Mechanisms of biological control of the damping-off fungus, *Pythium ultimum*, by binucleate *Rhizoctonia*.

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TABLE OF CONTENTS

DECLARATION

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ABSTRACT

Damping-off diseases are responsible for major production losses in agriculture and horticulture throughout Australia and overseas. In pre-emergence damping-off, the germinating seeds are killed soon after planting, whereas in post-emergence damping-off, seedlings collapse at soil level with a constricted stem base, watery soft rot or brown discolouration of the stem tissues. *Pythium ultimum* is a notorious damping-off pathogen because of its ability to exploit fresh plant tissue and cause an infection within 24 hours of sowing the seeds. The potential of binucleate *Rhizoctonia* spp. (BNR) to control *P. ultimum* var. *sporangiiferum,* and thereby reduce losses due to damping-off diseases in nursery seedlings, is currently under investigation. These fungi provided good disease control on *Capsicum* and *Celosia* spp. as well as on *Brassica oleracea* var. *gemmifera* (Brussels sprouts). This PhD project was undertaken to investigate the mechanism(s) involved in the protection of *Capsicum* seedlings by two isolates of BNR against the damping-off fungus, *P. u. sporangiiferum.*

Light- and scanning-electron microscopy studies demonstrated parasitism of *P. u. sporangiiferum* by BNR, both on agar media and on dead *Capsicum* seeds in sterilised potting mix.

The ability of BNR to produce soluble and volatile secondary metabolites was investigated in agar culture and in potting mix. There was no evidence of non-volatile antibiotic activity by BNR against the pathogen. However, lytic enzymes such as β-1,3-glucanase and cellulase were produced by BNR in synthetic liquid media with different carbon sources including dead cell walls of *P. u. sporangiiferum*, and in sterile potting mix. These enzymes were found to be responsible for inhibition of colony growth of *P. u. sporangiiferum* in agar media. Unidentified volatile secondary metabolites produced by BNR were also responsible for reduction of colony growth of *P. u. sporangiiferum* in agar media and in sterile potting mix.

Colonisation of *Capsicum* seeds and seedlings by BNR and *P. u. sporangiiferum* was investigated in pasteurised potting mix in a glasshouse. BNR produced dense hyphal mats on seeds and plant roots, especially in the meristematic region and the root tip. BNR also extensively colonised the epidermal cells of the host roots. *P. u. sporangiiferum* preferentially colonised the same plant tissues as did BNR. Histochemical studies were undertaken to determine if colonisation of *Capsicum* roots by BNR led to cell wall alterations in hypocotyls, the site of post-emergence damping-off by *P. u. sporangiiferum.* There was no indication of induced systemic resistance by BNR in *Capsicum* seedlings. However, lesions caused by the pathogen in *Capsicum* hypocotyls were colonised by BNR in preference to the non-infected hypocotyl tissues.

Studies on competitive saprophytic ability in sterilised potting mix revealed that colonisation of dead *Capsicum annuum* seeds by *P. u. sporangiiferum* was reduced in the presence of BNR. Complete displacement of *P. u. sporangiiferum* by BNR was observed in less than 50% of the seeds inoculated with both BNR and the pathogen. Growth of *P. u. sporangiiferum* from the remaining 50% of seeds, that were colonised by both the antagonist and the pathogen, was reduced in sterile potting mix. In a glasshouse experiment, when introduced on dead *Capsicum* seeds, *P. u. sporangiiferum* caused less disease of *Capsicum* seedlings in the presence of BNR than when introduced on its own.

Possible mechanisms of protection of *Capsicum* seedlings against pre- and post-emergence damping-off are discussed. Elimination of *P. u. sporangiiferum* from *Capsicum* seeds was apparently facilitated by mycoparasitism and/or inhibitory metabolites produced by BNR. Blockage of infection sites by dense hyphal mats of BNR is believed to result in the exclusion of *P. u. sporangiiferum* from *Capsicum* seeds or roots. Displacement of *P. u. sporangiiferum* from lesions by BNR evidently impeded further disease development in infected *Capsicum* hypocotyls.

CHAPTER 1 GENERAL INTRODUCTION

Biological control of plant pathogens was defined by Baker & Cook (1974) as "the reduction of inoculum density or disease-producing activities of a pathogen or parasite in its active or dormant state, by one or more organisms, accomplished naturally or through manipulation of the environment, host, or antagonist, or by mass introduction of one or more antagonists". Biological control is a natural phenomenon and it represents the associations of different, independent species in nature, such as pathogen-host- or pathogen-antagonist-interactions. These associations are dynamic processes and are, therefore, subject to environmental changes as well as to the adaptations, properties and limitations of the organisms involved (Snyder *et al.*, 1976).

In recent years, interest in biological control of plant diseases as a viable practice in modern agriculture and horticulture has increased dramatically. Not only government agencies but also the private sector of agricultural and horticultural industries, are undertaking research in this area (Still, 1982). There are several reasons for such accelerated interest in biological control. One significant factor is the general increase in public awareness of the potential ecological hazards posed by the use of chemical pesticides. Another reason is the development of resistance to chemical pesticides in many plant pathogens. It is also a result of increased knowledge of microbial ecology and the opportunities offered by techniques such as genetic engineering and immunological methods for identifying and tracking microorganisms released into natural or agricultural environments (Andrews, 1986; Nigam & Mukerji, 1988; Campbell, 1989*b*).

Biological control of fungal plant pathogens using antagonistic fungi has gained considerable attention as a promising alternative to chemical control

(Cook & Baker, 1983; Lumsden & Lewis, 1989). These antagonistic fungi control plant pathogens through different modes of action, including rapid colonisation of plants in advance of the pathogen or subsequent competition, which may lead to niche exclusion or displacement of the pathogen from infected host tissues. Moreover, antibiotics and other toxic substances may be produced, or there may be mycoparasitism or lysis of the pathogen. Some biocontrol agents may act simply by enhancing plant growth, thus reducing the susceptibility of the plant to an infection (Elad, 1986; Campbell, 1989*b*; Jarvis, 1992). Baker (1990) emphasised the importance of detailed studies on the mode of action of potential biocontrol agents against the target pathogen(s) as an essential step in enhancing the chances of success in the development and application of biocontrol strategies. Campbell (1986) also argued that several questions need to be answered before logical development of a delivery system can be achieved. For example, does the antagonist need to be on susceptible host tissue before the pathogen arrives? Does it spread on the plant or does the initial application have to achieve complete cover? Does the biocontrol agent persist in the environment or in association with the host? Does the antagonist have to come into contact with the pathogen or can it operate from a distance? In addition to the mode of action of the antagonist, the vulnerable stage in the life cycle of the pathogen must be also identified (Campbell, 1986).

While there are currently about 120 recognised species of *Pythium*, the most common plant pathogenic species are *P. ultimum*, in temperate climates, and *P. aphanidermatum*, in tropical climates (Bouhot, 1988; Campbell, 1989*b*; Dick, 1990*b*). Damping-off and root diseases in vegetable crops, bedding, foliage and woody plants, as well as cut flowers, caused by *Pythium* spp. are responsible for major economic losses, especially in the nursery industry throughout Australia and overseas. These economic losses are the result of decreased plant production and quality, and plant death, as well as increased management costs needed to minimise losses (Cook & Baker, 1983; Schisler *et al.*, 1994). Cultural and chemical control strategies are

commonly used in the nursery industry to control Pythium diseases, however, these vary in their effectiveness and are not always economically viable, especially for small-scale producers.

Biological control of fungal plant pathogens shows great promise as an alternative control strategy. Isolates of BNR have been studied recently as potential biocontrol agents of *Rhizoctonia solani* and *P. ultimum* (Cardoso & Echandi, 1986, 1987; Cubeta & Echandi, 1988, 1991; Escande & Echandi, 1991*b*; Harris *et al.*, 1991, 1993*b*, 1994; Harris & Adkins, 1993; Herr, 1988). Suggested mechanisms for biocontrol of *R. solani* by BNR include induced host resistance, protection of plant surfaces and competition for an ecological niche (Cardoso & Echandi, 1987; Jabaji-Hare *et al.*, 1993; Harris *et al.*, 1991, 1993*b*). Mechanisms of biocontrol of Pythium damping-off in nursery potting medium by BNR isolates are not well understood but may involve protection of plant surfaces or induced host resistance (Harris *et al.*, 1991, 1993*b*). A detailed study on the ecology and mode of action of BNR against *P. ultimum* is required to facilitate the successful commercial development of these antagonistic fungi as biocontrol "pesticides".

CHAPTER 2 LITERATURE REVIEW

2.1 INTRODUCTION

This literature review focuses on the biology of *Pythium* spp., the diseases they cause in agricultural crops, and on strategies applied to the control of Pythium damping-off and root rots. The advantages and disadvantages of biological control compared to other control measures and the mechanisms by which biocontrol agents reduce disease are discussed. Aspects of the biology of BNR spp., and their potential to control diseases caused by *Pythium* spp. are reviewed.

2.2 THE BIOLOGY OF *PYTHIUM*

2.2.1 Taxonomy

The genus *Pythium* was established by Pringsheim in 1985 with the description of *P. monospermum* Pringsh. as the type species. The genus *Pythium* is in the family Pythiaceae, order Peronosporales, class Oomycetes, and is now classified as a part of the Kingdom Chromista (Barr, 1992). The Union of Fungi, as proposed by Barr (1992), comprises organisms belonging to three different kingdoms, in order to overcome the taxonomic difficulties which exist in the "Kingdom Fungi". The most recent taxonomic description of the genus (Dick, 1990*a*) lists 120 recognised *Pythium* species.

2.2.2 Morphology

The mycelium of *Pythium* species is colourless, sometimes lustrous and occasionally slightly yellowish or greyish lilac (Van der Plaats-Niterink, 1981). The hyphae are

hyaline and usually 5-7 μm in diameter (occasionally up to 10 μm). They lack septa except in old hyphae or at the point of spore differentiation (Martin, 1992). Protoplasmic streaming is often visible in young hyphae (Van der Plaats-Niterink, 1981). The cell walls of *Pythium* species, as in most Oomycetes, are composed primarily of alkali-insoluble and cuprammonium-insoluble glucan(s) containing β-1,3- and β-1,6-linkages, with cellulose being a minor cell wall component and chitin found in non-significant amounts (usually less than 1%) in some fungi only (Bartnicki-Garcia, 1967; Hendrix, 1974).

Reproduction in *Pythium* species takes place by asexual or sexual means. Asexual reproduction occurs through the development of sporangia, and morphology of the sporangia is characteristic of a species (Van der Plaats-Niterink, 1981). Sporangia may germinate either directly by the formation of one or several germ tubes or indirectly by the formation of motile zoospores inside a vesicle formed at the end of a discharge tube (Martin, 1992). In the latter, the protoplasm passes from the sporangium into the vesicle, and there it forms biflagellate zoospores. After their release, in free water, the zoospores swarm about for a few minutes, come to rest, encyst and germinate by producing a germ tube which develops into mycelium. Sometimes the germ tube produces another vesicle in which secondary zoospores are formed, and this process may be repeated (Martin, 1992; Hendrix & Papa, 1974). In species which do not produce zoospores, the asexual structures are often referred to as conidia or hyphal swellings (Martin, 1992). Sexual reproduction involves the production of multinucleate antheridia and oogonia. The oogonia are usually spherical to limoniform, and are intercalary or terminal. The antheridia can be sessile, intercalary, or formed terminally on an antheridial stalk (Van der Plaats-Niterink, 1981). The oosphere within the oogonium is fertilised when the haploid male gametic nuclei enter the oogonial cavity through a fertilisation tube linking antheridium and oosphere. After fertilisation, reorganisation of protoplasmic contents of the oosphere results in the formation of a thick-walled, resistant oospore (Hendrix & Papa, 1974; Dick, 1990*b*). The

"constitutively" dormant oospores, which do not germinate because of the innate property of the dormant stage, are refered to as "dormant" or "thick-walled" oospores. On the other hand, the thin walled "exogenously" dormant oospores, which do not germinate because of an inappropriate environment (fungistasis), are referred to as "germinable" or "thin-walled" oospores (Lumsden & Ayers, 1975). The thin-walled oospores, in the absence of fungistasis, germinate by producing germ tubes, or vesicles in which zoospores are formed (Van der Plaats-Niterink, 1981). The vegetative hyphae of *Pythium* spp. are diploid and meiosis occurs in the gametangia. Homothallism predominates in *Pythium* spp., although the existence of several heterothallic species has been reported (Hendrix & Papa, 1974; Campbell & Hendrix, 1967; Van der Plaats-Niterink, 1968). Many *Pythium* spp. form appressoria which are sickle-, club- or sausage-shaped, or are subglobose hyphal- or germ tube-tips. The size and shape of appressoria are influenced by such factors as temperature, pH, carbohydrates and the nitrogen source (Van der Plaats-Niterink, 1981).

2.2.3 Distribution

Pythium spp. are found all over the world, including tropical, temperate and cold climates. They occur most commonly in cultivated soils (Van der Plaats-Niterink, 1975) and less frequently in non-cultivated or acid soils (Barton, 1958). *Pythium* spp. have been found in soil samples from arable land, pastures, forests, nurseries, marshes, swamps and in water. Dry sandy regions, dry forests and salt marshes are unfavourable to the growth of these fungi (Van der Plaats-Niterink, 1981; Dick & Ali-Shtayeh, 1986). *Pythium* spp. have been recovered from nests, intestines and feathers of free-living birds (Hubálek, 1974*a,b*), from bird droppings (Thornton, 1971), and from the gut (Hutchinson & Kamel, 1956), casts and external surfaces of earthworms (Thornton, 1970).

2.2.4 Pathogenicity

Not all *Pythium* spp. are plant pathogens; some are obligate saprophytes (Stanghellini, 1974), or parasites on mosquito larvae (Saunders *et al.*, cited in Martin, 1992). However, many *Pythium* species are facultative pathogens of plants (Hendrix & Campbell, 1973). These fungi are recognised as the most important cause of pre- and post-emergence damping-off of seeds and seedlings, as well as causing root- and stem-rots in more mature plants and soft-rots of mature fruits and vegetables (Hendrix & Campbell, 1973; Fletcher, 1984). Diseases caused by *Pythium* spp. occur mainly in nurseries of forest or garden plants, outdoor gardens and in greenhouses, and secondarily in cereal crops. Many species attack a variety of host plants while others are restricted to one host species (Hendrix & Campbell, 1973; Krupa & Dommergues, 1979).

2.3 SAPROPHYTIC AND PATHOGENIC ACTIVITIES OF *PYTHIUM ULTIMUM*

P. ultimum is a notorious damping-off and root-rot pathogen because it can rapidly colonise and exploit plant tissues, causing major crop losses, especially in the nursery industry (Cook & Baker, 1983; Fletcher, 1984). Symptoms of damping-off caused by this pathogen vary with the age and stage of development of the infected plant. Thus, juvenile or succulent tissues in which secondary wall thickenings have not yet developed are most likely to be infected by the pathogen (Wheeler, 1969). In pre-emergence damping-off, susceptible seeds fail to germinate in the soil due to rotting of the diseased tissues. Germinating seedlings which have not yet emerged above the soil level are also infected. The pathogen induces brownish, water-soak lesions on radicles or young hypocotyls, causing the seedlings to die (Wheeler, 1969). In post-emergence damping-off, emerged susceptible seedlings are attacked at or below the soil level. The fungus penetrates the tissues of the lower stem, causing water-soaked and brown

lesions. As the rotted basal part of the seedling stem cannot support the upper part of the plant, the seedling falls over on the soil surface and dies because of continued rotting of the tissue by the pathogen (Fletcher, 1984). Damping-off in mature plants is restricted mainly to the fine feeder roots or the root tips, with occasional small lesions being formed on the stems. The infected tissues are often killed by the fungus, resulting in stunting, wilting or even death of the above ground parts of the plant (Fletcher, 1984; Cook & Baker, 1983).

Population density of *P. ultimum* in soil increases either by saprophytic utilisation of organic residues or after an infection of a susceptible host (Lifshitz & Hancock, 1983). The severity of diseases caused by this, and other, *Pythium* spp. relates less to the initial inoculum density of the pathogen than to the amount of saprophytic growth of the fungus before the infection (Bouhot, 1979). However, the saprophytic activity of *Pythium* spp. in soil, generally, is restricted to an early colonisation of virgin substrates in advance of other microorganism. The inability of *Pythium* spp. to compete as secondary colonisers is attributed to their great sensitivity to toxic metabolites produced by competing microorganisms (Barton, 1961; Garrett, 1956). Thus, chemical or biological factors that suppress rapid colonisation of crop residues by *Pythium* spp. also reduce the saprophytic activity and inoculum potential of these fungi (Martin & Hancock, 1986). Oospores and sporangia of *P. ultimum* enable the fungus to survive in adverse soil conditions (Lumsden & Ayers, 1975; Stanghellini & Hancock, 1971*a*). The success of *P. ultimum* in colonising a substrate is dependent upon the percentage of the thick-walled oospores converting to thin-walled oospores, since it is the latter which act as the primary inoculum for the infection of a host plant (Lumsden & Ayers, 1975; Stanghellini, 1974).

2.3.1 Host range

P. ultimum has a wide host range including both nursery and field crops. Van der Plaats-Niterink (1981) provides a comprehensive summary of crops known to

be affected by the pathogen. For example, severe damping-off or root rots caused by *P. ultimum* have been reported for cotton (Arndt, 1943; Spencer & Cooper, 1967; Ogle *et al.*, 1993, 1995), beans and peas (Escobar *et al.*, 1967; Kraft & Burke, 1971; Short & Lacy, 1976; Tu *et al.*, 1993), cucumber, cabbage, beetroot, lettuce and tomato (Paulitz & Baker, 1987*a*; Gram & Weber, 1952). Moreover, *P. ultimum* has been isolated from roots of diseased tomato, cucumber and lettuce growing in greenhouse hydroponic systems (Jenkins & Averre, 1983).

2.3.2 Development of disease caused by *P. ultimum*

Germ tubes originating from zoospore cysts (Miller *et al.*, 1966; Chérif *et al.*, 1991), sporangia or oospores (Stanghellini & Hancock, 1971*b*; Nelson & Hsu, 1994), or saprophytic mycelium (Mellano *et al.*, 1970*b*) can initiate host infection. These propagules come into contact with susceptible seeds or seedling tissues in the soil either by chance or as a result of chemotropic stimulants, which are primarily host plant exudates (Endo & Colt, 1974; Nelson, 1990). *P. ultimum* enters the seed by direct penetration of the moistened, swollen seed coats or through cracks, then penetrates the embryo or emerging seedling tissues. Penetration may be accomplished by mechanical pressure or by enzymatic action upon host cell walls, or by a combination of both mechanisms (Endo & Colt, 1974). Because of its fast germination and growth, *P. ultimum* rapidly colonises the susceptible host tissues. For example, the fungus colonises the seed coat of pea in 10 hours, and the embryo in 24 to 48 hours, after the seeds are planted in infested soil (Lifshitz *et al.*, 1986). Osburn *et al.* (1989) also reported that, under ideal conditions, *P. ultimum* began to colonise the pericarp of sugar beet seeds within 4 hours, and colonised a great proportion of this tissue by 12 hours. When inside the seed or emerging radicle, pectinolytic and proteolytic enzymes secreted by *P. ultimum* dissolve the middle lamella and the protoplasts of invaded cells, respectively. As a result of enzymatic activity invaded tissue is macerated. The seeds are killed and turned into a rotten mass consisting mainly of the

fungus and substances such as suberin and lignin, which can not be broken down by the pathogen (Chérif *et al.*, 1991).

The infection of roots or hypocotyls of emerged young seedlings by *P. ultimum* progresses in a manner similar to that for seeds. The pathogen penetrates the host epidermal cells directly either by constricted hyphae (Chérif *et al.*, 1991), infection pegs (Nemec, 1972), or hyphae arising from appressoria, zoospore cysts (Mellano *et al.*, 1970*b*) or hyphal swellings (Endo & Colt, 1974). The zones of elongation and maturation of the root are most frequently penetrated by the pathogen (Mellano *et al.*, 1970*b*; Nemec, 1972; Endo & Colt, 1974). As in seed colonisation, *P. ultimum,* rapidly establishes in susceptible plant tissues. The fungus initiates host penetration in the first 2 to 10 hours, invades the cortical cells within the first 48 hours, and colonises the stele between 72 and 96 hours, after inoculation (Miller *et al.,* 1966; Mellano *et al.*, 1970*b*; Chérif *et al.*, 1991). Invasion of host cells by the pathogen is usually associated with the collapse of protoplasm and disintegration of organelles. Other host reactions may involve increased vacuolation of the protoplasm, deposition of an osmiophilic, electron-opaque material inside the cells, or occlusion of xylem elements by tyloses (Desilets *et al.*, 1994; Chérif *et al.*, 1991). Formation of sporangia, oogonia and oospores is observed frequently in diseased plant tissues, and these serve as secondary inoculum of the pathogen after the death of the host (Mellano *et al.*, 1970*b*; Nemec, 1972; Endo & Colt, 1974).

2.3.3 Environmental factors affecting the activities of *P. ultimum*

2.3.3.1 Moisture

Soil water is one of the most important aspects of the soil environment influencing the growth and survival of soilborne plant pathogens (Griffin, 1963; Cook & Papendick, 1970). High soil moisture content favours saprophytic activity of *P. ultimum* in soil. The population density of *P. ultimum* in natural soil increases as a direct response to increases in the soil water potential between -5 and -0.25 bar

(Lifshitz & Hancock, 1983). Saprophytic growth of *P. ultimum* is about the same at all water potentials from -0.02 to -1.0 bars, but no growth is detected in water-saturated soils (Paulitz & Baker, 1987*a*) or when water potential is -10 bars or lower (Lifshitz & Hancock, 1983). Conversion of thick-walled oospores of *P. ultimum* to thin-walled oospores also is determined by the moisture content of the soil. The maximal conversion of thick-walled to thin-walled oospores (about 96%) occurred in fully saturated soil (0 bar) incubated at 25°C for 6 weeks, using an agar-film technique (Lumsden & Ayers, 1975). Johnson *et al.* (1990), using a similar agar-film technique showed that high conversion rates for *P. ultimum* oospores occurred in soil with matric potentials of -0.3 to -0.1 bars. However, in contrast to Lumsden & Ayers (1975), they observed that only 18% of oospores were converted from the thick-walled to thin-walled form in soil near saturation. Johnson *et al.* (1990) argued that the results of Lumsden & Ayers (1975) can be considered as similar and comparable to their results, assuming that the full saturation in the former study was actually closer to -0.3 bars of matric potential, and that this discrepancy was due to differences in the methodology for precluding moisture loss during soil equilibration. Johnson *et al.* (1990) defended the criticism of using the agar-film technique as being too artificial and not providing a true representation of the behaviour of oospores in soil. He argued that results obtained in several other studies using this methodology did not appear to be influenced by the presence of agar films. Oospores and sporangia of *P. ultimum* can survive for relatively long periods of time in soils with low moisture contents, at low temperatures. This indicates a potential for prolonged survival of the pathogen during periods of drought and cool seasons (Hoppe, 1966; Stanghellini & Hancock, 1971*a*; Hancock, 1981).

