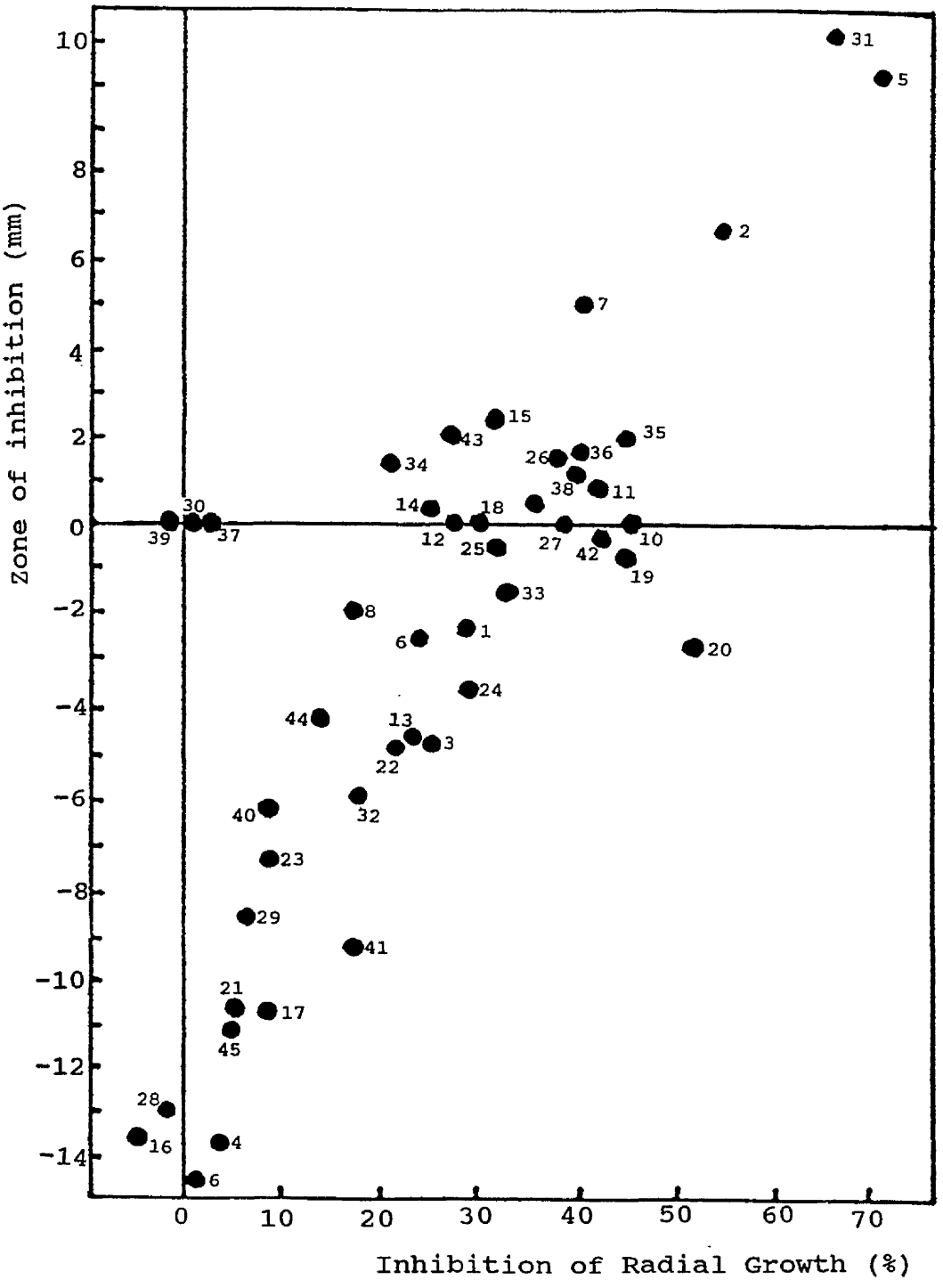
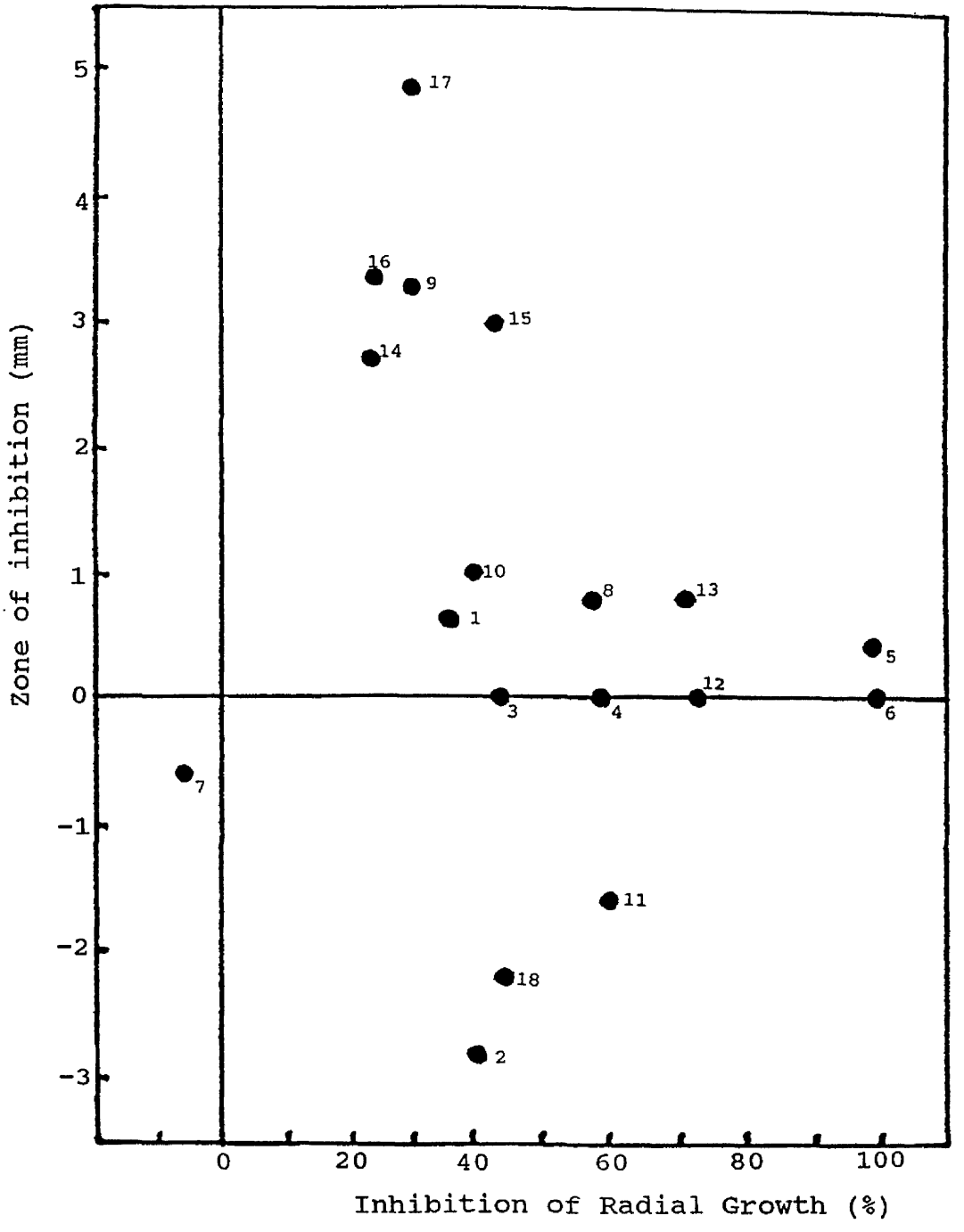

Zone of inhibition (mm)
Inhibition of Radial Growth (%)
10
8
6
4
2
0
-2
-4
-6
-8
-10
-12
-14
0
10
20
30
40
50
60
70
31
5
2
7
15
43
35
26
36
34
38
11
14
18
30
39
37
12
25
27
42
10
19
33
8
1
6
20
24
44
13
3
22
40
32
23
29
41
21
17
45
28
16
4
6

Fig. 3.6
Labelled fungal isolates screened for antagonism to *B. fabae*

Isolate	Number
MB2.F 45	1
MB2.S 52	2
Z1.S 22	3
MX1.F 62	4
MX1.L 32	5
MX1.L 34	6
AP1.S 19	7
AP1.S 20	8
AP2.R 15	9
AP2.R 16	10
AP2.R 18	11
AP2.R 19	12
AG1.R 4	13
AG1.R 5	14
AG1.R 8	15
AG1.R 13	16
AG1.F 4	17
DL1.F 19	18


Zone of inhibition (mm)
5
4
3
2
1
0
-1
-2
-3
17
16
9
15
14
10
8
13
1
5
12
3
4
6
7
11
18
2
0
20
40
60
80
100
Inhibition of Radial Growth (%)

It was essential to avoid condensation in the petri dishes, because surface water spreads both bacterial cells and fungal spores over the plate. This problem was more critical where the antagonists were being measured in dual cultures, and whole trials were ruined. Agar plugs of *B. fabae* rather than conidial suspensions were used to inoculate plates during screening, to try and reduce any problems related to surface water.

In comparison to control cultures, bacterial isolates LL1.F23, P1.S6 and P1.S13B with zones of inhibition of 11.2, -0.2 and 2.0 mm respectively, all reduced sclerotia formation (Table 3.4). Other isolates which showed some inhibition of sclerotial formation were T1.S1 (0 mm), T1.S14 (2.4 mm), A1.L2 (1.4 mm), AP2.R12A (2.0 mm) and AP2.R13B (1.8 mm) (average inhibition zone measurements of 5 replicates in brackets). Only one bacterial isolate (P1.S6) showed a reduction in sclerotial formation and exhibited a negative zone of inhibition on agar plates. A1.GS12, A1.R10, AP1.S6 and MB2.F33, displayed negative zones of inhibition of -0.4, -4.8, -5.6 and -4.4 mm respectively, and all enhanced sclerotial formation. When *B. fabae* grew over the bacterial colonies, sclerotial growth was concentrated on the site of the initial, inoculated bacterial streak. The results obtained from some bacterial isolates showed large variations in zones of inhibitions between replicates.

Two different bacteria were isolated from the initial inoculation of isolate LL1.F23 following subculturing. First, isolate LL1.F23 (M) which showed all the same qualities as the original isolate and LL1.F23 (W) which grew as discrete white colonies. The difference in colour of the colonies between

these isolates was clearest on PDA medium where LL1.F23 (M) was dark green and yellow in colour and LL1.F23 (W) was white, but not when routinely subcultured on NA medium. Isolate LL1.F23 (W) showed no antagonism towards *B. fabae*, whereas LL1.F23 (M) inhibited growth of *B. fabae* (Data not shown). Isolate LL1.F23 (W) was discarded, and Isolate LL1.F23 (M) was used in all further studies unless stated. Two bacteria were also isolated from the bacterium MB2.F5B also denoted as (M) and (W) in subsequent screening.

From the screening results it appears there was more likelihood of browning of *B. fabae* mycelium by isolates showing antagonism towards this pathogen than by isolates not showing antagonism.

The four bacterial isolates which caused greatest inhibition of radial growth (coded MB2.S7, MB2.F28, MB2.S5B & LL1.F23) also gave the largest zones of inhibition (Fig. 3.5). Bacterial isolate LL1.F23, completely suppressed sclerotial growth and gave the largest inhibition zone (11.2 mm), and also caused malformation of the edges of the *B. fabae* cultures (Plate 4b). Of all the bacterial isolates, P1.S13B and LL1.F23 caused the most severe browning of *B. fabae* mycelium. Over time, five bacteria, T1.S14, A1.L2, AP1.S6, MX1.L23, but especially MB1.S15, grew along the mycelium to eventually envelope the *B. fabae* colony. Many of the bacteria classified as antagonistic in the preliminary screen were not confirmed as such in this later test.

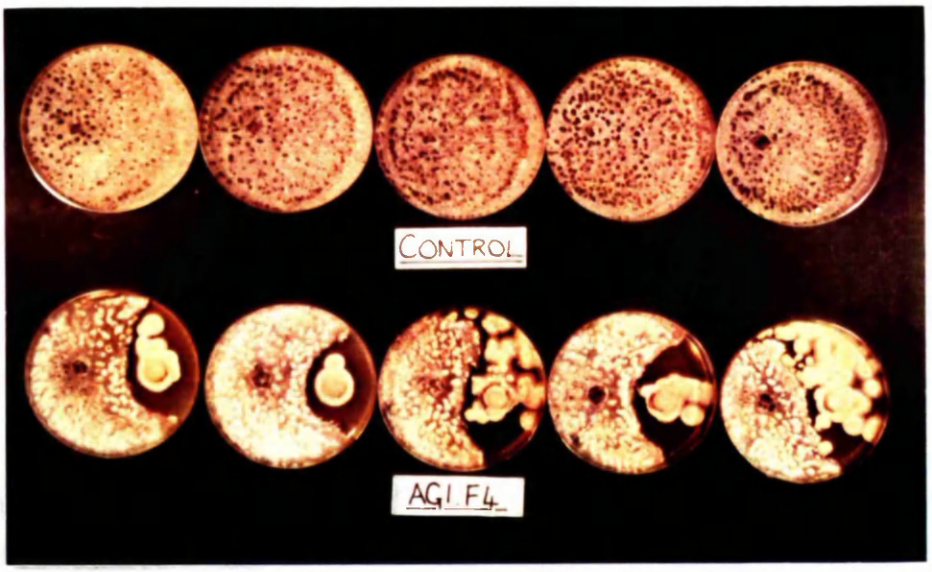
The two fungi showing most antagonistic potential (AG1.F4 and AG1.R8) had zones of inhibition of 4.8 and 3 mm respectively (Fig. 3.6; Plate 4a). Fungal antagonists generally produced

Plate 4: Antagonism tests.

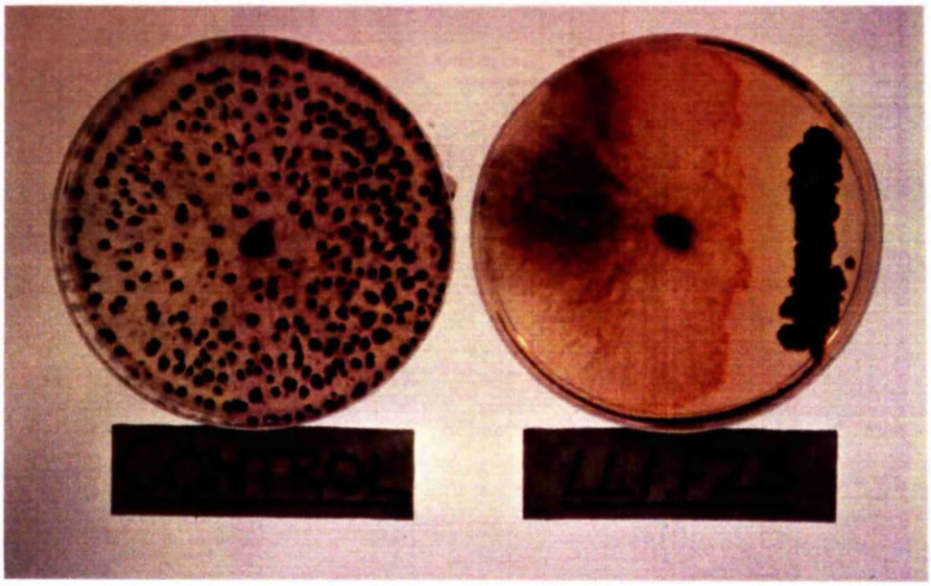
(a) Fungal isolate AG1.F4 showing significant inhibition of the radial growth of *B. fabae* of 31.2% and an average inhibition zone of 4.8 mm.

(b) Isolate LL1.F23 was the most impressive bacterium when screening *in vitro*, with an inhibition of radial growth of 66.07%, an inhibition zone of 11.2 mm and complete inhibition of sclerotia formation on the agar plate. The edge of the *B. fabae* colony was also malformed.

(a)



(b)



smaller inhibition zones than bacterial isolates in this study (Fig. 3.3). All but one of the fungal isolates screened here (DL1.F19) caused some degree of browning of *B. fabae* mycelium.

None of the fungal isolates screened for antagonism enhanced the pathogen's growth. Heavy sporulation by some fungal isolates soon after inoculation, gave rise to secondary colonies on the agar plates, distorting inhibition zones. In such cases the level of antagonism measured by the fungi could be altered or underestimated. It was therefore necessary to handle isolates carefully when measuring growth, but impossible to avoid spore release and spreading over the plates.

3.3.2.3 Antibiotic production

These experiments were conducted in order to determine if any antibiotics were released into the agar medium *in vitro*, and to measure their effects on the growth of *B. fabae* mycelium. There was a high loss of replicates, and even some treatments, through severe contamination of the agar plates containing fungal cultures (probably resulting from rapid sporulation). This occurred when removing the sterilised cling film and the mycelium of each sporing fungal isolate when assessing the production of non-volatile antibiotics.

Non-volatile antibiotic production for a number of fungal isolates was assessed by comparing mycelial growth of treated *B. fabae* to controls (Table 3.6). Only a few isolates reduced growth of *B. fabae* significantly, and at no time was complete mycelial inhibition induced. MX1.F62 reduced growth to 14% of the control after only 2 days growth from inoculation.

Table 3.6

Effects of non-volatile antibiotic production from selected fungal isolates, on the growth of *B. fabae in vitro*. Values are means of 5 replicates ± Standard error of the mean. (Percentage of the control in parentheses).

Isolate	Diameter of *B. fabae* colony (mm)		
	Day 2	Day 3	Day 5
Control	11.66 ± 3.00	26.00 ± 4.67	-
MX1.F62	1.67 ± 1.00 (14)[a]	4.35 ± 2.65 (17)	-
AP1.S20	5.22 ± 1.06 (45)[a]	22.68 ± 8.91 (87)[a]	-
AP2.R16	6.83 ± 1.50 (58)	14.15 ± 2.85 (54)	-
AG1.F4	3.90 ± 0.59 (33)[a]	15.00 ± 2.52 (58)	-
SED	2.40	7.50	-
Control	3.00 ± 0.18	17.60 ± 1.64	38.00 ± 0.45
MB2.F45	2.33 ± 0.14 (78)	17.32 ± 0.52 (93)	37.32 ± 0.43 (98)
Z1.S23	2.22 ± 0.11 (74)	17.63 ± 0.33 (99)	36.77 ± 0.39 (97)
MX1.F62[c]	-	-	-
MX1.L32	1.92 ± 0.34 (64)	4.30 ± 0.12 (24)	-
MX1.L33[c]	-	-	-
MX1.L34[c]	-	-	-
AP1.S20[c]	-	-	-
AP2.R16[c]	-	-	-
AG1.R4	1.92 ± 0.70 (64)	12.65 ± 4.27 (72)	27.82 ± 9.28 (72)[a]
AG1.R5	1.53 ± 0.08 (51)	10.92 ± 0.66 (62)[a]	36.54 ± 0.33 (96)
AG1.R7	2.75 ± 0.32 (92)	19.65 ± 0.90 (112)	37.00 ± 0.12 (97)
AG1.R13	2.00 ± 0.36 (67)	9.82 ± 2.35 (56)[b]	37.68 ± 0.77 (99)
AG1.R15	1.80 ± 0.23 (60)	13.14 ± 1.82 (75)[a]	35.86 ± 0.31 (94)
AGI.F4	1.80 ± 0.37 (60)	15.06 ± 1.40 (86)	33.68 ± 1.42 (89)
SED	1.29	2.37	3.70

a significant difference from control = P<0.1
b significant difference from control = P<0.01
c isolates contaminated by fungal antagonist colonies

Table 3.7

Effects of volatile antibiotic production from selected fungal isolates, on the growth of *B. fabae in vitro*. Values are the means of 5 replicates ± Standard errors of the mean. (Percentage of the control in parentheses).

Isolate	Diameter of *B. fabae* colony (mm)		
	Day 2	Day 3	Day 5
Control	3.67 ± 0.19	22.23 ± 0.77	33.03 ± 0.88
MX1.F62	-	-	-
AP1.S20	2.00 ± 0.33 (54)[b]	8.50 ± 0.20 (83)[a]	5.15 ± 2.15 (76)[a]
AP2.R16	2.22 ± 0.29 (60)[b]	14.00 ± 1.01 (63)[b]	23.17 ± 1.64 (70)[b]
AG1.F4	2.22 ± 0.11 (60)[b]	17.57 ± 0.87 (79)[a]	26.77 ± 1.62 (81)
SED	0.313	1.17	2.09
Control	-	34.00 ± 1.53	32.33 ± 0.67
MX1.F62	-	29.00 ± 2.00 (85)[a]	21.00 ± 4.00 (65)[a]
AP1.S20	-	-	-
AP2.R16	-	23.33 ± 4.37 (67)[a]	23.33 ± 3.38 (72)[a]
AG1.F4	-	28.00 ± 2.08 (82)	25.00 ± 1.73 (77)
SED	-	3.93	3.40
Control	1.84 ± 0.16	25.35 ± 0.35	20.65 ± 17.6
MB2.F45	1.00 ± 0.33 (54)[a]	12.70 ± 11.0 (50)	39.00 ± 1.00 (189)
AP2.R15	1.33 ± 0.00 (72)	23.65 ± 1.65 (93)	38.85 ± 0.85 (188)
AG1.F4	1.33 ± 0.00 (72)	22.70 ± 1.00 (90)	38.50 ± 0.20 (186)
SED	0.261	7.90	12.5

a significant difference from control = $P<0.1$
b significant difference from control = $P<0.01$

Volatile antibiotics produced by a number of fungal isolates reduced mycelial growth of *B. fabae* by up to 50% (Table 3.7). Several isolates, when screened for a second time, produced smaller inhibitions in growth of *B. fabae*. In general, nearly all treatments reduced the growth of *B. fabae in vitro* compared to the control, but these reductions were not always statistically significant (Table 3.7).

3.3.2.4 Spore germination tests

After 12 h, only one of the six isolates tested, LL1.F23, showed significant reduction in germination, germ-tube length and appressorium formation compared to the control, whereas after 36 h four isolates significantly reduced germination compared to the control. Two of these isolates were fungal; AP1.S20 and AG1.F4 ($P<0.05$), and 2 were bacterial; LL1.F23 ($P<0.001$) and MB2.S5B ($P<0.05$), (Table 3.8).

Measurements of germ-tube lengths showed that bacterial isolate LL1.F23 was the only antagonist to significantly reduce *B. fabae* germ-tubes at both recording times (54% of the control at 12 h ($P<0.05$) and 40% of the control at 36 h ($P<0.001$)), (Table 3.9). Three isolates increased germ-tube growth of *B. fabae* conidia significantly ($P<0.05$) after 12 h (MB2.F45, AG1.F4 and MB2.F45), while this was reduced to two isolates (AG1.F4 and MB2.S5B) after 36 h.

The numbers of appressoria formed at 12 h showed that all isolates significantly increased percentage formation compared to the control, except for LL1.F23, where no significant difference was observed (Table 3.9). After 36 h only one isolate still gave significantly greater appressorium formation

Table 3.8

Results of fungal and bacterial isolates on the germination, germ-tube development and appressorium formation of *B. fabae* conidia *in vitro* after (a) 12 h and (b) 36 h incubation (± Standard error of the mean).

(a)

Isolate	Germination (%)[a]	Germ-tube length (m)[a]	Appressorium formation[a]
Control	87.33 ± 2.29	1.73 ± 0.16	10.00 ± 2.58
MB2.F45	82.67 ± 1.69	2.56 ± 0.13 x	37.17 ± 3.65 x
AP1.S20	81.33 ± 3.37	2.02 ± 0.18	23.67 ± 4.52 x
AP2.S16	81.33 ± 3.37	2.02 ± 0.18	35.17 ± 1.78 x
AG1.F4	79.33 ± 2.30	2.89 ± 0.20 x	29.83 ± 7.69 x
MB2.S5B	85.00 ± 1.44	3.85 ± 0.33 x	26.67 ± 3.75 x
LL1.F23	69.50 ± 2.66 **	0.95 ± 0.07 *	4.67 ± 1.48

(b)

Isolate	Germination (%)[a]	Germ-tube length (m)[a]	Appressorium formation[a]
Control	85.17 ± 1.58	3.48 ± 0.17	33.50 ± 7.08
MB2.F45	79.00 ± 1.29	3.49 ± 0.62	17.83 ± 2.74 *
AP1.S20	75.33 ± 2.11 *	3.07 ± 0.24	27.83 ± 3.26
AP2.S16	79.00 ± 2.50	3.31 ± 0.32	51.67 ± 5.08 x
AG1.F4	68.33 ± 3.44 **	4.49 ± 0.20 x	33.83 ± 3.95
MB2.S5B	72.00 ± 3.43 **	4.33 ± 0.31 x	19.33 ± 2.95 **
LL1.F23	46.17 ± 7.77 ***	1.36 ± 0.23 ***	4.33 ± 1.50 ***

a Significance differences from the control are as follows: P<0.05 *, P<0.01 **, P<0.001 ***.
x significance greater than control (P>0.05)

Table 3.9

Results of fungal and bacterial isolates on the germination, germ-tube development and appressorium formation of *B. fabae* conidia *in vitro* after (a) 12 h and (b) 36 h incubation. Results expressed as a percentage of the control.