The activity of *P. ultimum* as a pathogen is also directly affected by soil water potential. Kraft & Roberts (1969) reported that rotting of pea roots by *P. ultimum* was most severe in soil with water potential fluctuating between -0.3 and -1 bar, at 18 or 24°C. Furthermore, drying of the soil to *ca* -5 and -15 bars prior to watering reduced

disease severity. Tammen (1962) observed that *P. ultimum* was able to parasitise *Lilium longiflorum* over the whole range of soil moisture contents from saturation (0 bar) to that equivalent to permanent wilting point (*ca* -15 bars). Similarly, Pieczarka & Abawi (1978) concluded, from their study, that soil water had significant effects on the severity of root rot of snap beans, as the activity of *P. ultimum* was directly related to soil water potential ranging from 0 to -12 bars.

Thus, saprophytic and pathogenic activities of *P. ultimum* occur over a wide range of soil water potentials, although disease severity appears to be most profound in conditions of high soil moisture. Consequently, by standardising soil moisture content, such that the activity of the pathogen is impeded but not that of the host, reduction in disease incidence is most likely to be attained under controlled experimental or nursery conditions.

2.3.3.2 Temperature

The soil temperature is the next most important environmental factor that influences the activities, such as survival, germination and dispersal, of *Pythium* spp. (Stanghellini, 1974). The rate of conversion of *P. ultimum* thick-walled to thin-walled oospores, and sporangial germination, are strongly affected by the soil temperature. For example, the germination of *P. ultimum* sporangia in soil amended with glucose occurs at 10 to 30^{o}C, with most germination taking place between 15 and 25^{o}C (Agnihotri & Vaartaja, 1967*b*). The germ tubes of sporangia are also affected by temperature, such that at 10^{o}C the germ tubes tend to be short and unbranched but healthy, but at 35^{o}C they are short and poorly branched, and eventually lyse. Furthermore, at 10, 15, 31 and 35^{o}C most sporangia produce a single germ tube, whereas at 20 and 25^{o}C one to six germ tubes are usually formed per sporangium (Agnihotri & Vaartaja, 1967*b*). Hancock (1977) reported that sporangial germination of *P. ultimum* in cotton leaves in field soil occurred between 9 and 23^{o}C, but not below 4^{o}C or above 30^{o}C. Similar effects of temperature on germinability of oospores

of *P. ultimum* were reported by Lumsden & Ayers (1975), who observed that maximal conversion from thick-walled to thin-walled oospores occurred at 25°C, with little conversion at 15 and 20°C, almost none at 10°C, and that above 30°C, the rate of both conversion and germination of oospores was significantly reduced. Saprophytic growth of *P. ultimum* on agar media and in soil is also influenced by temperature. For example, Lifshitz & Hancock (1983) observed that the optimal temperature for saprophytic development of *P. ultimum* in natural soils was below 27°C. However, in sterilised soil, the optimum temperature was between 27 and 30°C, similar to the range of temperatures at which maximum linear growth of this fungus occurred in culture. Lifshitz & Hancock (1983) suggested that the activity of antagonistic microflora was responsible for the reduction in the optimal temperature for growth and development of *P. ultimum*. Similarly, Paulitz & Baker (1987*a*) noted that optimal growth of *P. ultimum* on agar media and in pasteurised soil occurred between 25 and 30°C, which is in agreement with the findings of Leach (1947), Lifshitz & Hancock (1983) and Lifshitz *et al.* (1984*b*).

Results from the classic study by Leach (1947) showed that incidence and severity of damping-off of seedlings caused by *P. ultimum* at different temperatures is directly related to the relative influence of temperature on the growth rate of the plant and of the pathogen. In his study, spinach, a low-temperature crop, was most severely affected by the pathogen in the range 12 to 20°C, and escaped pre-emergence damping-off at 4°C. When the growth rate of *P. ultimum* in pure culture was compared with that of spinach, at 4°C as well as between 12 and 20°C, it became apparent that the plant grew better than the fungus at cooler temperatures and *vice versa* at warmer temperatures. Conversely, watermelon, a high-temperature crop, was not affected by *P. ultimum* at 35°C. This plant emerged most quickly, grew best, and showed little injury at soil temperatures above 20°C, and was most severely affected by the pathogen at soil temperatures below 20°C. On all hosts tested, pre-emergence infection by *P. ultimum* was most severe at temperatures less favourable to the host

than to the pathogen, as measured by the ratio of their growth rates (Leach, 1947). Conversely, temperatures that favoured the host more than the pathogen allowed expression of plant resistance, resulting in little or no disease. The influence of growth rate of the host versus that of *P. ultimum*, as affected by the environment, may explain the different effects of temperature on disease development by *P. ultimum* reported in a number of studies. For example, the pathogen was found to cause most severe pea root rot at soil temperatures of 18 or 24°C, with less disease occurring at 13°C (Kraft & Roberts, 1969). However, Pieczarka & Abawi (1978) reported that the fungus was most damaging to snap beans at 15°C, and that the root rot severity decreased and dry weight of plants increased at temperatures of 21 or 27°C. High temperatures were also unfavourable to disease development in cotton seedlings, such that more severe damping-off occurred in the greenhouse with day/night temperature regimes of 20/15, 25/20 and 30/25°C than at 35/30°C (Ogle *et al.*, 1995).

Due to of the apparent relationship between the growth of the pathogen and that of the host, the need for choosing an appropriate temperature in an experimental system is as great as that for soil water potential, so that neither the pathogen nor the plant has greater advantage, unless otherwise required. In terms of minimising the disease severity, the host should be grown at optimum temperatures in order to increase its vigour and, consequently, to reduce the susceptibility to infection.

2.3.3.3 pH

The soil pH influences the growth and germination of *P. ultimum* propagules in soil. Lumsden & Ayers (1975) reported that maximum conversion of thick-walled to thin-walled oospores of *P. ultimum*, on agar in contact with water-saturated soil, occurred at pH 7.0 after 8 weeks of incubation at 25°C. Oospore conversion was reduced at pH 7.8, and fewer than 10% of the oospores were converted at pH 4.7. However, germination of converted oospores on Mircetich's pimaricin-vancomycin medium with pH adjusted to 4.5 to 7.5 was very high (98-100%), whereas only 17.3%

germinated at pH 3.5 (Lumsden & Ayers, 1975). When thick-walled oospores of *P. ultimum* were buried in soils at various moisture contents and temperature alternating between 18 and 24°C, however, their conversion to thin-walled oospores at pH 4.3 was 79, 85, 92 and 96% after 21, 42, 63 and 84 days, respectively (Qian & Johnson, 1987). A positive correlation between soil pH and microbial lysis of thin-walled oospores of *P. ultimum* in greenhouse experiments was also reported. Oospore lysis was increased in an acid soil with low phosphate levels, conditions which are favourable for microbial growth, especially bacteria, including actinomycetes (Qian & Johnson, 1987). Hyphal growth of *P. ultimum* in culture media takes place in the pH range of 4.0 to 9.0, with most growth occurring between pH 5.0 and pH 7.0 (Roth & Riker, 1943; Griffin, 1958).

Griffin (1958) investigated the influence of soil pH on the host, the pathogen and the disease, by testing the hypothesis that the relationship between the three and the pH is similar to that described by Leach (1947) for temperature. He demonstrated that the incidence of damping-off of four host plants (*Beta vulgaris*, *Brassica campestris*, *Picea sitchensis* and *Pinus contorta*) by *P. ultimum* at soil pH in the range of 3.5 to 8.0 was strongly negatively correlated with the growth rate of the hosts. There was no positive correlation with the fungal growth rate or, with the exception of *Pinus contorta*, with the pathogen/host growth rate ratio. Griffin (1958) suggested that soil pH directly influences the vigour of the host, rather than the growth of the pathogen, and that the relationship between pre-emergence damping-off and pH is different to that described by Leach (1947) for temperature. Paulitz & Baker (1987*a*) reported that the incidence of damping-off of cucumber seedlings caused by *P. ultimum* as well as the inoculum density of the pathogen was significantly lower at pH 6.7 than at pH 5.0 or 6.0. The infection of wheat embryos by *P. ultimum* was also more frequent in soil with pH of 5.0 to 5.5 than in soil with pH of 7.0 to 8.0 (Fukui & Cook, 1988). Increased disease suppression in neutral to slightly alkaline soil was thought to be the

result of greater activity of soil bacteria competing with the pathogen for available nutrients.

As the saprophytic and pathogenic activities of *P. ultimum* are favoured by acidic conditions, adjustment of soil pH to more alkaline could lead to the reduction in disease incidence, either directly by affecting the fungal growth or through stimulated activity of antagonistic microorganisms that are responsible for the suppression of the pathogen (Simon & Sivasithamparam, 1988*a,b*).

2.3.3.4 Nutrition

Soluble and volatile host exudates stimulate the conversion of thick-walled to thin-walled oospores as well as germination of sporangia, permitting *P. ultimum* to increase its biomass and infectious capacity (Nelson, 1990). *P. ultimum* is very responsive to germinating seeds and seedlings. Sporangia of *P. ultimum* germinate within 1.5 to 3 hours after exposure to bean seed exudates, and extensive mycelial growth in soil and subsequent host infection by the fungus occur within 24 hours (Stanghellini & Hancock, 1971*a,b*). Johnson & Arroyo (1983) reported that conversion of thick-walled to thin-walled oospores of *P. ultimum* started within 12 hours of incubation in cotton rhizosphere, with 30% and 85% of the oospores having thin walls after 48 hours and 45 days, respectively. Despite the evidence that the pathogen is stimulated by seed exudates within hours of sowing, the role of specific molecules released by germinating seeds on the activity of *P. ultimum* has been given little attention. The exact nature of the stimulatory molecules has not yet been identified, however, it has been suggested that sugars and amino acids are the molecules most likely to be responsible for stimulating propagule germination in the spermosphere and rhizosphere (Nelson, 1990). For example, Agnihotri & Vaartaja (1967*a,b*) found mixtures of sugars and amino acids to be stimulatory to *P. ultimum*. Stanghellini & Hancock (1971*a*) observed that at least 30 µg glucose per g soil were needed to induce maximum germination of *P. ultimum* sporangia.

Schlub & Schmitthenner (1978) observed that as little as 12 µg glucose were capable of inducing high levels of *P. ultimum* germination in soil. Conversely, a mixture of unsaturated fatty acids and triglycerides of these fatty acids, present in cotton seed exudates, rather than the simple carbohydrates, were shown to be the molecules most stimulatory to *P. ultimum* (Nelson & Hsu, 1994). Sporangia of *P. ultimum* also germinate rapidly in response to volatile substances from germinating seeds. Germination is most rapid at 25°C, with germ tubes evident 2 hours after exposure to volatile substances and maximum germination occurring within 6 to 8 hours (Nelson, 1987). Increases of up to 60 fold in soil populations of *P. ultimum* were reported, by Norton & Harman (1985) to occur after exposing infested sterile soil to volatile substances from aged pea seeds. Ethanol is the primary volatile stimulant released from germinating cotton seeds, and is stimulatory at concentrations of 1 to 7 nmol/ml, but inhibitory at higher concentrations. Acetaldehyde, another seed volatile, is not stimulatory to germination of sporangia of *P. ultimum* at concentrations greater than *ca* 1 nmol/ml (Nelson, 1987). Understanding the nature and the role of host exudates in stimulating and regulating the activity of *P. ultimum* prior to host colonisation may allow appropriate manipulation of the spermosphere and rhizosphere environment in order to reduce or eliminate seed or root infection by the pathogen.

2.4 CONTROL STRATEGIES FOR PYTHIUM DISEASES

Different control measures for diseases of bedding plants are routinely practised in intensive greenhouse cropping systems. The various control methods are classified as cultural, chemical and biological (Jarvis, 1989).

2.4.1 Cultural strategies

Most cultural control strategies aim to help the plant to avoid contact with the pathogen and eradicate the pathogen in the host or in an area in which the host is

grown. Of the various sources of *Pythium* spp. in nurseries, the compost, soil or potting mix and growth containers are, by far, the most important (Stephens *et al.*, 1983). Sterilisation or pasteurisation of potting media before use can be applied to eliminate inoculum of the pathogen (Fletcher, 1984; Dixon, 1984). However, as argued by Baker (1962), the low-temperature aerated steaming at 60°C rather than treatment at above 80°C should be used to avoid creating a biological vacuum devoid of soil microorganisms that are potentially antagonistic to the pathogen. Solarisation is another form of pasteurisation that has been used to control *Pythium*. However, this method is not often practical as it requires up to 30 days of high solar radiation to be fully effective (Duff & Barnaart, 1992). Seed boxes, pots or punnets should be disinfected before being filled with sterilised or pasteurised potting media. Greenhouse benches should be disinfected during routine cleaning of the whole greenhouse, in order to remove dust and soil potentially contaminated with the pathogen (Fletcher, 1984). Diseased plants should be removed and burnt, as should any neighbouring apparently healthy seedlings which may harbour the pathogen (Fox, 1995). Mains water is generally free from damping-off pathogens but may become contaminated while being stored in the nursery (Stephens *et al.*, 1983). Thus storage tanks for water and nutrient solution as well as watering equipment, should be cleaned regularly, using chlorination if necessary (Fletcher, 1984).

The severity of diseases caused by *Pythium* spp. is closely related to the growing practices in many greenhouse crops, such as high soil moisture content, overcrowding of seedlings, low light intensity, defoliation, trimming and tying. These practices induce physical and metabolic strains on the plants, predisposing them to infection by the pathogen (Schoeneweiss, 1975). Furthermore, suboptimal environmental conditions, that might be present in some greenhouses, can prolong the seedling stage and, therefore, make the crop even more susceptible to disease (Jarvis, 1992). Hence, cultural practices that minimise plant stress and improve plant growth reduce the susceptibility of the crop to an infection (Jarvis, 1992). Consequently, good soil

drainage, improved soil texture, balanced nutrient supply, appropriate temperature, acidity, light and a moderate density of planting all favour fast plant growth and reduce the amount of disease caused by *Pythium* spp. (Dixon, 1984; Fox, 1995). Moreover, if overhead irrigation and pesticide sprays are used, then the droplet size must be as small as possible to reduce splashing which can aid the dispersal of the damping-off pathogens (Jarvis, 1992; Stephens *et al.*, 1983).

There is also some evidence in mature plants of varietal or cultivar differences in susceptibility to root rot diseases caused by *Pythium* spp. (Hendrix & Campbell, 1973), although varietal resistance in seedlings is lacking (Fox, 1995). As many *Pythium* spp. have a wide host range, and resistant cultivars generally remain disease-free for a relatively short time, this strategy to control Pythium diseases may not be successful on a large production scale (Fletcher, 1984).

Despite the efforts to control soilborne diseases caused by *Pythium* spp., the damping-off pathogens continue to cause severe losses in nursery industry. The main reason is the inadvertent introduction of these pathogens into the greenhouses via contaminated water or soil and dust transported by nursery operators and equipment. As a result, other means of controlling the pathogen have been employed, in particular the use of fungicides.

2.4.2 Chemical strategies

There are two main areas for the chemical control of damping-off diseases caused by *Pythium* spp. These are: 1) protection of healthy plants from infection; and 2) destruction of pathogen propagules present either on the plant seeds or in the potting medium (Dixon, 1984). Some fungicides have been tested and used for control of diseases caused by *Pythium* spp. in a variety of nursery and field crops. For example, seed treatment with metalaxyl, captan, benalaxyl, metalaxyl and thiabendazole is effective in controlling *P. u. sporangiiferum* and *P. u. ultimum*

responsible for causing pre-emergence damping-off of chickpea in both field and growth chamber tests. These seed treatments are at least as effective as fumigating the soil with methyl bromide or treating seeds with various biocontrol agents (Trapero-Casas *et al.*, 1990). Hering *et al.* (1987) observed that treatment of wheat seeds with carboxin in combination with thiram, captan or metalaxyl also reduces infection of the embryo by *P. u. sporangiiferum.* Cook (1985) reported that isolates of *P. u. sporangiiferum*, *P. u. ultimum*, *P. aristosporum* and *P. heterothallicum* have greater sensitivity to metalaxyl in *in vitro* tests than do isolates of *P. torulosum* and *P. irregulare*. He suggested that the failure of metalaxyl to inhibit growth of some *Pythium* spp. responsible for root rot of Pacific Northwest wheat, may account for failure to control the disease in the field using this fungicide. In another study, Cook *et al.* (1987) reported that treatment of commercial field plots in eastern Washington with methyl bromide, chloropicrin, or 1-3 dichloropropene and 17% chloropicrin eliminated the population of *Pythium* spp. by 95 to 99%. Propamocarb controlled damping-off caused by *P. u. sporangiiferum* in *Capsicum* but not in *Celosia* seedlings (Harris *et al.*, 1993*b*). Metalaxyl applied to carrot seeds protected the plant from pre- and post-emergence damping-off caused by *P. ultimum* and *P. acanthicum* both *in vitro* and in field experiments (Walker, 1991). Hymexazol has been used widely for the control of damping-off caused by *Pythium* spp. in a number of crops, including rice and sugar beet (Takahi *et al.*, cited in Kato *et al.*, 1990). Compounds other than registered fungicides, such as soluble silicon (Chérif *et al.*, 1994) or chitosan (El Ghaouth *et al.*, 1994) may control *Pythium* diseases, either by having a direct effect on the pathogen itself or by inducing defence mechanisms in the host prior to infection.

Chemical control, although successful in the management of certain diseases caused by fungi, has several problems. These include: 1) the negative attitudes of the public towards pesticides; 2) the possibility of phytotoxicity to some plant hosts; 3) environmental pollution; 4) the development of resistance to chemical control

agents in numerous plant pathogens; and 5) the increasing cost of development and registration of new or improved pesticides (Gilpatrick, 1976; Cook & Baker, 1983).

2.4.3 Biological control

Due to the problems associated with using chemicals to control Pythium diseases, biological control of the pathogens using antagonistic microorganisms has gained considerable attention as an alternative control measure.

2.4.3.1 Advantages and practical constraints of biological control

When applied successfully, biological control has numerous advantages over the chemical control methods used commonly today. Natural antagonists of plant pathogens are "self-powered, self-sufficient and self-regulating" (Burge, 1988). Biocontrol agents are usually endemic to habitats in which they are applied and do not threaten native fauna or flora, including other beneficial organisms (Burge, 1988; Nigam & Mukerji, 1988). As one application of biocontrol agent(s) should protect the plants for a considerable time, the need for frequent applications of chemical pesticides would be minimised. The reduced use of pesticides would benefit farmers and nursery operators by lowering production costs and reducing exposure to hazardous chemicals. Moreover, the consumers would benefit by having pesticide residue-free produce (Still, 1982; Upadhyay & Rai, 1988).

The economic success of biological control depends on its feasibility in terms of monetary returns. Biological control may never be adopted by farmers or nursery operators unless it provides them with more incentive than the conventional methods used today (Wilson & Huffaker, 1976). There are also other constraints that may reduce the feasibility of biological control. These include: 1) the inadequate technological development of biocontrol agents that can successfully outperform the chemical pesticides; 2) lack of assurance that the products delivered to the users have a high level of potency and purity; 3) recommendations for handling, transportation and

storage need to be followed, otherwise the antagonists may lose viability; 4) antagonists, in comparison to chemical pesticides, are more susceptible to environmental conditions; and 5) pesticides can control a variety of plant pathogens, whereas the biocontrol agents are more limited in their range (Wilson & Huffaker, 1976; Cook & Baker, 1983; Upadhyay & Rai, 1988). Although these practical constraints must be considered when developing a biocontrol product, the advantages of their use over chemical pesticides far outweigh their disadvantages.

2.4.3.2 Integrated control

The scope of integrated control is to blend biological, cultural and chemical strategies for controlling plant diseases. However, it is emphasised that the chemical pesticides are used in order to reinforce, rather than supplant, the action of biocontrol agents and they should be used only to help restore a favourable balance between the antagonists and the pathogens (Way, 1986). The need for integrated control is most prominent in situations where biological control, in particular the use of antagonists, is not capable alone of producing satisfactory crop protection (Cook & Baker, 1983). Some well known examples of integrated control of plant pathogens are control of: 1) *Armillaria mellea* on citrus by *Trichoderma* spp. plus methyl bromide; 2) *Rhizoctonia solani* on radish and eggplant by *T. harzianum* plus PCNB (pentachloronitrobenzene); 3) *Pythium ultimum* on pea by *T. harzianum* plus metalaxyl; and 4) *Phytophthora capsici* on pepper by *T. harzianum* plus Ridomil (Upadhyay & Rai, 1988).

2.4.3.3 Biological control agents

Specific characteristics must be met by an organism to be acceptable and feasible as a biocontrol agent, including: 1) genetic stability; 2) rapid multiplication rate; 3) high efficacy at low concentrations; 4) low nutrient requirement; 5) good survival ability under adverse environmental conditions; 6) wide range of activity against a variety of pathogens; 7) relatively risk-free; 8) resistant to pesticides; 9) non-pathogenic on the host plant; 9) low cost of marketing and research; and 10) comparable, in efficacy and

production costs, with available non-biological control strategies (Snyder *et al.*, 1976; Wilson & Wisniewski, 1989).

The ideal biological control organism, from a commercial viewpoint, is one that can be reproduced economically on a large scale, be formulated into a product that has a shelf life of at least 12 months at 25°C and can be applied using conventional farm machinery (Licastro, 1994). Some potential biocontrol agents and commercial biocontrol products already available on the international market are summarised by Upadhyay & Rai (1988) and Harris (1994). However, as noted by Campbell (1989*a*), there are numerous reports of control of diseases in the research literature, which have no possibility of becoming commercialised. One reason is that the organism does not perform consistently in the field or in conditions for which it has been intended. Moreover, there are potentially useful antagonists which have not been, and will not be, developed, either because the crop or the disease are not important enough to warrant the development of biocontrol agent or because the information regarding the identity of the antagonist has been published, preventing the protection of investments of the producers by means of patents (Campbell, 1989*a*).

2.4.3.4 Mechanisms of biological control

Cook (1981) grouped the mechanisms of biocontrol into three general categories: 1) reduction of inoculum of the target pathogen by antagonists; 2) protection of host plant surfaces with antagonists of the target pathogen; and 3) induced resistance and cross protection. Biological control of the target pathogen may involve one or a combination of these mechanisms, and different mechanisms may operate under different environmental conditions (Cook, 1981; Fravel & Keinath, 1991).

Biological control of pathogen inoculum by antagonists aims to exclude or eliminate the pathogen from soil or to keep it in a state of suppression (Cook & Baker, 1983). This can be achieved by: 1) destruction of pathogen propagules or biomass;

2) prevention or reduction of inoculum formation; 3) weakening or displacement of the pathogen in organic residue; and 4) reduction of pathogen vigour or virulence. The objective of biological protection of plant surfaces is to prevent the pathogen from colonising the host plant. This biocontrol strategy involves the establishment of the antagonist in or near the infection court in advance of the pathogen, and formation of a biological barrier against subsequent infection by the pathogen (Cook & Baker, 1983; Cook, 1981). Induced plant resistance and cross protection by the antagonist allow control of the target pathogen after host penetration has occurred. In cross protection, the antagonist acts directly on the target pathogen, eliminating or suppressing it within the plant tissues. Induced host resistance, however, involves a prior establishment of the antagonist in or on the plant, and induction of certain physiological processes in the host tissues that lead to inhibition of subsequent infection by the target pathogen (Cook, 1981; Cook & Baker, 1983; Heath, 1980).

Reduction of pathogen inoculum, as well as cross protection, by the fungal biocontrol agents is believed to be the consequence of one or more types of antagonistic activity, i.e. competition, antibiosis or mycoparasitism (Cook & Baker, 1983; Mehrotra *et al.*, 1988). Competition is one of the most important processes in nature and is as dynamic as the ecological niche itself. Competition describes any interaction between two or more species that is a consequence of shared demand for limited substrate(s), such as nutrients, space and oxygen, and which affects their growth and survival (Clark, 1965). Hence, competition determines and controls the population dynamics of individual organisms as well as whole populations within a given ecosystem (Campbell, 1989*b*; Cook & Baker, 1983). Antibiosis and mycoparasitism provide the antagonist with a selective advantage over its competitor(s), apart from more efficient nutrient uptake or oxygen acquisition, and are considered by a number of microbial ecologists to be the most common means of competition between microorganisms (Clark, 1965; Campbell, 1989*b*; Deacon, 1991).

Antibiosis is the inhibition or destruction of one microorganism by toxins or strongly inhibitory metabolites produced by another microorganism. Fungi are known to produce a variety of substances that are toxic to a range of plant pathogens (Faull, 1988). Antibiotics may diffuse in water, air or substrate to other microorganisms, eliminating the need for direct contact between organisms (Mehrotra *et al.*, 1988).

Mycoparasitism occurs when one fungus parasitises another fungus. Mycoparasites may be either necrotrophic or biotrophic and specific to their fungal host, or attack a wide range of fungi (Baker, 1987). There are several means by which mycoparasites attack their host and cause its death or retardation, including: 1) penetration of hyphae directly and growth within the host; 2) formation of coils around the hyphae of the host, with or without its penetration; and 3) excretion of extracellular enzymes that digest the walls of the host hyphae (Baker, 1987; Mehrotra *et al.*, 1988).

Lumsden & Lewis (1989) advocated the need to determine mechanisms of action of biocontrol agents, including factors that determine soil suppressiveness, to improve the action of antagonists against pathogens both through manipulation of their biology and their genetic constitution. A similar view is shared by the industry, in that before seeking a commercial partner, a research group should understand the organism's physiology, ecology and mode of action against the target pathogens and how these are affected by different environmental conditions. Furthermore, a knowledge of where the biocontrol product should be placed in relation to the host and the pathogen is critical to successful product development (Licastro, 1994; Scher & Castango, 1986). In contrast, Deacon (1991) argued that the route to practical success does not lie necessarily in the study of modes of action of potential antagonists, although this is valuable in its own right. His conclusions were based on the fact that for some biocontrol agents, the mechanisms of biocontrol have been discovered after their introduction into practice, without influencing their commercial usage in any way and

that, in some instances, detailed study of the mode of action of antagonists helped to refine a product that was already successful. Deacon (1991) also noted that it is important to establish how the *in vitro* studies of an antagonist's mode of action relate to microbial interactions *in vivo*, so that the ecological approach to the development of biocontrol agents can be adopted.

2.4.3.5 The role of *in vitro* assays in studies of biological control

One of the most critical problems with biocontrol products, to date, has been the variability in their performance in the field. This apparently has been due to many biocontrol agents being ecologically "ill-adapted" to the situations for which they are intended, as their ecological attributes relating to the microenvironments of pathogens have not been the basis of screening for antagonistic activity (Deacon, 1991). Similar views are shared by Lewis & Papavizas (1985), who noted that there is often little correlation between *in vitro* studies and the ability of potential biocontrol agents to reduce or prevent disease in the greenhouse or in the field. Researchers have postulated that evaluation of biocontrol ability should be carried out in an environment that closely resembles that in which the antagonist will be applied (Cook, 1985; Lumsden & Lewis, 1989; Deacon, 1991). Thus, if the target pathogen causes diseases of nursery plants, then assessment of biocontrol agents, including their modes of action, in greenhouses is appropriate (Lumsden & Locke, 1989). Furthermore, when greenhouse studies are performed, attempts should be made to ensure that assays are standardised with regard to environmental conditions and to the amount of inoculum of both the antagonist and the pathogen. This is necessary to achieve repeatable assays and to ensure that neither the antagonist nor the host is overwhelmed by the pathogen (Lumsden & Lewis, 1989). However, screening a large number of microorganisms for antagonistic activity against pathogens in the field is often unrealistic as it would require greater space, time and resources than laboratory screening. Moreover, candidate biological control agents must be screened in replicated disease assays that frequently require controlled-environment facilities that may not always be available in

the field (Duchesne *et al.*, 1989; Kloepper, 1991). It has been suggested by Duchesne *et al.* (1989), therefore, that initial screening be carried out *in vitro* and that any isolates with antagonistic capabilities should be selected for glasshouse and field assays. Kloepper (1991) promoted the use of *in vivo* "pre-screens", in which candidate biocontrol agents are evaluated for antagonistic activity against target pathogens on plant surfaces. It was argued that although the *in vivo* "pre-screens" are conducted in controlled conditions, their use overcomes some of the limitations of the *in vitro* "pre-screens", while maintaining the ability to screen rapidly a large number of biocontrol agents. It is, however, worth mentioning that many *in vitro* tests give valuable insights into mechanisms of biocontrol agents, including mycoparasitism, antibiosis, competition for substrates, as well as other saprophytic and pathogenic activities of the organisms tested (Lumsden & Lewis, 1989). Thus, experiments conducted *in vitro* may be useful in preliminary investigations of mechanisms involved in biocontrol. However, it must be remembered that these assays may be poor predictors of behaviour of antagonists in the field and, as such, should be interpreted in conjunction with results from *in vivo* studies.

2.4.3.6 Biocontrol agents of *Pythium* spp.

Representatives from various genera of fungi have been used experimentally to control diseases caused by *Pythium* spp., with *Trichoderma* spp., acting either via antibiosis, lysis, competition or mycoparasitism, or a combination of mechanisms (Papavizas, 1985), being the most extensively studied fungal antagonists. For example, Allen & Haenseler (1935) reported that damping-off of cucumber induced by *Pythium* sp. was appreciably reduced by infesting the soil with antagonistic *T. lignorum*. Damping-off of lettuce was also controlled when *T. viride* was added to soil conducive to *Pythium* spp. (Wood, 1951). Gregory *et al.* (1952) were able to prevent damping-off of alfalfa by *P. debaryanum* (= *P. ultimum*) by introducing *T. lignorum* and *Streptomyces* sp. into sterilised soil together with the pathogen, whereas damping-off of white mustard seedlings was partly controlled by dusting the seeds with spores of *T. viride* or

Penicillum sp. (Wright, 1956*c*). Other *Trichoderma* spp., including *T. harzianum* and *T. koningii* reduced the incidence of pre-emergence damping-off caused by *P. ultimum* after the application of conidia of each of the two antagonists to pea seeds (Lifshitz *et al.*, 1986).

Antagonistic *Pythium* spp. also have been shown to control diseases caused by pathogenic *Pythium* spp. For example, *P. nunn* has been reported, in a number of studies, to suppress seedling damping-off induced by *P. ultimum* (Lifshitz *et al.*, 1984*a*; Elad *et al.*, 1985; Paulitz *et al.*, 1985; Paulitz & Baker, 1987*a,b*). Results from the above *in vitro* and *in vivo* studies suggested that *P. nunn* may control damping-off via mycoparasitism, antibiosis or competition for organic substrates, with the latter being apparently the primary cause of disease suppression in soil (Paulitz & Baker, 1987*a,b*). *P. oligandrum* has also been shown to control Pythium damping-off in studies by Al-Hamdani *et al.* (1983), Martin & Hancock (1986) and Lewis *et al.* (1989). Although competition for substrates in disease control in the soil and spermosphere has been suggested (Martin & Hancock, 1986), mycoparasitism is strongly implicated as a major mode of action of *P. oligandrum* as a biocontrol agent (Lewis *et al.*, 1989).

The potential of *Pseudomonas fluorescens*, *P. putida* and *P. cepacia* to suppress pathogenic *Pythium* spp., with antibiosis being the likely mechanisms of control, has also been recognised by some researchers including Howell & Stipanovic (1980), Weller & Cook (1986), Elad & Chet (1987) and Walther & Gindrat (1988).

2.5 *PYTHIUM ULTIMUM* VAR. *SPORANGIIFERUM* DRECHSLER

The mycelium of *P. u. sporangiiferum* consists of hyphae which can be up to 11 μm wide. Sporangia and zoospores are formed readily by the fungus at room temperature

(*ca* 20°C). Sporangia may be subglobose, intercalary or terminal, with one or two discharge tubes. Oogonia are globose, smooth and usually terminal although sometimes intercallary. There can be one to three antheridia per oogonium; they are sac-like, mostly monoclinous, originating below the oogonium. Oospores are single, aplerotic, globose, with walls often 2 µm or thicker. The optimal temperature for fungal growth in culture is between 25 and 30°C, with the minimum temperature 5°C and maximum 35°C. *P. u. sporangiiferum* differs from other *Pythium* spp. in that it does not require special culture conditions to release zoospores at room temperature (Van der Plaats-Niterink, 1981).

P. u. sporangiiferum is pathogenic on a variety of both field and nursery crops including barley, peas and lentils (Ingram & Cook, 1990), chickpea (Trapero-Casas *et al.*, 1990), silver beat, capsicum and dwarf celosia (Harris *et al.*, 1993*b*). The fungus is also very pathogenic to wheat grown in areas of eastern Washington and northern Idaho, USA, receiving 40 to 45 cm of annual precipitation (Charmswarng & Cook, 1985; Cook *et al.*, 1987; Ingram & Cook, 1990).

The pathogenic activity of the fungus is influenced directly by soil moisture and indirectly, through the host plant, by temperature. For example, Hering *et al.* (1987) showed that, in pot experiments, infection of wheat embryos by *P. u. sporangiiferum* was maximal (45%) at -0.1 bar matric potential and that very little infection (less than 10%) occurred in soil drier than -0.4 to -0.5 bar at 20°C. *P. u. sporangiiferum* was also shown to cause severe pre-emergence damping-off in wheat at 15 to 25°C, in lentils at 10 to 25°C and in peas at 5 to 25°C, as well as post-emergence damping-off in wheat and peas at 10 to 25°C and in lentils at 5 to 25°C (Ingram & Cook, 1990). The pathogen was also reported to be very active in causing pre-emergence damping-off in chickpeas grown in the growth chamber at 10, 15, 20 and 25°C, and in an outdoor lath house at the average daily temperature of 16°C

(Trapero-Casas *et al.*, 1990). Thus, *P. u. sporangiiferum* is active over a wide temperature range and in conditions of high soil moisture content.

2.5.1 Control of diseases caused by *P. u. sporangiiferum*

Diseases caused by *P. u. sporangiiferum* in commercial nurseries and in the field are currently controlled using cultural and chemical methods, described in Sections 2.4.1 and 2.4.2, which often do not meet the needs of the growers or the consumers. An alternative control strategy, such as biological control by bacterial or fungal antagonists, has been concerned, to a large extent, with *P. ultimum*, with very little research being conducted on *P. u. sporangiiferum*. Becker & Cook (1988) conducted an *in vitro* study to determine the role of siderophore production by fluorescent *Pseudomonas* spp. in suppression of *Pythium* spp., including *P. u. sporangiiferum*, which are pathogenic to wheat. They found that 7% of nearly 5000 strains of bacteria (90% of which were pseudomonads) isolated from wheat roots produced a zone of inhibition (≥10 mm) against *P. u. sporangiiferum* on either King's medium B, potato dextrose agar or both. Moreover, a third of the inhibitory bacterial strains improved wheat growth when applied to seeds sown in a soil naturally infested with the pathogen. Based on their results, Becker & Cook (1988) suggested that, for wheat grown in soil naturally infested with *Pythium* spp., growth promotion of plants is a consequence of the suppression of the pathogens by siderophore-producing pseudomonads. In the study by Trapero-Casas *et al.* (1990), efficacy of various biocontrol agents against seed rot caused by *P. u. sporangiiferum* was compared to several chemical seed treatments in growth chambers and in the field. They reported that treating chickpea seed with *Pseudomonas fluorescens* strain Q29z-80 and *Penicillium oxalicum* resulted in plant yield equivalent to that obtained with any of the fungicide seed treatments, including metalaxyl, captan, benalaxyl, and metalaxyl plus thiabendazole. Biological seed treatment using *Pythium oligandrum* was also effective against pre- and post-emergence damping-off caused by *P. u. sporangiiferum* in the field but not in controlled temperature experiments in growth chambers.

Trapero-Casas *et al.* (1990) attributed this discrepancy to environmental conditions present in the growth chambers compared to those in the field, in particular the soil moisture, which apparently were favourable for the growth of the pathogen but not the antagonist. Although the potential of *P. fluorescens*, *P. oxalicum* and *P. oligandrum* to control Pythium damping-off of chickpea was addressed in this study, the mechanisms of biocontrol of *P. u. sporangiiferum* by the three antagonists were not investigated. Recently, BNR isolates have been shown to control damping-off of capsicum and dwarf celosia caused by *P. u. sporangiiferum* in nursery potting medium in a glasshouse and growth chamber, however, the mechanisms of biocontrol are not well understood (Harris *et al.*, 1991, 1993*b*).

2.6 BINUCLEATE *RHIZOCTONIA* SPP.

Teleomorphs of BNR isolates fall into one of two genera of the Basidiomycotina: *Ceratobasidium* (anamorph = binucleate *Rhizoctonia* spp.) and *Waitea* (anamorph = *R. zeae* Voorhees and *R. oryzae* Ryker and Gooch). Characteristics of BNR isolates include: 1) branching near the distal septum of cells in young vegetative hyphae; 2) formation of a septum in the branch near the point of origin; 3) constriction of branch hyphae at the point of origin; 4) presence of the dolipore septal apparatus; 5) absence of clamp connections; 6) absence of conidia; 7) sclerotial tissue (when produced) not differentiated into rind and medulla; and 8) absence of rhizomorphs (Carling & Sumner, 1992; Ogoshi, 1987). BNR isolates, as other *Rhizoctonia* spp., can be subdivided according to anastomosis reaction (Carling & Sumner, 1992; Sneh *et al.*, 1991). Dimensions of hyphal cells vary among isolates of *Rhizoctonia*, with cell diameter ranging from 3 to 17 μm, and cell length of 50 to 250 μm. Many isolates of BNR produce chains of hyaline or brown monilioid cells, which arise as buds or blown-out ends of pre-existing cells mainly on the surface of a host or a substrate, but also within the host tissues. The function of monilioid cells is not well understood,

although it is commonly accepted that they are important in the survival of the fungus (Ferriss *et al.*, 1984; Sneh *et al.* 1991).

BNR comprise a group of fungi that are saprobes, soil-borne plant pathogens, symbionts of orchids, and potential biocontrol agents. For example, Cubeta *et al.* (1995) obtained 300 strains of BNR from organic debris, roots and soil, and evaluated these strains over a period of 8 years for pathogenicity on 20 different cultivated plants commonly grown in North California. Ten percent of these strains were non-pathogenic and were subsequently shown to suppress diseases induced by *Rhizoctonia* spp., but not *Pythium* spp., in cucumber, potato and snapbean. In another study, 35 isolates of BNR were collected from 30 nurseries or potting mix suppliers in South Australia (Schisler *et al.*, 1994). Isolations from bait plants accounted for 74% of the BNR isolates. Six of the 21 BNR isolates tested had a deleterious effect on a number of bedding plants, while others either increased plant growth or had no effect on shoot growth (Schisler *et al.*, 1994).

Pathogenic BNR are responsible for causing diseases in commercial and wild strawberry plants (Drozdowski & Manning, 1988), bean, pea, radish, onion, lettuce and tomato (Burpee *et al.*, 1980), wheat (Roberts & Sivasithamparam, 1987), cereals (Lipps & Herr, 1982), subterranean clover (Wong & Sivasithamparam, 1985), turf grasses (Burpee, 1980), azaleas (Frizina & Benson, 1987), pine seedlings (English *et al.*, 1986; Huang & Kuhlman, 1989) and other cultivated plants (Sneh *et al.*, 1991). Effective biocontrol of pathogenic BNR is most likely to require a biocontrol agent to colonise the host as efficiently as the pathogen, without causing significant harm to the plant. Thus, if the non-pathogenic isolates of BNR share characteristics with the pathogenic BNR with respect to host colonisation and nutrient specialisation, then competitive interactions between the two are more likely to occur, and may be a facet of successful biocontrol.

BNR isolates are also known to form symbiotic associations with orchids. Masuhara *et al.* (1993) reported that all or some isolates of BNR obtained from non-orchid sources and belonging to nearly all anastomosis groups, induced symbiotic germination of seeds of *Spiranthes sinensis* var. *amoena* in *in vitro* experiments. The role of the mycorrhizal fungus is to provide the tiny orchid seeds with phosphate and other minerals, as well as a carbon source, vitamins and growth factors, that are essential for seed germination and subsequent plant development (Uetake *et al.*, 1992; Sneh *et al.*, 1991). This attribute of BNR to form such close association with its host may have implications for biocontrol, such that improved plant vigour may lead to an increased tolerance of the host to disease.

Very little research has been conducted on the ecology of non-pathogenic BNR. Cubeta *et al.* (1991) reported that BNR can survive in field soil in snapbean stems for 11 months and in ground oat kernels for up to 9 months, and that recovery of the fungus was higher in material obtained from soil depth of 15 cm than from 1cm. The increased survival of BNR in soil at 15 cm compared to that at 1 cm was attributed to cooler and drier conditions at the greater depth, although no experimental attempts were made to evaluate the effect of temperature or soil moisture on the survival of BNR in soil. Sumner & Bell (1986) and Bell & Sumner (1987) found that isolates of BNR survived for 2 years in fumigated soil and for up to 213 days in pasteurised soil in clay pots buried in the field. However, Sumner & Bell (1986) did not account for the possible introduction of other BNR on the incorporated peanut debris and the experimental soil was not assayed after fumigation to determine the presence of indigenous *Rhizoctonia* spp. Escande & Echandi (1991*a*) studied the effects of growth medium, storage environment, soil temperature and means of delivery to soil on two isolates of BNR for protection of potato from Rhizoctonia canker. They reported that both isolates of BNR retained about 89% viability in whole oat kernels and 100% viability in chopped oat kernels and, in vermiculite amended with potato broth, viability of one isolate remained at 100% while that of the other isolate was only 28%,

after 17 weeks of storage at 5°C in air. Generally, viability of both BNR isolates was found to be higher at 5°C than at 24°C regardless of the substrate used. Escande & Echandi (1991*a*) also reported that the protective activity against Rhizoctonia canker of potato by BNR isolates was not affected by soil temperature of 11, 17 and 23°C, suggesting that BNR can be effective in most potato growing areas of the world. The effects of soil moisture or soil pH on both viability and biocontrol activity of BNR isolates, however, have not been addressed in this study.

2.6.1 Diseases controlled by BNR

Non-pathogenic BNR isolates have been reported in several studies to control diseases caused by *R. solani* and *Pythium* spp. on a variety of plant species. For example, BNR-like fungi protected bean seedlings from Rhizoctonia root rot (Cardoso & Echandi, 1986, 1987) and cucumber seedlings from damping-off caused by *R. solani* (Cubeta & Echandi, 1988) in both the greenhouse and the field. Similarly, biological control of Rhizoctonia crown and root rot of sugar beet has been obtained in greenhouse tests by using BNR as biocontrol agents (Herr, 1988). Escande & Echandi (1991*b*) reported that isolates of BNR protected potato from Rhizoctonia stolon canker in artificially infested soil in the greenhouse and in potato fields naturally infested with *R. solani* (AG-3). Moreover, protection of potato cultivars by BNR was similar to that by PCNB and was better than thiophanatemethyl (Tops 2.5D®). Survival of capsicum, dwarf celosia and viola seedlings in the presence of pathogenic *R. solani* (AG-4) has been significantly improved by amending the potting medium with two antagonistic BNR isolates (Harris *et al.*, 1991, 1994; Harris & Adkins, 1993). A non-pathogenic BNR has also been shown to control brown patch disease on leaves of creeping bentgrass caused by pathogenic BNR (Burpee & Goulty, 1984).

BNR are also effective in suppressing diseases caused by *Pythium* spp., although research in this area of biological control is very limited. Harris & Adkins (1993) and Harris *et al.* (1993*b*) reported that two isolates of BNR suppressed damping-off

diseases caused by *P. u. sporangiiferum* and *P. irregulare* in capsicum seedlings grown in pasteurised potting medium. Moreover, one of the two isolates provided better protection against *P. u. sporangiiferum* than did the fungicide propamocarb (Harris *et al.*, 1991). In contrast, Cubeta & Echandi (1991) reported that control of damping-off of cucumber caused by *Pythium* spp. in a greenhouse and in the field requires an integrated approach, since BNR alone did not protect the seedlings against the pathogen. Protection of cucumber was achieved using BNR in combination with metalaxyl and this combined treatment protected cucumber plants from Pythium damping-off as effectively as did the recommended fungicide, captan.