(a)

Isolate	Germination (%)[a]	Germ-tube length (%)[a]	Appressorium formation (%)[a]
MB2.F45	95	114 x	372 x
AP1.S20	94	113	237 x
AP2.S16	97	109	352 x
AG1.F4	91	161 x	289 x
MB2.S5B	98	215 x	267 x
LL1.F23	80 **	54 **	47

(b)

Isolate	Germination (%)[a]	Germ-tube length (%)[a]	Appressorium formation (%)[a]
MB2.F45	93	101	54 *
AP1.S20	89 *	89	83
AP2.S16	93	95	155 x
AG1.F4	81 **	129 x	101
MB2.S5B	85 **	125 x	58 **
LL1.F23	55 ***	40 ***	13 ***

a Significance differences from the control are as follows: P<0.05 *, P<0.01 **, P<0.001 ***.
x significance greater than control (P>0.05)

compared to the control (fungal isolate AP2.S16), whereas isolates MB2.F45 ($P<0.05$), LL1.F23 ($P<0.001$) and MB2.S5B ($P<0.01$) significantly reduced appressorium formation. AP1.S20 and AG1.F4 were no different from control cultures. This would suggest that the initial rate of appressorium formation in the control was much slower than in most of the samples containing antagonists and either its rate of appressorium formation increased, or that the rate of appressorial formation in the presence of the antagonists (MB2.F45, MB2.S5B and LL1.F23) declined, between the 12 h and 36 h recordings. Isolate LL1.F23, gave similar levels of appressorium formation to the control at 12 h, but after 36 h from inoculation, only 13% of *B. fabae* conidia ($P<0.001$) formed appressoria (Table 3.2).

3.3.3 ANTAGONISM TO UROMYCES VICIAE-FABAE

3.3.3.1 Spore germination bioassay

Of the 14 fungal isolates screened, only two (AP2.R16 and AG1.F5) did not significantly affect germination of the control (Table 3.10). Indeed isolate AG1.F5 was not antagonistic to *B. fabae* when screened *in vitro*. Out of the 16 bacterial isolates screened (Table 3.10), 11 significantly affected germination, while the following isolates did not: MB2.F28, MX1.F2, P1.S13B, AP2.R13A and AP2.R12A.

Isolates LL1.F23 (W) and T1.S11A stimulated germination of the rust uredospores (Table 3.10). In this experiment the controls for these isolates displayed germination of uredospores of only 16%, while germination of LL1.F23(W) and T1.S11A was 84% and 62% respectively. Indeed, the level of germination of the

Table 3.10
Results of fungal and bacterial isolates germination of rust uredospores (*Uromyces viciae-fabae*) *in vitro*.

Fungal isolate	Percentage of control[a]		Bacterial isolate	Percentage of control[a]	
MB2.F45	90	*	MB2.F28	72	
Z1.S23	76	*	MB2.S19	37	*
MX1.F62	59	*	TFW.GS2	25	*
MX1.L32	84	*	T1.S1	44	*
MX1.L33	61	*	MX1.F2	82	
MX1.L34	68	*	MB2.S27	19	*
AP1.S20	78	*	LL1.F23(M)	10	*
AP2.R13	83	*	LL1.F23(W)	548[b]	
AP2.R15	79	*	MB2.S5B(M)	24	*
AP2.R16	100		A1.L2	24	*
AG1.R5	85	*	MB2.S20	76	*
AG1.R7	78	*	T1.S11A	404[b]	
AG1.F4	82	*	P1.S13B	118	
AG1.F5	95		AG1.R3		*
			AP2.R13A	89	
			AP2.R12A	61	

a significant difference from the control are shown as follows: $P<0.05$ *.
b significantly greater than the control ($P<0.05$)

controls varied greatly between experiments. This was likely to be due to variation in the age of uredospores and the environmental conditions prior to spore collection from bean plants, together with the variation of expression of self inhibitors present in the uredospores. It was very difficult to obtain spores of similar age and development in each batch of experiments. However, the potential of some fungal and bacterial isolates to reduce the germination of *U. viciae-fabae* uredospores significantly is displayed.

3.3.4 INFECTION AND LEAF STAINING STUDIES

3.3.4.1 *Uromyces viciae-fabae*

During the staining of leaf sections inoculated with bean rust, following the technique described by Shipton & Brown (1962), great difficulty was experienced when trying to successfully clear the leaf material before microscopic examination of the stained infection structures.

The length of time that leaf samples were kept in the lactophenol solution, together with the time allowed for boiling and simmering the solutions were varied, followed by clearing in chloral hydrate. Fifty minutes in the chloral hydrate did not always clear the leaves sufficiently for light microscopy. Stronger solutions were more successful.

Examination of mature pustules on bean leaves was easier, but at this stage of infection little data could be collected on the process of initial infection of the leaf and the subsequent sequence and timing of infection development. Bean leaves were stained and cleared 3, 6 and 24 h after inoculation of leaves.

Unfortunately, little signs of infection were visualised, with only a few uredospores and germ-tubes located even after the use of high inoculation densities. It seems likely that during the process of heating, or just through general handling of the leaf samples for staining, the fungal structures were washed off the leaves. Preliminary experiments using lactophenol without heat after leaf clearing, as described in Section 3.2.4.2, did not show any more success.

3.3.4.2 *Botrytis fabae*

As shown previously in this study, staining of infection structures *in vitro* proved very successful prior to examination (Section 3.3.2.4). Of course, with leaf disc studies, the leaf sample must be cleared for viewing of infection development of the pathogen. Chloral hydrate cleared the leaves but the tissue became brittle, proving difficult to handle without further damage. Lactophenol did not seem to clear the leaves fully to allow easy examination. The use of methanol, for at least 3 days, was the most successful at clearing the leaves without any structural damage to the leaf.

Samples were subsequently placed in lactophenol cotton-blue for staining. On microscopic examination, conidia and germ-tubes were visible on the leaf surface, but due to stain still on the samples, their examination was difficult. Leaf samples placed back into methanol for further clearing before mounting were the most successful, with infection structures most clearly visible. Either of the mounting medias used were successful.

Appressorial structures were visible on the leaves, with

their characteristic brown hyphal walls. Little data on germination of conidia, germ-tube growth and other infection structures were collected with antagonists.

3.4 Discussion

The foliage and soil samples collected and screened for antagonists are listed in Table 3.1. Omar *et al.*, (1989) found that in the phyllosphere of faba beans, bacteria and fungal populations increased with plant age to crop maturity and that the levels of bacteria greatly out numbered fungal colonies. The samples collected in this study were all taken at crop maturity before harvesting in the Autumn. The majority of isolates in this study were bacterial (82%), so the use of antibiotics for selection in the culture media was essential to enable a reasonable number of fungi to be isolated.

Any applied organism must be harmless to plant tissue that it is to protect, both as a growing plant and through the subsequent storage of the crop. This includes many potential antagonists which are weak parasites or which produce toxic or tainting metabolites (Skidmore, 1976).

The use of detached leaves to assess host pathogenicity was of value, allowing the rejection of 19% of potential antagonists early in the screening process. The variability of the results from these tests could be explained by the use of only two replicate petri dishes per treatment and with an uncontrolled inoculum size, although a total of 12 inoculation

sites were used per treatment. More replication was not feasible because of the large number of isolates to be screened at this stage. It is also possible that some of the necrosis resulted from physical damage to the detached leaves during sterilisation or inoculation of samples. Hanounik & Maliha (1984) classified infection of bean leaves by *B. fabae* on a scale of 1 to 10 for assessment.

Interactions between test isolates of bacteria and fungi, and *B. fabae* resulted in either: the production of a zone of inhibition, contact inhibition or no inhibition. Measuring radial growth of *B. fabae* and inhibition zones created by each isolate allows discrimination between different types of interaction, such as inhibition at a distance or by contact. Thus, Fokkema (1973) pointed out that variation in growth rates of saprophytes can affect the radial growth of the pathogen independent of their antagonistic actions, whereas the zone of inhibition remains unaffected. On the other hand, the width of the inhibition zone may also be affected by the retardation of the growth of the saprophytes. A significant inhibition of radial growth is therefore not on its own, necessarily a sign of antagonism. The width of the inhibitory zone measured on agar is not necessarily related to the size of the bacterial or fungal colony produced (Baker & Cook, 1974). In the present study, inhibition plate tests were carried out until the growth of the isolates reached a plateau or equilibrium of growth (after 12-16 days) and only those which inhibited both parameters measured significantly, were considered to be antagonistic towards the pathogen (Melgarejo, Carrillo & M-Sagasta, (1985).

Appressorial structures of plant pathogens are thought to be more resistant to antagonism than other structures and are used by fungi for short term survival (Lenne & Parbery, 1976). These authors also reported that appressorium formation by the pathogen was enhanced by the presence of bacteria and reduced in nutrient solutions. The presence of high nutrient levels can cause germ-tubes of some fungi to elongate and branch instead of remaining short producing appressorium (Emmett & Parbery, 1975).

In this series of germination experiments carried out on *B. fabae* conidia, there was reduced appressorium formation after 36 h compared to 12 h. This possibly results from nutrient leakage and diffusion from *B. fabae* conidia in these test solutions over time and so suppressing appressorium formation (Austin *et al.*, 1977). Lenne & Parbery (1976) commented, that any treatment with an antagonist which decreases appressorium production could be important in control of diseases caused by such pathogenic fungi, where the addition of nutrients enhanced disease control by stimulating bacterial multiplication with deleterious effects on the fungus. Appressoria are structures enabling a fungus to establish a nutritional relationship with its host where initiation of appressoria is possibly a response to exhaustion of endogenous energy reserves (Emmett & Parbery, 1975).

Lenne & Parbery (1976) applied nutrients which enhanced disease control, where the nutrients applied stimulated bacterial multiplication. Bacteria and fungi can create a steep concentration gradient of nutrients towards conidia by absorbing them. During these germination tests, there was an accumulation of bacterial cells around the *Botrytis* conidia and their germ-

tubes. Austin *et al.*, (1977) noticed a similar build up of bacteria around the pathogen's conidia and germ-tubes, causing a reduction in spore germination and a reduced rate of elongation. They found antagonism was increased in the presence of additional nutrients possibly increasing bacterial populations, but emphasised that other interactions beside nutrient competition were involved.

Removal of nutrients from the phyllosphere or interaction site is a reliable means of biological control of foliar diseases (Elad, 1990). Nutrient competition is intense on aerial plant surfaces and is an important aspect of antagonism towards necrotrophic pathogens, including *B. cinerea* (Blakeman & Fokkema, 1982), as discussed earlier (Section 1.5.4). *B. cinerea* has a nutrient dependent phase of mycelial development on the leaf surface (Fokkema, 1978) and so the pre-penetration growth stage is likely to be the most antagonised stage. The greater the delay in penetration, the greater the opportunity for antagonism. There are less requirements for exogenous nutrients to promote germination of conidia of those *Botrytis* spp. that are host-specialised such as *B. fabae* (Rossall & Mansfield, 1980), which can achieve almost 100% germination in water *in vitro* (Last, 1960).

The majority of isolates screened in this study displayed inhibition at a distance, which is commonly ascribed to production of antibiotics (Fokkema, 1973). Initial studies here showed that a number of isolates have the potential to produce antibiotic substances *in vitro*, reducing *B. fabae* growth significantly. However, nutrition can affect antibiotic production, and other factors including the depth of agar, age

of plates, amount of inoculum and incubation temperature may also be important (Fravel, 1988).

Plate antibiosis tests have shown variation in screening results where different agar media have been used (Whipps, 1987). A screening programme based on utilising a selection of media would loose the major advantages of simplicity and cheapness (Renwick, Campbell & Coe, 1991). In the present work it can be concluded that antibiotics were produced by fungal isolates *in vitro* which significantly reduced the mycelial growth of *B. fabae*. However, the technique used in this study was possibly not sensitive enough to measure differences in volatile or non volatile antibiotic production between isolates.

Both leaf washings and diffusates from detached leaves of faba bean plants were shown to have strong inhibitory effects on chocolate spot development (Hanounik & Hasanain, 1986). Bacteria have also been isolated that can suppress *B. fabae in vitro* (Verdie & Le Normand, 1984).

Unfortunately, the preliminary studies of leaf staining were not successful for the examination of rust infection structures. A similar technique for staining rust uredospores to that of Shipton & Brown (1962) was outlined (White & Baker, 1954) when assessing infection of another biotrophic pathogen-host system, powdery mildew of barley. The use of a fixative, e.g. absolute alcohol-glacial acetic acid (1:1 v/v) solution (Erb *et al.*, 1973) prior to leaf clearing and staining should be examined.

Petersen (1956) used collodion to study the distribution

of stomata and their penetration by germ-tubes of *Puccinia graminis* f.sp. *tritici*. This impression technique used cellulose acetate as the impression medium and allowed the successful examination of infection structures across the leaf.

These methods used for infection and leaf staining studies *in vivo* do show potential, but clearly further work is required before we obtain detailed assessment of the infection structures of both pathogens on faba bean leaves, relative to antagonist populations.

The main problem with screening micro-organisms is the decision to disregard potential antagonists on the basis of failure to cause inhibition on agar (Fokkema, 1976). Blakeman & Fokkema (1982) point out that failure of candidates to produce inhibition zones *in vitro* should never lead to rejection, because nutrient competition is seldom detected in this way. Selection on agar plates *in vitro* has caused concern to various researchers, relating to nutrient solubility/ availability, concentration and distribution in space (Dowding, 1978). Saprophyte antagonists are often better able to use readily available nutrients in agar than the pathogen (Baker & Cook, 1974). Both Dowding (1978) and Elad (1990) point out that there are high nutrient concentrations *in vitro*, which are in different proportions to natural conditions in the phyllosphere. This could mask the effects of nutrient competition in pure cultures. It is unlikely that any 2 members being assessed in dual culture will respond similarly to these high nutrient concentrations (Dowding, 1978). It should also be remembered that *in vitro*, antagonists are free from any competition from other microbes. Not all interactions, of course, involve

competition for available nutrients.

The use of *in vitro* techniques allows large numbers of candidate micro-organisms to be assessed and screened reasonably quickly compared to studies on the leaf surface (Baker & Cook, 1974), increasing the chances of isolating antagonists. The advantages of the agar plate technique include its striking visual effects with zones of inhibited growth, being easy to conduct and interpret, and that environmental conditions can be easily adjusted and controlled (Andrews, 1985). The agar techniques should be included in any screening programme because: (i) *in vivo* techniques can be too severe, rejecting potentially useful candidates, (ii) agar plate method gives clues to presumptive mode of antagonism and the system for further study and (iii) agar plates are a convenient way to compare isolates (Andrews, 1985).

On the other hand, Elad (1990) points out that in the search for biological control agents, experiments carried out *in vitro* are poor predictors of *in vivo* performance, as there is no significant correlation demonstrable between culture and effectiveness in the field (Andrews, 1985). Mechanisms other than those measured in petri dishes can be important in field crop conditions, for example, the ability of saprophytes to live and grow on the plant surface. This not selected when screening *in vitro* (Elad, 1990).

Nevertheless, agar plate methods of selection and screening can be useful as an integrated approach. The judicious use of agar media has a useful function in preliminary tests on individual antagonists before subsequent testing in the glasshouse and out in field conditions (Baker & Cook, 1974).

Indeed, the programme discussed in this thesis to select antagonists against *B. fabae* has yielded some potentially useful isolates. The *in vivo* screening of those isolates will be discussed later.

4.

4. IN VIVO SCREENING OF BACTERIAL AND FUNGAL ISOLATES FOR ANTAGONISM TO BOTRYTIS FABAE AND UROMYCES VICIAE-FABAE

4.1 Introduction

After screening potential antagonists *in vitro*, the next logical step was to screen selected isolates *in vivo*. Those isolates showing greatest antagonism towards *B. fabae* and *U. viciae-fabae* *in vitro* need to be further investigated to determine which of their characteristics are related to their ability to control either or both of the pathogens. The reduced level of available nutrients in the field situation compared to *in vitro* agar screens will possibly affect the ability of antagonists to reduce disease levels. Here, the effects of both fungal and bacterial isolates in disease development and symptom expression will be examined on the bean leaf surface, giving a more realistic assessment of any potential antagonism that might occur in the field.

The use of antagonists to control plant diseases can depend on whether high populations of saprophytes can be created on the leaf surface. Often such high densities of saprophytes do not occur naturally (Fokkema *et al.*, 1979) and thus large inoculum levels are required. The natural buffering capacity of the microflora in the faba bean phyllosphere will possibly have a influence on any potential antagonists and their ability to reduce disease levels.

4.2 Materials and Methods

4.2.1 CHOCOLATE SPOT SCREENING

Plant material used in the following experiments was grown as described in Section 2.1 and *B. fabae* maintained and conidia collected as described earlier (Section 2.3.1).

Potential fungal and bacterial antagonists selected from *in vitro* trials, were applied to the leaf surface, either simultaneously with *B. fabae* conidia, or 2 days prior to the application of *B. fabae* conidia. Spore concentrations of fungal antagonists of 1×10^6 spores ml^{-1}, and bacterial antagonists at a concentration of 1×10^9 cells ml^{-1} were applied to the plants. Control treatments used concentrations of *B. fabae* conidia of either 5×10^5 or 1×10^5 spores ml^{-1}. There were 2 controls, one for each inoculation time of the antagonists.

Fungal isolates were grown on PDA media for 7 days. Spores were dislodged from the cultures with a scalpel, followed by filtration through four ply cheesecloth to remove any mycelial debris. Bacterial isolates were grown on NA media, inoculated with sterilised loops using the streak plate method. After 7 days growth, the plates were flooded with distilled water containing Tween 80 (0.01%) as a surfactant and the bacterial cells dislodged using a glass rod (Lenne & Parbery, 1976). Conditions utilised for growth of fungal and bacterial isolates were as described earlier (Section 2.4).

Aerosol sprayers (Fisons Ltd.) were used for application of antagonists to the plant surface, spraying targeted leaves until run-off. After each inoculation time, the plants were covered for 48 h to allow germination and infection of *B. fabae*

conidia and fungal antagonist spores or growth of antagonistic bacterial cells.

For each isolate screened, there were 4 replicate plants with 6 leaves per plant for each of 3 treatments:-

(1) Control: *B. fabae* conidia only

(2) *B. fabae* conidia, and fungal spores or bacterial cells applied simultaneously

(3) *B. fabae* conidia, with fungal spores or bacterial cells applied 2 days earlier

The development of *B. fabae* infection on each leaf (percentage leaf area covered with lesions) was recorded between 4 and 10 days after inoculation with *B. fabae* conidia.

4.2.1.1 Screen 1.

The following fungal isolates were screened: MX1.L32, AP1.S20, AP2.R16 and AG1.F4, with the following bacterial isolates: MB2.S5B, MB2.S7, MB2.F28 and LL1.F23. The potential antagonists were only applied to the leaf surface 2 days prior to the inoculation of *B. fabae* conidia.

4.2.1.2 Screen 2.

This screen was identical to Screen 1. Fungal isolates: MX1.L32, AP1.S20, AP2.R16 and AG1.F4, were screened with the following bacterial isolates: MB2.S5B, MB2.S7, MB2.F28 and LL1.F23. The potential antagonists were only applied to the leaf surface 2 days prior to the inoculation of *B. fabae* conidia.

4.2.1.3 Screen 3.

The following fungal isolates were screened: AP1.S20, AG1.R8 and AG1.F4. The potential antagonists were applied to the leaf surface 2 days prior to, or simultaneously with, *B. fabae* conidia.

4.2.1.4 Screen 4.

The following fungal isolates were screened: MB2.F45, MX1.L32, AP2.R16 and AG1.F4, with the following bacterial isolates: MB2.S5B, LL1.F23. The potential antagonists were applied to the leaf surface 2 days prior to, or simultaneously with, *B. fabae* conidia.

4.2.1.5 Screen 5.

The following fungal isolates were screened: Z1.S23, MX1.L33, MX1.L34, MX1.F62, AP2.R15, AP2.R19, AG1.R5 and AG1.R13, with the following bacterial isolates: AP2.R8 and P1.S13B. The potential antagonists were applied to the leaf surface 2 days prior to, or simultaneously with, *B. fabae* conidia.

4.2.2 RUST SCREENING

Plant material used in the following experiments was grown as described in Section 2.1 and *Uromyces viciae-fabae* maintained and uredospores collected as described previously (Section 2.2).

Potential fungal and bacterial antagonists selected from *in vitro* trials were applied to the leaf surface, either simultaneously with, or 2 days after, bean rust uredospores.

Concentrations of fungal antagonists of 1×10^6 spores ml^{-1} and bacterial antagonists of 1×10^9 were applied to the plants. Control treatments of 5×10^4 rust uredospores ml^{-1} were used.

Fungal isolates were grown on PDA media for 7 days. Spores were dislodged from the cultures using a scalpel followed by filtration through four ply cheesecloth to remove any mycelial debris. Bacterial isolates were grown on NA media, inoculated with sterilised loops using the streak plate method. After 7 days growth, the plates were flooded with distilled water containing Tween 80 (0.01%) as a surfactant and the bacterial cells dislodged using a glass rod (Lenne & Parbery, 1976). Conditions utilised for growth of fungal and bacterial isolates were as described earlier (Section 2.4).

Aerosol sprayers (Fisons Ltd.) were used for application of antagonists to the plant surface, spraying targeted leaves until run-off. After each inoculation time the plants were covered for 48 h to allow germination and infection of rust uredospores, fungal antagonist spores or the growth of antagonistic bacterial cells.

For each isolate there were 4 replicate plants, using 6 leaves for each of 3 treatments:-

(1) Control: rust uredospores only

(2) Rust uredospores, and fungal spores or bacterial cells applied simultaneously

(3) Rust uredospores, with fungal spores or bacterial cells applied 2 days later

The following measurements were recorded between 13 and 15 days after inoculation with *U. viciae-fabae* uredospores:-

(1) Assessment of infection on each leaf (% leaf cover with pustules)
(2) Number of pustules cm^{-2} on each leaf (3 measurements per leaf)

4.2.2.1 Screen 6.

The following bacterial isolates were screened: MB2.F28, MB2.S5B(M), MB2.S5B(W), MB2.S6, MB2.S7, T1.S5B, T1.S14, A1.L2, LL1.F23(M), LL1.F23(W) and P1.S13B. The antagonists were applied simultaneously with, or 2 days after, inoculation with rust uredospores. In this experiment separate controls were inoculated for each bacterial isolate screened.

4.2.2.2 Screen 7.

The following bacterial isolates were screened: MB2.S5B(M), MB2.S5B(W), MB2.S7, T1.S1, TFW.GS2, LL1.F23(M), LL1.F23(W), AP2.R12A and AP2.R13A. The antagonists were only applied simultaneously with rust uredospores. Again, in this experiment separate controls were used for each bacterial isolate screened.

4.2.2.3 Screen 8.

The following fungal isolates were screened: MX1.F62, MX1.L34, AP1.S20, AP2.R16 and AG1.F4. The antagonists were applied simultaneously with, or 2 days after, the inoculation with rust uredospores. In this experiment there were 2 controls, one for each inoculation time of the antagonists.

4.2.2.4 Screen 9.

The following fungal isolates were screened: MB2.F45, Z1.S23, MX1.L32, MX1.L33 and AP2.R15. The antagonists were applied simultaneously with, or 2 days after, inoculation with rust uredospores. In this experiment there were 2 controls, one for each inoculation time of the antagonists.

4.2.2.5 Screen 10.

The following fungal isolates were screened: AP2.R19, AG1.R5, AG1.R8 and AG1.R13, with the following bacterial isolates: MB2.S19, MB2.S7, MB2.S20, LL1.F21, AP2.R8 and AG1.R3. The antagonists were only applied simultaneously with the rust uredospores.

4.2.3 EFFECT OF FUNGAL ANTAGONIST CONCENTRATION ON THE DEVELOPMENT OF RUST INFECTION

The following fungal antagonists were selected for this experiment: MX1.F62, MX1.L34, AP1.S20 and AP2.R16. An examination was made of the effects of different spore concentrations on rust pustule development. Five concentrations of antagonists were applied, 2 days after inoculation with rust uredospores: 1×10^4, 1×10^5, 1×10^6, 1×10^7 and 1×10^8 spores ml^{-1}.

4.2.4 FUNGAL AND BACTERIAL ANTAGONIST IDENTIFICATION

Fungal and bacterial antagonists were selected for identification following *in vitro* and *in vivo* screening against

bean rust and chocolate spot. Fungal isolates were identified by the International Mycological Institute, Bakeham Lane, Egham, Surrey and bacterial isolates were identified by Mrs J.E. Sellwood (Bacterial Diagnosis) at the Central Science Laboratory (MAFF). The analysis of the bacteria was based on cellular fatty acid profiles which were compared to known profiles in their extensive library. Only 2 bacterial isolates were identified.

4.3 Results

4.3.1 CHOCOLATE SPOT SCREENING

4.3.1.1 Screen 1.

The results from this screen are displayed in Table 4.1. All of the isolates except MX1.L32 and MB2.F28, a fungal and bacterial isolate respectively, showed significant reductions in disease levels on the bean leaf surface ($P<0.05$). Three fungal isolates; AP1.S20, AG1.F4 and AP2.R16 showed the greatest inhibition ($P<0.01$) in lesion formation, reducing symptom expression to as little as 16% of the untreated control plants. Bacterial isolate, LL1.F23 reduced disease levels by 68%.

Table 4.1

Results from Screen 1 for antagonism to *B. fabae in vivo* by fungal and bacterial isolates, applied simultaneously with *B. fabae* conidia. Disease was estimated as the percentage leaf area covered with lesions (± Standard error of the mean).

Isolate	Infection (%)	Percentage of control[a]
Control	6.75 ± 1.42	
MX1.L32	4.25 ± 1.42	63
AP1.S20	1.08 ± 0.36	16 **
AP2.R16	1.50 ± 0.87	22 **
AG1.F4	1.25 ± 0.63	19 **
MB2.S5B	2.58 ± 1.24	38 *
MB2.S7	2.75 ± 1.13	41 *
MB2.F28	4.50 ± 1.42	67
LL1.F23	2.17 ± 0.96	32 *
SED	1.54	

a significant differences are shown as follows: P<0.1 *; P<0.01 **; P<0.001 ***.

4.3.1.2 Screen 2.

The isolates studied in Screen 1 were screened again to examine whether the methods used in this study (for *in vivo* screening and the inoculation procedure of bean plants with *B. fabae* conidia) could be considered consistent and standardised between batches. It was important to establish the repeatability of experiments on the leaf surface in a glasshouse environment, as there are so many factors that could influence micro-organism interactions in the phyllosphere.