2.6.2 Modes of action of BNR

Although the potential of BNR in controlling diseases caused by *Rhizoctonia* and *Pythium* spp. has been widely recognised, in few studies has the mode of action of the biocontrol agents been investigated. Cardoso & Echandi (1987) studied the nature of protection of bean seedlings from Rhizoctonia root rot by BNR in laboratory and greenhouse experiments. They reported that the most likely mechanism of biocontrol was competition for infection sites and/or induction of defence mechanisms in the host, because hyperparasitism and antibiosis were not observed in *in vitro* tests and seedlings remained protected against infection by *R. solani* after BNR had been eradicated from colonised tissues. Cardoso & Echandi (1987), however, did not account for possible failure of surface sterilisation to eliminate BNR from soil particles or debris attached to the plant and, thus, interaction between BNR and the pathogen in soil or on the host, rather than the induced host resistance, that could have been responsible for disease suppression. Jabaji-Hare *et al.* (1993) studied the anatomical and cytological changes in tissues of bean seedlings treated with BNR prior to inoculation with *R. solani*. They reported that an electron-dense material was deposited along the host cell walls in response to colonisation by BNR, and that this material may act as a protective barrier against infection by the pathogen. Protection of plant surfaces and competiton for an ecological niche were the suggested mechanisms

of biocontrol of damping-off caused by *R. solani* in pasteurised potting mix (Harris *et al.*, 1991; 1994).

With respect to diseases caused by *Pythium* spp., however, no comprehensive study has been undertaken, to date, to investigate the mode of action of antagonistic BNR. The ability of two BNR isolates to control diseases caused by two unrelated pathogens, *R. solani* and *Pythium* spp., on unrelated plant hosts (Harris *et al.*, 1991; 1993*b*) makes their use as biocontrol agents of seedling damping-off potentially important in the nursery industry. Thus, research on the mechanisms involved in the protection of seedlings by the two isolates of BNR against the damping-off fungus, *P. ultimum*, was undertaken in this thesis.

2.7 SUMMARY

Pathogenic *Pythium* spp. infect seeds and seedlings of many plant species, causing pre- and post-emergence damping-off in nearly every country of the world (Bouhot, 1988). Damping-off can be prevented, to some extent, in nursery plants by using pasteurised soil and pots, fungicide drenches and good hygiene. Satisfactory soil drainage and environmental conditions that favour fast growth of seedlings can also reduce the risk of *Pythium* diseases. There are, however, several problems associated with the use of current control methods, especially the fungicides. Hence, nursery producers are keen to try alternative control methods, in particular, biological control (Harris, 1995). Isolates of BNR have potential as biocontrol agents of diseases caused by *Pythium* spp. Information on the biology and mode of action of BNR may provide a means for managing the biocontrol agents and implementing effective control measures for Pythium diseases in a range of nursery situations.

2.8 OBJECTIVES OF THE PROJECT

The main aim of this project was to ascertain the mechanisms involved in the protection of *Capsicum* seedlings by the two isolates of BNR against *P. u. sporangiiferum* in nursery potting mix. Experiments presented in this thesis emphasised ecological characteristics of BNR in relation to the pathogen, in conditions resembling those of the nursery environment for which biological control is intended. The objectives were, therefore:

1) to determine whether BNR parasitise *P. u. sporangiiferum* in agar media and on germinating *Capsicum* seeds;

2) to investigate the involvement of antibiotics and lytic enzymes in the suppression of *P. u. sporangiiferum* by BNR in culture media and in autoclaved potting mix;

3) to study the process of colonisation of *Capsicum* seedlings by *P. u. sporangiiferum* and BNR *in vivo*, and to determine if protection of plant surfaces by the biocontrol agents is involved in the suppression of *P. u. sporangiiferum*;

4) to ascertain whether induced resistance, by BNR, prevents or reduces infection by *P. u. sporangiiferum in vitro* and *in vivo*;

5) to determine whether cross protection is involved in the suppression of *P. u. sporangiiferum* after infection of *Capsicum* by the pathogen has occurred;

6) to compare the competitive saprophytic ability of *P. u. sporangiiferum* with that of BNR in potting mix, and the effects of BNR on saprophytic and pathogenic activities of the pathogen *in vitro* and *in vivo*.

CHAPTER 3 GENERAL MATERIALS AND METHODS

3.1 FUNGAL ISOLATES

The two isolates of BNR (BNR1 and BNR2) were obtained from potting mixes and plants collected from potting mix suppliers and plant nurseries in South Australia (Schisler *et al.*, 1994). Their potential to control damping-off of nursery seedlings had been demonstrated by Harris *et al.* (1991, 1993b, 1994). *P. u. sporangiiferum* Drechsler (isolate 2) was cultured in 1987 from roots of a diseased bait seedling of silver beet (*Beta vulgaris* L.) grown in a used potting medium from a plant nursery near Adelaide, South Australia (Harris *et al.*, 1993*b*).

Fungal cultures were stored at -18°C on gamma-irradiated, autoclaved millet seeds (Harris *et al.*, 1993*a*). For experimental purposes, fungal cultures were established on either 1/4-strength potato dextrose agar (1/4 PDA, Difco Laboratories, Michigan, USA) or deionised water agar (WA, Difco). To start active cultures, four millet seed-inocula of each fungus were placed on *ca* 20 ml of either 1/4 PDA or WA in sterile plastic Petri dishes (90 mm diam., Disposable Products, South Australia), and incubated at 25°C in the dark. Further subculturing was achieved by cutting plugs with a sterile cork borer (8 mm diam.) from the edges of actively growing colonies on agar medium and transferring these to fresh agar medium (one to two plugs per plate). Fungi were grown at 25°C in the dark in all experiments unless stated otherwise. Fungal cultures on agar media were stored at 4°C for a maximum of 2 weeks, after which time new cultures were established from millet seeds. All culture media and equipment were sterilised by autoclaving at 121°C for 20 minutes unless stated otherwise.

3.2 THE HOST PLANT

Capsicum annuum "Green Giant" (*Capsicum* syn. bell pepper) was used as a 'model' host plant in *in vitro* and *in vivo* experiments. Seeds of *Capsicum annuum* were supplied by New World Seeds Pty. Ltd., Galston, N.S.W, and were not fungicide-treated but were dusted with calcium hypochlorite ($CaClO_2$) as a standard procedure. When these treated seeds were placed on 1/4 PDA (eight seeds per plate), after soaking for 15 minutes in sterile deionised water, they showed no visible signs of bacterial or fungal contamination after 5 days of incubation at 25°C in the dark. For that reason, no further surface sterilisation of *Capsicum* seeds was considered necessary.

3.3 POTTING MIX

The potting mix was provided by Falg Nurseries Pty. Ltd., Uraidla, South Australia. The potting mix consisted of sphagnum peat moss, sand and rice hulls in the ratio 70:15:15 (by volume), respectively, supplemented with essential macro- and micro-nutrients (Harris *et al.*, 1993*b*).

3.3.1 Sterilisation and pasteurisation of potting mix and sand

Sterilisation was achieved by autoclaving 1 kg-lots of the potting mix or sand (coarse, washed, sieved river sand), in metal trays covered with aluminium foil, at 121°C for 1 hour on three successive days. Pasteurisation involved treating *ca* 10 kg lots of the potting mix with aerated steam at 65°C for 30 minutes. If not used within the first few hours after pasteurisation, the potting mix was stored at 4°C and used within 24 hours.

3.3.2 Determination of pH of potting mix

Approximately 50 g samples of potting mix were wetted thoroughly with deionised water and placed in 125 ml Erlenmeyer flasks with 75 ml of 0.01 M $CaCl_2$. The flasks were placed on a magnetic stirrer at 10 rpm for 1 hour. The pH of the suspension was measured to the nearest 0.1 of a pH unit using an Activon pH meter (Scientific Product Company, Sydney). There were four replicate samples for each pH determination. The pH of the potting mix was in the range of 5.4 to 5.6.

3.3.3 Determination of moisture content of potting mix

Between 5 and 100 g of potting mix were placed in aluminium weighing trays and weighed before and after drying the potting mix for 24 hours at 110°C in a fan oven. The difference in weight of the potting mix before and after drying represented the water in the sample. The moisture content of the potting mix was calculated by the formula:

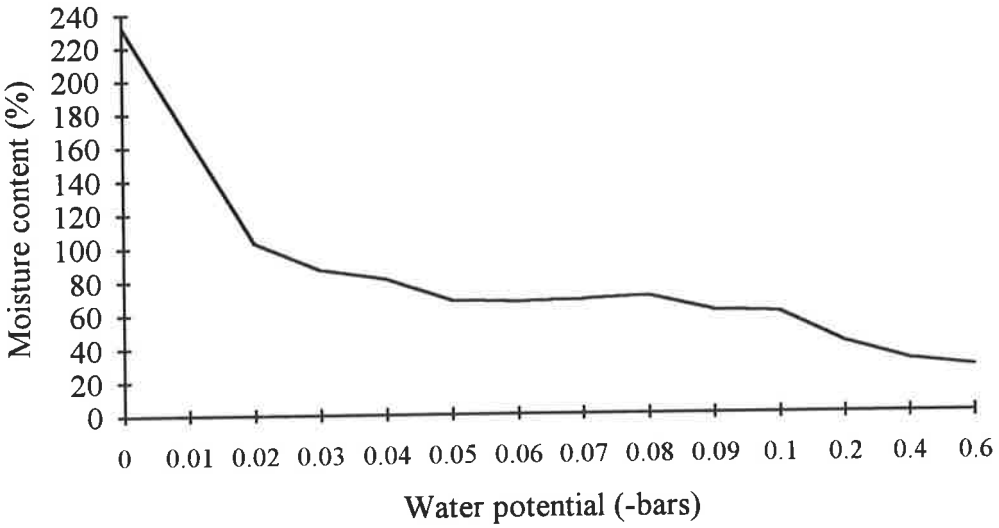
$$(\%)\ \text{moisture} = \frac{\text{Weight of water (g)}}{\text{Oven-dry weight (g)}} \times 100$$

3.3.4 Determination of Moisture Characteristic Curve

A suction plate was used to determine the relationship between the water potential and water content of the potting mix (Papendick & Campbell, 1978). The suction plate consisted of a porous plate that was permeable to water and solutes, but not to air, connected to a water reservoir which was placed under various suctions (i.e. water potentials). Approximately 6 g of freshly pasteurised potting mix were placed in small plastic containers (*ca* 10 ml capacity), watered thoroughly with deionised water and placed on the suction plate covered with a lid. The water in the potting mix was

allowed to equilibrate with the water in the reservoir for 24 hours at suctions ranging from 0 cm (0 bar) to 620 cm (-0.62 bar) as determined by the height of the water column in the reservoir. After the potential of the water in the reservoir was equal to the pressure on the water in the potting mix, the samples were oven-dried at 110°C for 24 hours. The percentage moisture (water) in sampled potting mix at a given water potential was determined as described in Section 3.3.3. There were four replicate potting mix samples for each water potential tested. The relationship between the water potential and the water content for the potting mix is represented by the soil moisture characteristic curve:

Moisture Characteristic Curve for Falg's Potting Mix



3.3.5 Adjustment of water content in potting mix

Moisture content of the potting mix used in all *in vitro* experiments (i.e. in Petri dishes) was adjusted to *ca* -0.03 bars. This was done by 1) determining the moisture content of the potting mix to be used (see Section 3.3.3), 2) calculating the amount of water to be added to that potting mix to obtain the required percent soil moisture

(% SM) and 3) converting the percentage soil moisture to water potential units (bars) using the moisture characteristic curve. The following calculations were used to determine the amount of water needed to obtain a given % SM:

(i) potting mix to be used (g) ÷ [1 + (% SM in that potting mix ÷ 100)] ⇒ dry soil (g)

(ii) potting mix to be used (g) - dry soil (g) ⇒ water present in the potting mix (g)*

(iii) dry soil (g) × [% SM required ÷ 100] - water present in the potting mix (g) ⇒ amount of water (g) to give the required % SM.

(* One g of water was considered to be equivalent to 1 ml of that water at 25^{o}C).

CHAPTER 4 PARASITISM OF *P. U. SPORANGIIFERUM* BY BINUCLEATE *RHIZOCTONIA*

4.1 INTRODUCTION

Parasitism, a direct attack of one organism on another, is generally termed mycoparasitism if it occurs among fungi, and when the host fungus itself is parasitising a plant or an animal host, the parasite is a hyperparasite (Sundheim & Tronsmo, 1988). There are at least 50 fungal species known to parasitise other fungi. Some of these mycoparasites, such as *Trichoderma* spp., have a very broad host range while others, such as some representatives of the chytrids, are host specific (Lumsden, 1981).

Barnett & Binder (1973) classified mycoparasitic interactions into two groups based on the mode of action of the mycoparasite. The biotrophic or balanced mycoparasites obtain nutrients from living cells without causing significant harm to the host fungus in the early stages of parasitism, as in case of *Rhizopus oryzae* being parasitised by *Syncephalis californica* (Hunter *et al.*, 1977). The parasites may obtain their nutrients either through absorptive cells or haustoria formed from appressorium-like hyphal swellings, or by growing within the host cells. The necrotrophic or destructive mycoparasites, on the other hand, kill the host cells before, or just after invasion, and utilise nutrients released from the dying or dead host. Infection by the necrotrophic mycoparasites may involve hyphal contact and coiling around fungal cells, as well as direct penetration and invasion of host structures. Some examples of such necrotrophic mycoparasites include *Verticillium biguttatum* inactivating sclerotia of *Rhizoctonia solani* on potato tubers (Jager & Velvis, 1988), *Coniothyrium minitans* parasitising *Sclerotinia sclerotiorum*, a major cause of sunflower wilts in Canada (Huang, 1980), and *Sporidesmium sclerotivorum* known as an obligate parasite of *Sclerotinia minor*,

S. sclerotiorum, *S. trifolium*, *Sclerotium cepivorum* and *Botrytis cinerea* (Ayers & Adams, 1981).

Most studies relating to ecological and biocontrol aspects of mycoparasitism have investigated destructive mycoparasites and soilborne plant pathogens, including *Pythium* spp. (Lumsden, 1981). Some of the more well known destructive mycoparasites include *Pythium nunn, P. oligandrum* and *Trichoderma* spp. For example, *Pythium nunn* suppressed cucumber damping-off caused by *P. ultimum* in a raw soil amended with ground bean leaves (Paulitz & Baker, 1987*a*), as well as in aerated steamed soil in growth room experiments (Paulitz & Baker, 1987*b*). When *Pythium oligandrum* was added to treatments containing *P. ultimum* in non-sterile soil, the antagonists reduced the incidence of damping-off in emerging sugar beet seedlings and significantly increased the dry weights of emerged plants (Veselý, 1977). *Trichoderma hamatum* has been reported by Sivan & Chet (1982) to control *Pythium aphanidermatum* in pea, cucumber, tomato, pepper and gypsophila, and *Trichoderma virens* has been shown to suppress damping-off of zinnia, cotton and cabbage caused by *P. ultimum* in non-sterile potting mix in the greenhouse (Lumsden & Locke, 1989). *P. periplocum* and *Laetisaria arvalis* also have been shown to be effective in controlling damping-off caused by *Pythium* spp., with mycoparasitism being the postulated mechanism (Hockenhull *et al.*, 1992; Hoch & Abawi, 1979).

Although many fungi appear to be mycoparasites in *in vitro* experiments, not all have been shown to be parasites under natural conditions. Boosalis (1956) was among the first to investigate mycoparasitism in a natural system by estimating the frequency of parasitism of *R. solani* hyphae by *Penicillium vermiculatum* in natural soil. Similarly, Huber *et al.* (1966) observed invasion of *R. solani* by an unknown fungus, as well as parasitism of *Fusarium solani* f. sp. *phaseoli* by soil actinomycetes. Sclerotia of *Sclerotinia trifoliorum* and *S. sclerotiorum* were found to be mycoparasitised by *Coniothyrium minitans* in field soil and inside pit cavities of wilted sunflower plants,

by Tribe (1957) and Huang (1976), respectively. The presence of sporangia of parasitic *Hyphochytrium catenoides* within infected oospores of *Pythium spp.*, in a natural soil, was considered by Ayers & Lumsden (1977) as an indication of parasitism of the pathogen under field conditions. In studies by Henis *et al.* (1978, 1979), frequent isolation of the mycoparasite *Trichoderma harzianum* was reported from mycelial mats of *R. solani* incubated in suppressive soil obtained from radish monoculture. Introduction of *T. harzianum* into conducive soil at the same densities as were found in the suppressive soil induced suppression of the pathogen. Furthermore, Henis *et al.* (1978, 1979) re-isolated *T. harzianum* from mycelial mats of *R. solani* introduced into soil previously infested with the mycoparasite.

For some potential biocontrol agents, including *Trichoderma* spp., *Gliocladium* spp. and *Pythium nunn,* the control of pathogens is thought to be achieved through a combination of mechanisms such as parasitism and antibiosis or competition for substrates (Fravel & Keinath, 1991), with parasitism being either a primary or a facilitative mechanism of biological control (Deacon, 1991).

In this chapter, observations on hyphal interactions between *P. u. sporangiiferum* and BNR1 or BNR2, in artificial media as well as on *Capsicum* seeds in sterilized potting mix, are presented. Light- and scanning electron microscopy were used to explore in detail the hyperparasitic behaviour of the two BNR isolates, which apparently has not been reported previously.

4.2 MATERIALS AND METHODS

4.2.1 Fungal isolates

BNR1, BNR2 and *P. u. sporangiiferum* were cultured and maintained as described in Section 3.1.

4.2.2 Hyphal interactions in dual culture

Hyphal interactions between *P. u. sporangiiferum* and either BNR1 or BNR2 were studied in plastic Petri dishes (90 mm diam.), on cellophane membranes placed on 1/4 PDA or WA, or on 1/4 PDA alone. The cellophane membranes were boiled in distilled water for 20 min to remove any surface residues that could interfere with fungal growth, then autoclaved for 15 min at 121°C before being placed aseptically on the media. Mycelial plugs (8 mm diam.) of the test fungi were taken from the margins of actively growing colonies on 1/4 PDA and placed at opposite sides of 1/4 PDA plates, or on the surface of cellophane membranes overlaying 1/4 PDA or WA. *P. u. sporangiiferum* was paired with each BNR isolate, and control plates were inoculated with BNR1, BNR2 or *P. u. sporangiiferum* alone. Dual cultures and controls were incubated in the dark at 25°C. The interaction zones began to form after 24 hours. The expanding fungal interaction regions were observed at various magnifications *in situ* using a Leitz Laborlux S compound microscope every day for 10 days. The interaction regions on cellophane membranes overlaying 1/4 PDA were collected after 5 days of incubation and processed for scanning electron microscopy. There were 10 replicate plates for each antagonist × pathogen combination and for controls, and the experiment was carried out three times.

4.2.3 Scanning electron microscopy (SEM)

Mycelial samples on cellophane membranes were cut into *ca* 5 mm^2 pieces (four pieces from each plate) and placed in ceramic vials (Selby Scientific Ltd., Australia) of 5 ml capacity. Fixation of specimens was achieved by immersion in 2.5% glutaraldehyde (Sigma Chemical Co., St. Louis, MO) in 0.1 M phosphate buffer (pH 7.0) for 12 hours at room temperature (*ca* 25°C). This was followed by dehydration of specimens through a graded ethanol series in deionized water (Glauert, 1974). The specimens were washed first in 0.1 M phosphate buffer (pH 7.0) for 15 min, then in 30%, 50%, 70%, 90%, 96% and 100% ethanol, each concentration for 10 min. The final wash was in a second volume of 100% ethanol for at least 15 min, after which the specimens

were critical point dried in an Emscope CPD 750 unit with CO_2 as the transitional fluid, then mounted on aluminium stubs and sputter-coated with 30 nm of gold in an Emscope SC 500 unit. Specimens were examined with a Cambridge Stereoscan S 250 scanning electron microscope.

4.2.4 Hyphal interactions on *Capsicum* seeds

Hyphal interactions between *P. u. sporangiiferum* and either BNR1 or BNR2 on germinating *Capsicum* seeds in sterilized potting mix were investigated. Approximately 20 g of autoclaved potting mix, with water potential of *ca* -0.03 bar and pH of 5.6 (see Section 3.3), were placed aseptically into each plastic Petri dish (90 mm diam.). Twenty *Capsicum* seeds were soaked in sterile, deionized water for 15 min before being placed on the surface of the potting mix in four rows of five seeds, approximately 1 cm apart. Mycelial plugs (8 mm diam.) of either BNR1 or BNR2 and *P. u. sporangiiferum* were taken from the margins of actively growing colonies on 1/4 PDA, and placed at opposite sides of each plate, approximately 1.5 cm from the outer rows of seeds. The control plates consisted of twenty *Capsicum* seeds which were inoculated with only BNR1, BNR2 or *P. u. sporangiiferum*. The plates were incubated at 25°C in the dark until all the seeds were colonized by both fungi (*ca* 5 days). *Capsicum* seeds were removed from the plates, cleared in 10% KOH for 30 min, washed in sterile deionized water, rinsed in 10% KCl and washed again in sterile deionized water. The seeds were then soaked in a wetting solution (see Appendix) for 30 min and stained with trypan blue in lactoglycerol (see Appendix). The seed coats were removed and their surface examined for mycoparasitic interactions using light microscopy. There were 10 replicate plates for each antagonist × pathogen combination and for controls, and the experiment was repeated once.

4.3 RESULTS

4.3.1 Hyphal interactions in dual culture - Light microscopic observations

Both BNR1 and BNR2 extensively parasitised *P. u. sporangiiferum* on 1/4 PDA and on cellophane membranes overlaying 1/4 PDA or WA. Prior to contact between *P. u. sporangiiferum* and either BNR1 or BNR2, growth of runner hyphae of paired fungi towards each other was observed. There was no visible change in the cytoplasm of either fungus as they approached one another. Upon contact, the runner hyphae of both fungi grew around one another, forming lateral branches. As the density of both fungi increased in the interaction zone (*ca* 20 min from the time of contact), the specific parasitic behaviour towards the pathogen by either BNR1 or BNR2 increased. Parasitism of *P. u. sporangiiferum* was similar for both antagonists and consisted of the following events: a) parallel growth of BNR and *P. u. sporangiiferum* hyphae (Plate 4.1.A); b) formation of hook-shaped hyphal tips by BNR on the surface of *P. u. sporangiiferum* hyphae (Plate 4.1.B); c) formation of "simple coils" by a BNR runner hypha or a lateral branch on pathogen hyphae (usually one to four coils) (Plate 4.1.C); d) formation of "complex coils" on pathogen hyphae (usually more than 10 coils), originating from more than one lateral branch of one or more BNR runner hyphae (Plate 4.1.D); e) penetration of *P. u. sporangiiferum* hyphae (Plate 4.1.E), appressoria and sporangia (Plate 4.1.F) by BNR, and occasional growth of BNR hyphae within these structures. Oospores of *P. u. sporangiiferum* were not penetrated by BNR. The hook-shaped BNR hyphae, with swollen, appressorium-like tips, formed either simple coils or initiated penetration of pathogen structures and growth within them. Hyphal densities of both BNR1 and BNR2, and *P. u. sporangiiferum*, were lower when grown on cellophane membranes overlaying WA than on 1/4 PDA. Also, parallel growth, hook-shaped hyphal tips, simple and complex coils, as well as penetration of pathogen structures by both BNR, were observed less frequently when WA was used.

Plate. 4.1 Light micrographs of interactions between *P. u. sporangiiferum* and either BNR1 or BNR2 in dual culture on 1/4 PDA.

P. u. sporangiiferum was paired with each BNR isolate on 1/4 PDA in plastic Petri dishes (90 mm diam.) and for controls, 1/4 PDA was inoculated with either BNR1, BNR2 or *P. u. sporangiiferum* alone. Dual cultures and controls were incubated in the dark at 25°C, and the expanding fungal interaction regions observed *in situ* with a Leitz Laborlux S compound microscope every day for 10 days.

A. Parallel growth of BNR2 hypha (arrowhead) and *P. u. sporangiiferum* hypha (double arrowheads). Bar = 10 μm.

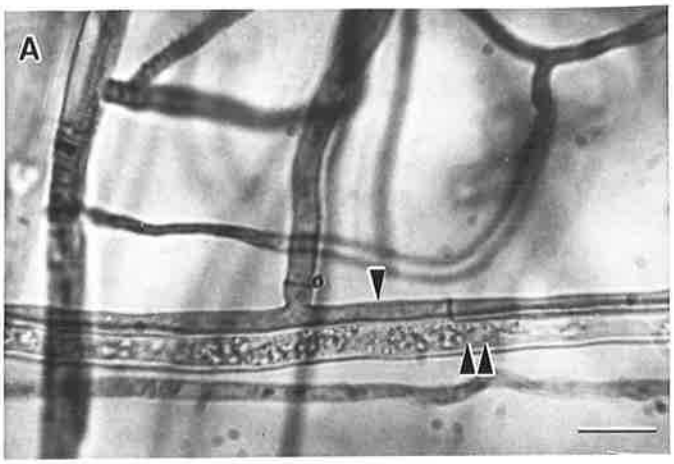
B. Hook-shaped hyphal tip (arrowhead) of BNR2 on the surface of *P. u. sporangiiferum* hypha. Bar = 10 μm.

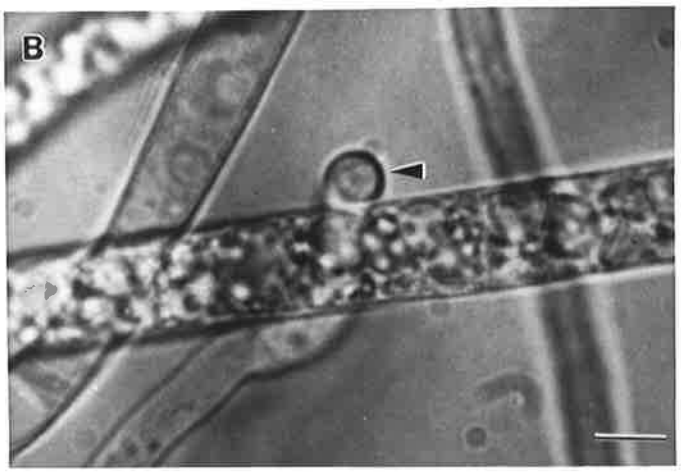
C. Simple coil formed by BNR1 on *P. u. sporangiiferum* hypha. Bar = 10 μm.

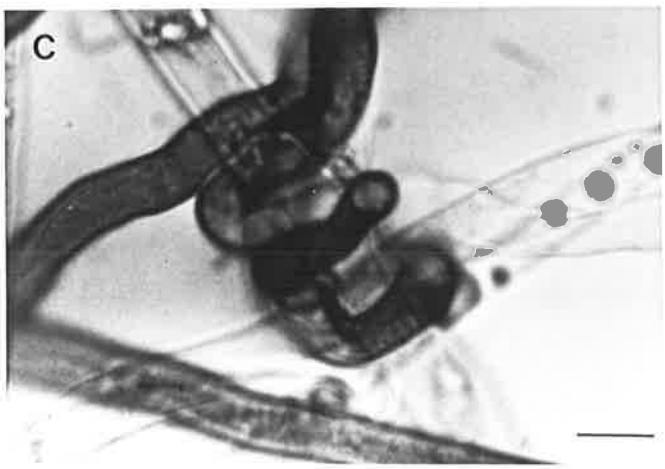
D. Complex coil (arrowheads) formed by BNR2 on *P. u. sporangiiferum* hypha. Bar = 25 μm.

E. BNR1 hyphae penetrate (arrowhead) and grow inside (double arrowheads) *P. u. sporangiiferum* hypha. Bar = 10 μm.

F. BNR1 hyphae penetrate (arrowheads) a sporangium of *P. u. sporangiiferum.* Bar = 10 μm.


A


B


C

Noticeable signs of stress in *P. u. sporangiiferum* hyphae due to direct mycoparasitic action by either BNR1 or BNR2 (*ca* 3-5 hours from the first hyphal contact) included increasingly vacuolated cytoplasm, as well as cytoplasmic streaming convulsively changing its direction of flow. This response of *P. u. sporangiiferum* hyphae was even greater when grown with either BNR isolate on cellophane membranes overlaying WA than on cellophane membranes overlaying 1/4 PDA. In contrast, in healthy *P. u. sporangiiferum* hyphae (controls) cytoplasmic streaming occurred mainly towards the hyphal tips and was relatively uniform in its flow. Approximately 48 hours from the time of contact with BNR hyphae, disorganisation and disappearance of *P. u. sporangiiferum* cytoplasm were observed, without obvious hyphal lysis. The remaining empty hyphae disappeared eventually from the media (*ca* 7-10 days after hyphal contact) as indicated by observations of BNR hyphae remaining coiled around the "ghost-hyphae" of the pathogen. After 10 days, fewer hyphae and sporangia of *P. u. sporangiiferum* were observed in the presence of BNR than in the control plates without BNR.

4.3.2 Hyphal interactions in dual culture - SEM observations

Scanning electron microscopy revealed, in more detail, the nature of hyphal interactions between *P. u. sporangiiferum* and the two BNRs. Both BNR1 and BNR2 formed tight coils on *P. u. sporangiiferum* hyphae (Plate 4.2.A), such that constriction of the host cell surface was sometimes visible. On closer examination of the pathogen hyphae enveloped by either BNR1 or BNR2, extensive wrinkling of the cell surface was observed. Such alteration of the pathogen cell walls was either local (Plate 4.2.B), or whole sections of the hyphae or appressoria displayed this wrinkled appearance (Plate 4.2.D). Hyphae of *P. u. sporangiiferum* in the absence of BNR (controls) did not show such changes. There was also evidence of digestion or penetration of cell walls of *P. u. sporangiiferum* by the swollen hyphal tips of either BNR1 or BNR2 associated with these wrinkled areas (Plate 4.2.C & D).

Plate 4.2 Scanning electron micrographs of interactions between *P. u. sporangiiferum* and either BNR1 or BNR2 in dual cultures on cellophane membranes overlaying 1/4 PDA.

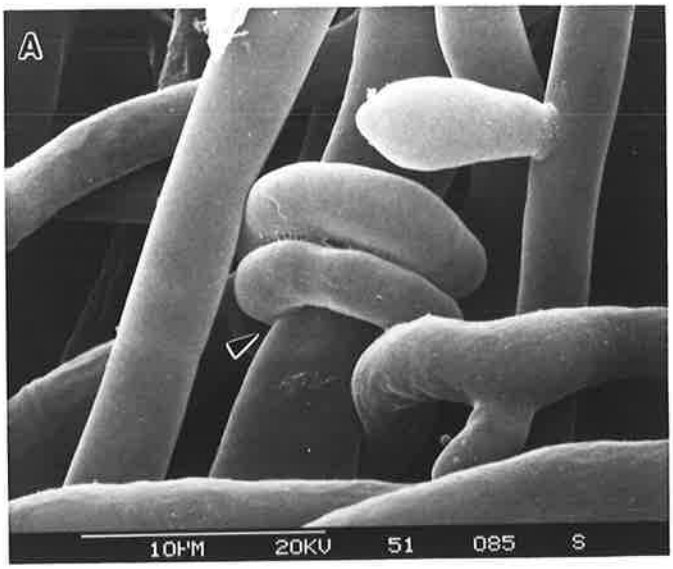
P. u. sporangiiferum was paired with each BNR isolate on the surface of cellophane membranes overlaying 1/4 PDA in plastic Petri dishes (90 mm diam.). For controls, 1/4 PDA was inoculated with BNR1, BNR2 or *P. u. sporangiiferum* alone. Dual cultures and controls were incubated in the dark at 25°C for 5 days. Mycelial samples on cellophane membranes (*ca* 5 mm^2 pieces) were fixed, dehydrated, critical-point-dried and sputter-coated with 30 nm of gold. Specimens were examined with a Cambridge Stereoscan S 250 scanning electron microscope.

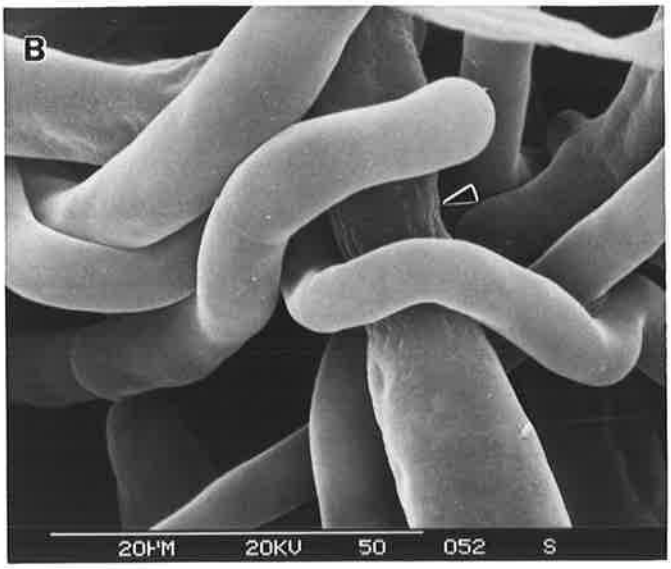
A. Tight double coil formed by a BNR2 hypha on a *P. u. sporangiiferum* hypha. Constriction of *P. u. sporangiiferum* cell surface (arrowhead) is visible.

B. Wrinkled surface of a *P. u. sporangiiferum* hypha (arrowhead) underneath two coiling hyphae of BNR2, indicating early signs of cell wall alteration.

C. Cell wall of *P. u. sporangiiferum* digested (arrowhead) by a swollen hyphal tip of BNR1.

D. Penetration of *P. u. sporangiiferum* appressorium (double arrowheads) by BNR1 hyphal tip. The wrinkled surface of an appressorium and hyphae of *P. u. sporangiiferum* (arrowheads) are visible.


A
10UM 20KV 51 085 S


B
20UM 20KV 50 052 S


C
10µM 20KV 43 044 S

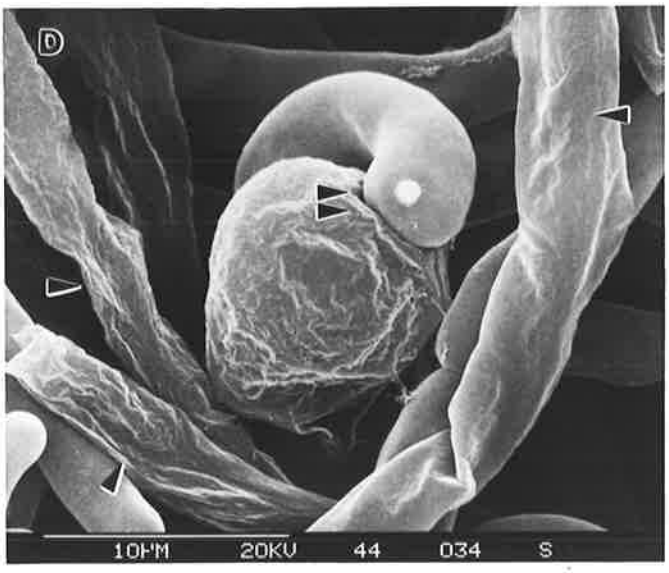

D
10µM 20KV 44 034 S

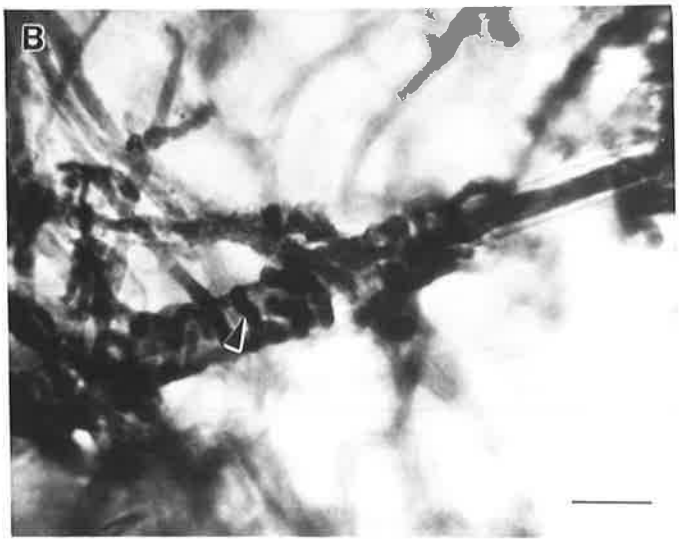
Plate 4.3 Light micrographs of interactions between *P. u. sporangiiferum* and either BNR1 or BNR2 on *Capsicum* seed coats.

P. u. sporangiiferum was paired with each BNR isolate in sterilized potting mix in the presence of germinating *Capsicum* seeds. For controls *Capsicum* seeds in potting mix were inoculated with BNR1, BNR2 or *P. u. sporangiiferum* alone. *Capsicum* seeds were removed after 5 days, cleared in 10% KOH for 30 min and washed in 10% KCl before being stained with trypan blue in lactoglycerol. The seed coats were removed and their surfaces examined for mycoparasitic interactions, using a Leitz Laborlux S compound microscope.

A. A BNR2 hypha coiling around a *P. u. sporangiiferum* hypha. Bar = 10 μm.

B. BNR1 hyphae (arrowhead) coiling around a *P. u. sporangiiferum* hypha. Bar = 31 μm.


A


B

Many of the parasitised *P. u. sporangiiferum* hyphae appeared ruptured and collapsed, whereas hyphae of controls remained intact.

4.3.3 Hyphal interactions on *Capsicum* seeds - Light microscopic observations

Both BNR1 and BNR2 parasitised *P. u. sporangiiferum* on *Capsicum* seed coats in sterilized potting mix (Plate 4.3.A & B). Mycoparasitic activity was most intensive on the *Capsicum* seeds most densely colonized by both the antagonist and the pathogen. Formation of dense mycelial mats consisting of short, swollen hyphal cells on the seed coats by either BNR was also associated with increased parasitism of *P. u. sporangiiferum.* Interactions between *P. u. sporangiiferum* and either BNR1 or BNR2 included: a) parallel growth of BNR and pathogen hyphae; b) formation of hook-shaped hyphal tips by BNR, some of which appeared to penetrate the surface of pathogen hyphae; c) formation of simple or complex coils by BNR on pathogen hyphae, and d) partial or total lysis of pathogen hyphae. In addition, mycoparasitic behaviour such as parallel growth and hook-formation were observed across the entire seed surface, whereas coiling was most frequent between and within the deep grooves of the seed coat, a region preferentially colonized by both fungi.

4.4 DISCUSSION

BNR1 and BNR2 displayed similar parasitic behaviour towards *P. u. sporangiiferum* in artificial media. There were four distinct stages that overlapped in time: a) apparent location and recognition of host hyphae, reproductive or resting structures; b) contact with the host and subsequent formation of hook-shaped hyphal tips and coils; c) softening and degradation of host cell walls; and d) penetration of host structures and occasional growth within them. A similar order of events has been recorded for a number of well known mycoparasites of *Pythium* spp., including *Pythium nunn* (Lifshitz *et al.*, 1984*a*), *P. oligandrum* (Lewis *et al.*, 1989) and *Trichoderma hamatum*

(Chet *et al.*, 1981). In contrast, Cardoso & Echandi (1987) reported an absence of hyphal interactions between an antagonistic BNR-like fungus and pathogenic *R. solani* in dual culture on agar media.

Light microscopic observations provided no evidence of "pre-contact" effects on *P. u. sporangiiferum* hyphae which might have been due to action of extracellular metabolites produced in advance by either BNR1 or BNR2. Thus, inhibition of hyphal tip growth, visible alteration to the host cytoplasm (e.g. increased vacuolation or cytoplasm aggregation) or hyphal lysis were not observed prior to contact with the antagonists. These observations were consistent with findings of Lewis *et al.* (1989) in their study of hyphal interactions between the mycoparasite *Pythium oligandrum* and several host fungi, including *P. ultimum*, *P. intermedium* and *P. debaryanum*. On the other hand, Chérif & Benhamou (1990) observed marked wall alterations, rapid collapse and loss of cell turgor in hyphae of *Fusarium oxysporum* f. sp. *radicis-lycopersici*, in areas that were not in direct contact with the hyphae of a parasitic strain of *Trichoderma* sp.

In many "post-contact" interactions, coiling of the mycoparasite around its host is a common occurrence, although the extent of coiling varies among different host-parasite combinations (Whipps *et al.*, 1988). It has been suggested that extensive coiling may be indicative of host resistance, rather than susceptibility, to a mycoparasitic attack, enabling the mycoparasite to establish closer contact with more resistant host hyphae, thus allowing antibiotics or extracellular enzymes to exert greater and more localized impact (Deacon, 1976; Lifshitz *et al.*, 1984*a*; Laing & Deacon, 1991). The observations reported here appear to agree with the above hypothesis. Both BNR1 and BNR2 produced two distinct coiling patterns, i.e. simple and complex coils, which were found in the same region on the Petri dish but usually on different hyphae of *P. u. sporangiiferum*. Simple coils were formed on younger, narrower runner hyphae as well as on lateral branches. Complex coils were mostly

associated with the older and larger runner hyphae of the pathogen. In addition, many *P. u. sporangiiferum* hyphae, probably the most susceptible, appeared dead as a consequence of mere parallel growth or hook-formation by either BNR isolate. From these observations, it seems likely that formation of complex coils enabled both BNR1 and BNR2 to mycoparasitise more effectively the older, more resistant hyphae of *P. u. sporangiiferum*. Similar observations were also reported by Dennis & Webster (1971*c*) in their study of hyphal interactions between mycoparasitic isolates of *Trichoderma* and several host fungi, including *P. ultimum*.

The most frequently observed reactions in mycoparasitised hyphae of *P. u. sporangiiferum* were rapid changes in the flow of cytoplasmic streaming and increased vacuolation of the cytoplasm. The convulsive movements of *P. u. sporangiiferum* cytoplasm were usually found in regions of direct contact with BNR hyphae, through parallel growth, hook-formation or coiling. Increased vacuolation, however, was observed throughout mycoparasitised hyphae of the pathogen. The convulsive behaviour of *P. u. sporangiiferum* cytoplasm could have resulted from changes in internal turgor or osmotic pressure gradients, caused by inhibitory substances released by the antagonists, that interfered with the cell wall or cell membrane structure (Benhamou & Chet, 1993). Alternatively, puncture or penetration of the cell wall and attached cell membrane could have disrupted the ecto- and endoplasm that are involved in producing a contracting force responsible for cytoplasmic motion, as suggested by Kamiya's hypothesis (Burnett, 1976). Similarly, alteration of the pathogen cell wall, cell membrane, or the cytoplasm itself, through either chemical or physical means, could have been responsible for the increased vacuolation of *P. u. sporangiiferum* cytoplasm in response to the antagonists. Similar cytoplasmic reactions, such as vacuolation and jerky shifts in cytoplasmic organelles, were also observed by Hoch & Fuller (1977) in several fungal hosts of *Pythium acanthicum*. Walther & Gindrat (1987) also reported that intensive vacuolation of the cytoplasm in hyphae of *P. oligandrum* and *P. ultimum* was observed in the presence of *R. solani*,

and binucleate *R. cerealis* and *R. fragariae*. Likewise, Laing & Deacon (1991) reported increased vacuolation and a conspicuous surge of protoplasm in hyphae of different hosts, including *Pythium* spp., of the parasitic *Pythium nunn* and *P. oligandrum*.

Penetration of host hyphae by the parasite, with the aid of lytic or cell-wall degrading enzymes, appears to be a probable cause of death in many mycoparasitic interactions (Whipps *et al.*, 1988). Cell-wall degrading enzymes such as glucanases, chitinases and cellulases are believed to be responsible for the degradation of pathogen hyphae via alteration of cell wall components, β-glucans, chitin and cellulose, respectively. These lytic enzymes have been produced, mainly in *in vitro* experiments, by a number of well known hyperparasites, including *Trichoderma harzianum* (Elad *et al.*, 1982), *T. hamatum* (Elad *et al.*, 1983), *Pythium nunn* (Elad *et al.*, 1985), and *P. oligandrum* (Lewis *et al.*, 1989). In this study of hyphal interactions between *P. u. sporangiiferum* and either BNR1 or BNR2, the most commonly described indicators of lytic enzyme action (Chérif & Benhamou, 1990; Deacon & Berry, 1992; Benhamou & Chet, 1993; Lifshitz *et al.*, 1984*a*; Walther & Gindrat, 1987) were also observed. These included wrinkling of the *P. u. sporangiiferum* hyphae and appressoria underneath coiling hyphae of BNR, as well as degradation of pathogen cell walls by the BNR at possible sites of penetration. Lytic enzyme production by BNR1 and BNR2 will be considered in detail in Chapter 5 of this thesis.