The results in Table 4.2 show that all eight isolates screened reduced infection significantly compared to the controls ($P<0.05$). There was some variation in the order of antagonism among the isolates compared to Screen 1 (Table 4.1), but fungal isolate AP1.S20 showed the greatest reduction in lesion formation on bean leaves in both Screens 1 and 2. In this screen, disease levels were reduced to 20% of the control treatments by isolate AP1.S20. Bacterial isolate MB2.S7 reduced disease by 77%, compared with a 59% reduction in Screen 1 (Table 4.1). It was concluded that such small variations were acceptable in such experiments. In this screen there was a much higher level of infection in the control, rising from 6.75% in Screen 1, to 13.75% in Screen 2.

Table 4.2

Results from Screen 2 for antagonism to *B. fabae in vivo* by fungal and bacterial isolates, applied simultaneously with *B. fabae* conidia. Disease was estimated as the percentage leaf area covered with lesions (± Standard error of the mean).

Isolate	Infection (%)	Percentage of control[a]
Control	13.75 ± 2.89	
MX1.L32	3.75 ± 1.91	27 **
AP1.S20	2.83 ± 1.13	20 **
AP2.R16	4.92 ± 1.60	36 **
AG1.F4	6.42 ± 2.09	47 *
MB2.S5B	6.58 ± 3.63	48 *
MB2.S7	3.33 ± 2.13	23 **
MB2.F28	4.42 ± 0.96	32 **
LL1.F23	6.83 ± 3.29	50 *
SED	2.74	

a significant differences are shown as follows: P<0.1 *; P<0.01 **; P<0.001 ***.

4.3.1.3 Screen 3.

Table 4.3 and Fig. 4.1 show the results of the third screen of fungal isolates against chocolate spot formation by *B. fabae*. Potential antagonists were applied either 2 days prior to, or simultaneously with, *B. fabae* conidia. Applying the antagonists prior to *B. fabae* conidia reduced lesion development, with two of the fungal isolates (AP1.S20 and AG1.F4) reducing the % leaf area covered with lesions by 44% and 30% respectively, to the control ($P<0.01$ and $P<0.05$, respectively). When applied simultaneously, isolates AP1.S20 and AG1.R8 reduced disease symptoms on bean leaves. Isolate AP1.S20 reduced disease development in both treatments ($P<0.01$), even though levels of *B. fabae*, measured as the % leaf area covered with lesions, were considerably greater in this screen compared to the disease levels in Screens 1 and 2 (Table 4.1 and 4.2). Depending upon the time of inoculation with antagonists (i.e. prior to or simultaneously with *B. fabae*), experimental plants were probably covered with plastic bags (for conidial germination) for different periods of time. Since this covering has altered the micro-environment on the leaf surfaces of experimental plants, it was decided that a separate control was required at each time of inoculation of the antagonist.

Table 4.3

Results from Screen 3 for antagonism to *B. fabae in vivo* by fungal isolates applied either 2 days prior to, or simultaneously with, *B. fabae* conidia. Disease was estimated as the percentage leaf area covered with lesions (± Standard error of the mean).

	Different[a]	Simultaneous[b]
Isolate	Infection[c] (%)	Infection[c] (%)
Control	28.10 ± 2.87	23.32 ± 3.33
AP1.S20	15.80 ± 1.06 **	12.52 ± 1.24 **
AG1.R8	25.22 ± 3.90	15.28 ± 1.36 *
AG1.F4	19.80 ± 1.47 *	20.75 ± 2.25
SED	2.74	2.74

a antagonist inoculated 2 days prior to *B. fabae* conidia
b antagonist and *B. fabae* conidia inoculated simultaneously
c significant differences are shown as follows:
P<0.1 *; P<0.01 **; P<0.001 ***.

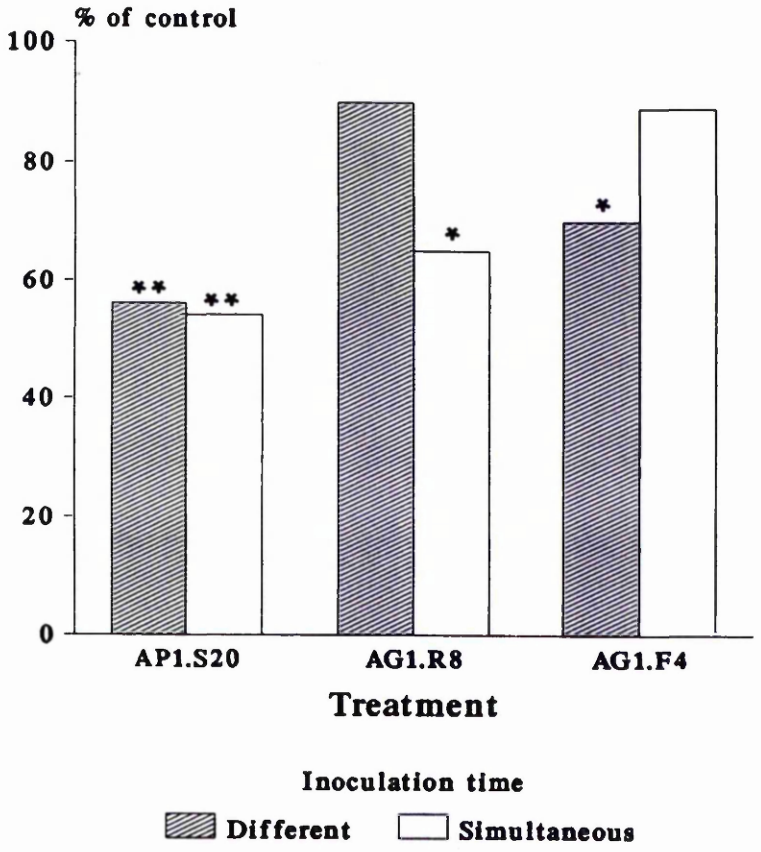


Fig. 4.1

Results from Screen 3 for antagonism towards *B. fabae in vivo* by fungal isolates applied either 2 days prior to, or simultaneously with, *B. fabae* conidia. Disease was estimated as the percentage leaf area covered with lesions, but are expressed here as % of the controls.

4.3.1.4 Screen 4.

The results from Screen 4 are shown in Table 4.4. It can be seen that all isolates, whether fungal or bacterial, when inoculated simultaneously with the *B. fabae* conidia, gave significant reductions in lesion development compared to the control ($P<0.05$). Bacterial isolate LL1.F23 produced the greatest effect, reducing disease to 49% of the control ($P<0.01$). Only two isolates, MB2.F45 (fungal) and LL1.F23 (bacterial), reduced infection significantly when applied prior to inoculation with *B. fabae* conidia (Table 4.4).

There is a possibility that the fungal spores or bacterial cells were desiccated when the bags were removed before the *B. fabae* conidia were applied. From the estimates of infection levels in both control treatments, there was no significant difference between them, suggesting that the conditions of infection were similar under both treatments. To try and make the conditions the same between the two different inoculation times, all the plants were covered with bags over the 4 day inoculation period.

Table 4.4

Results from Screen 4 for antagonism to *B. fabae in vivo* by fungal and bacterial isolates applied either 2 days prior to, or simultaneously with, *B. fabae* conidia. Disease was estimated as percentage leaf area covered with lesions (± Standard error of the mean).

	Different[a]		Simultaneous[b]	
Isolate	Infection (%)	Percent of control[c]	Infection(%)	Percent of control[c]
Control	21.02 ± 1.29		18.92 ± 0.66	
MB2.F45	11.45 ± 0.83	54 **	14.05 ± 1.28	74 *
MX1.L32	17.80 ± 2.78	85	14.92 ± 0.89	79 *
AP2.R16	19.62 ± 1.15	93	13.98 ± 0.96	74 *
AG1.R4	19.68 ± 2.49	94	14.58 ± 0.89	77 *
MB2.S5B	19.10 ± 0.77	91	4.42 ± 0.73	76 *
LL1.F23	11.30 ± 0.68	54 *	9.20 ± 0.90	49 **
SED	1.88		1.88	

a antagonist inoculated 2 days prior to *B. fabae* conidia
b antagonist and *B. fabae* conidia inoculated simultaneously
c significant differences are shown as follows:
P<0.1 *; P<0.01 **; P<0.001 ***.

4.3.1.5 Screen 5.

Results from this experiment were recorded on two separate days after inoculation (3 and 10). Three days after inoculation with *B. fabae*, severe necrosis of some infected leaves was observed. As a result, disease levels were recorded at this time in case of leaf loss, as well as 10 days after inoculation.

From the results displayed in Tables 4.5 and 4.6 only three isolates reduced disease significantly after 3 days, and four isolates 10 days after inoculation. The isolates which significantly reduced disease on the second recording day were Z1.S23, MX1.L34, AG1.R13 ($P<0.05$) and P1.S13B ($P<0.01$), when applied simultaneously with *B. fabae* conidia. The bacterial isolate P1.S13B was especially antagonistic, showing a reduction in disease levels to 16% and 12% of controls, 3 and 10 days after infection respectively.

Some isolates reduced disease levels when applied 2 days prior to *B. fabae* conidia, but not significantly. In fact, many isolates actually increased disease levels (Tables 4.5 and 4.6). These results are similar to those recorded in Screen 4, where isolates applied prior to *B. fabae* conidia, in general, were much less likely to reduce levels of chocolate spot.

Table 4.5

Results from Screen 5 for antagonism to *B. fabae in vivo* by fungal and bacterial isolates applied either 2 days prior to or simultaneously with *B. fabae* conidia. Disease was estimated as percentage leaf area covered with lesions (± Standard error of the mean), 3 days from inoculation.

	Different[a]		Simultaneous[b]	
Isolate	Infection (%)	Percent of control[c]	Infection(%)	Percent of control[c]
Control	6.88 ± 1.95		8.63 ± 1.32	
Z1.S23	7.33 ± 1.43	106	5.21 ± 1.39	60
MX1.L33	5.00 ± 1.52	72	8.71 ± 0.73	101
MX1.L34	9.50 ± 1.72	138	4.21 ± 1.02	61 *
MX1.F62	9.46 ± 1.32	138	8.00 ± 1.29	93
AP2.R15	7.12 ± 1.88	103	6.79 ± 0.92	79
AP2.R19	10.71 ± 1.55	155	5.86 ± 2.88	69
AG1.R5	8.62 ± 0.46	125	5.38 ± 1.02	63
AG1.R13	6.46 ± 1.14	93	3.79 ± 0.39	44 *
AP2.R8	5.08 ± 1.00	74	6.12 ± 2.34	71
P1.S13B	3.71 ± 1.08	54	1.37 ± 0.42	16 **
SED	2.03		2.03	

a antagonist inoculated 2 days prior to *B. fabae* conidia
b antagonist and *B. fabae* conidia inoculated simultaneously
c significant differences are shown as follows:
P<0.1 *; P<0.01 **; P<0.001 ***.

Table 4.6

Results from Screen 5 for antagonism to *B. fabae in vivo* by fungal and bacterial isolates applied either 2 days prior to or simultaneously with *B. fabae* conidia. Disease was estimated as percentage leaf area covered with lesions (± Standard error of the mean), 10 days from inoculation.

	Different[a]		Simultaneous[b]	
Isolate	Infection (%)	Percent of control[c]	Infection(%)	Percent of control[c]
Control	8.42 ± 1.74		11.22 ± 1.60	
Z1.S23	6.95 ± 1.63	83	5.28 ± 1.17	47 *
MX1.L33	6.95 ± 1.93	83	9.12 ± 0.85	81
MX1.L34	11.42 ± 1.58	136	6.08 ± 1.69	54 *
MX1.F62	12.70 ± 0.93	151	7.78 ± 1.45	69
AP2.R15	9.72 ± 1.78	115	7.22 ± 0.08	64
AP2.R19	13.38 ± 1.60	160	7.00 ± 2.25	62
AG1.R5	11.75 ± 1.05	140	9.60 ± 1.99	86
AG1.R13	9.02 ± 0.98	107	5.60 ± 1.22	50 *
AP2.R8	7.32 ± 1.02	87	10.08 ± 3.09	96
P1.S13B	5.70 ± 1.37	68	1.28 ± 0.34	12 **
SED	2.20		2.20	

a antagonist inoculated 2 days prior to *B. fabae* conidia
b antagonist and *B. fabae* conidia inoculated simultaneously
c significant differences are shown as follows:
P<0.1 *; P<0.01 **; P<0.001 ***.

4.3.1.6 Summary of results - Chocolate spot

From the results obtained in Screens 3, 4 and 5, the largest and most consistent reductions in chocolate spot infection were obtained with simultaneous inoculation of the antagonist with conidia of *B. fabae*. The fungal isolate AP1.S20 showed the greatest potential in these limited studies. This isolate provided the greatest reduction in disease levels in both Screen 1 and 2, of 74% and 80% respectively, together with significant reductions in disease in Screen 3. Isolate AG1.F4 also controlled chocolate spot, reducing disease to 19% of the control, respectively. Of the bacterial isolates, P1.S13B and MB2.S7 gave the greatest reduction in disease, reducing infection to 12% and 23% of the control. All of these isolates provided control when applied simultaneously with the pathogen.

With both bacterial and fungal isolates, the greatest levels of disease control were obtained with simultaneous inoculation of the antagonist with the pathogen. As discussed earlier, this could be a result of a failure in the methodology used in this study, possibly affecting the environmental conditions on the leaf surface, allowing the antagonist to be desiccated before the *B. fabae* conidia were applied to the leaf. On the other hand, these results may reflect the actual mechanism of antagonism displayed by these isolates.

The level of infection symptoms on bean leaves varied over each of the screens from 6.75 up to 28.10 percent of the leaf surface covered with lesions. The results are consistent throughout this studies, with isolates AP1.S20 and LL1.F23 both showing significant reductions in disease in different screens, ruling out the possibility of chance reductions in disease in

these treatments in individual screens.

4.3.2 RUST SCREENING

4.3.2.1 Screen 6.

From the results shown in Table 4.7, only a few of the bacterial isolates screened gave a significant decrease in rust infection. The following 3 isolates gave significant reductions ($P<0.05$) in measurements of the percentage of leaf area infected: T1.S14, LL1.F23(M) and MB2.F28. The isolate T1.S14 reduced rust infection significantly to 27% and 40% of the control, when applied simultaneously or 2 days after the rust uredospores, respectively. Isolate MB2.S5B(M), MB2.S7 and T1.S5B increased the levels of disease significantly ($P<0.05$) compared to the controls. Interestingly, when rust infection was determined as pustule number cm^{-2}, no significant difference between treatments and controls were observed. There was substantial variation between the controls for the different treatments, which could possibly have masked actual disease control by some bacterial isolates.

Table 4.7

Results from Screen 6 for antagonism to *U. viciae-fabae in vivo* by bacterial isolates applied either simultaneously with or 2 days after rust uredospores. Disease was estimated as percentage leaf area covered with pustules and pustule number cm^{-2} ± the standard error of the mean (See page 121 for footnotes).

Isolate	Infection (%)	Percent of control[c]	Pustule No.	Percent of control[c]
Control	10.00 ± 0.00		10.84 ± 3.40	
MB2.F28[a]	2.75 ± 1.13	28 *	7.92 ± 2.95	73
MB2.F28[b]	10.00 ± 0.00	100	16.92 ± 1.86	156
Control	6.50 ± 2.18		9.00 ± 2.88	
MB2.S5B(M)[a]	5.25 ± 1.84	81	11.67 ± 3.57	130
MB2.S5B(M)[b]	11.25 ± 4.73	173[d]	19.25 ± 5.38	214[d]
Control	4.00 ± 1.00		7.67 ± 1.92	
MB2.S5B(W)[a]	4.00 ± 1.00	100	9.58 ± 2.26	125
MB2.S5B(W)[b]	2.00 ± 1.00	50	4.83 ± 1.27	63
Control	7.50 ± 1.44		11.00 ± 1.66	
MB2.S6[a]	7.75 ± 2.25	103	15.83 ± 4.23	144
MB2.S6[b]	5.00 ± 0.00	67	9.92 ± 0.97	90
Control	6.26 ± 1.25		9.30 ± 1.91	
MB2.S7[a]	8.75 ± 1.25	140	16.33 ± 2.48	176[d]
MB2.S7[b]	6.50 ± 2.18	104	12.34 ± 2.97	133
Control	3.00 ± 1.15		6.75 ± 1.45	
T1.S5B[a]	7.50 ± 1.44	250[d]	13.08 ± 2.46	194
T1.S5B[b]	2.00 ± 1.00	66	5.50 ± 1.32	81
Control	7.50 ± 1.44		11.42 ± 1.71	
T1.S14[a]	3.00 ± 1.15	40 *	8.83 ± 2.93	77
T1.S14[b]	2.00 ± 1.00	27 *	5.40 ± 1.74	47
Control	7.50 ± 1.44		13.66 ± 2.62	
A1.L2[a]	7.50 ± 1.40	100	15.33 ± 3.29	112
A1.L2[b]	10.00 ± 0.00	133	16.66 ± 1.88	122
Control	8.75 ± 1.25		13.25 ± 2.41	
LL1.F23(M)[a]	12.50 ± 4.33	143	18.16 ± 4.07	137
LL1.F23(M)[b]	3.00 ± 1.15	34 *	7.50 ± 2.05	56
Control	3.00 ± 1.15		5.50 ± 1.32	
LL1.F23(W)[a]	5.00 ± 0.00	167	10.92 ± 1.45	198
LL1.F23(W)[b]	5.00 ± 0.00	167	6.59 ± 1.56	120
Control	6.50 ± 2.18		8.34 ± 3.71	
P1.S13B[a]	7.50 ± 1.44	115	7.92 ± 0.34	95
P1.S13B[b]	3.00 ± 1.15	46	10.92 ± 5.39	131
SED	2.10		3.14	

Notes on **Table 4.7**

a antagonist inoculated simultaneously with rust uredospores
b antagonist inoculated 2 days after rust uredospores
c significant differences are shown as follows:
P<0.1 *; P<0.01 **; P<0.001 ***.
d significantly greater than the control (P<0.05)

4.3.2.2 Screen 7.

In this experiment, 9 bacterial isolates were screened, with the antagonists applied simultaneously with the rust uredospores. Two isolates gave significant reductions in disease, estimated either as: number of pustules cm^{-2} or percentage leaf area infected. These isolates were LL1.F23(M) and MB2.S7, which both gave large reductions in disease, especially MB2.S7 which gave greater than 80% control of rust infection (Table 4.8; Fig 4.2). Some of the other isolates only showed significance with one of the measurements recorded.

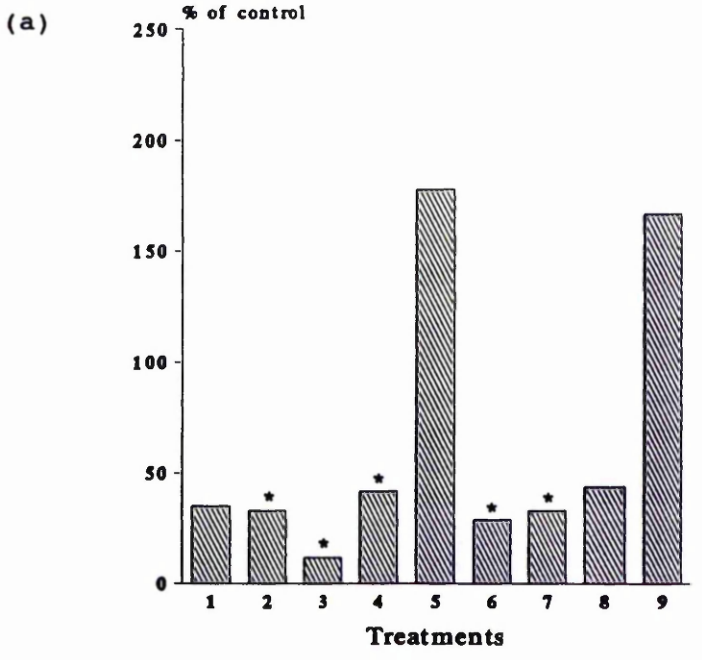
Table 4.8

Results from Screen 7 for antagonism to *U. viciae-fabae in vivo* by bacterial isolates applied simultaneously with rust uredospores. Disease was estimated as the percentage leaf area covered with pustules and pustule number cm^{-2} (± Standard error of the mean).

Isolate	Infection[a] (%)	Pustule No.[a]
Control	2.21 ± 0.69	5.42 ± 1.94
MB2.S5B(M)	0.75 ± 0.37	1.79 ± 0.54 *
Control	2.62 ± 1.21	2.46 ± 0.72
MB2.S5B(W)	0.88 ± 0.35 *	2.12 ± 1.03
Control	3.12 ± 0.76	6.83 ± 2.03
MB2.S7	0.38 ± 0.18 *	1.21 ± 0.30 *
Control	4.25 ± 1.08	5.33 ± 1.85
T1.S1	2.00 ± 0.54 *	2.75 ± 0.55
Control	1.12 ± 0.44	2.25 ± 0.78
TFW.GS2	2.00 ± 0.38	3.42 ± 0.58
Control	2.62 ± 1.21	5.17 ± 2.09
LL1.F23(M)	0.75 ± 0.37 *	2.00 ± 0.86 *
Control	2.62 ± 0.65	4.17 ± 0.96
LL1.F23(W)	0.88 ± 0.35 *	1.58 ± 0.60
Control	2.00 ± 0.54	1.96 ± 0.60
AP2.R12A	0.88 ± 0.35	1.67 ± 0.60
Control	1.12 ± 0.30	0.96 ± 0.44
AP2.R13A	1.88 ± 0.58	2.08 ± 0.58[b]
SED	0.79	1.32

a significant differences are shown as follows: $P<0.05$ *; $P<0.01$ **; $P<0.001$ ***.

b significantly greater than the control ($P<0.05$)



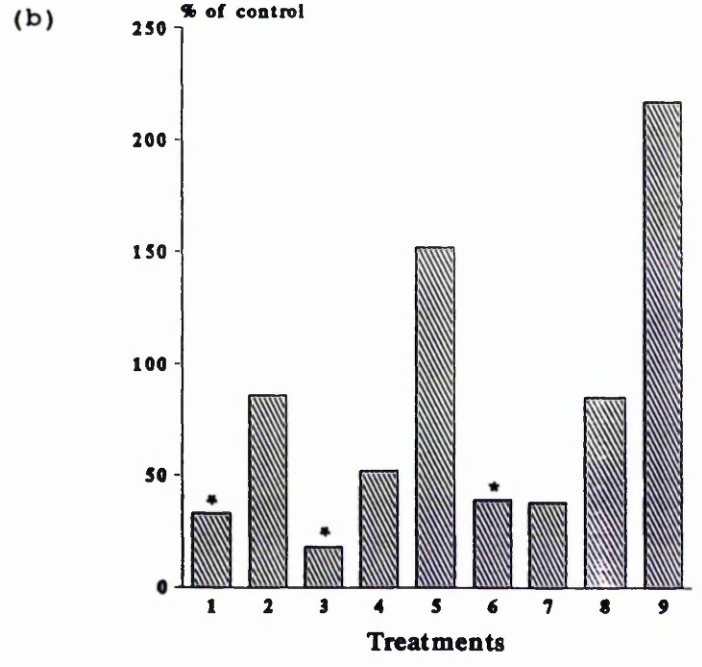


Fig. 4.2

Results from Screen 7 for antagonism towards *U. viciae-fabae in vivo* by bacterial isolates applied simultaneously with the inoculation of rust uredospores. Disease was measured as (a) the percentage leaf area covered with pustules and (b) pustule number cm^{-2}, but are expressed here as the % of the controls.

Isolates: 1, MB2.S5B(M); 2, MB2.S5B(W); 3, MB2.S7; 4, T1.S1; 5, TFW.GS2; 6, LL1.F23(M); 7, LL1.F23(W); 8, AP2.R12A; 9, AP2.R13A.

4.3.2.3 Screen 8.

The fungal isolates screened here were selected from *in vitro* germination tests (Section 3.3.3.1). In general, taking into account both measurements of disease levels, there was better control of rust when the antagonists were applied simultaneously with the rust spores rather than two days later. However, the greatest and most significant reductions in disease were obtained when antagonists were applied 2 days after rust uredospores (Table 4.10). Isolate AP1.S20 reduced the pustule numbers on the bean leaf surface to 18% of the control ($P<0.01$). All the isolates showing significant reduction in disease development when applied simultaneously and when disease was estimated as percentage leaf cover (Table 4.9), also showed significant reductions in the pustules formed on the leaf surface (Table 4.10). Isolate AP2.R16 gave the most consistent results when applied simultaneously, reducing rust infection by 57-78% ($P<0.01$; Tables 4.9 and 4.10).

Table 4.9

Results from Screen 8 for antagonism to *U. viciae-fabae in vivo* by fungal isolates applied either simultaneously with or 2 days after the inoculation of rust uredospores. Disease was estimated as the percentage leaf area covered with pustules (± Standard error of the mean).

	Simultaneous[a]		Different[b]	
Isolate	Infection (%)	Percent of control[c]	Infection(%)	Percent of control[c]
Control	4.29 ± 0.84		3.50 ± 0.42	
MX1.F62	2.29 ± 0.86	53 *	-	-
MX1.L34	2.50 ± 0.35	58 *	2.08 ± 0.32	59
AP1.S20	3.16 ± 0.49	74	1.33 ± 0.14	38 *
AP2.R16	1.08 ± 0.20	25 **	2.21 ± 0.57	63
AG1.F4	3.87 ± 0.21	90	5.46 ± 1.24	156
SED	0.86		0.86	

a antagonist inoculated simultaneously with rust uredospores
b antagonist inoculated 2 days after rust uredospores
c significant differences are shown as follows:
P<0.05 *; P<0.01 **; P<0.001 ***.

Table 4.10

Results from Screen 8 for antagonism to *U. viciae-fabae in vivo* by fungal isolates applied either simultaneously with or 2 days after the inoculation of rust uredospores. Disease was estimated as pustule number cm^{-2} (± Standard error of the mean).