One of the main criticisms of studying mycoparasitic interactions *in vitro* is the lack of evidence that these occur in a natural system (Ayers & Adams, 1981; Whipps *et al.*, 1988; Stewart, 1995). For that reason, experiments were conducted to show that both BNR1 and BNR2 parasitised *P. u. sporangiiferum* on a natural substrate, that is germinating *Capsicum* seeds in a sterilized potting mix, since the seed is a primary site of infection by this pathogen. In this study, a sterilized potting mix was used in Petri dishes instead of pasteurized potting mix in punnets in a glasshouse. This was done

mainly to exclude microorganisms that might interfere with the assessment of mycoparasitism of *P. u. sporangiiferum* by BNR. Results from these experiments confirmed the *in vitro* observations of hyphal interactions between *P. u. sporangiiferum* and either BNR1 or BNR2, and provided direct evidence of mycoparasitism of the pathogen on germinating *Capsicum* seeds.

The question now arises as to the role of the mycoparasitic activity of BNR1 and BNR2 in biocontrol of *P. u. sporangiiferum* in nature. The most intensive hyphal interactions between the antagonists and the pathogen were observed to occur on substrates that had the highest relative density of both fungi, i.e. either on cellophane membranes overlaying 1/4 PDA or on densely colonised *Capsicum* seeds. This observation led to the hypothesis that mycoparasitism by the saprophytic BNR1 and BNR2 may have a significant role in facilitating competition for substrates by the antagonists on agar media and on *Capsicum* seeds. Apparently, these nutrient rich substrates allowed the initial increase of density of both fungi which, in turn, increased the pressure on resources available for their growing colonies. Due to diminishing access to nutrients and space, competition between the fungi for the remaining substrates intensified. By exerting mycoparasitic activity, both BNR isolates had a "competitive advantage" over the pathogen that resulted in greater "possession" of the substrates and reduced inoculum potential of *P. u. sporangiiferum.* Similar views on the facilitative role of mycoparasitism in biocontrol of plant pathogens are shared by a number of microbial ecologists, including Clark (1965), Campbell (1989*b*) and Deacon (1991).

CHAPTER 5 ANTIBIOSIS AND BIOCONTROL OF *P. U. SPORANGIIFERUM* BY BINUCLEATE *RHIZOCTONIA*

5.1 INTRODUCTION

Antibiosis is generally recognised as the principal mechanism of interference competition by which fungi exclude other organisms from resources potentially available to each of the competitors (Wicklow, 1981). For the purpose of this thesis, antibiosis is considered to be mediated by specific or non-specific metabolites of microbial origin, by lytic enzymes, volatile compounds or other toxic substances (Jackson, 1965).

Antibiotic production may confer a selective advantage to the producers in competition for nutrients and space within their ecological niches (Fravel, 1988). This assumption was based on the study by Bruehl *et al.* (1969), in which all of the several hundred cultures of *Cephalosporium gramineum*, freshly isolated from stems of diseased plants, produced antibiotics, whereas isolates maintained in culture at 6^{o}C for 2 to 5 years, showed little or no antibiotic activity. It has been suggested also that antibiotic production is an important mode of action for some fungal biocontrol agents against their target pathogens. For example, Dennis & Webster (1971*a*) reported that many isolates of *Trichoderma* spp. produced chloroform-soluble antibiotics, including trichodermin and peptide antibiotics. They have also shown that volatile antibiotics with a "coconut" odour, identified as alkyl-pyrones by Claydon & Allan (1987), were produced by species of *Trichoderma*, and that these compunds inhibited growth of many fungi including *P. ultimum* and *R. solani* (Dennis & Webster, 1971*b*). In another study, microscopic observations revealed that a debilitating factor ("routing factor")

was apparently produced by antagonistic *Trichoderma* spp. and was responsible for the lysis of hyphal tips of *P. ultimum* in dual culture tests (Lifshitz *et al.*, 1986). Howell & Stipanovic (1983) found a correlation between protection of cotton seeds from damping-off caused by *P. ultimum* and production of an antibiotic, gliovirin, by the antagonistic *Gliocladium* (*Trichoderma*) *virens*. A.R. Harris & R.D. Lumsden (1996, pers. comm.) also observed that *G. virens* G-20 retarded growth of *P. ultimum* from germinated sporangia in non-sterile potting medium and caused cytoplasmic leakage. They also observed a reduced branching of mycelium of *R. solani*, consistent with the production of gliotoxin by the antagonist. *Pythium oligandrum* was reported not to produce vast quantities of antibiotics, although it did secrete non-volatile metabolites that were inhibitory to certain fungal hosts *in vitro* (Deacon, 1976; Whipps, 1987*a*; Lewis *et al.*, 1989).

Antibiotic activity has also been associated with sclerotia and mycelia of some *Rhizoctonia* spp. Most of the antibiotic-producing *Rhizoctonia* spp. were identified by Burton & Coley-Smith (1985) as being binucleate *R. cerealis, R. oryzae-sativae* and *R. tuliparum*. The sclerotia of *R. tuliparum* were found to contain a pyrone antibiotic effective against a wide range of bacteria and certain fungi, including *Mucor*, *Fusarium* and *Trichoderma* spp. (Gladders & Coley-Smith, 1980). Antibiotics produced by *R. cerealis* and *R. oryzae-sativae* are thought to be closely related to the pyrone compound of *R. tuliparum* (Burton & Coley-Smith, 1985). Cardoso & Echandi (1987), however, reported that an isolate of BNR, which protected bean seedlings from *Rhizoctonia* root rot, failed to show any inhibitory effect towards *R. solani* in *in vitro* tests.

The involvement of enzymes in biological control is thought to obscure the distinction between parasitism and antibiosis. This stems from the observations that lytic enzymes produced by an antagonist could be involved simultaneously in both parasitism and antibiosis, or they may act independently as antibiotics (Fravel & Keinath, 1991).

For example, Elad *et al.* (1983) demonstrated an increased enzymatic activity of *Trichoderma harzianum* during parasitism of *Sclerotium rolfsii* or *R. solani*, particularly at the point where *T. harzianum* attached itself to host hyphae with clamps or by coiling. In addition, in many host-mycoparasite interactions, penetration of host hyphae is believed to occur as a result of cell wall hydrolysis by extracellular enzymes produced by the mycoparasite, for example *T. harzianum* (Elad *et al.*, 1982), *T. hamatum* (Elad *et al.*, 1983), *Pythium nunn* (Elad *et al.*, 1985) and *P. oligandrum* (Lewis *et al.*, 1989). Bélanger *et al.* (1995) investigated the relative roles of antibiosis and parasitism during mycoparasitism of *Botrytis cinerea* by *T. harzianum*. They concluded that *T. harzianum* antagonised first and foremost by antibiosis, leading to cell death, followed by degradation of the cell by means of chitinolytic enzymes. In contrast, cellulolytic activity alone is thought to be a probable explanation of antagonism of *P. oligandrum*, *P. ultimum* and *Aphanomyces cochlioides* by binucleate *Rhizoctonia* spp. and *R. solani* (Walther & Gindrat, 1987).

In this chapter, production of soluble and volatile antibiotics by BNR1 and BNR2 in agar media, liquid cultures and in sterile potting mix is described. Evidence for the production of lytic enzymes by the two antagonists on different substrates, including a mycelial preparation of *P. u. sporangiiferum* as well as in sterile potting mix, is presented.

5.2 MATERIALS AND METHODS

5.2.1 Antibiosis by soluble metabolites - culture filtrates

Liquid cultures of BNR1, BNR2 and *P. u. sporangiiferum* were prepared by placing four plugs (8 mm diam.), taken from the edges of 2-day-old colonies on 1/4 PDA, into either a) 50 ml Erlenmeyer flasks containing 10 ml of full strength potato dextrose broth (PDB) (Difco) or b) 250 ml Erlenmeyer flasks containing 100 ml of PDB.

Cultures were shaken at 100 rpm for 4 days in treatment (a) and for 14 days in treatment (b), at 25°C in the dark. Mycelial mats were removed from each flask with a sterile spatula, the liquid centrifuged at 5000 rpm for 20 min, and the supernatants sterilised by filtration through 0.22 µm Milipore filters. The cell-free culture filtrates were incorporated at 200 ml/l and 500 ml/l into autoclaved and cooled WA to give a final concentrations of 20% and 50% (v/v). The media were then poured into plastic Petri dishes (90 mm diam.), *ca* 20 ml per plate, and allowed to set. A WA plug (8 mm diam.) of *P. u. sporangiiferum* was then placed in the centre of each plate containing culture filtrates of either BNR1 or BNR2, at the two concentrations, and incubated at 25°C in the dark. Similarly, plates containing culture filtrates of *P. u. sporangiiferum* were inoculated with plugs of either BNR1 or BNR2. Controls consisted of plates of WA containing sterile PDB at 20% and 50% (v/v) inoculated as described above with one plug of *P. u. sporangiiferum*, BNR1 or BNR2. The radial growth of each fungus, determined as a mean of four radii at right angles, was measured after *ca* 25 hours for *P. u. sporangiiferum* and 48 hours for BNR1 and BNR2, at which time more then half of the plate was covered by each fungus. At the end of the experiment, colony morphology of the fungi was examined at ×320 and ×400 *in situ*, with a Leitz Laborlux S compound microscope. There were twenty replicate plates for each pathogen × antagonist, and antagonist × pathogen combination, for each concentration of culture filtrates and for controls. The experiment was conducted twice with similar results, and data from each experiment analysed with one-way analysis of variance. The Tukey's HSD test (Zar, 1984) was used to determine pairwise-mean-differences between the treatments.

5.2.2 Antibiosis by soluble metabolites - cellophane membranes

Production of soluble metabolites by BNR1 and BNR2 and their effect on the radial growth of *P. u. sporangiiferum* were investigated on cellophane membranes using a method modified from Dennis & Webster (1971*a*). Cellophane membranes (90 mm diam.) were washed in boiling deionised water for 20 min, autoclaved for 15 min at

121°C in glass Petri dishes (180 mm diam.) between layers of wet Whatman No. 2 filter papers, and placed aseptically on the surface of *ca* 20 ml of either WA, 1/4 PDA or 3/4 PDA in plastic Petri dishes (90 mm diam.). Agar plugs (8 mm diam.) of either BNR1 or BNR2, taken from the margins of 2-day-old colonies grown on 1/4 PDA, were placed on the cellophane membranes and incubated at 25°C in the dark for 3 days. The cellophane membranes together with BNR1 or BNR2 were removed from each plate and replaced with a 1/4 PDA plug (8 mm diam.) of *P. u. sporangiiferum*, which was placed directly onto the medium. Plates were incubated in the dark at 25°C for *ca* 31 hours, or until three quarters of the plate area was covered by the fungus. The effect of soluble metabolites produced by *P. u. sporangiiferum* on the growth of BNR1 and BNR2 was also investigated on cellophane membranes overlaying WA, as described above. Controls consisted of cellophane membranes on either WA, 1/4 PDA or 3/4 PDA that were inoculated with sterile 1/4 PDA plugs (8 mm diam.), which were removed after 3 days of incubation at 25°C in the dark, and replaced with a 1/4 PDA plug of either *P. u. sporangiiferum,* BNR1 or BNR2. The radial growth of each fungus was determined as the mean of four radii at right angles on each medium tested, and compared with the growth in the control treatment. The percentage reduction in the radial growth of *P. u. sporangiiferum*, BNR1 and BNR2, was then calculated for each medium using the formula:

$$\%\ \text{RRG (medium A)} = \frac{[\ \text{RG (control A)} - \text{RG (treatment A)}]}{\text{RG (control A)}} \times 100$$

where:

% RRG (medium A) is the percentage reduction in radial growth on medium A;

RG (control A) is the radial growth on medium A in a control plate;

RG (treatment A) is the radial growth on medium A in a treatment plate.

Fungal colonies were also examined at ×320 and ×400 *in situ*, using a Leitz Laborlux S compound microscope, at the end of the experiment. There were ten replicate plates for each antagonist × pathogen and pathogen × antagonist combination, and controls. The experiment was repeated twice with similar results, and data from each repeated experiment analysed with one-way analysis of variance. The Tukey's HSD test was used to determine pairwise-mean-differences between the treatments.

5.2.3 Antibiosis by volatile metabolites

The method described by Dennis & Webster (1971*b*) was used to study the production of volatile metabolites by BNR1 and BNR2, inhibitory to the growth of *P. u. sporangiiferum*, in culture media and in sterile potting mix.

5.2.3.1 Production of volatile metabolites in agar media

Plugs (8 mm diam.) of either BNR1 or BNR2 were taken from the edges of 2-day-old colonies growing on 1/4 PDA, and placed in the centre of Petri dishes (90 mm diam.) containing *ca* 20 ml of either WA or 1/4 PDA. Plates were incubated at 25°C in the dark for 2 days, during which time each BNR covered approximately half of the agar surface. Another set of 1/4 PDA plates was then inoculated with 1/4 PDA plugs (8 mm diam.) of *P. u. sporangiiferum*, one plug in the centre of each plate, and then inverted and taped to the bases of WA or 1/4 PDA plates containing the 2-day-old colonies of either BNR1 or BNR2. Plates were incubated at 25°C in the dark. The radial growth (measured as the mean of four radii at right angles) of *P. u. sporangiiferum* was determined after 31 hours, by which time the fungus covered approximately three quarters of the plate area. In control treatments, the radial growth of *P. u. sporangiiferum* was determined after growing the fungus above sterile 1/4 PDA plugs, placed instead of BNR1 or BNR2, on either WA or 1/4 PDA plates. Colony morphology of the pathogen was examined at ×320 and ×400 *in situ*, using a Leitz Laborlux S compound microscope, at the end of the experiment. There were ten replicate plates for each pathogen × antagonist × medium combination, and fifteen

replicate plates for each control × medium combination. The experiment was repeated once, with similar results, and data analysed with one-way analysis of variance. Tukey's HSD test was used to determine pairwise-mean-differences between the treatments.

5.2.3.2 Production of volatile metabolites in sterile potting mix

Approximately 20 g of autoclaved potting mix, with water potential of *ca* -0.03 bar and pH of 5.6 (see Section 3.3 for details), were placed aseptically in plastic Petri dishes (90 mm diam.) and inoculated with 1/4 PDA plugs (8 mm diam.) of either BNR1 or BNR2. Plates were incubated at 25°C in the dark for 2 days. Fresh plates containing *ca* 20 ml of 1/4 PDA were then inoculated with 1/4 PDA plugs (8 mm diam.) of *P. u. sporangiiferum*, inverted and taped to the bases of plates containing either BNR1 or BNR2 grown on potting mix for 2 days. Plates were incubated at 25°C in the dark for *ca* 31 hours and the radial growth (mean of four radii at right angles) of *P. u. sporangiiferum* measured. For controls, plugs of sterile 1/4 PDA were applied to the potting mix. Colony morphology of the pathogen was examined at ×320 and ×400 *in situ*, using a Leitz Laborlux S compound microscope, at the end of the experiment. There were twenty replicate plates for each pathogen × antagonist combination and for controls. The experiment was repeated once, with similar results, and data analysed with one-way analysis of variance, and Tukey's HSD test used to determine pairwise-mean-differences between the treatments.

5.2.4 Enzymatic activity by BNR1 and BNR2

5.2.4.1 Production of β-1,3 glucanase and cellulase in liquid synthetic medium on specific carbon sources

Production of β-1,3 glucanase and cellulase by BNR1 and BNR2 was investigated using liquid cultures on synthetic medium (SM) supplemented with sole carbon sources such as laminarin (Sigma; substrate for β-1,3 glucanase), carboxymethyl

cellulose (CMC; Sigma; substrate for cellulase), or mycelial preparation of *P. u. sporangiiferum* (see Section 5.2.4.3). Laminarin and CMC were used as specific substrates for induction of β-1,3 glucanase and cellulase, respectively, based on their chemical structures, which consisted mainly of β-1,3 linkages for laminarin and β-1,4 linkages for cellulose.

Preliminary experiments were conducted to establish a) whether 4 or 10 days of incubation of BNR1 and BNR2 were better for production of β-1,3 glucanase and cellulase on laminarin and CMC, respectively, and b) the effect of dialysis on cellulase activity during partial purification of the enzyme. Bateman (1964) showed that dialysis involved in partial purification of culture filtrates of *Rhizoctonia solani* decreased the cellulase activity up to 60%. As production of β-1,3 glucanase and cellulase by each BNR had decreased significantly by the 10th day of incubation (data not shown), the 4-day incubation period was chosen for subsequent experiments. Similarly, the activity of cellulase produced by BNR1 and BNR2 was significantly reduced by the partial purification procedure and, as a result, crude enzyme extracts were used subsequently.

Four plugs (8 mm diam.) of BNR1 or BNR2, taken from the edges of 2-day-old colonies grown on WA, were placed in 50 ml Erlenmeyer flasks containing SM and either laminarin, CMC or mycelial preparation of *P. u. sporangiiferum* at 2% (w/v). The SM consisted of (grams per litre): $MgSO_4.7H_2O$, 0.2; K_2HPO_4, 0.9; KCL, 0.2; NH_4NO_3, 1.0; $FeSO_4$, 0.002; $MnCl_2$, 0.002; $ZnCl_2$, 0.002; in deionized water with pH adjusted to 6.3 (Elad *et al.*, 1982). The medium plus laminarin or CMC was sterilised using 0.22 μm Millipore membranes before being inoculated with either BNR1 or BNR2. Due to the coarser nature of *P. u. sporangiiferum* cell walls, these material was first added to 0.5 ml of SM and autoclaved separately at 121°C for 20 min, before being pipetted into filter-sterilised SM. Cultures were incubated for 4 days at 30°C in the dark on a shaker at *ca* 100 rpm. Cultures (without the mycelial mat) were decanted into 10 ml sterile, plastic syringes and filtered through 0.2 μm filters (Gelman).

The cell-free culture filtrates were collected into sterile 50 ml Erlenmeyer flasks and the crude enzyme solutions used in enzyme assays. Controls were prepared by boiling crude enzyme solutions for 10 min prior to enzyme assays (see Section 5.2.4.7).

5.2.4.2 Partial purification of β-1,3 glucanase and cellulase

The cell-free culture filtrates in 50 ml Erlenmeyer flasks were precipitated by gradual addition of 80% (w/v) ammonium sulphate. The suspension was centrifuged at 12,500 rpm for 15 min at 4°C. The resulting precipitate was then diluted to half of the original volume in cold (4°C) distilled water. Dialysis was conducted in *ca* 20 cm long dialysis tubes (Sigma Chemical Co., USA) against distilled water, on a magnetic stirrer at 4°C for approximately 24 hours. During the first 4 to 5 hours of dialysis, the water was changed hourly to hasten the process of salt release. Nestler's reagent (Sigma) was used to detected the ammonium sulphate in the dialysis water by adding 1 drop of the reagent to 2 ml of water. Formation of an orange precipitate indicated the presence of salt. There were two additional water changes after no more salt was detected in the dialysis water. The contents of dialysis tubes were decanted into sterile 50 ml Erlenmeyer flasks and used in subsequent enzyme assays.

5.2.4.3 Preparation of mycelia of *P. u. sporangiiferum*

Liquid cultures of *P. u. sporangiiferum* were prepared by placing four 1/4 PDA plugs (8 mm diam.), taken from the edges of 2-day-old colonies, into 250 ml Erlenmeyer flasks containing 150 ml of full strength PDB. Cultures were incubated at 25°C in the dark for *ca* 30 days. The flasks were autoclaved at 121°C for 20 min on three successive days. Hyphal mats were removed from the flasks, dried at 80°C for 3 days and ground to a fine powder using a mortar and pestle. The mycelial preparation of *P. u. sporangiiferum* was then autoclaved for 20 min at 121°C in a small glass McCartney bottle and stored at 4°C.

5.2.4.4 Production of β-1,3 glucanase and cellulase on cellophane membranes overlaying agar media

Production of β-1,3 glucanase and cellulase by BNR1 and BNR2 was investigated using cellophane membranes overlaying agar media (see Section 4.2.2 for preparation of cellophane membranes). Agar plugs (8 mm diam.) of either BNR1 or BNR2, taken from the margins of 2-day-old colonies growing on 1/4 PDA, were placed on cellophane membranes overlaying either WA, 1/4 PDA or 3/4 PDA. Plates were incubated at 25°C in the dark for 3 days during which time more than half of the agar surface was covered by each fungus. The cellophane membranes together with either BNR1 or BNR2 were removed from each plate and six plugs from each plate were cut from the medium underneath, approximately 1.0 cm away from where the inoculum plug was originally placed, using a sterile (8 mm diam.) cork borer. Agar plugs, containing enzymes released by either BNR1 or BNR2, were placed in a glass extraction tube, six plugs per tube, with 4 ml of citrate buffer (0.1 M) pH 5.0, ground eight times, and 1.0 ml of this crude extract was used in the enzyme assays. Controls were prepared by boiling crude enzyme solutions for 10 min prior to enzyme assays (see Section 5.2.4.7).

5.2.4.5 Production of β-1,3 glucanase and cellulase in full strength potato dextrose broth

Cultures of BNR1 and BNR2 were prepared by placing four 1/4 PDA plugs (8 mm diam.), taken from the edges of 2-day-old colonies, into 50 ml Erlenmeyer flasks containing 10 ml of full strength PDB. Fungi were grown for 4 days at 25°C in the dark on a shaker at *ca* 100 rpm. In addition, 250 ml Erlenmeyer flasks were prepared with 150 ml PDB and inoculated with either BNR1 or BNR2 (as described above), and incubated for 14 days at 25°C in the dark on a shaker at *ca* 100 rpm. Crude enzyme solutions and controls were prepared as described in Section 5.2.4.1.