	Simultaneous[a]		Different[b]	
Isolate	Pustule No.	Percent of control[c]	Pustule No.	Percent of control[c]
Control	6.12 ± 1.32		7.70 ± 1.24	
MX1.F62	3.76 ± 1.55	61 *	-	-
MX1.L34	3.18 ± 0.53	52 *	2.74 ± 0.60	35 **
AP1.S20	5.24 ± 0.84	85	1.40 ± 0.26	18 ***
AP2.R16	1.36 ± 0.34	22 **	3.33 ± 1.36	43 **
AG1.F4	5.48 ± 0.84	89	7.46 ± 1.76	97
SED	1.15		1.15	

a antagonist inoculated simultaneously with rust uredospores
b antagonist inoculated 2 days after rust uredospores
c significant differences are shown as follows:
P<0.05 *; P<0.01 **; P<0.001 ***.

4.3.2.4 Screen 9.

When rust infection was estimated as the % leaf area covered by pustules, infection level in the control was similar for both simultaneous application with the pathogen or application 2 days after the pathogen (Table 4.11). This was not, however, the case when rust infection was determined as pustule numbers per cm^2 (Table 4.12), where the levels of infection in the control was three times greater when the antagonists were applied after the uredospores, compared to simultaneous application.

When rust was estimated as % leaf cover infected, all five fungal isolates gave highly significant reductions in disease levels, whether applied simultaneously or after 2 days. This was especially true for isolate, AP2.R15, which gave reductions of 74% and 56% when applied simultaneously or after the rust uredospores, respectively. But when measuring the pustule number on the leaf surface, significant differences were obtained only when the antagonists were applied 2 days after the rust spores (Table 4.12). This difference between simultaneous application and application post pathogen inoculation was not observed on previous screens. This variation was possibly due to changes in environmental conditions and the influence of the conditions on the pathogen and antagonists.

Table 4.11

Results from Screen 9 for antagonism to *U. viciae-fabae in vivo* by fungal isolates applied either simultaneously with or 2 days after the inoculation of rust uredospores. Disease was estimated as the percentage leaf area covered with pustules (± Standard error of the mean).

	Simultaneous[a]		Different[b]	
Isolate	Infection (%)	Percent of control[c]	Infection(%)	Percent of control[c]
Control	3.80 ± 0.27		3.58 ± 0.34	
MB2.F45	0.88 ± 0.34	23 ***	1.58 ± 0.34	44 **
Z1.S23	2.40 ± 0.68	63 *	2.45 ± 0.30	68 *
MX1.L32	1.20 ± 0.24	32 **	1.82 ± 0.34	51 **
MXI.L33	1.38 ± 0.28	36 **	2.30 ± 0.30	64 *
AP2.R15	0.99 ± 0.46	26 ***	1.58 ± 0.38	44 **
SED	0.53		0.53	

a antagonist inoculated simultaneously with rust uredospores
b antagonist inoculated 2 days after rust uredospores
c significant differences are shown as follows:
P<0.05 *; P<0.01 **; P<0.001 ***.

Table 4.12

Results from Screen 9 for antagonism to *U. viciae-fabae in vivo* by fungal isolates applied either simultaneously with or 2 days after the inoculation of rust uredospores. Disease was estimated as pustule number cm^{-2} (± Standard error of the mean).

	Simultaneous[a]		Different[b]	
Isolate	Pustule No.	Percent of control[c]	Pustule No.	Percent of control[c]
Control	3.65 ± 0.88		9.80 ± 1.76	
MB2.F45	1.42 ± 0.40	54	3.75 ± 1.21	38 **
Z1.S23	4.62 ± 1.62	174	5.52 ± 1.31	56 *
MX1.L32	2.68 ± 0.79	101	4.18 ± 0.62	43 **
MX1.L33	2.20 ± 0.51	83	4.25 ± 0.34	43 **
AP2.R15	3.32 ± 1.42	125	3.28 ± 1.14	33 **
SED	1.57		1.57	

a antagonist inoculated simultaneously with rust uredospores
b antagonist inoculated 2 days after rust uredospores
c significant differences are shown as follows:
P<0.05 *; P<0.01 **; P<0.001 ***.

4.3.2.5 Screen 10.

In this experiment, the antagonists were inoculated simultaneously with rust uredospores (Table 4.13). None of the isolates tested, neither bacterial nor fungal, gave any significant ($P<0.05$) reductions in disease development. This could be due to the low level of infection of the bean leaves, as seen when comparing the controls in Table 4.13 with those of the previous rust screen. Many of the isolates increased disease levels, especially when measured as percentage leaf area infected, although not significantly.

4.3.2.6 Summary of results - Rust

In general, the most consistent reductions in disease levels were obtained with simultaneous inoculation of fungal antagonists with rust uredospores. Isolates AP2.R16 and AP1.S20 reduced the % leaf area covered with rust by 78% ($P<0.01$) and 82% ($P<0.001$) following simultaneous application, or 2 days after inoculation with the rust, respectively. Isolate AP1.S20 also reduced rust infection, but this was not as significant as recorded in screens earlier against *B. fabae* (Section 4.3.1.6).

When data from both measurements of infection, pustule number versus percentage infection were plotted against one another, there were high correlation values in all of the screens (between 0.551 and 0.876), calculated from regression analysis (Table 4.14). This analysis highlights the reliability of predicting one of the measurements of infection from the other, and suggests that both parameters measured are similar. Often pustules were not evenly distributed over the leaf

Table 4.13

Results from Screen 10 for antagonism to *U. viciae-fabae in vivo* by fungal isolates applied simultaneously with rust uredospores. Disease was estimated as percentage leaf area covered with lesions and pustule number cm^{-2} (± Standard error of the mean).

Isolate	Infection (%)	Percent of control[a]	Pustule No.	Percent of control[a]
Control	1.00 ± 0.50		0.74 ± 0.42	
AP2.R19	1.08 ± 0.46	108	0.99 ± 0.65	75
AG1.R5	1.28 ± 0.15	128	0.70 ± 0.29	106
AG1.R8	1.56 ± 0.29	156	1.00 ± 0.29	74
AG1.R13	0.89 ± 0.24	89	0.39 ± 0.20	52
MB2.S19	1.05 ± 0.15	105	0.64 ± 0.15	86
MB2.S7	1.22 ± 0.22	122	0.50 ± 0.20	68
MB2.S20	1.55 ± 0.40	155	1.37 ± 0.10	185
LL1.F21	1.33 ± 0.00	133	0.68 ± 0.03	109
AP2.R8	1.28 ± 0.36	128	0.59 ± 0.22	80
AG1.R3	1.56 ± 0.11	156	1.37 ± 0.51	185
SED	0.96		0.85	

a significant differences are shown as follows:
P<0.05 *; P<0.01 **; P<0.001 ***.

Table 4.14

Regression analysis (r^2, coefficient of determination) of growth measurements (Percentage infection of the leaf surface with pustules and the number of pustules per cm^2) of Screens 6 to 10.

Rust screen	r^2
6	0.860
7	0.737
8	0.831
9	0.551
10	0.876

surface, making counting pustules difficult and possibly unreliable as a means of assessing infection. In some experiments, the bags used to facilitate spore germination touched the leaf surface, encouraging either a greater number of pustules or a concentration of pustules at the leaf tip. Thus, given the results of the regression analysis (Table 4.14), in future experiments rust infection could be estimated as the % leaf area infected.

4.3.3 EFFECT OF FUNGAL ANTAGONIST CONCENTRATION ON THE DEVELOPMENT OF RUST INFECTION

Fungal isolates selected for this experiment had already shown significant inhibition of *U. viciae-fabae* both during *in vitro* screening and on the bean leaf surface, as seen previously in this chapter. All four isolates (MX1.F62, MX1.L34, AP1.S20 and AP2.R16) gave significant control of rust infection (up to $P<0.001$) (Table 4.15; Fig. 4.3). Isolates AP2.R16 and MX1.L34 reduced infection significantly at all the concentrations applied, even as low as 1×10^4 antagonist spores ml^{-1}. The other two isolates, MX1.F62 and AP1.S20, did not provide significant control at the lowest concentration, but did at all the other concentrations: 1×10^5 to 1×10^8. Isolate MX1.L34 showed the most dramatic reductions in disease levels, reducing infection to 8% that of the control. In general, the higher the concentration of antagonist inoculum, the greater the reduction of rust on the leaf surface. The levels of disease control at 1×10^6 spores ml^{-1} were similar to those in earlier screens in this chapter.

This experiment reinforced the suitability of these fungal

Table 4.15

Control of rust infection using different spore concentrations of selected fungal isolates, applied 2 days after rust uredospores. Disease was estimated as the percentage leaf area covered with lesions and pustule number cm^{-2} (± Standard error of the mean).

Isolate	Conc[a]	Infection[b] (%)	Pustule No.[b]
Control	-	4.21 ± 0.91	5.48 ± 0.38
MX1.F62	1	3.42 ± 0.55	2.39 ± 0.48 **
	2	1.63 ± 0.70 **	1.08 ± 0.48 ***
	3	1.34 ± 0.28 **	1.07 ± 0.34 ***
	4	1.46 ± 0.38 **	2.62 ± 0.93 **
	5	0.86 ± 0.04 ***	1.00 ± 0.21 ***
MX1.L34	1	2.58 ± 0.31 *	2.42 ± 0.20 **
	2	1.75 ± 0.16 **	2.44 ± 0.41 **
	3	1.83 ± 0.39 **	2.22 ± 0.67 **
	4	1.42 ± 0.21 **	2.72 ± 0.34 **
	5	0.34 ± 0.15 ***	0.26 ± 0.12 ***
AP1.S20	1	4.12 ± 0.63	4.28 ± 0.74
	2	2.24 ± 0.35 **	2.71 ± 0.19 **
	3	1.33 ± 0.24 **	1.01 ± 0.33 ***
	4	1.67 ± 0.39 **	2.78 ± 0.94 ***
	5	0.54 ± 0.18 ***	0.47 ± 0.17 ***
AP2.R16	1	2.46 ± 0.52 *	2.89 ± 0.62 *
	2	1.84 ± 0.35 **	2.32 ± 0.34 **
	3	1.12 ± 0.18 ***	1.37 ± 0.41 **
	4	1.50 ± 0.23 **	2.08 ± 0.62 **
	5	0.66 ± 0.22 ***	0.71 ± 0.52 ***
SED		0.563	0.834

a Concentration of antagonist applied 1 = 1×10^4
2 = 1×10^5
3 = 1×10^6
4 = 1×10^7
5 = 1×10^8

b significant differences are shown as follows:
$P<0.05$ *; $P<0.01$ **; $P<0.001$ ***.

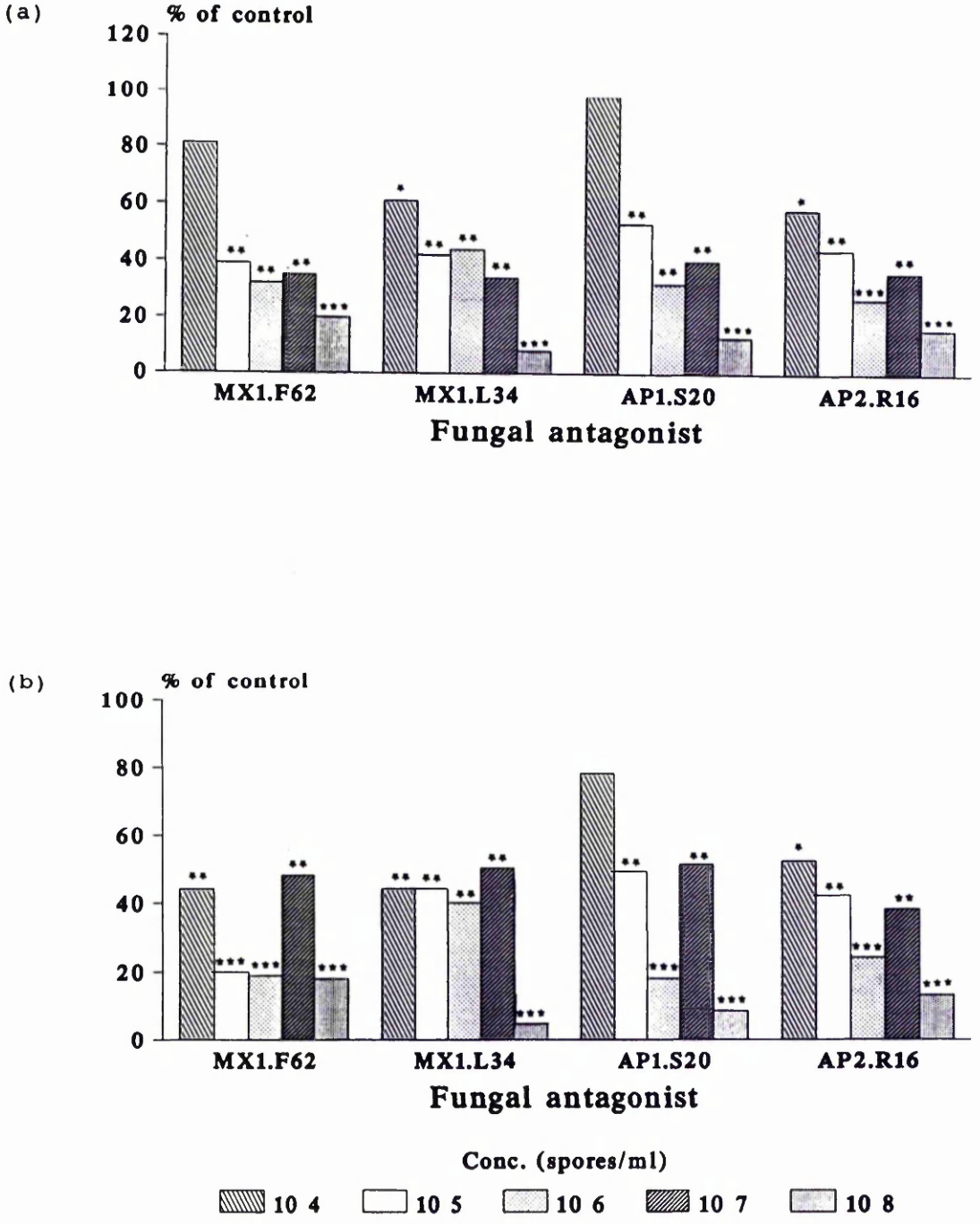


Fig. 4.3

Results from studying the effect of different fungal antagonists applied at a range of concentrations to the development of rust infection. Disease was measured as (a) the percentage leaf area covered with pustules and (b) the pustule number cm^{-2}, but expressed as the % of the control.

isolates for antagonism of rust uredospores, through direct application to the leaf surface, even at concentrations as low as 1 x 10^4 spores ml^{-1} (Fig 4.3).

4.3.4 ANTAGONIST IDENTIFICATION

4.3.4.1 Fungal isolates

Tabulated results obtained from the International Mycological Institute are presented in Table 4.16.

P. chrysogenum is one of the most widespread species of *Penicillium*. *P. brevicompactum* is an ubiquitous species, found in decaying vegetation, food and cereals. *C. cladosporioides* is commonly found in the phyllosphere.

4.3.4.2 Bacterial isolates

Tabulated results obtained from MAFF Central Science Laboratory are shown in Table 4.17. Only a few of the most promising bacteria have been identified at this stage.

Table 4.16
Results of identification of antagonistic fungal isolates.

Isolate[a]	IMI Number	Genus	Species
MB2.F45	352094	*Cladosporium*	*cladosporioides* (Fresen.) De Vries agg.
Z1.S23	352095	*Penicillium*	*chrysogenum* Thom.
MX1.F62	352098	*Penicillium*	*brevicompactum* Dierckx.
MX1.L32	352096	*Penicillium*	*brevicompactum* Dierckx.
MX1.L33	352097	*Penicillium*	*brevicompactum* Dierckx.
MX1.L34	354587	*Penicillium*	*brevicompactum* Dierckx.
AP1.S20	352091	*Penicillium*	*chrysogenum* Thom.
AP2.R15	352093	discarded	
AP2.R16	352093	*Penicillium*	*chrysogenum* Thom.
AP2.R19	354588	*Penicillium*	*chrysogenum* Thom.
AG1.R4[b]	-		
AG1.R5[b]	-		
AG1.R8[b]	-		
AG1.R13[b]	-		
AG1.F4	352090	*Geomyces pannorus*	(Link) Singler & J.W. Carmich.

a see Table 3.1 for details of sample origin and source
b isolates awaiting identification

Table 4.17
Results of identification of bacterial isolates.

Isolate[a]	Genus	Species
MB2.S5B	(growth unsuccessful)	
LL1.F23	*Pseudomonas*	*fluorescens*
P1.S13B	*Bacillus*	*cerus*

a see Table 3.1 for details of sample origin and source

4.4 Discussion

The biological control of *B. fabae* and *U. viciae-fabae* has been envisaged as the colonisation of aerial plant parts by antagonistic saprophytes, thus precluding subsequent invasion by these pathogens and infection of plant material (Renwick *et al.*, 1991). In this study, isolates were selected initially by their antagonism during *in vitro* screening (Chapter 3), followed by direct inoculation to the plant surface in glasshouse conditions, as described in this chapter. This allowed initial experiments to be carried out in a relatively controlled environment (Andrews, 1985), as the glasshouse environment is considerably more favourable for the growth of saprophytic populations of micro-organisms than field conditions.

Any *in vivo* screening methodology should be focused to make it more uniform, interpretable and amenable to large scale screening, thus facilitating accurate assessment of antagonistic potential. Andrews (1985) suggested the development of the 'model leaf' to incorporate more realistic conditions than agar tests, giving reproducability and ease of observation not usually attainable in glasshouse screening or *in situ* phyllosphere assays in the field. The best model system would seem to be detached leaf culture. It is important to optimise conditions for selection because, as mentioned earlier, selection of antagonistic micro-organisms underpins virtually all bio-control programmes.

In *in vivo* tests the amount of disease control is measured regardless of the mode of action regardless of the mode of action (Campbell, 1989). The overwhelming problem with *in vivo* screening is the time, effort, growing space and the

financial cost.

It has been suggested that tests assessing divergent properties on the leaf surface will be more complementary than those measuring essentially the same response (Andrews, 1985). In this study with the rust, 2 measurements of overall symptom development (% leaf area covered with pustules and the number of pustules in a given area of the leaf), were recorded. As both of these measurements were closely related, only one measure of infection really needs to be recorded. The estimation of % leaf area infected with pustules would save valuable time, as it is easier to record.

Newhook (1951a; 1951b), when studying the control of *B. cinerea* on lettuce, found that a reasonable period was needed for establishment of saprophytes on the leaf surface, together with a high humidity and moderate temperatures for effective biological control. Similar conditions were required in this study, where a 48 h period was needed for uniform artificial infection of rust or chocolate spot on the leaf surface, with the same conditions given to allow the antagonist population to establish. Newhook also found that alternation of temperature in glasshouse experiments reduced the efficiency of biological antagonism, although such alternation would be normal in the field. Failure of antagonists to perform *in vivo* may be solely due to rapid death of the antagonists on the phylloplane (Andrews, 1985).

We can see that in screening against chocolate spot and rust *in vivo*, considerable variation was observed among the isolates, with some displaying high levels of control of one or both pathogens. Baker & Stavely, (1985) used glasshouse

experiments to test the potential of suspensions of *Bacillus subtilis* on bean plants, and found that under field conditions, frequent application of the antagonist (say, once per week), was not sufficient to control the rust. Either this bacterium did not survive well on the foliage, or the inhibitory material controlling the pathogen was not produced under such conditions (Stavely *et al.*, 1981). *B. subtilis* has shown continued success in the control of a range of soil-borne pathogens (Baker *et al.*, 1983). Obviously these isolates will need to be screened on a larger scale, in either small plots or field trials here at Auchincruive.

Fokkema *et al.*, (1979) found that different nutrients sprayed simultaneously with micro-organisms, successfully increased the microflora on wheat. Exogenous nutrients of a natural origin, like pollen (Chou & Preece, 1968), or other nutrients e.g. yeast extract (De Cal *et al.*, 1990), have been shown to sustain and increase the microflora substantially. Where bio-control in the field has proved successful, nutrients were also applied to the plants (Bhatt & Vaughan, 1963; De Cal *et al.*, 1990). Again, this is a very important area for research on the formulation and application of any antagonists, and has a large bearing on antagonist establishment and growth in the phyllosphere. A drawback of enhanced saprophytic microflora might be an acceleration of leaf senescence due to microbial activity (Baddeley, 1971). This phenomenon has been discounted by Fokkema *et al.*, (1979).

Of course, variability in environmental conditions is a greater problem in field tests, where the degree of survival of the antagonist is a much more critical issue than in glasshouse

trials (Baker *et al.*, 1983). The population dynamics of any antagonist inoculated onto the leaf surface needs to be monitored and assessed independently, along and with the disease symptom development and disease severity (Melgarejo *et al.*, 1985). The experiments carried out here screened for isolates displaying good colonisation on the leaf surface. At this stage they are not being selected on known modes of antagonism, only presumptive ones. Organisms not colonising the leaf would be less likely to be selected for good disease control in these experiments (Renwick *et al.*, 1991). Further studies could include counts of the applied antagonists on the leaves over time, together with leaf staining and light microscopy.

The harmful influences of 2 climatic factors namely, water availability and sunlight are the main causes of failure to establish introduced saprophytic bacteria on leaves as part of attempted bio-control (Leben *et al.*, 1965). Filamentous fungi associated with aerial plant surfaces are predominantly dark pigmented like *C. cladosporioides*, which may give protection against the harmful effects of uv radiation (Blakeman, 1993). Some fungi also persist on the leaves in the form of spores which are more resistant to desiccation than their mycelium. *In vivo* assays integrate a measure of various properties of any candidate antagonist, leading to the supreme test of the bio-control potential of the organism in the field. The distinction between antagonistic activity *per se* and biological control potential needs also to be stated more clearly than is presently the case in current literature. A micro-organism may be an outstanding antagonist through many preceding *in vitro* trials, but still show no activity in the field, simply because it can not colonise the host to reduce disease levels (Andrews, 1985).

Bacteria can prevent spore germination or reduce germ-tube growth of necrotrophic pathogens more effectively than filamentous fungi because of the slower growth rate and hyphal habit of fungi, preventing the development of such a close association between antagonist and pathogen (Blakeman, 1993), as well as bacteria having a higher surface-to-volume ratio (Paton, 1960).

In general, a higher inoculum concentration leads to greater disease control (De Cal *et al.*, 1990), as seen in this chapter, where 2 different *Penicillium* spp. were studied. This is probably due to isolates having a higher chance of surviving in the phyllosphere, or higher levels of inhibitory compounds being produced. If an organism does not grow very fast or colonise well, the inoculum may have to be applied in massive doses to give cover of the leaf surface without depending on growth or movement of the organism in the environment (Campbell, 1989). The susceptibility of the bean cultivar used could also be an important factor when assessing via *in vivo* screening, as could cultural effects on pathogen expression. Nevertheless, De Cal *et al.*, (1990) found that time of application of antagonists was crucial in bio-control, with success depending on the rate of colonisation and the mode of antagonism, together with environmental conditions.

From these studies, the isolates showing greatest disease control must be subjected to further studies to determine which of their characteristics are related to their ability to control the pathogen and reduce disease levels.

5.

5. ISOLATION AND PARTIAL PURIFICATION OF ANTIFUNGAL AND ANTIBIOTIC SUBSTANCES FROM SELECTED ANTAGONISTS

5.1 Introduction

As discussed previously (Section 1.5.3.1), antibiotic production is an important method of antagonism towards fungal pathogens. On undamaged leaves, antagonism through the production of antibiotics, probably plays a greater role than competition for nutrients, in the interactions between saprophytes and fungal pathogens (Blakeman & Fokkema, 1982). The action of antibiotics produced by saprophytes on plant pathogens can take many forms, including: alteration of cell walls or membrane permeability, uncoupling agents, inhibition of nucleotides, DNA or protein synthesis, and changes in the transcription of genetic information (Newton, 1965).

Modern fungal taxonomy is based on three different areas: classic morphological taxonomy, physiological taxonomy and chemotaxonomy. The latter is based on specific production of both intracellular and extracellular secondary metabolites, with any given fungal or bacterial isolate giving a metabolite profile which is unique for any species (Anderson, 1991). Some metabolites are only produced under very specific conditions, whereas the production of others are less dependent on the substrate and other external factors (Anderson, 1991). These metabolite profiles can be visualised by thin layer chromatography (tlc), tlc scanning (tlc/s) and high performance liquid chromatography with uv-diode array detection (hplc-dad) (Anderson, 1991). It is often seen that two different fungal species have one or more secondary metabolites in common, but

the presence or absence of other metabolites can discriminate between two fungal species (Anderson, 1991).

A large number of *Penicillium* spp. are known for their antibiotic production, including the production of lytic enzymes and the degeneration of hyphae in dual cultures by *P. purpurogenum* (Larena & Melgarejo, 1993; Melgarejo & M-Sagasta, 1986). *P. chrysogenum* and other *Penicillium* spp. were isolated and screened, producing clear inhibition zones on agar plates (Melgarejo *et al.*, 1985) suggesting the presence of antibiotic agents (Fokkema, 1976). Anderson (1991) reports of a range secondary metabolites produced by *P. brevicompactum* including mycophenolic acid and the Raistrick phenols. There appears to be no information of the antagonistic properties of these metabolites.

Cladosporium spp. are the most common airborne fungi (Baker & Cook, 1974) and are widely referenced as being successful biological agents (Baker & Cook, 1974; Blakeman & Fokkema, 1982). No literature on previous research has been located on the production of antibiotics or antifungal agents by *C. cladosporioides*.

Production of antibiotic active agents against fungi by leaf surface bacteria has not been widely reported (Blakeman & Brodie, 1976). However, rhizosphere isolates of *Pseudomonas fluorescens* have produced antibiotics inhibiting pathogen development and their potential role in the phyllosphere has been suggested, where it occurs widely as an epiphyte (Blakeman & Fokkema, 1982). There has been much work on the potential of two antibiotics, Pyoluteorin and Pyrrolnitrin, produced by *P. fluorescens*, for disease control (Howell & Stipanovic, 1980;

Keel *et al.*, 1992; Levy *et al.*, 1992; Krans & Loper, 1992; Renwick *et al.*, 1991).

Where antibiotic production has been identified as the main or major cause of antagonism by a saprophyte, a cell-free culture filtrate or semipurified antibiotic preparation may be used for bio-control purposes, as opposed to using live inoculum (Blakeman & Fokkema, 1982). This aspect is important especially if the antagonist is not an effective coloniser of the phyllosphere, or is a weak pathogen.

The growing of an antagonist and pathogen in dual cultures *in vitro* allows detailed examination on the effects of antagonists on the morphology and growth of the pathogen. Dual culturing is the classical method used to examine in detail the effect of extracellular metabolites on hyphal morphology of fungi (Kope & Fortin, 1989).

The affects of potential antifungal compounds produced by antagonistic fungal isolates was recorded earlier (Section 3.3.2.3). Their production in culture and effects on the pathogen *B. fabae* need to be examined closely to assess their potential role in biological control of chocolate spot.

5.2 Materials and Methods

5.2.1 PRODUCTION OF ANTIFUNGAL COMPOUNDS

5.2.1.1 Fungal cultures

Fungal cultures of the following antagonists: MB2.F45 (*C. cladosporioides*), MX1.L34 (*P. brevicompactum*), AP1.S20 (*P.*

chrysogenum) and AG1.F4 (*Geomyces pannorus*) were grown in liquid culture to allow the extraction of antifungal compounds. Their potential was indicated in earlier experiments *in vitro* (Section 3.3.2.3) Studies were carried out on one from each of the four different fungal species identified (Table 4.16).

Potato dextrose broth (PDB; 150 ml), which was prepared by boiling 200 g of diced potatoes for one hour in a 250 ml conical flask and filtering through muslin (2 ply). Twenty g of dextrose was added and the final solution made up to 1 litre, before autoclaving for 15 min at 120°C, 1 kg cm^{-2} (Anon, 1974).

Each flask was inoculated with three discs of mycelium (8 mm diameter), cut from the edges of actively growing colonies and placed stationary in an incubator at 20°C for 20 days (De Cal *et al.*, 1988). The growing medium was separated from the mycelium and spores by filtration through a Buchner funnel (Whatman filter paper No. 1), followed by centrifugation at 15000g for 15 min. The supernatant (fungal culture filtrate) was then sterilised by filtration through 0.22 μm Millipore membranes.

Crude antibiotics isolated from the culture filtrates of the isolates (MB2.F45, MX1.L34, AP1.S20 and AG1.F4) were bioassayed for their effects on the germination of conidia and on germ-tube growth of *B. fabae* over a 24 h period.

5.2.1.2 Bacterial cultures

Bacterial isolate LL1.F23 (*P. fluorescens*) was grown in Medium 523 (Howell & Stipanovic, 1979) containing 10 g sucrose, 8 g casein hydroslate, 4 g yeast extract, 2 g K_2HPO_4, 15 g

$MgSO_4.7H_2O$ and 15 g agar in 1 litre distilled water (pH 6.9 after autoclaving).

Agar plates containing Medium 523 were inoculated with a cell suspension of bacterial isolate LL1.F23 and incubated for 10 days at $20^{o}C \pm 1^{o}C$.

5.2.2 ISOLATION AND PARTIAL PURIFICATION OF ANTIBIOTICS

5.2.2.1 Fungal cultures

5.2.2.1.1 Method 1.

Crude preparations of cell-free culture filtrates were obtained from crude filtrates of 20 day old stationary cultures of fungal antagonists (MB2.F45, MX1.L34, AP1.S20 and AG1.F4; Section 5.2.1.1), by liquid:liquid partitions as described by Birkinshaw (1952) and De Cal *et al.*, (1988) (Fig. 5.1). At each purification step, the relative antibiotic activity was bioassayed by examining the germination of *B. fabae* conidia and their germ-tube growth.

In addition to purification of culture filtrates from these fungal isolates, an uninoculated sample of PDB was placed with the fungal cultures in the incubator and put through the same purification steps of liquid:liquid partitions. Such a control allows the determination of the effects of the solvents alone used during the purification procedure and bioassay on *B. fabae*.

The organic fractions were evaporated to complete dryness in a sample concentrator (Dri block 08-3, Techne) at $40^{o}C$ under a steady flow of Nitrogen gas (N_2, oxygen free, BOC Ltd.) and

the residue was dissolved in 0.4 ml chloroform. To this solution, 1 ml phosphate buffer was added (0.01 M phosphate, pH 6.0). The chloroform was bubbled off in the sample concentrator to leave the extracted samples dissolved in the buffer solution. All 6 solutions extracted during the purification of the culture filtrate of isolate MX1.L34 and the uninoculated control medium (Fig. 5.1) were bioassayed for their effects on the germination and germ-tube development of *B. fabae* conidia *in vitro*.

5.2.2.1.2 Method 2.

A second procedure to separate the active principle from culture filtrates was based on Purkayastha & Bhattacharyya (1982). Selected fungal cultures were grown in stationary liquid cultures as described above (5.2.1.1). Equal volumes of ethyl acetate, chloroform, hexane or petroleum ether and the cell-free culture filtrate were mixed twice (1:1) in separating flasks to isolate any antifungal substances, and were then separated into aqueous and organic fractions. These fractions were evaporated to complete dryness in a rotary evaporator (Rotavapor R110, Buchi) at 40^{o}C. The residue was redissolved in 0.6 ml phosphate buffer (0.01 M, pH 6.0) for germination tests and bioassays, or 0.6 ml chloroform for chromatography. These final extracted solutions were concentrated around 100 fold, relative to the original culture filtrate.

To test the effects of the organic solvent residues alone, similar volumes of each solvent as used for the extraction of culture filtrate, were evaporated and the residues redissolved in 0.6 ml phosphate buffer or chloroform. These acted as controls, and were compared to the extracts already obtained

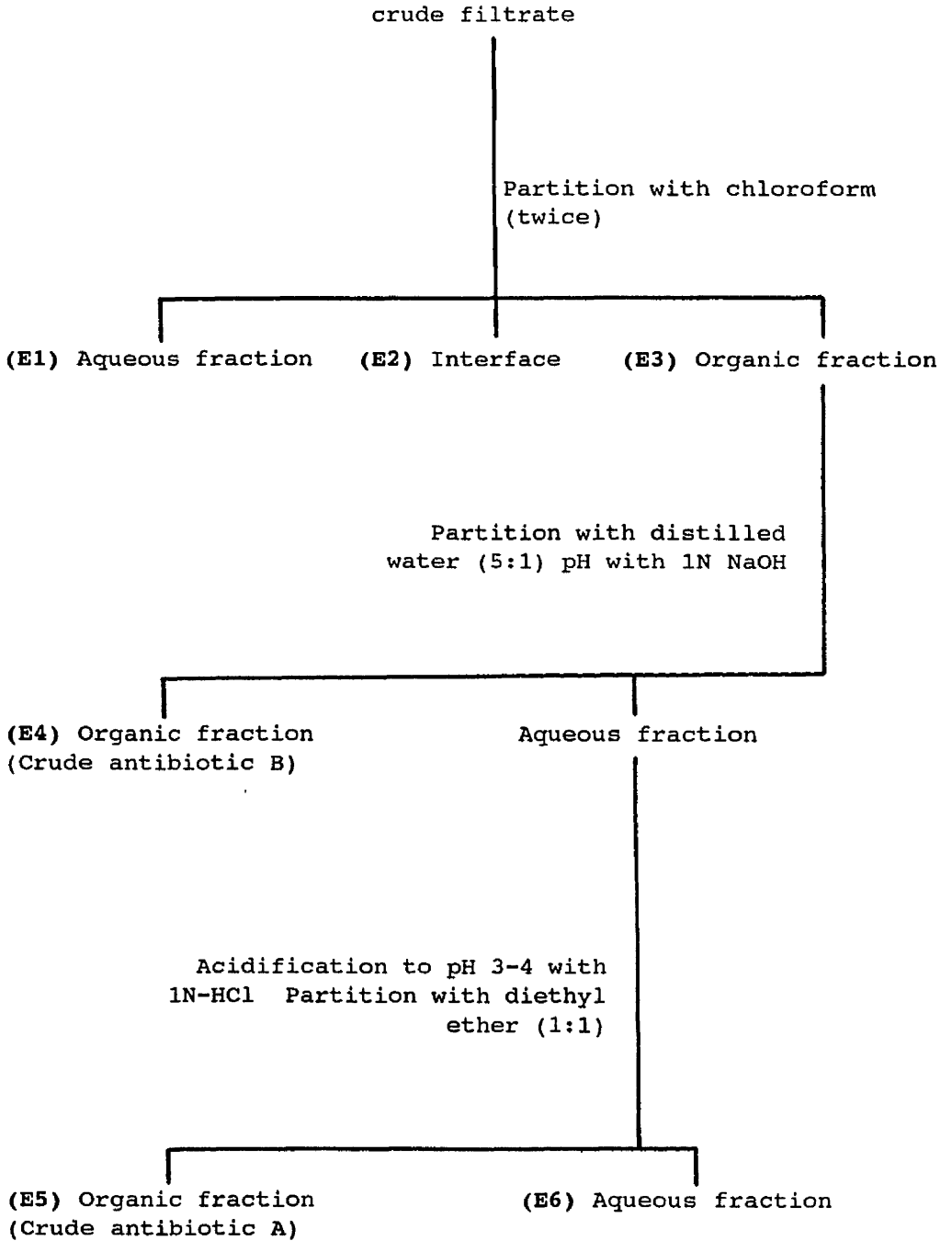


Fig. 5.1
Procedural outline of the biochemical steps (Method 1, Section 5.2.2.1.1) for the isolation and purification of antibiotics of selected fungal isolates (De Cal *et al.*, 1988).

using each solvent with the fungal isolate.

5.2.2.2 Bacterial cultures

After incubation (5.2.1.2) each of ten plates were cut into 10 mm^2 and extracted with 200 ml of 80% aqueous acetone. The agar medium and liquid were then filtered through muslin and centrifuged at 10000 rpm for 10 min to remove any particulate matter (Howell & Stipanovic, 1979). The supernatant fluids were then condensed in a rotary evaporator at 40°C *in vacuo*. Five g of NaCl were added to each 100 ml of the aqueous concentrate and then extracted twice with 100 ml volumes of ethyl ether (Howell & Stipanovic, 1979). The ethyl ether extracts were then combined and taken to dryness *in vacuo*. The residue was dissolved in chloroform to yield a concentration of 100 fold that of the original culture volume.

5.2.3 DUAL CULTURE TESTS

Agar plates (PDA medium) with dual cultures of the fungal isolates MB2.F45, MX1.L34, AP1.S20 and AG1.F4, together with mycelial plugs of *B. fabae*, were set up as described earlier (Section 3.2.2.3), to examine the effects of fungal antagonists in more detail. Here, one antagonistic isolate was chosen from each of the different species of the identified antagonistic fungi (Table 4.16). The cultures were incubated at 20°C for 30 days and examined both macroscopically (x10 to x40 magnification) for growth patterns on the agar surfaces and microscopically (x100 to x400 magnification), to examine for reductions in germ-tube growth and alterations of germ-tube morphology.

At regular intervals, small samples of *B. fabae* mycelium (2x2x1 mm) were removed from control cultures at colony edges, and also from *Botrytis* colony edges growing with the antagonists, from both contact and non-contact areas with the antagonist (Melgarejo & M-Sagasta, 1986). These samples were soaked in lactophenol cotton-blue solution before being fixed in glycerol-gelatin mounting medium (Section 3.2.4.2) for microscopic examination (x10 to x400).

5.2.4 BIOASSAYS

5.2.4.1 in vitro

5.2.4.1.1 Germination tests

Bioassays were set up *in vitro* in a solution of Czapek Dox Liquid Medium (Oxoid Ltd.). Sterile glass slides were placed on a glass rod in sterile petri dishes and lined with moist filter paper as described earlier (Section 3.2.2.2). On each slide a 15 μl droplet of a *B. fabae* conidial suspension (1×10^5 ml^{-1}) in Czapek Dox Liquid Medium and a 30 μl droplet of each treatment solution were placed. These solutions were incubated at 20°C for 24 h (De Cal *et al.*, 1988).

Results were subject to analysis of variance using the statistical data package Minitab.

5.2.4.1.2 Agar diffusion test

The effectiveness of crude cell-free culture filtrates from fungal samples on mycelial growth of *B. fabae in vitro* were tested using an agar diffusion test (Howell & Stipanovic, 1979;

Swinburne, Barr & Brown, 1975). Wells 5mm in diameter were cut centrally in PDA plates (90mm diameter) and 100 µl aliquots of each culture filtrate were added and left to settle for 2 hours at room temperature before seeding the plates with a suspension of *B. fabae* conidia and incubating at 20°C. Four plate replicates were used per treatment.

Plates were examined to identify mycelium free zones around the central well after two and five days. The mean inhibition zone diameter in millimetres was recorded. Samples of mycelium around the well site were dissected, stained in lactophenol-cotton blue and mounted on microscope slides to view patterns of mycelial formation and growth of *B. fabae*.

5.2.4.1.3 Dual culture test

Dual cultures were set up with cell-free culture filtrates in a well (100µl) placed equidistant to a disc of *B. fabae* mycelium on a 90mm Petri dish containing PDA (Section 3.2.2.3) incubated at 20°C and 4 replicates were used per treatment. Plates were examined after 2 and 6 days for visible zones of inhibition of *B. fabae* mycelial growth. Samples of mycelium around the well were stained and examined under a light microscope as described above. Measurements of growth of *B. fabae* cultures were made as detailed in Fig. 3.2 and the percentage inhibition of radial growth of *B. fabae* was calculated from these measurements.

5.2.4.1.4 Dose response test

The organic fraction E4 (crude antibiotic B, as detailed in Fig. 5.1) from the fungal isolate MX1.L34, and from the medium of the corresponding uninoculated control (Section 5.2.2.1.1),

were dissolved in phosphate buffer and used to create a dilution series of 1, 0.1, 0.01, 0.001, and 0.0001. The solutions were then bioassayed as described in Section 5.2.4.1.1, on conidia of *B. fabae*, to determine the dilution end point of fungitoxicity of this extract.

5.2.4.2 in vivo

This test was devised to compare the effects of a spore suspension and the culture filtrate of *P. chrysogenum* (AP1.S20) on lesion development caused by *B. fabae* on detached bean leaves, based on the procedure set out by Purkayastha & Bhattacharyya, (1982). This isolate was tested because of its consistent performance as an antagonist and the activity of its cell-free culture filtrate. Washed leaves were placed in petri dishes lined with moist filter paper (Section 3.2.2.2), before being sprayed with one of the following treatments: control-water only, control-uninoculated PDB medium, the antagonist spore suspension or its cell-free culture filtrate. After 24 h, 10 μl droplets of a *B. fabae* conidial suspension (1×10^5 conidia ml^{-1}) were placed on each treated leaf (8 per leaf, with 8 replicates). Production of lesions (% leaf cover) on the inoculated areas was assessed after 2 days, and their subsequent lesion spread after 4 days was recorded, using the key to infection grades of *B. fabae* outlined by Mansfield & Deverall, (1974).

5.2.5 THIN LAYER CHROMATOGRAPHY

Samples of the concentrated antifungal extracts (15 l) were spotted onto 20 x 20 cm silica gel glass plates (Silica Gel 60A

K6F, Whatman Ltd.). Plates used for tlc analysis were activated at 110°C for 2 h (Scott, Lawerence & Walbeek, 1970). Plates were developed with three different solvent mixtures: (i) Cyclohexane/ chloroform/diethyl ether (5:4:1, by vol., CCD), (Howell & Stipanovic, 1979), (ii) Toluene/ethyl acetate/90% formic acid (5:4:1, by vol., TEF) and (iii) Chloroform/acetone/2-propanol (85:15:20, by vol., CAP), (Anderson, 1991). Plates were examined, and bands visualised under long and short ultraviolet light.

For localisation of the active bands on the plates, each was bioassayed by spraying a concentrated suspension of *B. fabae* conidia onto the plate, followed by incubation in a moist chamber. Areas showing inhibition of the germination of *B. fabae* conidia were scraped off and eluted in 0.2 ml chloroform. Extracts from plates developed in CCD and TEF were mixed on a rotamix before 25 µl samples were applied to a high resolution tlc channelled plate (Silica Gel 60A LK6DF, Whatman Ltd.) and run with original extract samples and standards (listed below) using the CCD solvent mixture. The following standard antibiotics were selected from literature (Anderson, 1991; Scott *et al.*, 1970; Sigma chemical Catalogue, 1993): Citrinin ($C_{13}H_{14}O_5$), Gliotoxin ($C_{13}H_{14}N_2O_4S_2$), Griseofulvin ($C_{17}H_{17}Cl$), Mycophenolic acid ($C_{11}H_{20}O_6$), Ochratoxin A ($C_{20}H_{18}ClNO_2$), Patulin ($C_7H_6O_5$), Penicillic acid ($C_8H_{10}O_4$). It was hoped that the sample extraction from the plates would allow the inhibitory compound to be tentatively identified.

Samples (1 ml) of the extracts from bacterial isolate LL1.F23 were also studied on tlc plates (Silica Gel 60A LK6 preabsorbent strip, preparative, Whatman Ltd.) using solvent

mixture CCD, and compared to controls. Plates were examined and bands visualised under long and short ultraviolet light, as described above.

5.3 Results

5.3.1 DUAL CULTURE TESTS

5.3.1.1 Isolate MB2.F45 (*C. cladosporioides*)

At the edge of the *B. fabae* colony adjacent to the fungal antagonist, there was a dense growth of hyphae, both on the agar surface and with aerial hyphae up to the colony edge of *Botrytis*. This contrasted with the control cultures where the aerial mycelium followed about 10 mm behind the front of the *B. fabae* mycelium on the agar surface. A clear zone of 2-3 mm was measured after the equilibrium between pathogen and antagonist growth was established (8 days), although there was further browning of pathogen mycelium nearest to the antagonist colony. Younger secondary colonies of the antagonist, establishing nearer to the pathogen, did not form clear inhibition zones. Sclerotia formation was similar to control cultures.

The mycelium along the *B. fabae* colony edge adjacent to the antagonist culture was less dense, with longer, less branched mycelial growths than the control hyphae. Nine days after inoculation, coagulation of pathogen cytoplasm, and cytoplasm leakage was evident (Plate 7c). There were also some convoluted and twisted *B. fabae* hyphae (Plate 7b).

5.3.1.2 Isolate MX1.L34 (*P. brevicompactum*)

Secondary spores of this isolate from the original inoculation in dual cultures were readily spread over the agar plates, resulting in colonies forming randomly over the plate surface. Their physical presence affected the interaction between the pathogen and antagonist, where a mass of aerial mycelium was produced by the pathogen. There was severe browning at the edge of the *Botrytis* colony on the agar surface and also of the mycelium growing below, in the agar. A clear zone of 4-6 mm was measured after the growth of the antagonist and pathogen were in equilibrium.

Samples of mycelium taken from the edge of the *B. fabae* colony and examined microscopically exhibited shortened, branched, and stubby hyphae compared to the controls (Plates 5a and 5b). After 9 days the mycelium became coagulated, shortened in cell length through increased septation (Plate 7a) with bulging cells. From 9 days after inoculation, there was cytoplasm leakage into the surrounding medium (Plate 7d). After 18 days, secondary branching of hyphae was recorded, together with the appearance of severe convolutions of the hyphae. Mycelial debris was scattered across the slide after 25 days.

5.3.1.3 Isolate AP1.S20 (*P. chrysogenum*)

From visual examination of the dual cultures using a binocular microscope, a clear zone of 3-4 mm formed between the pathogen and antagonist, after 4-5 days. There was no direct contact between the two fungal colonies on the plates, with an obvious change in the growth habit at the colony edge of *B. fabae*. Sclerotial formation and development was similar to the control.

Areas of the pathogen close to the isolate showed a thinning of hyphae with an increase in branching and a reduction in the length of mycelium relative to control cultures after 3 days (Plates 5a and 5c). With age, the mycelium became more twisted and less dense (Plates 5e and 5f). Mycelial tips at the colony edge were shortened, and ramified, when the mycelium became coagulated after 6 days (Plate 6a). In most of the mycelial samples, there was evidence of cell wall lysis (Plate 6c), with cell cytoplasm leakage after 9 days (Plates 6d). There were localised areas on the samples strewn with ruptured cell material and mycelial debris. After 18 days, many hyphae had cells with little or no contents, which had collapsed and shrunk (Plate 6f). There was also some localised secondary branching of hyphae (Plate 6e).

5.3.1.4 Isolate AG1.F4 (*G. pannorus*)

Ten days after inoculation, dense mycelial growth of *B. fabae* along its colony edge, with a clear zone of 3-4 mm developing, was recorded. Spores of the antagonist from the initial inoculated agar plug, germinated on the agar surface, forming secondary colonies which then spread over the plate, causing at least a physical barrier to the growth of *B. fabae* mycelium. Where the cultures touched, there was a visible interaction, with the production of a mass of aerial mycelium of *B. fabae*. On other colony edges, clear zones were up to 1-2 mm.

B. fabae mycelium showed an increase in the number of hyphal strands, which were thinner and more branched than the controls (Plates 5a and 5d). On closer examination, they were also stubby and twisted in form, with much coagulation of

Plate 5: Mycelial growth of B. fabae in dual cultures in vitro with antagonistic fungal isolates (results recorded 3 days from inoculation).

(a) Control culture (x428).

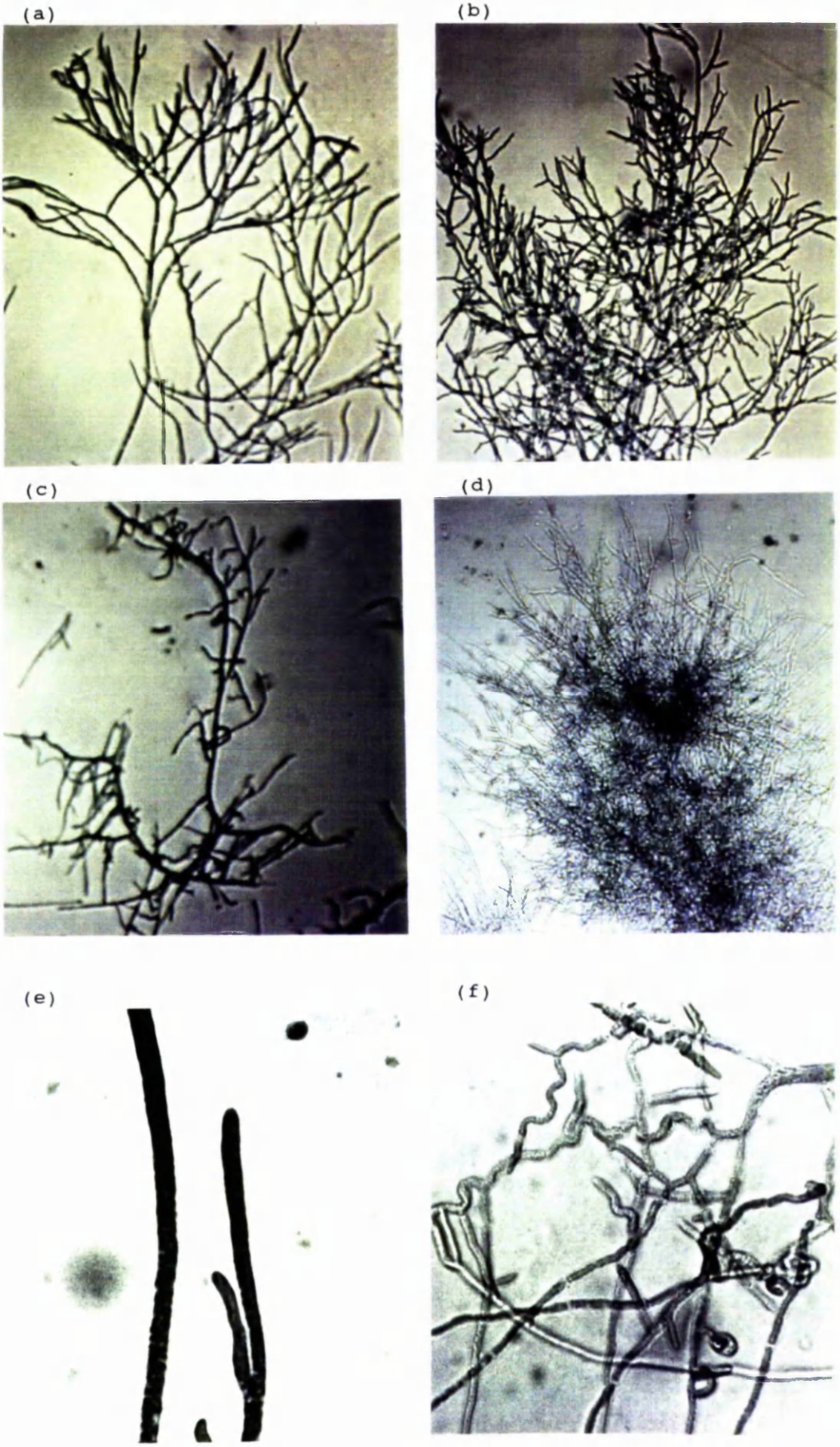
(b) In the presence of isolate MX1.L34 (*P. brevicompactum*), there was shortening and stunting of hyphae (x428).

(c) In the presence of isolate API.S20 (*P. chrysogenum*), there was a reduction in mycelial growth, and hyphae were twisted (x428).

(d) In the presence of isolate AG1.F4 (*G.pannorus*) there was an increase in the number of hyphal strands, which were thinner than the control (x428).