5.2.4.6 Production of β-1,3 glucanase and cellulase in sterile potting mix

Approximately 20 g of autoclaved potting mix, with water potential of *ca* -0.03 bar and pH of 5.6 (see Section 3.3) was placed aseptically into plastic Petri dishes (90 mm diam.) and inoculated with 1/4 PDA plugs (8 mm diam.) of either BNR1 or BNR2, one plug per plate. Plates were incubated at 25°C in the dark for 6 days. The potting mix from each plate was mixed with 15 ml of citrate buffer (0.1 M) pH 5.0 on a shaker at *ca* 200 rpm for 30 min. The preparation was centrifuged for 15 min at 6,500 rpm, then the supernatant filtered through 0.2 µm filters (Gelman) into sterile 50 ml Erlenmeyer flasks. This crude enzyme solution was than used in the enzyme assays and controls were prepared by boiling the crude enzyme solution for 10 min prior to enzyme assays (see Section 5.2.4.7).

Experiments presented in Sections 5.2.4.1, 5.2.4.4, 5.2.4.5 and 5.2.4.6 were conducted twice, with six replicates per treatment and their corresponding controls in the first run and four replicates plus controls in the second run. Data for each experiment were analysed by the two-way analysis of variance to determine if results of the first and second sets could be pooled, then further analyses were performed on the combined data.

For the experiment in Section 5.2.4.4, the relationship between the production of lytic enzymes by BNR1 or BNR2 on either WA, 1/4 PDA or 3/4 PDA (see Section 5.3.4.2) and the percentage reduction in radial growth of *P. u. sporangiiferum* on the three media after the removal of cellophane membranes with the antagonists, was investigated. Regression analysis was performed on enzyme units of β-1,3 glucanase or cellulase produced by BNR1 or BNR2, and the percentage reduction in radial growth of the pathogen on the three media tested. The percentage reduction in radial growth of *P. u. sporangiiferum* was chosen, rather than the enzyme activity, as the independent variable (X) since the former was measured with greater accuracy. Thus, the assumption of the regression hypothesis, that values of the variable X should be

obtained with negligible error (Zar, 1984) was fulfilled. Regression lines were forced through the origin due to 1) regression constants being not significant ($P > 0.05$), and 2) the assumption that in controls there is zero enzyme activity and zero reduction in radial growth of the pathogen.

5.2.4.7 Enzyme assay

The activity of β-1,3 glucanase and cellulase was determined by measuring the release of free glucose from laminarin and CMC, respectively, during a glucose oxidase reaction (Sigma) according to the manufacturer's directions. The reaction mixture consisted of 1.0 ml crude enzyme solution (test or control), 2.0 ml citrate buffer (0.1 M) pH 5.0 and 0.2% (w/v) laminarin or CMC in sterile 10 ml scintillation vials. The reaction mixture was incubated for 1.5 hours at 40°C and the reaction stopped by boiling the vials for 10 min. Each enzyme test solution had a corresponding control prepared by taking 1.0 ml of the test solution and boiling it for 10 min prior to the above reaction. The reaction mixture (i.e. 3.0 ml of the test or control enzyme solution) was then incubated with 14.4 ml of 0.21 mM o-Dianisidine solution for 10 min at 35°C while being purged with oxygen. Any loss in volume of the reaction mixture, due to previous boiling, was replaced with boiled distilled water to make exactly 3.0 ml. Following the incubation, 1.74 ml of the reaction mixture was pipetted into five plastic 3.0 ml cuvettes (Bio-Rad Laboratories, USA) including one blank, and 60 µl of peroxidase enzyme solution was then added to each one. In addition, 60 µl of sodium acetate buffer (50 mM) pH 5.1 was added to the blank. The contents were mixed by inverting the cuvettes five times and incubating at 35°C for 5 min. After that time, 60 µl of glucose oxidase solution was added to each cuvette at 30 seconds intervals, starting with the blank, mixed five times by inversion and read at 500 nm (DU Series 600 Spectrophotometer) at 0, 2 and 5 min. Enzyme activity (enzyme units, EU) expressed as mmoles of glucose per ml of culture filtrate per hour, was calculated using the formula:

$$\text{Enzyme activity} = \frac{\Delta A'}{7.5} \times \frac{1.86}{0.1} \times \frac{17.4}{1} \times 60$$

where:

A' is the optical density (i.e. absorbance)

ΔA' is the change in the absorbance per min and is determined as:

the maximal ΔA/min (test) - maximal ΔA/min (control);

7.5 is the millimolar extinction coefficient of oxidized o-Dianisidine at 500 nm;

1.86 / 0.1 is the dilution factor of the enzyme in the cuvette;

17.4 / 1 is the dilution factor of the enzyme in the reaction mixture;

60 is the conversion factor from minutes to an hour.

5.3 RESULTS

5.3.1 Antibiosis by soluble metabolites - culture filtrates

Culture filtrates of either BNR1 or BNR2, harvested after 4 or 14 days of incubation, did not reduce ($P > 0.05$) the radial growth of *P. u. sporangiiferum* when compared with that of controls (Table 5.1.A & B). There was no inhibitory effect on the pathogen at either low (20%) or high (50%) concentrations of the filtrates from 4-day-old or 14-day-old cultures of either BNR1 or BNR2. However, radial growth of *P. u. sporangiiferum* was stimulated ($P \leq 0.05$) by the filtrates from 14-day-old cultures of each of the two BNR, at both concentrations tested (Table 5.1.B). No changes in *P. u. sporangiiferum* colony morphology were observed in the presence of filtrates of either 4-day- or 14-day-old cultures of BNR1 and BNR2 when compared with that in controls. Filtrates of the 4-day-old and 14-day-old cultures of *P. u. sporangiiferum* had no effect ($P > 0.05$) on radial growth or colony morphology of either BNR1 or BNR2 at the two concentrations tested (data not shown).

Table 5.1.A & B Mean radial growth of *P. u. sporangiiferum* (mm) after 25 hours in the presence of soluble metabolites produced in PDB by BNR1 and BNR2, after incubation at 25°C for 4 and 14 days.

A.

	final concentration of culture filtrate	
filtrate from 4-day-old cultures of:	**20%**	**50%**
BNR1	35.5 a	35.5 x
BNR2	35.5 a	35.9 x
Control	35.9 a	35.6 x

B.

	final concentration of culture filtrate	
filtrate from 14-day-old cultures of:	**20%**	**50%**
BNR1	31.4 a (9 %)	31.7 x (9 %)
BNR2	30.9 a (8 %)	31.3 x (8 %)
Control	28.5 b	28.7 y

The cell-free culture filtrates were incorporated into WA at 200 ml/l (20%) and 500 ml/l (50%). Controls consisted of sterile PDB incorporated into WA at 20 % and 50%. Data are means of twenty replicate plates. Percentage stimulation of radial growth of *P. u. sporangiiferum*, when compared with that of controls, is given in parenthesis. Values, for each concentration of culture filtrate, for each incubaton period, followed by the same letter are not significantly different ($P > 0.05$) by Tukey's HSD test.

5.3.2 Antibiosis by soluble metabolites - cellophane membranes

Radial growth of *P. u. sporangiiferum* was significantly ($P \leq 0.05$) reduced by soluble metabolites produced by either BNR1 or BNR2, on cellophane membranes overlaying WA, 1/4 PDA or 3/4 PDA, compared to controls (Fig. 5.1.A). Inhibition of radial growth of *P. u. sporangiiferum* by BNR1 was greatest on WA (38%), followed by 1/4 PDA (18%) and 3/4 PDA (8%) (Fig. 5.1.B). A similar trend in reduction of radial growth of *P. u. sporangiiferum* by soluble metabolites produced by BNR2 was observed, such that the pathogen was inhibited to the extent of 51% on WA, 23% on 1/4 PDA and 10% on 3/4 PDA (Fig. 5.1.B).

There was also a visible difference in colony morphology of *P. u. sporangiiferum* when grown on WA in the presence of soluble metabolites of either BNR1 or BNR2, when compared with colony morphology on 1/4 PDA or 3/4 PDA, which contained soluble metabolites of BNR, and in controls on all three media. Growth of *P. u. sporangiiferum* on WA in the presence of soluble metabolites of either BNR1 or BNR2 was very sparse, with hyphae being less branched than on 1/4 PDA and 3/4 PDA containing soluble metabolites of each of the two BNR isolates, and in WA controls. When viewed under a compound microscope (Plate 5.1), an intense cytoplasmic vacuolation and hyphal lysis were clearly visible throughout the pathogen colony on WA in the presence of soluble metabolites of either BNR1 or BNR2. Growth of *P. u. sporangiiferum* on 1/4 PDA in the presence of soluble metabolites of each of the two BNR isolates was more dense than that on WA, but it was less dense than the growth on 3/4 PDA in the presence of soluble metabolites of BNR. Growth of *P. u. sporangiiferum* on 3/4 PDA containing soluble metabolites of either BNR1 or BNR2, closely resembled that of 3/4 PDA controls. There was no inhibition ($P > 0.05$) of radial growth of either BNR1 or BNR2 by soluble metabolites produced by *P. u. sporangiiferum* while the pathogen was grown on cellophane membranes overlaying WA (data not shown).

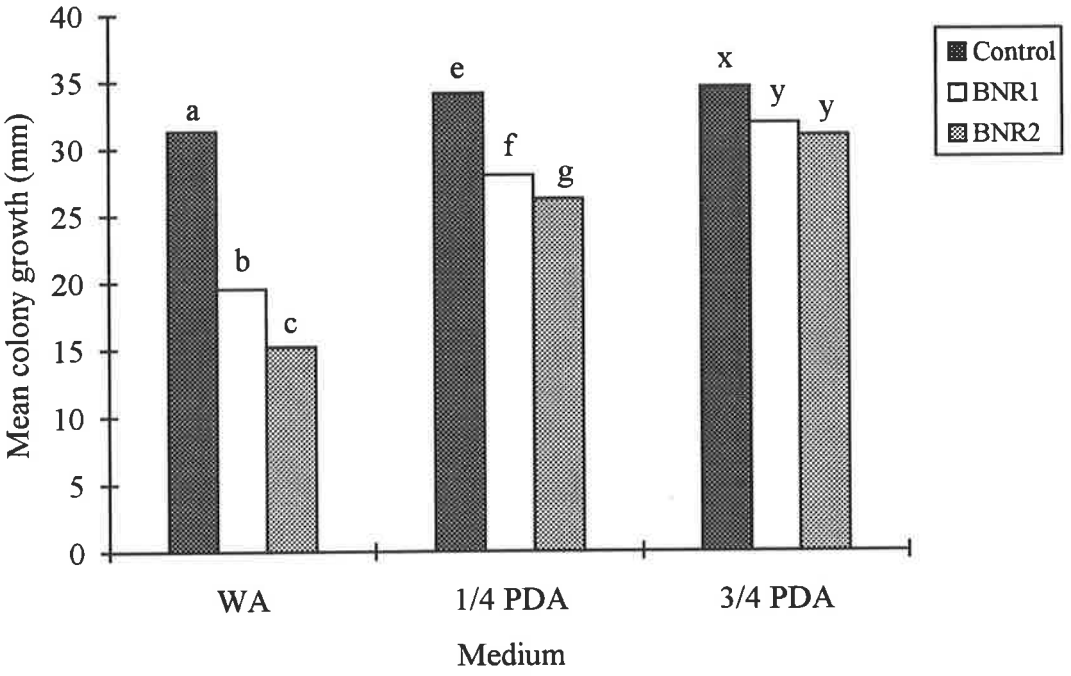
Figure 5.1 Mean radial growth of *P. u. sporangiiferum* on agar media after removal of cellophane membranes and BNR.

BNR1 and BNR2 were grown in Petri dishes on cellophane membranes overlaying WA, 1/4 PDA or 3/4 PDA, for 3 days at 25°C. Membranes plus the antagonists were removed and the media inoculated with *P. u. sporangiiferum*. Plates were incubated at 25°C until 3/4 of the plate area was covered by the fungus. Controls consisted of *P. u. sporangiiferum* growing on agar media from which cellophane membranes with sterile 1/4 PDA plugs were removed. Radial growth (mm) of *P. u. sporangiiferum* was measured and compared with that of controls (see Section 5.2.2) to determine the percentage reduction in growth. Data are means of ten replicate plates for each antagonist × pathogen combination. Values in Fig. 5.1.A, for each medium, followed by the same letter are not significantly different ($P > 0.05$) by Tukey's HSD test. Values in Fig. 5.1.B, for each BNR isolate, followed by the same letter are not significantly different ($P > 0.05$) by Tukey's HSD test.

Figure 5.1.A Mean radial growth of *P. u. sporangiiferum* on agar media after removal of cellophane membranes with BNR1 or BNR2.

Figure 5.1.B Percentage reduction in radial growth of *P. u. sporangiiferum* on agar media after removal of cellophane membranes with BNR1 or BNR2.

A.


Mean colony growth (mm)
40
35
30
25
20
15
10
5
0
a
b
c
e
f
g
x
y
y
Control
BNR1
BNR2
WA
1/4 PDA
3/4 PDA
Medium

B.

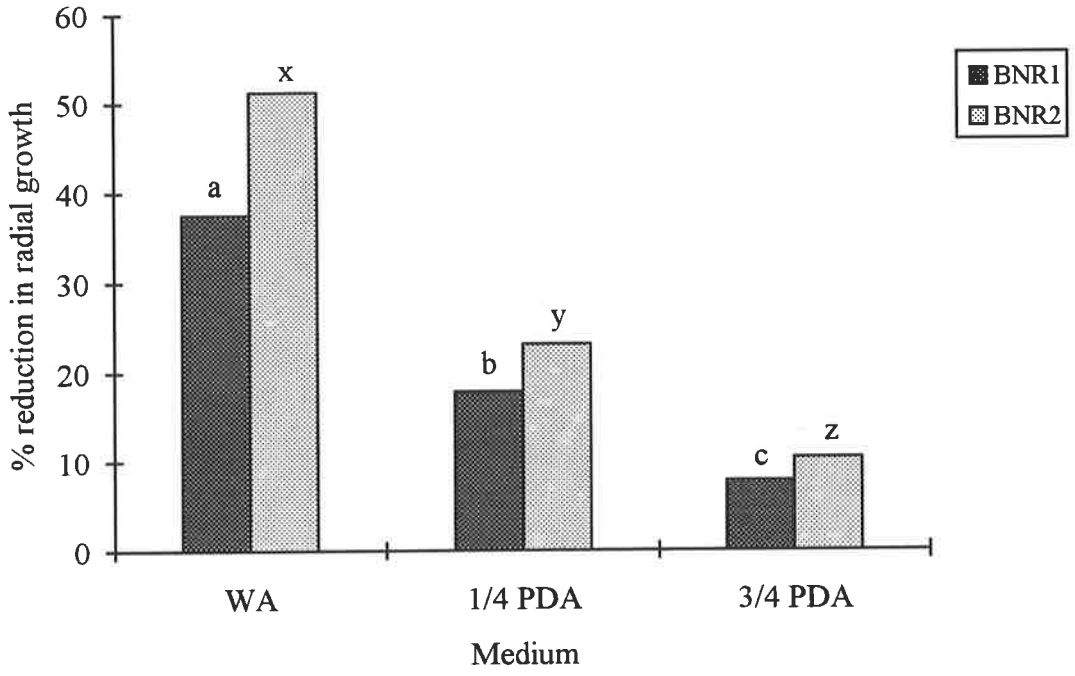
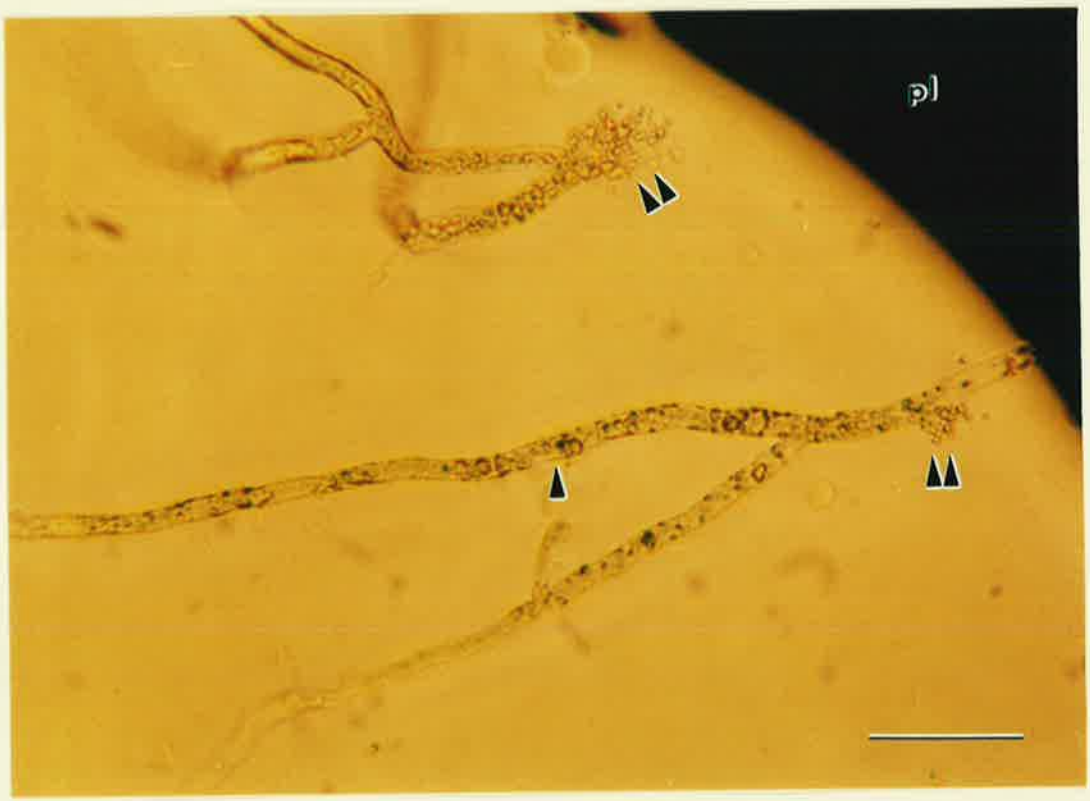

% reduction in radial growth
60
50
40
30
20
10
0
a
x
b
y
c
z
BNR1
BNR2
WA
1/4 PDA
3/4 PDA
Medium

Plate 5.1 Hyphae of *P. u. sporangiiferum* grown in the presence of soluble metabolites produced by BNR2 in WA.

BNR2 was grown in Petri dishes on cellophane membranes overlaying WA for 3 days at 25°C. Membrane plus the antagonist was removed and the medium inoculated with a 1/4 PDA plug (pl) of *P. u. sporangiiferum.* Plates were incubated at 25°C for *ca* 31 hours and colony morphology of the pathogen examined *in situ* using a Leitz Laborlux S compound microscope. An intense vacuolation (arrowhead) and lysis (double arrowheads) are visible in the hyphae of *P. u. sporangiiferum*. Bar = 62 μm.


pl

5.3.3 Antibiosis by volatile metabolites

Radial growth of *P. u. sporangiiferum* was not inhibited ($P > 0.05$) by volatile metabolites produced by BNR1 on WA, however, it was significantly reduced ($P \leq 0.05$) on 1/4 PDA in comparison with controls (Fig. 5.2). On the other hand, volatile metabolites produced by BNR2 significantly reduced ($P \leq 0.05$) the growth of the pathogen on WA and 1/4 PDA (Fig. 5.2). Radial growth of *P. u. sporangiiferum* was also affected ($P \leq 0.05$) by volatile metabolites produced by BNR1 and BNR2 in sterile potting mix (Fig. 5.3). Both BNR1 and BNR2 produced volatile substances that inhibited the growth of the pathogen by *ca* 11% each. No definite odour was detected in plates containing either BNR1 or BNR2 on WA, 1/4 PDA or sterile potting mix at the end of each experiment.

There was no difference in colony morphology of *P. u. sporangiiferum* when the pathogen was grown either in the presence of volatile metabolites produced by BNR1 or BNR2 on WA, 1/4 PDA or sterile potting mix, or in controls.

5.3.4 Enzymatic activity by BNR1 and BNR2

5.3.4.1 Production of β-1,3 glucanase and cellulase in liquid synthetic medium on specific carbon sources

BNR1 and BNR2 produced extracellular β-1,3 glucanase and cellulase while growing in liquid SM cultures with sole carbon source laminarin and CMC, respectively, as well as in the presence of the mycelial preparation of *P. u. sporangiiferum* (Table 5.2). The amount of β-1,3 glucanase produced by BNR1 on laminarin was not significantly different ($P > 0.05$) from that on the mycelial preparation of *P. u. sporangiiferum*. In contrast, BNR2 produced significantly more ($P \leq 0.05$) β-1,3 glucanase when grown on the mycelial preparation of the pathogen (*ca* two fold) than on laminarin. Production of cellulase by BNR1 in the presence CMC was, again, not significantly different ($P > 0.05$) from that produced on the mycelial preparation of the pathogen.

Figure 5.2 Mean radial growth of *P. u. sporangiiferum* on agar media inverted over cultures of BNR1 or BNR2 to test for inhibition by volatile metabolites.

BNR1 and BNR2 were grown in Petri dishes containing either WA or 1/4 PDA or for 2 days at 25°C. Petri dishes inoculated with *P. u. sporangiiferum* were inverted and taped to the bases of plates containing either BNR1 or BNR2, and incubated at 25°C for *ca* 31 hours. Data are means of ten replicate plates for each antagonist × pathogen × agar media combination. Values, for each medium, followed by the same letter are not significantly different ($P > 0.05$) by Tukey's HSD test.