(e) Mycelial tip of *B. fabae* in the control culture (x 1712).

(f) Hyphal formations in dual cultures with isolate AP1.S20. Here the mycelium is thinner than the control, shortened, twisted with increased branching (x1712).


(a)
(b)
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(f)

Plate 6: Mycelial growth of B. fabae in dual cultures in vitro with the fungal isolate AP1.S20 (P. chrysogenum).

(a) *B. fabae* mycelium displaying a breakdown and coagulation of hyphae (after 3 days; x1712).

(b) *B. fabae* exhibiting twisting hyphae (after 3 days; x1712).

(c) *B. fabae* hyphal wall ruptured with subsequent leakage of the cell contents (after 9 days; x1712).

(d) Collapsed *B. fabae* hyphae with released cell contents (after 18 days; x1712).

(e) *B. fabae* mycelium displaying increased secondary branching in the presence of the antagonist (after 18 days; x1712).

(f) *B. fabae* collapsed and convoluted (arrowed) hyphae with empty cells (after 18 days; x4280).

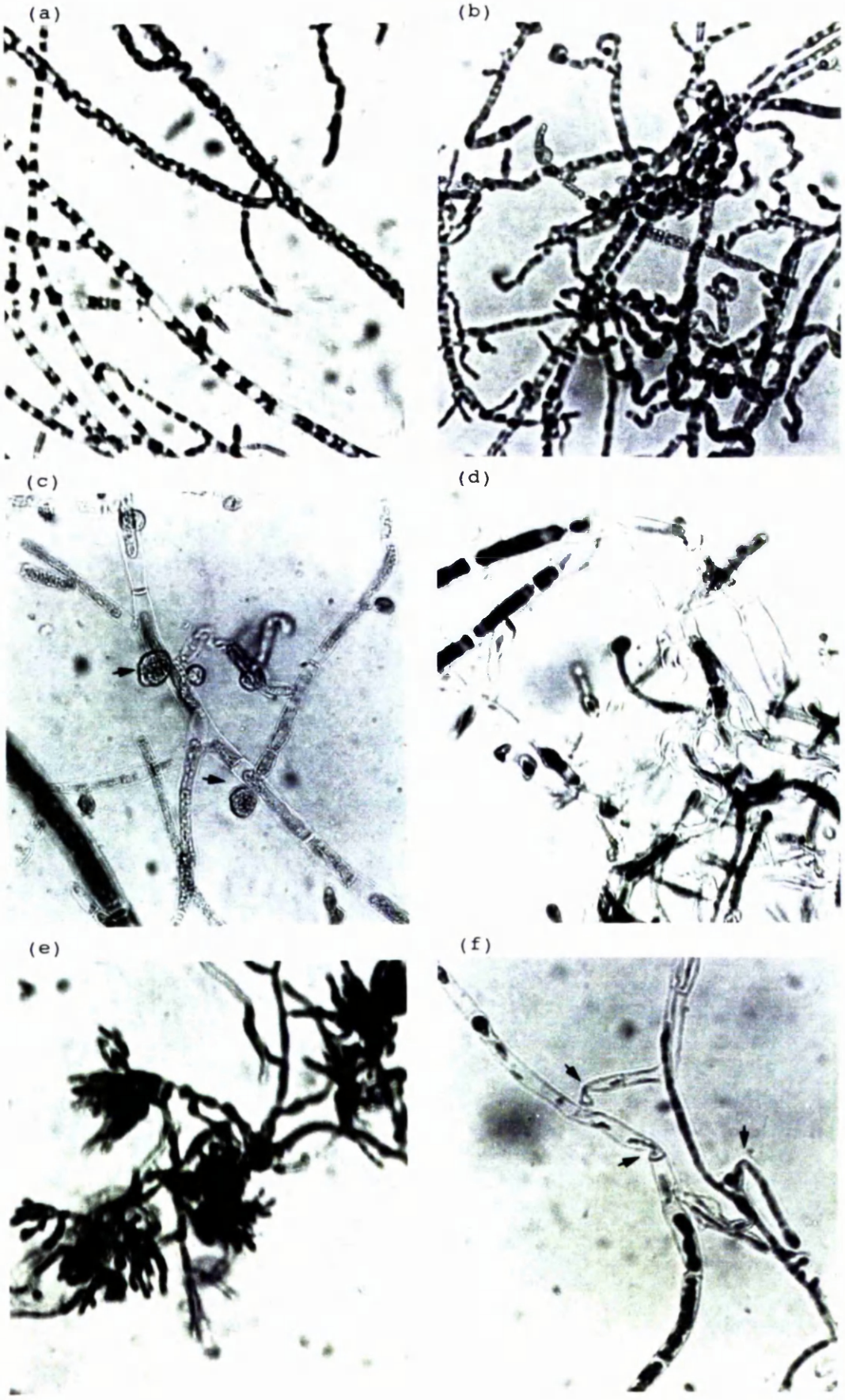
(a)
(b)
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Plate 7: Mycelial growth of B. fabae in dual cultures in vitro with fungal antagonists.

(a) *B. fabae* hyphae exhibiting increased cell septation (after 18 days; x1712) in the presence of isolate MX1.L34 (*P. brevicompactum*).

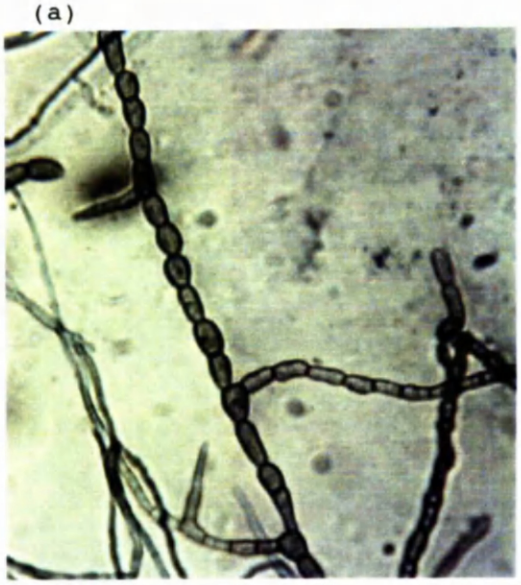
(b) *B. fabae* hyphae showing a twisted formation in the presence of isolate MB2.F45 (*C. cladosporioides*).

(c) *B. fabae* hyphae showing a release of the cells cytoplasmic contents in the presence of *C. cladosporioides* (MX1.L34), after 10 days (x1712).

(d) *B. fabae* hyphae displaying release of the cell contents, together with stunted cell growth in the presence of isolate MX1.L34 (*P. brevicompactum*) after 10 days (x1712).

(e) *B. fabae* hyphae displaying an increase in secondary branching in the presence of the cell-free culture filtrate of *P. chrysogenum* (after 10 days; x1712).

(a)



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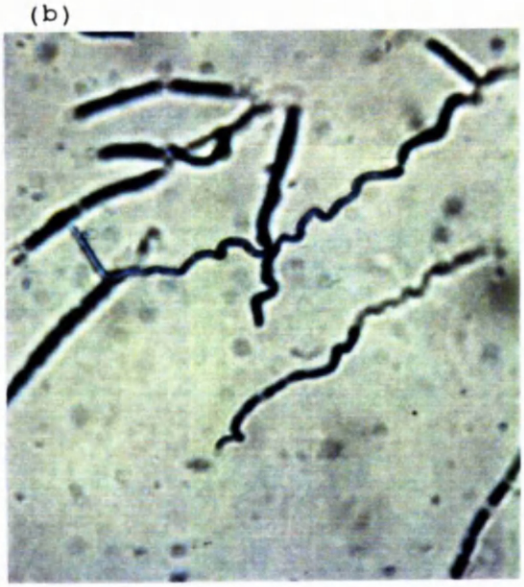
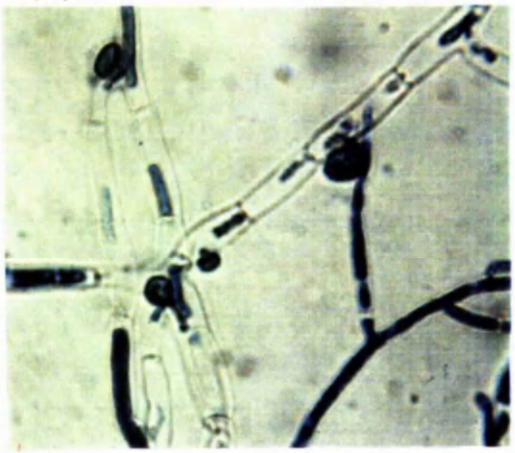


(c)



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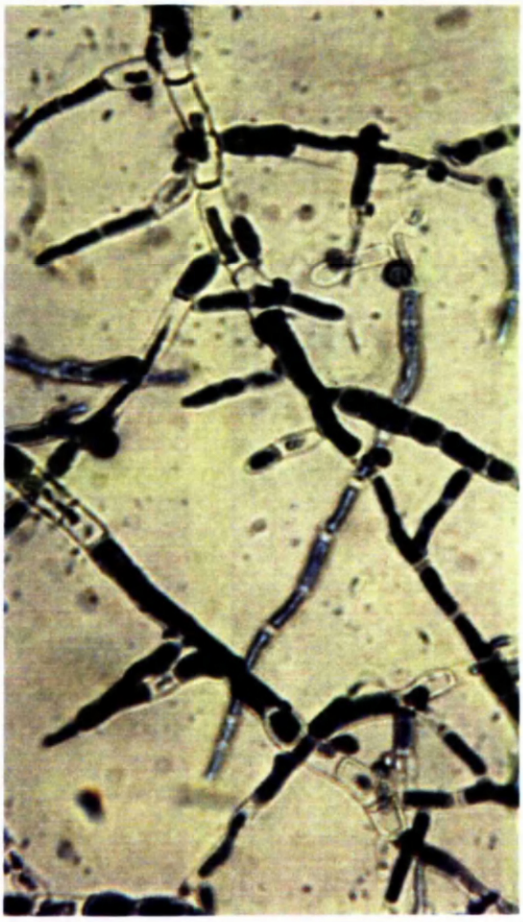

(e)

mycelium after 10 days. Convoluted mycelium, cell rupturing prior to cell collapse and bulging cells were also an evident effect of the presence of this isolate.

5.3.2 ANTIBIOTIC PRODUCTION AND SCREENING

5.3.2.1 *in vitro*

5.3.2.1.1 Germination tests

Of the culture filtrates assayed against *B. fabae* conidia *in vitro*, MX1.L34, AP1.S20 and AG1.F4 all showed significant reductions in germination of *B. fabae* conidia (Table 5.1). Isolate MB2.F45 had no effect on germination of *B. fabae* conidia. Culture filtrates of all four isolates reduced germ-tube growth ($P<0.001$). Fungal isolate AP1.S20 was the most effective at inhibiting germination and germ-tube growth of *Botrytis* conidia, reducing germination and germ-tube growth by 31% and 12% respectively (Table 5.1).

The relative abilities of different forms of isolate AP1.S20 to reduce germination of conidia of *B. fabae* were assessed (Table 5.2). The cell-free culture filtrate collected from liquid cultures gave significant inhibition ($P<0.001$) of both percentage germination and germ-tube growth, with reductions of 76% and 33% respectively, relative to the *B. fabae* control (Table 5.2). A spore suspension of isolate AP1.S20 (1×10^6 spores ml^{-1}) reduced germination to 65% of the control with no significant reduction in germ-tube growth (Table 5.2). The active principle present in the culture filtrate appeared to be partially thermolabile, but even after being autoclaved, the sample still reduced germination to 37% ($P<0.001$) of the

Table 5.1

Effect of cell-free culture filtrate of selected fungal isolates on the spore germination and germ-tube lengths of *B. fabae in vitro* (± Standard errors of the mean).

Isolate	Germination (%)	Percent of control[a]	Germ-tube length (µm)	Percent of control[a]
Control	86.33 ± 1.52	-	10.31 ± 0.91	-
MB2.F45	80.33 ± 2.50	93	3.70 ± 0.34	36 ***
MX1.L34	68.17 ± 2.94	79 **	2.99 ± 0.36	29 ***
AP1.S20	26.83 ± 3.17	31 ***	1.20 ± 0.06	12 ***
AG1.F4	71.33 ± 2.36	82 **	4.14 ± 0.24	40 ***
SED	4.46		0.75	

a significant differences are shown as follows: P < 0.05 *; P < 0.01 **; P < 0.001 ***.

Table 5.2

Effect of a spore suspension of isolate AP1.S20 and its cell-free culture filtrate, sampled before and after autoclaving, on spore germination and germ-tube growth of *B. fabae in vitro* (± standard errors of the mean).

Treatment	Germination (%)	Percent of control[a]	Germ-tube length (µm)	Percent of control[a]
Control	91.00 ± 2.38	-	7.234 ± 0.497	-
Spore suspension	59.50 ± 5.19	65 **	6.443 ± 0.393	89
Culture filtrate:				
filter sterile	22.00 ± 2.68	24 ***	4.830 ± 0.993	67 *
autoclaved	33.50 ± 6.80	37 ***	4.260 ± 0.348	59 **
SED	6.56		0.87	

a significant differences are shown as follows: P < 0.05 *; P < 0.01 **; P < 0.001 ***.

control. Filter sterilised culture filtrate reduced spore germination by 76% ($P<0.001$). Measurements of germ-tube growth showed that the filter sterilised culture filtrate was most inhibitory, followed by the autoclaved culture filtrate, with the spore suspension not exhibiting any significant reduction compared to the control (Table 5.2).

The culture filtrates of the four fungal isolates were used to extract and purify any potential antifungal compounds following the procedure described by De Cal *et al.*, (1988). Samples of solutions obtained at each stage of the purification procedure for isolate MX1.L34, were assayed for relative antibiotic activity against germination and germ-tube growth of *B. fabae* conidia (Table 5.3). All of the extracts, except E5, showed significant reductions in germination ($P<0.001$) after 24 h and all extracts reduced germ-tube extension relative to the control ($P<0.001$).

De Cal *et al.*, (1988) who outlined this purification scheme, found that the organic fractions, extracts E3, E4 and E5 were most toxic, giving greatest reductions in both germination and germ-tube growth compared to the control. With isolate MX1.L34, extracts E3 and E4 inhibited germination of *B. fabae* conidia to the greatest extent, to 3% and 4% of the controls respectively. Extract E5 did not significantly reduce the germination of *B. fabae* conidia, but did reduce the germ-tube lengths significantly. De Cal *et al.*, (1988) classified extracts E4 and E5 as crude antibiotics. In the present study it was found that all the extracts significantly altered growth and development of conidia in some way (Table 5.3). Previous studies (De Cal *et al.*, 1988) showed that extracts E1 and E2

Table 5.3

Activity of different extracts of the fungal isolate MX1.L34 against spore germination and germ-tube length of *B. fabae* conidia *in vitro* (± standard errors the the means).

Extract	Germination (%)	Percent of control[a]	Germ-tube length (µm)	Percent of control[a]
Control	86.33 ± 1.52	-	10.31 ± 0.91	-
E1	66.17 ± 6.36	77 ***	3.87 ± 0.23	38 ***
E2	15.00 ± 4.80	17 ***	4.41 ± 1.30	43 ***
E3	3.00 ± 1.46	3 ***	1.17 ± 0.07	11 ***
E4	3.33 ± 1.28	4 ***	1.03 ± 0.12	10 ***
E5	77.17 ± 1.96	89	4.42 ± 0.35	43 ***
E6	21.50 ± 2.38	25 ***	1.80 ± 0.15	17 ***
SED	4.46		0.75	

a Significant differences are shown as follows: $P < 0.05$ *; $P < 0.01$ **; $P < 0.001$ ***.

had little effect on germination and germ-tube growth. This was not the case in this study (Table 5.3) where highly significant reductions in germination and germ-tube growth were obtained with extract E1 ($P<0.001$). Extract E2 was the interface between liquid:liquid partitions and some of the sample would probably be similar to the inhibitory organic fraction E3.

Studies by De Cal *et al.*, (1988) compared pathogen spore germination in the presence of antifungal extracts (Fig. 5.1) to controls of sterile distilled water. The results detailed in Table 5.4 compare both sterile distilled water and PDB controls to different extracts (M) of uninoculated PDB culture media, put through the same procedural steps as each of the cell-free culture filtrates of the fungal isolates. Significant differences were determined and compared between these M solutions and water and PDB, and then between each control extract (M3, M4, M5 & M6) and its equivalent from isolate MX1.L34 (E3, E4, E5 & E6). This indicates that in this series of experiments germination of *B. fabae* conidia was low relative to previous germination experiments. Analysis of these results suggests that there is some degree of inhibition resulting from the the solvents used for the initial extraction (Fig. 5.1).

Extracts E4 and E5 (Table 5.4), both considered to be the more active extracts containing crude antibiotics (De Cal *et al.*, 1988), showed significant inhibition of germination (Table 5.4) compared to the sterile distilled water control ($P<0.01$) and PDB medium control ($P<0.001$), as well as to the relative controls E4 to M4 ($P<0.01$) and E5 to M5 ($P<0.01$).

Table 5.4

Activity of selected extracts of the fungal isolate MX1.L34 compared to various sample controls against spore germination of *B. fabae in vitro* (± standard errors of the mean).

		Percentage of control[a]					
Extract	Germination	Water	PDB	M3	M4	M5	M6
Controls							
Water	47.00 ± 4.04	-	90				
PDB	52.50 ± 2.22	112	-				
M3	36.50 ± 5.32	78	70 *	-			
M4	50.50 ± 5.32	107	96		-		
M5	47.50 ± 3.10	101	90			-	
M6	31.50 ± 5.56	67 *	60 *				-
MX1.L34							
E3	32.50 ± 4.99	69	62 *	89			
E4	22.00 ± 4.55	47 **	42 ***		43 **		
E5	19.00 ± 4.36	40 **	36 ***			40 **	
E6	32.50 ± 10.70	68	62 *				103
SED	7.71						

a significant differences are shown as follows: $P < 0.05$; $P < 0.01$ **; $P < 0.001$ ***.

Comparing extracts of fungal isolates to extracts obtained from uninoculated media controls using the same procedure, is indicative that substances inhibiting the growth and development of *B. fabae* were present in the culture filtrate of the antagonistic isolate.

Results detailed in Tables 5.5 and 5.6 compare the crude antibiotic samples from each fungal isolate to PDB and extracts of uninoculated media (Section 5.2.1.1). Table 5.5a shows that extract E4 of all four fungal isolates significantly reduced germination ($P<0.01$) of *B. fabae* conidia compared to the M4 control. None of the four extracts significantly reduced the extension of *B. fabae* germ-tubes when compared to this M4 media control (Table 5.5b). The M4 control significantly reduced both conidial germination and germ-tube growth ($P<0.001$) relative to the PDB control (Table 5.5a and 5.5b).

The results of screening extract E5 relative to the control extract obtained from uninoculated media, are listed in Table 5.6. Extract E5 from AP1.S20 and AG1.F4 did not reduce the germination of *B. fabae* conidia relative to the M5 control, although the AP1.S20 extract, E5 significantly reduced germination compared to the PDB media control (Table 5.6a). MB2.F45 and MX1.L34 reduced conidial germination to 61% and 63% of the control ($P<0.01$) respectively (Table 5.6a).

Relative to the PDB control the extracts from uninoculated samples of PDB (M4 and M5), reduced the germination significantly, to 15% and 89% of the PDB control (Table 5.5a and 5.6a). Germ-tube growth of the same extracts from uninoculated samples of PDA showed reductions of 9.2% and 35% relative to the PDB control (Table 5.5b and 5.6b).

Table 5.5

Activity of extracts (crude extract antibiotic B, Fig. 5.1) of selected fungal isolates compared to controls, against: (a) spore germination and (b) germ-tube growth of *B. fabae* conidia *in vitro* (± standard errors of the mean).

(a)

Extract	Germination (%)	Percent of control[b] PDB	M4
Controls			
PDB	94.50 ± 0.96	-	675
M4	14.00 ± 6.22	15.0 ***	-
MB2.F45, E4	0.50 ± 0.50	0.5 ***	4 **
MX1.L34, E4	1.50 ± 0.96	2 ***	1 **
AP1.S20, E4	0.50 ± 0.50	0.5 ***	4 **
AG1.F4, E4	4.00 ± 1.41	4 ***	28 **
SED	3.79		

(b)

Extract	Germ-tube[a]	Percent of control[b] PDB	M4
Controls			
PDB	5.44 ± 0.22	-	1088
M4	0.50 ± 0.21	9.2 ***	-
MB2.F45, E4	0.53 ± 0.30	9.7 ***	106
MX1.L34, E4	0.00 ± 0.00	0 ***	0
AP1.S20, E4	0.00 ± 0.00	0 ***	0
AG1.F4, E4	0.33 ± 0.24	6 ***	66
SED	0.28		

a germ-tube length measured in μm
b Significant differences are shown as follows:
$P < 0.05$ *; $P < 0.01$ **; $P < 0.001$ ***.

Table 5.6

Activity of extracts (crude extract antibiotic A, Fig. 5.1) of selected fungal isolates compared to controls, against: (a) spore germination and (b) germ-tube growth of *B. fabae* conidia *in vitro* (± standard error of the mean).

(a)

Extract	Germination (%)	Percent of control[b] PDB	M5
Controls			
PDB	94.50 ± 0.96	-	112
M5	84.50 ± 2.06	89	-
MB2.F45, E5	51.50 ± 8.22	54 **	61 **
MX1.L34, E5	53.50 ± 10.3	57 **	63 **
AP1.S20, E5	76.50 ± 5.68	81 *	90
AG1.F4, E5	85.00 ± 3.00	90	100
SED	8.57		

(b)

Extract	Germ-tube[a]	Percent of control[b] PDB	M5
Controls			
PDB	5.44 ± 0.22	-	280
M5	1.94 ± 0.46	35 ***	-
MB2.F45, E5	0.89 ± 0.13	16 ***	46 **
MX1.L34, E5	1.58 ± 0.09	29 ***	81
AP1.S20, E5	0.93 ± 0.12	17 ***	48 **
AG1.F4, E5	1.06 ± 0.14	20 ***	55 *
SED	0.33		

a germ-tube length measured in μm

b Significant differences are shown as follows:
$P < 0.05$ *; $P < 0.01$ **; $P < 0.001$ ***.

A range of solvent extracts of the culture filtrate of AP1.S20 obtained using extraction methodology 2 (Section 5.2.2.1.2), produced significant reductions in germination of *B. fabae* conidia and subsequent germ-tube extension (Table 5.7). However, controls containing solvent alone and processed identically to the extracts, all reduced germination of *B. fabae* conidia, but to a much smaller degree than the solvent extracts. A similar phenomenon was also observed when extraction method 1 was used previously. Hexane, petroleum ether and ethyl acetate controls produced conidial germination similar to the phosphate buffer control, but germination in the chloroform control was 34% (Fig. 5.2). Each extract was therefore compared to the appropriate control extract sample. All the solvent extracts were highly effective at reducing conidial germination ($P<0.001$) compared to their respective controls (Fig. 5.2). Inhibition ranged from 86% - 92% with chloroform, hexane and petroleum ether extracts, and 96% with the ethyl acetate extract.

The effects of these extracts on germ-tube extension of *B. fabae* germlings were less marked, with only the petroleum ether extract showing a significant reduction (81%) compared to the control ($P<0.01$), (Table 5.7). The aqueous extracts, which were dried down and dissolved in phosphate buffer, all showed significant inhibition of germination of *B. fabae* conidia ($P<0.001$) relative to the buffer control (Fig. 5.3). This suggests that either the extraction procedure was inefficient at removing the inhibiting substance, and that different extraction techniques might be required for optimum extraction, or there is other inhibitory agents in the aqueous samples.

Table 5.7

Bioassay of solvent and aqueous extracts of the culture filtrate of isolate AP1.S20 (*P. chrysogenum*) on spore germination and germ-tube growth of *B. fabae* (± standard errors of the mean).

Treatment	Germination[c]	Germ-tube[ac] (μm)
Controls		
Ethyl acetate	52.00 ± 7.16	1.918 ± 0.495
Chloroform	34.00 ± 3.08	2.790 ± 0.602
Hexane	69.50 ± 2.06	2.466 ± 0.325
Petroleum ether	67.75 ± 3.77	7.238 ± 0.375
Culture extracts[b]		
Ethyl acetate	2.25 ± 0.85 ***	0.062 ± 0.062 ns
Chloroform	4.75 ± 2.50 ***	2.000 ± 2.000 ns
Hexane	9.00 ± 3.03 ***	0.518 ± 0.148 ns
Petroleum ether	5.75 ± 1.65 ***	1.411 ± 0.612 **
Controls		
Sterile water	90.75 ± 2.66	9.318 ± 1.742
Phosphate buffer	72.00 ± 2.16	7.544 ± 0.612
Cul fil[d]	22.00 ± 2.68 ***	-
Cul fil[d] (conc)	4.50 ± 2.63 ***	-
Aqueous extracts		
Ethyl acetate	23.00 ± 3.79 ***	-
Chloroform	9.50 ± 3.20 ***	-
Hexane	15.25 ± 6.45 ***	-
Petroleum ether	24.50 ± 6.65 ***	-
SED	4.69	1.315

a germ-tube lengths were not determined for aqueous extracts or culture filtrate samples

b significance compares each culture extract with its relative solvent control

c significant differences are shown as follows: $P < 0.05$ *; $P < 0.01$ **; $P < 0.001$ ***.

d culture filtrate sample of isolate AP1.S20

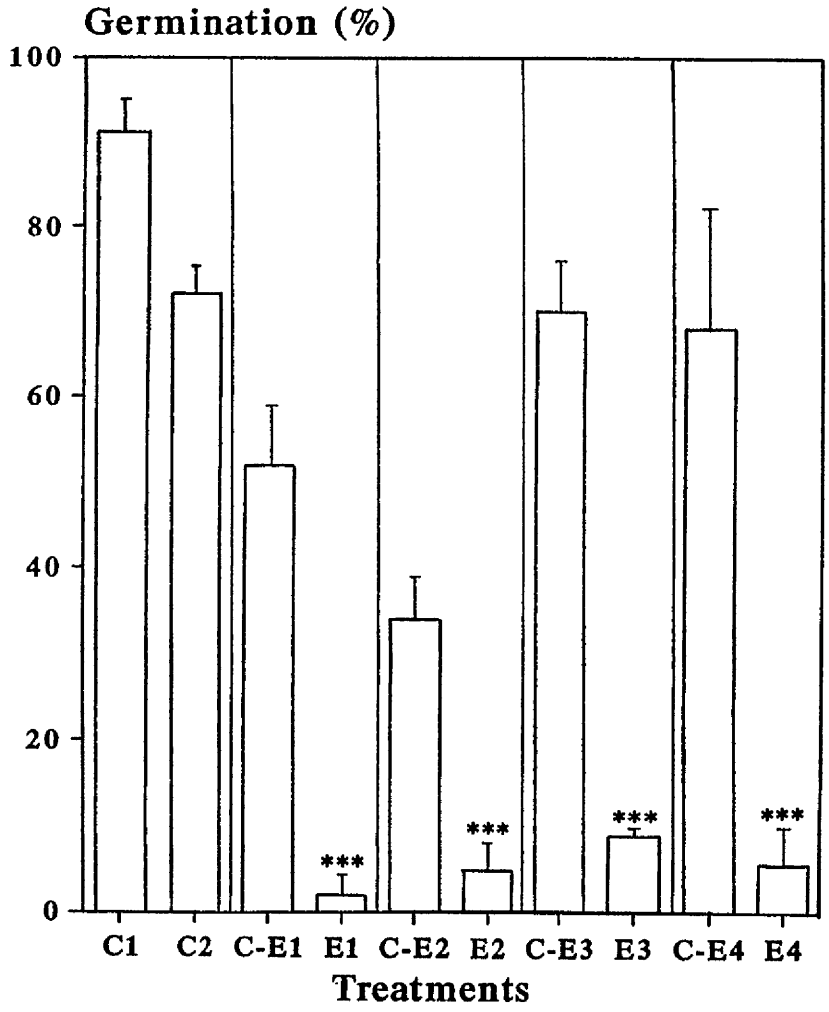

Fig. 5.2

Germination of *B. fabae* conidia *in vitro* in the presence of four solvent extracts (ethyl acetate, chloroform, hexane and petroleum ether) of the culture filtrate of isolate AP1.S20 (20 days old).

[Treatments: C1 (Control water), C2 (Control buffer); C-E1, C-E2, C-E3, C-E4, are control extracts (solvent sample alone) using ethyl acetate, chloroform, hexane and petroleum ether respectively; E1, E2, E3 and E4 are solvent extracts of the culture filtrate of isolate AP1.S20, using ethyl acetate, chloroform, hexane and petroleum ether respectively].

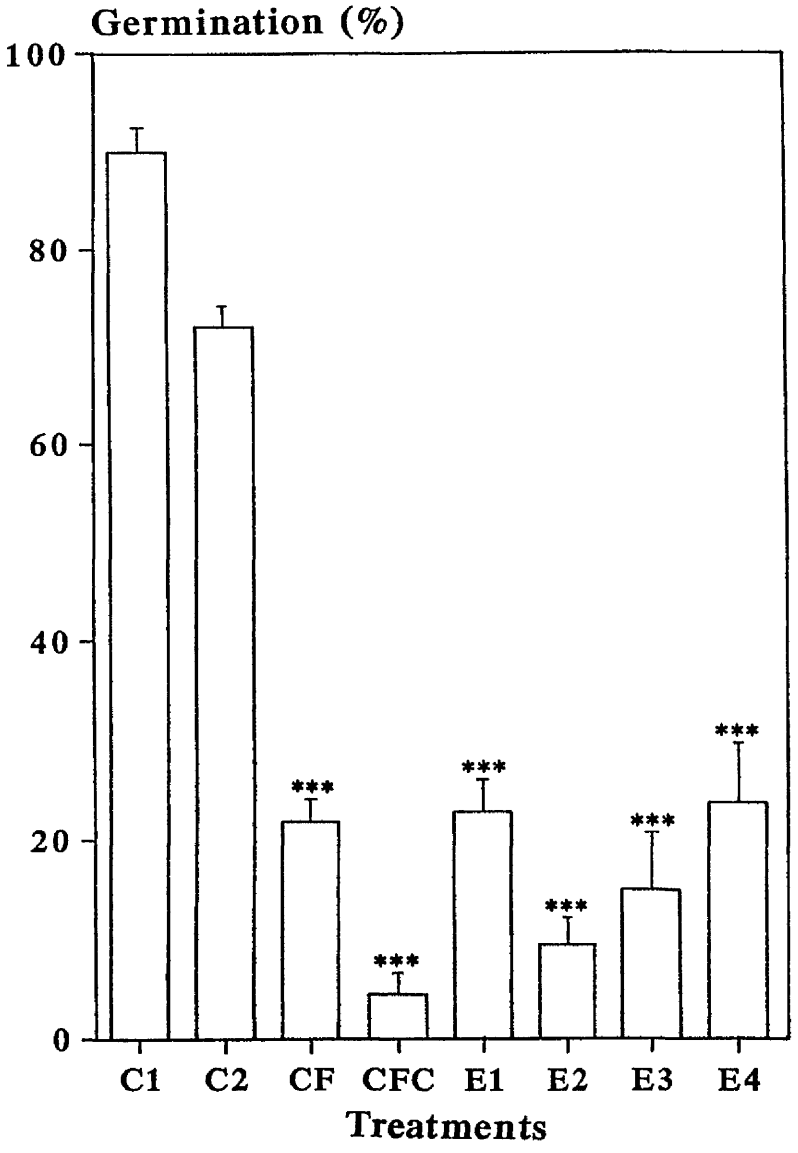

Fig. 5.3

Germination of *B. fabae* conidia *in vitro* in the presence of four aqueous solvent extracts: ethyl acetate, chloroform, hexane and petroleum ether (E1, E2, E3 and E4 respectively) and also a culture filtrate and a concentrated culture filtrate extract. All samples were collected from a 20 day old suspension culture of isolate AP1.S20, and compared to two controls: water and phosphate buffer.

[Treatments: C1 (control water), C2 (control buffer), CF (culture filtrate), CFC (culture filtrate concentrated); E1, E2, E3 and E4 for aqueous solvent extracts of ethyl acetate, chloroform, hexane and petroleum ether, respectively].

5.3.2.1.2 Agar diffusion test

At each examination, none of the fungal isolates, all of which displayed antifungal properties in germination tests (Section 5.3.2.1.1), produced any visibly clear zones of *B. fabae* mycelial growth in the area around each of the wells, nor did they produce visual differences in mycelium or sclerotial formation relative to the control. However, microscopic examination of the stained sections of mycelium revealed some alterations in morphology of *B. fabae* mycelium similar to that described earlier in dual cultures (Section 5.3.1), with shortening of hyphal extension growth.

5.3.2.1.3 Dual culture test

Radial growth was calculated from measurements of growth of *B. fabae* mycelium *in vitro*, taken on 2 and 6 days after inoculation. By day 6, *B. fabae* mycelium was at or approaching the edge of the petri plates. All the plates showed similar sclerotial formation, in both size and colour, to the controls. The isolates MB2.F45 and AP1.S20 produced significant inhibition of radial growth of *B. fabae* after 6 days ($P<0.05$), (Table 5.8). From slide preparations of mycelial samples taken from around the wells containing cell-free culture filtrate, a variety of morphological changes were noted when compared to the control. All four fungal isolates produced some visible changes in growth habit of *B. fabae*, but MB2.F45 produced the most striking changes, with shortening of mycelial branches and different orientation of hyphal growth relative to the control, as discussed earlier (Section 5.3.1.1). After 6 days, the culture filtrate of AP1.S20 had shortened and twisted

the *B. fabae* hyphae and there was leakage of its cell contents, and some localised secondary branching of mycelium (Plate 7e). AG1.F4 exhibited dense, thinned and shortened hyphae with increased branching of fine hyphae. Similar alterations in morphology have previously been observed and discussed in detail (Section 5.3.1).

Table 5.8
Percentage inhibition of radial growth of *B. fabae* produced by cell-free culture filtrates of fungal antagonists in dual cultures *in vitro* (± standard errors of the mean).

	Day 2	Day 6
Isolate	Inhibition[a]	Inhibition[a]
Control	3.65 ± 4.99	6.93 ± 2.59
MB2.F45	-3.16 ± 5.00	-1.92 ± 0.54 *
MX1.L34	-8.73 ± 10.40	3.70 ± 0.94
AP1.S20	4.93 ± 11.00	-1.23 ± 2.93 *
AG1.F4	2.69 ± 7.13	10.54 ± 3.83
SED	11.00	3.55

a Inhibition of radial mycelial growth of *B. fabae*
b Significant differences are shown as follows: $P < 0.05$ *; $P < 0.01$ **; $P < 0.001$ ***.

5.3.2.1.4 Dose response test

The strong inhibitory effect of the control M4 on the germination and germ-tube growth of *B. fabae* can be seen in Table 5.9. The undiluted control, inhibited conidial germination to 53% ($P<0.001$) of the phosphate buffer control. All the concentrations of this control, serially diluted up to 0.0001 of the original extract, still showed some inhibition of germination, although not all dilutions were significantly different from the buffer control. The extract from isolate MX1.L34 completely inhibited germination of *B. fabae* conidia, with a 10-fold dilution of the extract inhibiting germination by 90%. In dilutions 0.01 and 0.001 of the fungal extract, germination was inhibited by 40% and 34% respectively ($P<0.01$).

Germ-tube extension was greatly reduced by serial dilutions ($P<0.001$) of control M4 and MX1.L34, over all concentrations. There was however, greater inhibition produced by the antibiotic extract from the fungal isolate MX1.L34 (Table 5.9).

Table 5.9

Response of spore germination and germ-tube growth of *B. fabae* conidia to different doses of extracted antifungal substances from the fungal isolate MX1.L34 (*P. brevicompactum*), compared to controls (± standard error of the mean).

Extract	Germination (%)	Percent of control[a]	Germ-tube length (µm)	Percent of control[a]
Control buffer	96.00 ± 2.16	-	3.46 ± 0.10	-
M4				
1.x	50.50 ± 13.60	53 ***	0.45 ± 0.18	13 ***
0.1x	65.50 ± 9.43	68 **	0.30 ± 0.09	8.8 ***
0.01x	92.50 ± 1.50	96	0.32 ± 0.09	9.1 ***
0.001x	86.00 ± 2.00	90	0.25 ± 0.03	7.3 ***
0.0001x	87.59 ± 5.80	91	0.34 ± 0.04	9.9 ***
MX1.L34				
1x	0.00 ± 0.00	0 ***	0.00 ± 0.00	0 ***
0.1x	10.00 ± 3.56	10 ***	0.26 ± 0.13	7.4 ***
0.01x	57.50 ± 6.40	60 **	0.61 ± 0.12	18 ***
0.001x	63.50 ± 5.85	66 **	0.55 ± 0.09	16 ***
0.0001x	80.50 ± 2.63	84	0.61 ± 0.14	18 ***
SED	8.66		0.15	

a Significant differences are shown as follows: $P < 0.05$ *; $P < 0.01$ **; $P < 0.001$ ***.

5.3.2.2 *in vivo*

The culture filtrate or a spore suspension of fungal isolate AP1.S20 (*P. chrysogenum*) produced large reductions ($P<0.001$) in the percentage leaf area covered by lesions on detached bean leaves (Table 5.10). This isolate was chosen because it gave the largest reduction in both germination and germ-tube length in earlier work (Table 5.1).

Both of these treatments also reduced the subsequent spread of the lesions ($P<0.05$) on bean leaves (Table 5.10). On leaves sprayed with uninoculated culture medium, lesion formation was significantly reduced compared to the control ($P<0.05$), whereas the spread of chocolate spot lesions on the leaf surface was slightly increased (Table 5.10), although this difference was not significant.

5.3.3 THIN LAYER CHROMATOGRAPHY

Each tlc solvent system separated the each extract (E1 - E4) into a different number of localised spots on the plates, visible under short-wave and long-wave uv light (Figs. 5.4 and 5.5). On close examination, each of the tlc plates showed large variation both in the number of discrete spots and in their location on the plates. This variation was dependent both on, ultraviolet wavelength the plates were viewed under and the solvent system used. When visualised under long-wave uv, the ethyl acetate and chloroform extracts showed the best separation, into 13 - 15 discrete spots. Less separation was achieved with the other extracts.

Table 5.10

Effect of applying a foliar spray of a spore suspension or cell-free culture filtrate of *P. chrysogenum* (isolate AP1.S20), on lesion production and spread of *B. fabae* on detached bean leaves (Results ± standard error of the mean).

Treatment	% leaf area with lesions (48 hours)	Percent of control	Spread of lesions (mm) (96 hours)	Percent of control
Control (water)	83.64 ± 1.22	-	1.786 ± 0.32	
Control (medium)	68.03 ± 5.29	81*	1.875 ± 0.59	105
Spore suspension	53.71 ± 5.63	64***	0.679 ± 0.27	38*
Culture filtrate	57.09 ± 4.56	68***	0.679 ± 0.18	38*
SED	6.42		0.487	

b significant differences are shown as follows: $P < 0.05$ *; $P < 0.01$ **; $P < 0.001$ ***.

Table 5.11

Zones of inhibition recorded from bioassays of thin layer chromatography plates using a spore suspension of *B. fabae* conidia (Rf values in parentheses)[a].

Solvent	Inhibition zone (mm)			
	Ethyl acetate	Chloroform	Hexane	Petroleum ether
1. TEF[b]	20 (0.653)	21 (0.598)	13 (0.590)	16 (0.590)
2. CAP[c]	22 (0.974)	16 (0.958)	13 (0.958)	10 (0.966)

a results not available for solvent mixture (CCD; cyclohexane/chloroform/diethyl ether)
b TEF, Toluene/Ethyl acetate/90% Formic acid
c CAP, Chloroform/acetone/2-propanol

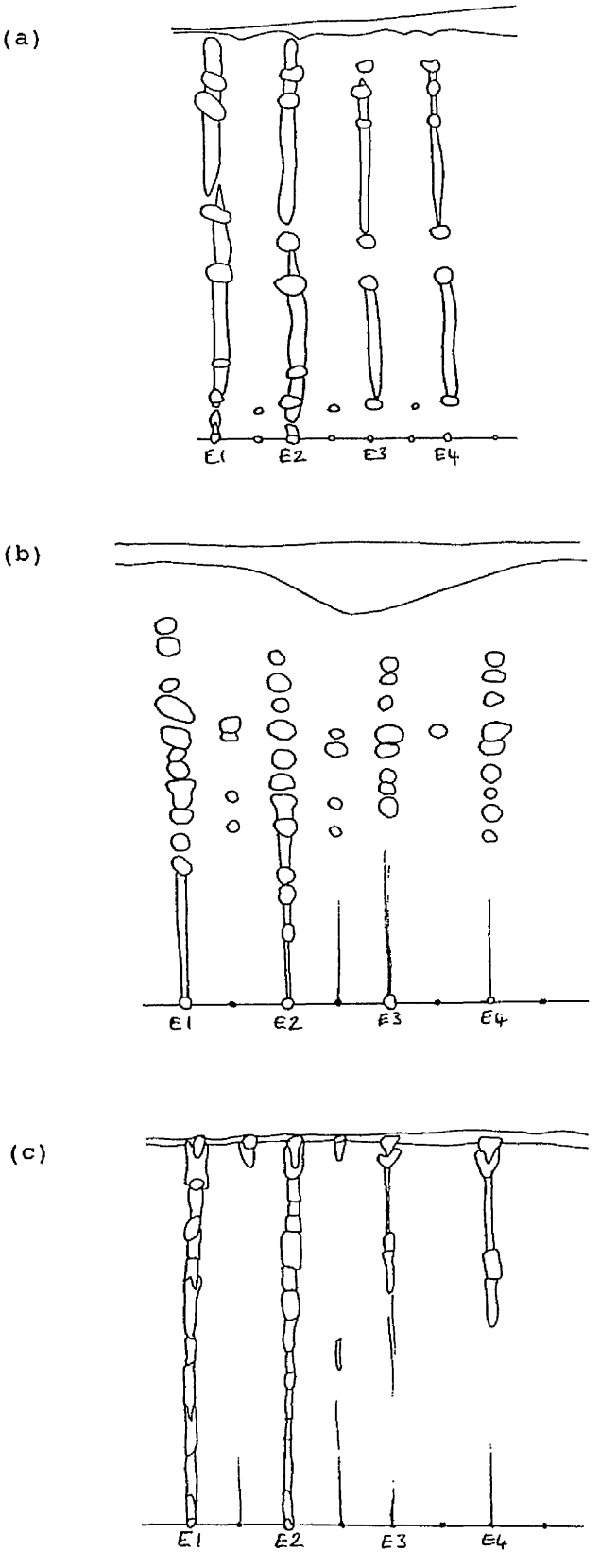

Fig. 5.4

Overlay of discrete spots on tlc plates separated using solvent mixtures (a) CCD, (b) TEF and (c) CAP (Section 5.2.5), visualised under long-wave ultraviolet light. Samples extracted from fungal isolate AP1.S20 are: E1 ethyl acetate extract, E2 chloroform extract, E3 hexane extract and E4 petroleum ether extract, [control extract samples were spotted to the right of each solvent extract sample].

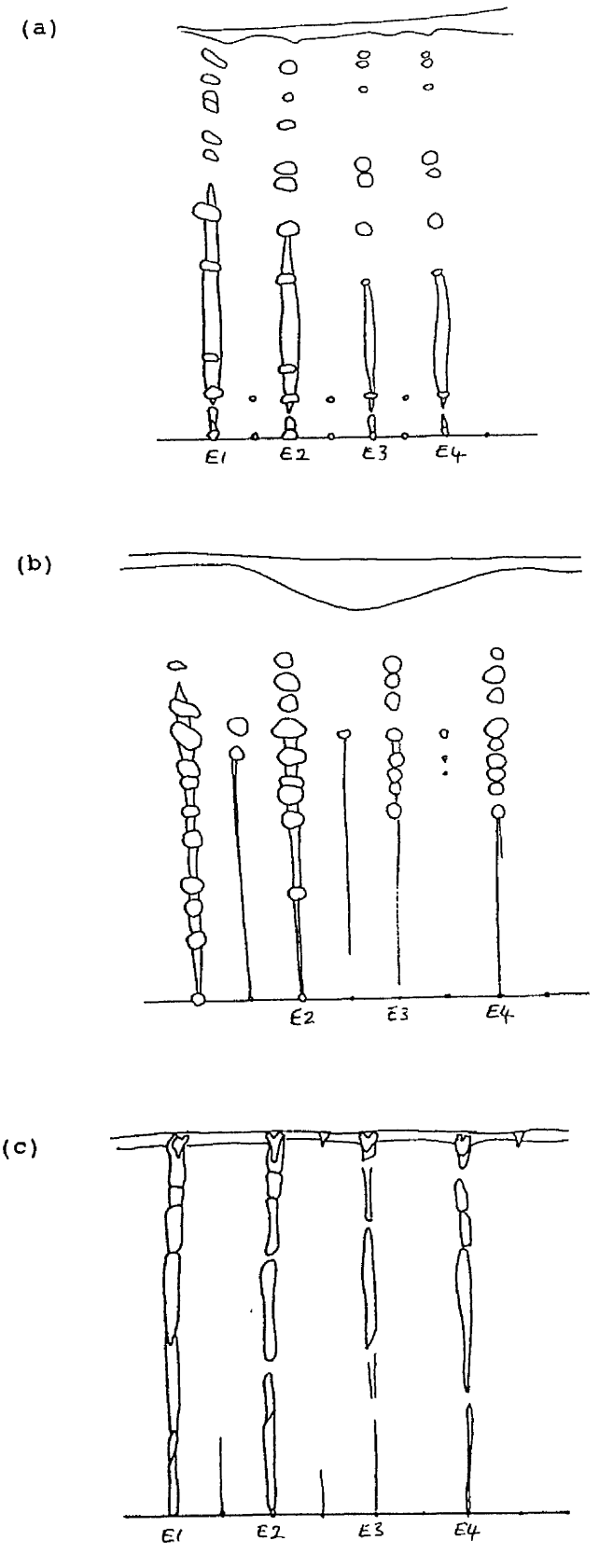

Fig. 5.5

Overlay of discrete spots on tlc plates separated using solvent mixtures (a) CCD, (b) TEF and (c) CAP (Section 5.2.5), visualised under short-wave ultraviolet light. Samples extracted from fungal isolate AP1.S20 are: E1 ethyl acetate extract, E2 chloroform extract, E3 hexane extract and E4 petroleum ether extract, [control extract samples were spotted to the right of each solvent extract sample].

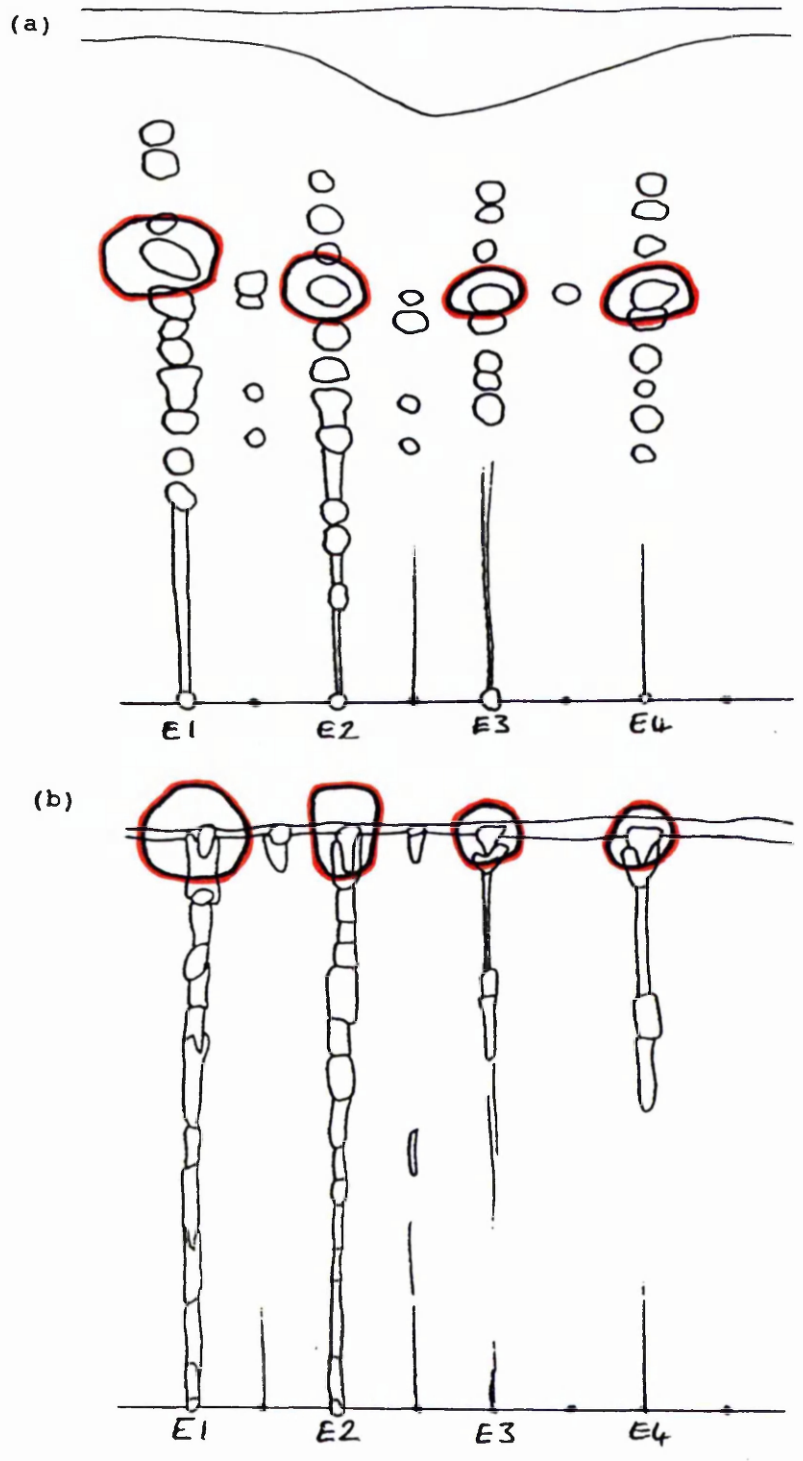

Fig. 5.6

Overlay of discrete spots on the tlc plates separated using solvent mixtures (a) CCD and (b) CAP (Section 5.2.5), visualised under long-wave uv light, where the red outlined area reflects the extent of the inhibition zone of the germination of *B. fabae* conidia. Samples extracted from fungal isolate AP1.S20 are: E1 ethyl acetate extract, E2 chloroform extract, E3 hexane extract and E4 petroleum ether extract, [control extract samples were spotted to the right of each solvent extract sample].

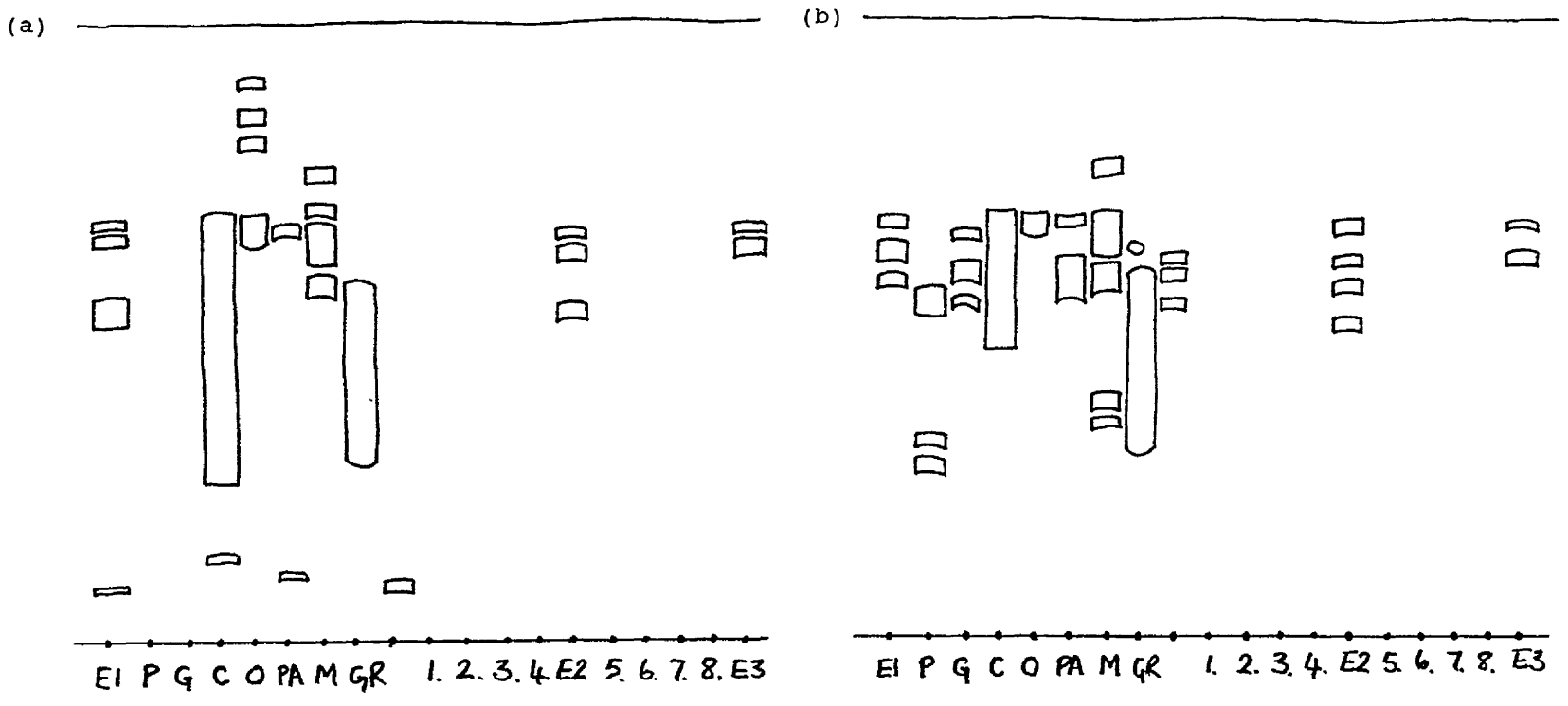

Fig. 5.7

Overlay of discrete banding on the tlc plates using the solvent mixture CCD (Section 5.2.5), visualised under (a) long-wave and (b) short-wave uv light. Samples extracted from fungal isolate AP1.S20 are: E1 ethyl acetate extract, E2 chloroform extract and E3 hexane extract; Standards are: P patulin, G gliotoxin, C citrinin, O ochratoxin A, PA penicillic acid, M mycophenolic acid and GR griseofulvin; Samples 1 to 4 are the eluted samples from TEF plate, and 5 to 8 from CAP solvents ethyl acetate, chloroform, hexane and petroleum ether respectively.

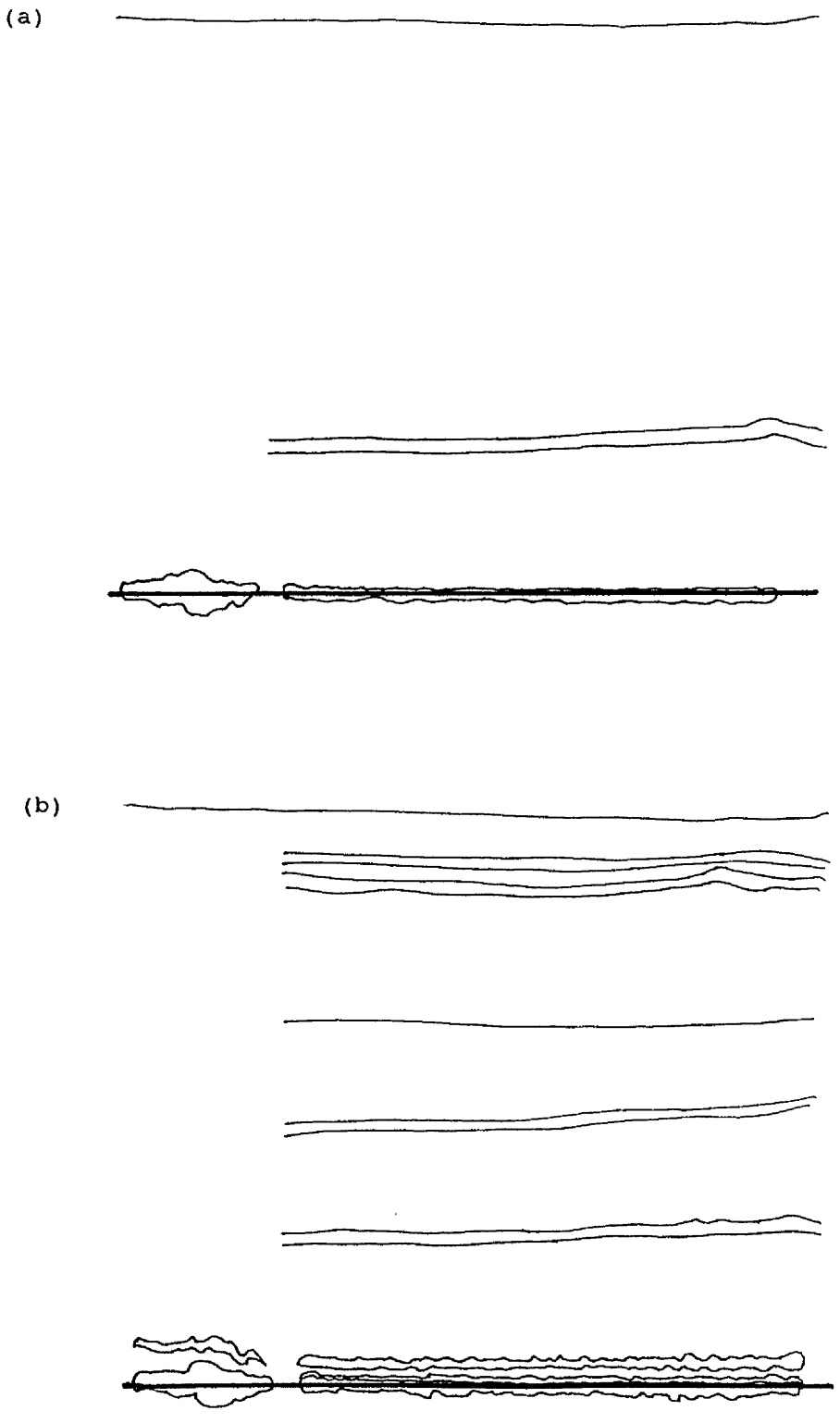

Fig. 5.8

Overlay of discrete spots on tlc plates of an aqueous extract (left) and an ethyl ether solvent extract (right) from an isolate of *P. fluorescens*, separated using solvent CCD, visualised under (a) long-wave and (b) short-wave ultraviolet light.

All of the plates sprayed with a suspension of *B. fabae* conidia showed large areas around an individual spot previously seen under uv light, where germination of *B. fabae* conidia were inhibited. With each solvent extract, this inhibition was localised to discrete spots with similar Rf values (0.590-0.653 for TEF, and 0.958-0.974 for CAP; Table 5.11; Fig. 5.6). From the measurements of the diameter of the inhibition zone around the spots, the ethyl acetate and chloroform extracts gave the largest areas of inhibition (Table 5.11).

The inhibition zones, scraped and eluted in chloroform, were run alongside seven standards often found in *Penicillium* spp. (Fig. 5.7), and also with E1, E2 and E3. The standards were run successfully with the extracts, but unfortunately the eluted samples did not display any banding when viewed under uv light. There was some similarity in the banding of the extracts and the standards.

When the ethyl ether solvent and aqueous extracts from *P. fluorescens* were chromatographed a number of bands were visualised with the ethyl ether extracts, especially under short uv light (Fig. 5.8), while only one band occurred with the aqueous extract.

5.4 Discussion

Throughout this study on the production of antifungal substances by fungal and bacterial antagonists, their effects on the germination and germ-tube growth of *B. fabae* conidia *in vitro* were recorded. This technique allowed a quick initial assessment of each of the extracted fractions obtained from

culture filtrates. Each fraction could easily be compared with others from the came culture filtrate or even different extraction methods, and presented as percent of the control. The fractions obtained from Method 1 all restricted the growth of *B. fabae* significantly, with *P. chrysogenum* (Isolate AP1.S20) showing the greatest inhibitory effects. Similarly, all the extracted fractions obtained from Method 2 reduced germination of *B. fabae* conidia significantly, compared to control samples.

Inhibition at a distance in agar tests is commonly ascribed to antibiotic production (Blakeman & Fokkema, 1982; Fokkema, 1973). However, the ability to produce antibiotics in culture does not necessarily mean that the antibiotic will be produced *in vivo* (Baker & Cook, 1974). Even if it is produced *in vivo*, it may not necessarily be responsible for the disease protection properties of a particular isolate, even if high levels of inhibition are shown *in vitro* (Blakeman & Brodie, 1976). Inhibition zones on agar, as seen in Chapter 3, we can conclude are due to antibiotic production, but are not a direct sign of any antibiotic role in nature (Fokkema, 1976). The production of such antibiotics in plate cultures varies depending on environmental factors, including: nutrition, depth and age of agar, size of inoculum and the temperature of incubation (Fravel, 1988). The nutrients necessary for production of antibiotics may be present in agar, but they may not necessarily be available in the phyllosphere.

In vitro assays for assessment of antibiosis have been found to be more valuable in some systems than others. Indeed, in the present study, the screening of different fractions of antagonists against *B. fabae* has yielded successful inhibition.

Two different methodologies for the extraction of antifungal agents from cell-free culture filtrates were examined here (5.2.2.1). The first, outlined by De Cal *et al.*, (1988), allowed the isolation of a range of antifungal extracts which reduced *B. fabae* germination and germ-tube development. Initial studies compared extracted fractions with sterile distilled water controls, as indicated by De Cal *et al.*, (1988). It was later discovered that the uninoculated culture medium, processed in a manner similar to the culture filtrates, also significantly reduced the germination of *B. fabae* conidia. However, the extracted active fractions from the fungal culture filtrates still caused very significant reductions when compared to these controls.

The other extraction method, based on work by Purkayastha & Bhattacharyya (1982), allowed the study of one isolate (AP1.S20), with extracts obtained using four different solvents. Again, it was found that controls of uninoculated medium extracted with the solvents produced significant reductions in conidial germination when compared to water or buffer controls. Nevertheless, there were still significant differences in conidial germination when controls from uninoculated media were compared to the fungal isolate extracts, as discussed in the results.

The symptoms described earlier (Section 5.3.1), resulting from dual culture experiments, are similar to growth patterns and structures noted by various authors in previous studies (Melgarejo & M-Sagasta, 1986; Kope & Fortin, 1989; Dennis & Webster, 1971c; Skidmore & Dickinson, 1976). It is best to assess the dual cultures when they have reached an equilibrium

of growth where there is with no further alteration to the growth pattern of the colonies. On this basis all four of the fungal cultures displayed clear zones of inhibition *in vitro*.

Studies of mycelial growth of *B. fabae* in dual culture with antagonists cultures, suggest the presence of inhibitory compounds produced by these fungal isolates. This was confirmed by the effects of the cell-free culture filtrates from these isolates which severely inhibited conidial germination and germ-tube extension of *B. fabae in vitro*. In this study the symptoms of all the fungal isolates altered throughout the period of culturing over a time-scale; from coagulation and convolution of mycelium, shrinkage and collapse of the hyphal walls and finally with the mycelium thoroughly destroyed into pieces after 25 days.

The morphological changes seen here, both in agar diffusion tests and dual cultures with cell-free culture filtrates, e.g., the initial distortion in growth along the edge of *B. fabae* mycelium at the line of inhibition, followed by severe lytic action with the eventual collapse and death of hyphae, have been well documented in previous studies (De Cal & Melgarejo, 1992; Melgarejo & M-Sagasta, 1986). The lysis recorded is reflected in permeability changes in protoplasmic membranes caused by the action of antibiotics, enzymes or metabolites (Austin *et al.*, 1977). Hyphal lysis is normally the breakdown of the fungal cytoplasm before there is any noticable effect on the cell walls, i.e. coagulation of the cell cytoplasm (Malajczuk, Newsbitt & Glenn, 1977). The equilibrium in wall metabolism could be displaced in the direction of wall degradation by either an increase in wall lysis, or a decrease

in wall synthesis (Skidmore & Dickinson, 1976), elicitated by external application of a wall splitting enzyme (lysozyme) or an inhibitor of wall synthesis (Bartnicki-Garcia & Lippman, 1972). Digestion with hydrolytic enzymes causes apical disintegration of hyphae.

Such morphological changes in *B. fabae* growth *in vitro*, result from the action of extracellular metabolites produced by the antagonists into the culture medium. *Penicillium* spp. have been widely documented as antagonists with the potential to control a broad spectrum of pathogenic fungi (Purkayastha & Bhattacharyya, 1982). Melgarejo & M-Sagasta, (1986) focused on the ability of *P. frequentans* to produce antifungal substances, and in this present study, antibiotics were produced by a number of isolates, particularly Isolate AP1.S20 (*P. chrysogenum*). They were active in advance of the hyphal growth of the antagonist, maintaining zones of inhibited growth *in vitro* (Kope & Fortin, 1989), or from extracted culture medium. Morris & Lane (1990) reported intense vacuolation and lysis of *B. fabae* by *Trichoderma viride* in plate cultures, with a significant zone free of mycelial growth. Similar symptoms were observed here, with all the extracts from medium greatly reducing the germination and germ-tube extension of *B. fabae*.

Young secondary colonies of fungal antagonists that arose after inoculation in dual cultures did not produce inhibition zones. This is possibly because they were not actively producing any inhibitory substances when first initiating growth. Melgarejo & M-Sagasta (1986) found that activity from their antagonist cultures *in vitro* has little effect on growth of the pathogen 7-10 days from inoculation, but that the

symptoms became more evident and extreme over time in the dual cultures. This would probably be due to the accumulation of metabolites to a level toxic towards *B. fabae* with the continued destruction of the pathogen's hyphae.

The lack of any physical contact or interference between the pathogen and the antagonists rules out hyperparasitism as a mode of antagonism. It is unlikely that the antagonist altered the pH of the growing medium, which could result in reduced growth of *B. fabae*, as no significant changes in pH of the medium by the fungal antagonists were recorded.

A possible reason for the lack of inhibition zones in the agar diffusion tests is that the crude culture filtrate was not concentrated enough to inhibit mycelial development. Evaluation of the dosage response for a parameter such as inhibition of germination, can provide information about activity not apparent from a single dose bioassay. From the dose response test we can conclude that although the isolate fraction reduced growth to the greatest extent, the relative control severely affected growth only at high concentrations. In contrast, germ-tube extension was very sensitive to inhibition over all concentrations of both samples.

In this study, the culture filtrate of *P. chrysogenum*, an organism that colonises the phyllosphere of peach trees (Melgarejo *et al.*, 1985), successfully lowered disease levels, reducing lesion formation and subsequent spread on detached leaves. This suggests that there could be potential for some of these antibiotic fractions in biological control. When *B. fabae* conidia were applied to detached bean leaves in PDB medium, there was an increase in infection. This was probably due to

the higher nutrient levels in the PDB medium compared to water, as used in the controls. On the other hand, fungal antagonists certainly have been shown to reduce nutrients on plant surfaces. Thus for necrotrophic pathogens, such as *B. fabae*, the pre-penetration stage of development is the most vulnerable stage in growth and the site most likely to result in successful biological control (Baker & Cook, 1974).

Samples of culture filtrates and solvent extracts on tlc plates were fractionated into a number of spots. Following a bioassay with *B. fabae* conidia, one active spot was visualised on each of the tlc plates developed in the different solvent systems. The active spots from each of the four solvent extracts, on each plate, had very similar Rf values. It appears therefore, that only one compound from each extract was active in reducing germination of *B. fabae* conidia and changes in the morphology of the mycelium. The concentration of inhibitor varied with each solvent extract as seen by the variation in the diameter of the inhibition zones. Blakeman & Atkinson (1976) found that from a range of organic solvents tested, chloroform extracted the largest number of component compounds. It is possible that other potential inhibitors were present, but at a concentration too low to be effective in this bioassay.

The results presented here highlight the powerful effects that antibiotic metabolites, produced by saprophytic fungi, can have on plant pathogens. The identification of the different, semi-purified active compounds would facilitate a more detailed study of its interaction with *B. fabae* than has been possible in this study.

6.

6. GENERAL DISCUSSION AND CONCLUSIONS

From this study, a protocol has been described allowing the isolation of both bacterial and fungal isolates from samples of foliage and soil, for use in screening tests. It has been emphasised previously (Fermor & Lynch, 1988; Blakeman & Fokkema, 1982) that micro-organisms to be screened as biological control agents should be isolated from areas where the disease could occur, but where no symptoms have been found. Most isolates screened in this study were, for this reason, isolated from leaf and soil samples collected mainly from faba bean crops. All bacteria and fungi selected after initial screening *in vitro*, except for one bacterium (P1.S13B), were isolated from foliage or soil samples collected from faba bean cropped areas.

Extensive screening, both *in vitro* and *in vivo*, was carried out in the course of this study. When screening and selecting antagonists, it is important to try and prevent antagonists being discarded, or the retention of non-antagonists. Obviously, the objective of any screening procedure is to minimise the likelihood of these two outcomes. To reduce the probability of discarding valuable microbes, which is the more serious consequence, we must accept an increasing probability of keeping non-antagonists for further screening (Andrews, 1985), before final selection. Decisions on selecting antagonists, given the above constraints, are further complicated by the fact that *in vitro* assays are often poor predictors of performance *in vivo* (Blakeman & Fokkema, 1982), as discussed in Chapter 3.

A quick preliminary screen allowed the symptoms of

antagonism towards *B. fabae in vitro* to be identified for a large number of isolates, before more detailed analysis using replication. Here, the visual symptoms were striking and easy to assess. The series of *in vitro* screening tests allowed the original 690 bacterial and fungal isolates in this study to be reduced to a more manageable number for further screening and application to the leaf surface. Such studies of antagonism using pure cultures *in vitro* can be misleading, as the conditions cannot always mimic the diversity and complexity of the phyllosphere, where the nutritional levels *in vitro* are not comparable to the non-enriched phyllosphere (Blakeman & Fokkema, 1982).

The ideal environment for studies on the effects of phyllosphere antagonists on the development of foliar pathogens would be the surface of healthy attached bean leaves. Technical problems of using whole plants for screening were avoided initially in this study by using parallel systems, e.g., agar plates and detached leaves. Thirty five isolates (20 bacterial and 15 fungal), selected from the *in vitro* studies, were screened against *B. fabae* conidia and rust uredospores on bean plants in the glasshouse.

Screening on the plant surface gave a much better assessment of antagonism, and of the potential of these antagonists to control disease in the field. *In vitro* screening methods did not select for parameters relevant to their potential ability as successful bio-control agents on the crop, e.g. the ability of isolates to survive and colonise the phyllosphere, reducing disease development on the plant foliage. *In vivo* techniques are often found to be too severe, rejecting

potentially useful candidates (Andrews, 1985; Baker & Cook, 1974). It has been suggested that the apparent disparity between *in vitro* and *in vivo* performance when screening, may represent nothing more than differential survival in two quite different environments (Andrews, 1985). Certainly, in this study, a number of the most antagonistic isolates *in vitro* also controlled the diseases to a significant degree on the plant.

Various authors have concluded that the mode of antagonism of fungal and bacterial isolates towards necrotrophic pathogens like *B. cinerea* is mainly through nutrient competition (Mansfield & Hutson, 1980; Brodie & Blakeman, 1976). Morris & Lane (1990) suggested that antibiotic production was a major method of antagonism of *B. fabae* conidia, rather than nutrient competition. *B. fabae*, unlike *B. cinerea*, has larger conidia with more food reserves, giving a better growth rate and germ-tube extension (Purkayastha & Deverall, 1965). Such reserves reduce the need for exogenous nutrient supplies for infection. Obligate biotrophic pathogens such as rust, have no requirement for nutrients prior to forming its specialised relationship with cells of the host plant (Blakeman, 1993).

Studies of mycelial growth of *B. fabae* in dual cultures with fungal antagonists, indicated the presence of inhibitory compounds, active in advance of the hyphal growth of the antagonist, maintaining inhibition zones. Here, abnormal germ-tube growth and interference with the development of *B. fabae* colonies was observed. The presence of inhibitory agents produced by these antagonists was confirmed through *in vitro* assay tests on cell-free culture filtrates.

Antibiosis often acts in concert with competition and/or

parasitism, but generally it is not an exclusive one (Fravel, 1988). Nevertheless, the ability to produce antibiotics in culture, as demonstrated in this study, does not necessarily mean that the antibiotic will be produced *in vivo*. Even if it is produced *in vivo*, it may not necessarily be responsible for the disease protection properties of a particular isolate (Blakeman & Brodie, 1976). However, Smith, Wilcox & Harman, (1990) used evidence of antibiosis in a preliminary *in vitro* screen to assess potential bio-control capabilities of isolates, as this was an easily identifiable characteristic to measure. Isolates not exhibiting antibiosis were not tested further.

There was little evidence of hyperparasitic interactions of the two pathogens studied in dual cultures with antagonists. Most of the isolates selected displayed clear inhibition zones *in vitro*, which were likely to result from the production of antibiotics.

A good antagonist could be converted into a good bio-control agent by assessing and promoting the colonisation ability of these antagonists, rather than further screening to seek for more candidate antagonists. If an antagonist controls disease through the production of antifungal agents, then their production *in vitro*, followed by their application to the leaf surface, might give better disease control than the application of live inoculum (Baker, 1987).

We can conclude that the extraction, isolation and partial purification of antifungal substances has proved successful, controlling chocolate spot in germination tests *in vitro* and subsequently on detached bean leaves, measuring lesion development and spread.

There is increasing interest in biological control of plant diseases because of a greater knowledge of microbial ecology, helping us understand the interactions involved in bio-control in the phyllosphere (Campbell, 1989). The increasing use of bio-control agents in the future will help to reduce the environmental hazards of pesticide application. This study has discussed the initial assessments on the use of antagonistic micro-organisms or their by-products to control chocolate spot and rust. It is important to remember that we are dealing here with a dynamic and diverse system, compared to the precision and specificity of action of modern chemical control.

7.

7. FURTHER STUDIES

This study, initiated 3 years ago, to investigate the potential of biological control of foliar diseases of faba beans, has yielded some interesting and promising results, following an extensive isolation and screening programme. A number of identified bacterial and fungal antagonists have shown much potential in these studies. If this potential is to be realised, a great deal of additional experimental work will be required.

Studies in this thesis suggesting the presence of lytic activity from the culture filtrates of *Penicillium* spp. agree with previous research (Melgarejo & M-Sagasta, 1986; De Cal *et al.*, 1988). Further work using a number of chromatographic techniques (tlc, hplc, column chromatography) would allow the identification of the inhibitory substance isolated on the tlc plates from the culture filtrate of isolate AP1.S20 (*Penicillium chrysogenum*), (Chapter 5). The isolation of antifungal substances from a number of fungal and bacterial species involving the use of various media combinations and solvent extractants is well documented (De Cal *et al.*, 1988; Kraus & Loper, 1992; Levy, Eyal & Chet, 1988; Madrigal, Tadeo & Melgarejo, 1991). Further experimental work preparing extracts, followed by isolation of inhibitory substances from the fungal and bacterial isolates identified in this study is proposed, based on the methods above. Identification of such compounds involves biochemical techniques, including: the measurement of melting point, infrared spectrum, nuclear magnetic resonance spectrum and the mass spectrometry profile, before comparing the

results with database libraries (Madrigal *et al.*, 1991; Levy *et al.*, 1992).

Infection structures at the host/pathogen/antagonist interface, should be studied at a cellular level, using leaf staining with light and electron microscopy (Spurr, 1981). The use of sellotape strip samples, leaf impressions and leaf discs would allow the inhibition levels and activity of antagonists on the leaf surface to be defined (Fokkema, 1978; Sleesman & Leben, 1976). This could pin-point the mode of antagonism and its mechanisms.

Knowledge of the establishment and colonisation, as well as the survival of antagonistic micro-organisms in the phyllosphere of faba beans, is an important area of research, extending the screening work detailed in Chapter 4. The optimum application of any biological control agent is crucial for effective disease control (Levy *et al.*, 1992), and the formulation of any bio-control agent/compound applied can greatly influence their success in the phyllosphere. Such formulations are numerous and include spores, spores with nutrients, mycelium, inoculum in wheat bran cultures or even mycelial plugs (De Cal *et al.*, 1990; Fokkema, 1979). Formulation of antagonists in alginate pellets for control of soil-borne diseases has been well documented (Knudsen *et al.*, 1991; Lewis & Papavizas, 1987), with some initial studies on formulation allowing their application in the phyllosphere (e.g. *Trichoderma harzianum*), through milling dried PEG granules to specified sizes (Knudsen & Eschen, 1991).

As such novel agents must remain stable and in an active form, extensive screening is required at the whole plant level.

Field scale trials of isolates and compounds showing most potential would allow the efficient assessment of their suitability as bio-control agents on field crops.

The levels of sensitivity of the antagonists to the chemical fungicides commonly used on faba beans should be determined. This would examine the potential for integrated disease management, with the potential advantage of reducing the volume of chemicals applied to control diseases (Morris & Lane, 1990), possibly reducing the selection pressure for resistance of the pathogen to fungicides.

8.

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