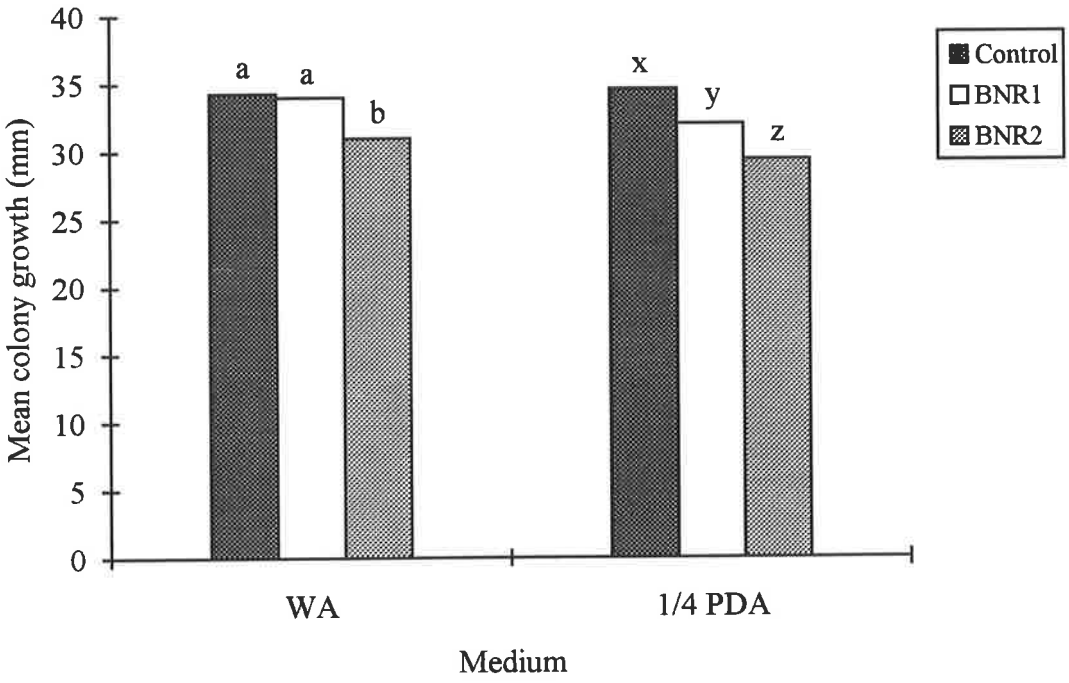

40
35
30
25
20
15
10
5
0
Mean colony growth (mm)
a
a
b
x
y
z
Control
BNR1
BNR2
WA
1/4 PDA
Medium

Figure 5.3 Mean radial growth of *P. u. sporangiiferum* on 1/4 PDA inverted over cultures of BNR1 or BNR2 on sterile potting mix to test for inhibition by volatile metabolites.

BNR1 and BNR2 were grown in Petri dishes in sterile potting mix for 2 days at 25°C. Petri dishes containing 1/4 PDA were inoculated with *P. u. sporangiiferum*, inverted and taped to the bases of plates containing either BNR1 or BNR2, and incubated at 25°C for *ca* 31 hours. Data are means of twenty replicate plates for each antagonist × pathogen combination. Values followed by the same letter are not significantly different ($P > 0.05$) by Tukey's HSD test.

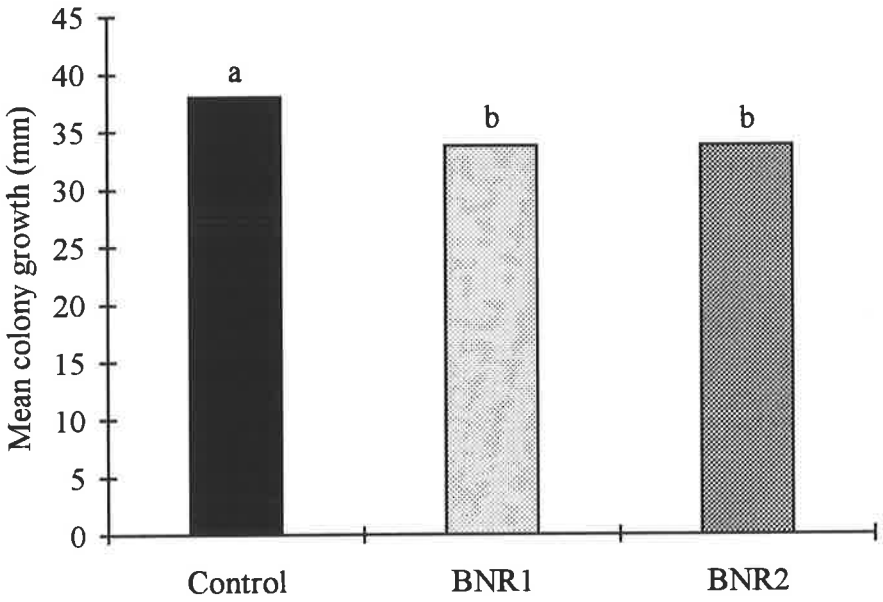

45
40
35
30
25
20
15
10
5
0
Mean colony growth (mm)
a
b
b
Control
BNR1
BNR2

Table 5.2 The amount* of β-1,3 glucanase and cellulase produced by BNR1 and BNR2 in liquid SM supplemented with laminarin, CMC or mycelial preparation of *P. u. sporangiiferum*, after incubation at 30°C for 4 days.

Enzyme	Sole carbon source	BNR1	BNR2
β-1,3 glucanase	Laminarin	0.91* a	0.62 x
	Mycelial preparation of *Pythium*	1.50 a	1.48 y
Cellulase	CMC	2.26 A	2.07 X
	Mycelial preparation of *Pythium*	1.50 A	1.06 Y

Laminarin, CMC or mycelial preparation of *P. u. sporangiiferum* (*Pythium*) was added to liquid SM at 0.2% (w/v). Liquid cultures were filtered through 0.2 μm filters and 1.0 ml of the crude enzyme solutions assayed for enzymatic activity. *Enzyme units are expressed in mmol ml^{-1} h^{-1}. Data are means of two experiments (pooled) with a total of ten replicates. Values, for each enzyme, for each BNR isolate, followed by the same letter are not significantly different ($P > 0.05$) by Tukey's HSD test.

In contrast, cellulase activity by BNR2 was generally less ($P \leq 0.05$) on the mycelial preparation of *P. u. sporangiiferum* than on CMC.

Preliminary experiments indicated that the production of β-1,3 glucanase and cellulase by either BNR1 or BNR2 on laminarin and CMC, respectively, was significantly ($P \leq 0.05$) affected by the period of fungal incubation. The cellulase activity decreased by approximately 99% for BNR1 and 73% for BNR2, after 10 days of incubation when compared to that of 4 days. Similarly, the activity of β-1,3 glucanase was reduced by about 64% for BNR1 and 38% for BNR2. Partial purification of cellulase that had been produced on CMC by either BNR1 or BNR2, resulted in significant ($P \leq 0.05$) reduction of enzymatic activity, by about 54% for BNR1 and 79% for BNR2.

5.3.4.2 Production of β-1,3 glucanase and cellulase on cellophane membranes overlaying agar media

β-1,3 glucanase and cellulase were produced by both BNR1 and BNR2 while growing on cellophane membranes overlaying WA, 1/4 PDA or 3/4 PDA (Table 5.3). Production of β-1,3 glucanase and cellulase by both BNR1 and BNR2 followed a trend such that the highest enzyme activities were recorded on water agar followed by 1/4 PDA and 3/4 PDA. The activity of β-1,3 glucanase by BNR1 on 1/4 PDA was 39% of that on WA, and for BNR2, activity on 1/4 PDA was 45% of that on WA. Cellulase activity was also less on 1/4 PDA than on WA, reduced by 58% and 74% for BNR1 and BNR2, respectively. When BNR1 or BNR2 were grown on 3/4 PDA there were 44% and 73% less activity of β-1,3 glucanase and 80% and 94% less cellulase activity, respectively, compared to when grown on WA. This trend was significant ($P \leq 0.05$) for cellulase production by BNR1 and for both enzymes produced by BNR2. The non-significant differences ($P > 0.05$) in enzyme units of cellulase for BNR1, and β-1,3 glucanase for BNR2, between cultures grown on WA

Table 5.3 The amount* of β-1,3 glucanase and cellulase produced by BNR1 and BNR2 on cellophane membranes overlaying agar media, after incubation at 25°C for 3 days.

	BNR1		BNR2	
Cellophane membranes on:	**β-1,3-glucanase**	**Cellulase**	**β-1,3-glucanase**	**Cellulase**
WA	0.99* a	1.32 x	1.76 A	2.13 X
1/4 PDA	0.60 a (39%)	0.55 xy (58%)	0.96 AB (45%)	0.55 Y (74%)
3/4 PDA	0.55 a (44%)	0.26 y (80%)	0.47 B (73%)	0.13 Y (94%)

*Enzyme units are in mmol ml^{-1} h^{-1}. Data are means of two experiments (pooled) with a total of ten replicates. Values, for each enzyme, for each BNR isolate, followed by the same letter are not significantly different ($P > 0.05$) by Tukey's HSD test. Percentage reduction in enzyme units for 1/4 PDA and 3/4 PDA, when compared with that on WA, is given in parenthesis.

and 1/4 PDA, are thought to be caused by relatively high standard error values (data not shown).

Regression analysis showed a positive relationship between the activity of either β-1,3 glucanase or cellulase, produced by BNR1 and BNR2 on WA, 1/4 PDA and 3/4 PDA with cellophane overlays, and the percentage reduction in *P. u. sporangiiferum* radial growth on the three media (Fig. 5.4). The relationship between increasing enzyme units and increasing percentage reduction in *P. u. sporangiiferum* radial growth was significant ($P \leq 0.05$) for β-1,3 glucanase and cellulase produced by BNR1 (Fig. 5.4.A & B) and for β-1,3 glucanase and cellulase produced by BNR2 (Fig. 5.4.C & D).

5.3.4.3 Production of β-1,3 glucanase and cellulase in PDB

BNR1 and BNR2 produced β-1,3 glucanase and cellulase while growing in full strength PDB for 4 and 14 days (Table 5.4). There was no significant difference ($P > 0.05$) in the amount of these enzymes produced by the two antagonists during 4 and 14 days of incubation.

5.3.4.4 Production of β-1,3 glucanase and cellulase in sterile potting mix

β-1,3 glucanase and cellulase were produced by BNR1 and BNR2 while growing in sterile Falg's potting mix for 6 days (Table 5.5). There was no significant difference ($P > 0.05$) in the number of enzyme units of either β-1,3 glucanase or cellulase produced by each BNR isolate.

Figure 5.4 Regressions of units of lytic enzymes produced by BNR1 and BNR2 on the percentage reduction in radial growth of *P. u. sporangiiferum*.

Regressions of enzyme units of β-1,3-glucanase and cellulase, produced by BNR1 and BNR2 on cellophane membranes overlaying WA, 1/4 PDA or 3/4 PDA, was performed on the percentage reduction in radial growth of *P. u. sporangiiferum* on the three media after removal of antagonists. Enzyme units are expressed in mmol ml^{-1} h^{-1}. *Regression of enzyme units of β-1,3 glucanase produced by BNR1 on the percentage reduction in radial growth of the pathogen was not significant ($P > 0.05$), prior to the regression line being forced through the origin. s.e. = standard error of the regression line.

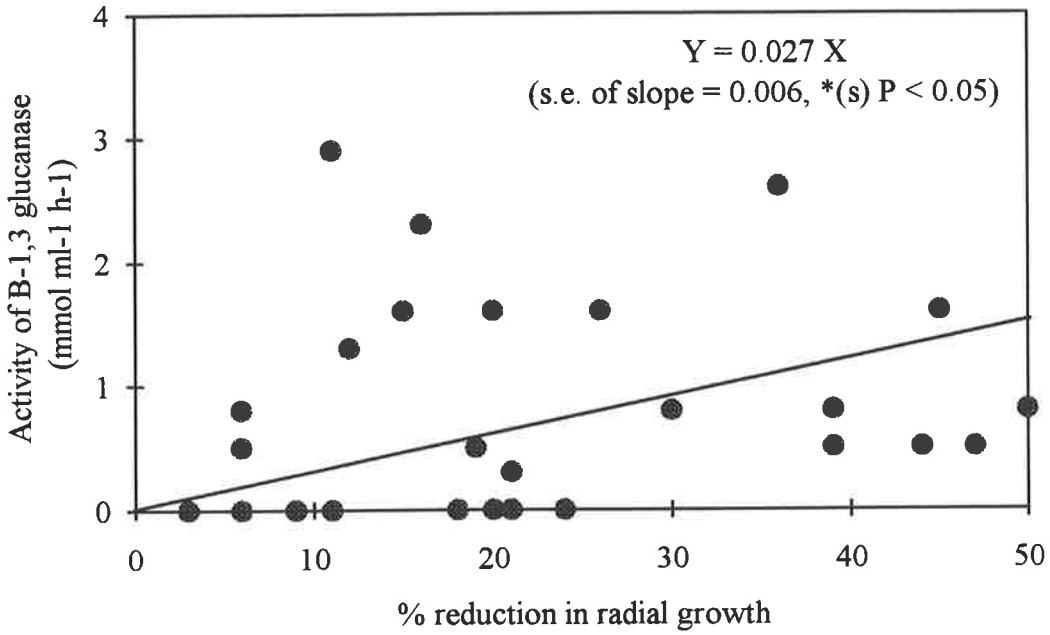
Figure 5.4.A Regression of enzyme units of β-1,3-glucanase produced by BNR1 on the percentage reduction in radial growth of *P. u. sporangiiferum*.

Figure 5.4.B Regression of enzyme units of cellulase produced by BNR1 on the percentage reduction in radial growth of *P. u. sporangiiferum*.

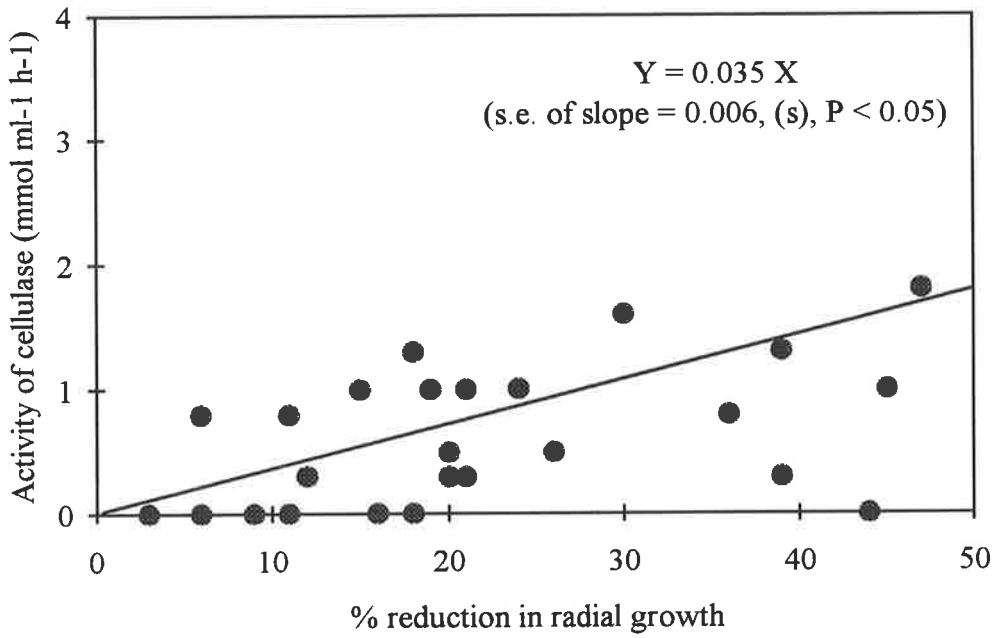
Figure 5.4.C Regression of enzyme units of β-1,3-glucanase produced by BNR2 on the percentage reduction in radial growth of *P. u. sporangiiferum*.

Figure 5.4.D Regression of enzyme units of cellulase produced by BNR2 on the percentage reduction in radial growth of *P. u. sporangiiferum*.

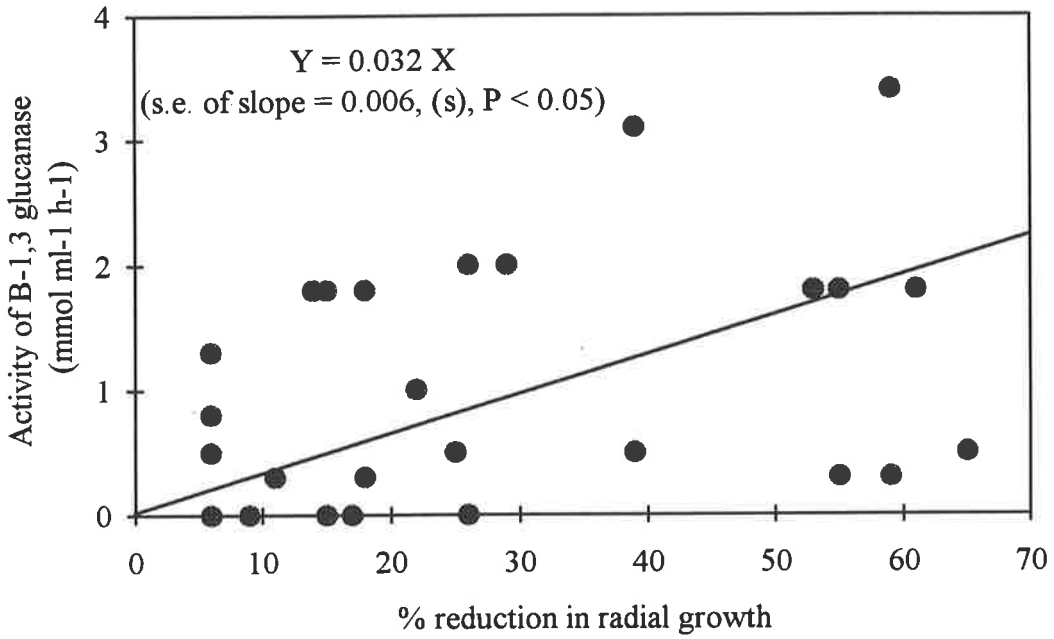
A.


Y = 0.027 X
(s.e. of slope = 0.006, *(s) P < 0.05)
Activity of B-1,3 glucanase (mmol ml-1 h-1)
% reduction in radial growth
0
1
2
3
4
0
10
20
30
40
50

B.


Y = 0.035 X
(s.e. of slope = 0.006, (s), P < 0.05)
Activity of cellulase (mmol ml-1 h-1)
% reduction in radial growth
0
1
2
3
4
0
10
20
30
40
50

C.


Y = 0.032 X
(s.e. of slope = 0.006, (s), P < 0.05)
Activity of B-1,3 glucanase (mmol ml-1 h-1)
% reduction in radial growth
0
1
2
3
4
0
10
20
30
40
50
60
70

D.

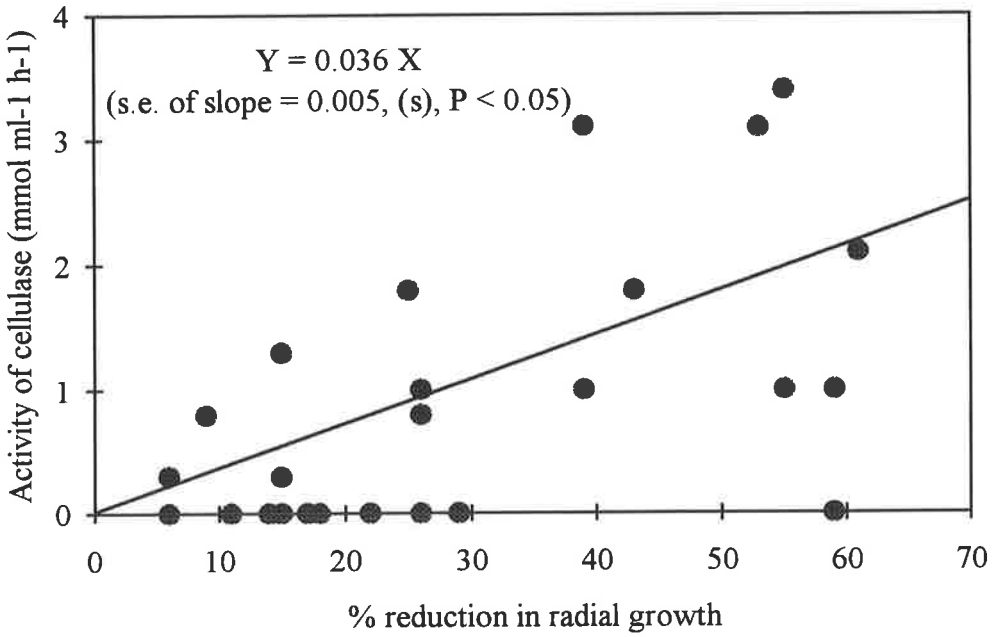

Y = 0.036 X
(s.e. of slope = 0.005, (s), P < 0.05)
Activity of cellulase (mmol ml-1 h-1)
% reduction in radial growth
0
1
2
3
4
0
10
20
30
40
50
60
70

Table 5.4 The amount* of β-1,3 glucanase and cellulase produced by BNR1 and BNR2 in cultures on PDB, after incubation at 25°C for 4 or 14 days.

	BNR1		BNR2	
Substrate (incubation period)	**β-1,3-glucanase**	**Cellulase**	**β-1,3-glucanase**	**Cellulase**
PDB (4 days)	2.85* a	1.92 x	1.06 A	1.56 X
PDB (14 days)	2.01 a	3.11 x	1.68 A	2.85 X

Liquid cultures were filtered through 0.2 μm filters and 1.0 ml of the crude enzyme solutions assayed for enzymatic activity. *Enzyme units are expressed in mmol ml^{-1} h^{-1}. Data are means of ten replicates for the 4-day incubation period and four replicates for the 14-day incubation period. Values, for each enzyme, for each BNR isolate, followed by the same letter are not significantly different ($P > 0.05$) by Tukey's HSD test.

Table 5.5 The amount* of β-1,3 glucanase and cellulase produced by BNR1 and BNR2 in sterile potting mix, after incubation at 25°C for 6 days.

Enzyme	BNR1	BNR2
β-1,3 glucanase	0.99* a	1.27 x
Cellulase	1.01 a	1.32 y

The crude enzyme extract was prepared by shaking 20 g of potting mix with 15 ml citrate buffer on a shaker at *ca* 200 rpm for 30 min. The mixture was then centrifuged at 6,500 rpm for 15 min, filtered through 0.2 μm filters, and assayed (1.0 ml) for enzymatic activity. *Enzyme units are expressed in mmol ml^{-1} h^{-1}. Data are means of two experiments (pooled) with a total of 10 replicates. Values, for each BNR isolate, followed by the same letter are not significantly different ($P > 0.05$) by Tukey's HSD test.

5.4 DISCUSSION

No substances inhibitory to growth of *P. u. sporangiiferum* were detected in culture filtrates of either BNR1 or BNR2 grown in full strength PDB. Burton & Coley-Smith (1993) also reported very low or no antibiotic activity, against *Bacillus subtilis* and *Escherichia coli*, in filtrates of cultures of binucleate *Rhizoctonia cerealis*, *R. oryzae-sativae* and *R. tuliparum* grown in Czapek Dox liquid medium. Similarly, Cardoso & Echandi (1987) found that filtrates of 10-day-old cultures of BNR-like fungus, grown in full strength PDB, did not inhibit a virulent isolate of *R. solani*. Filtrates of 14-day-old cultures of either BNR1 or BNR2 increased rather than decreased the growth of *P. u. sporangiiferum*. Such stimulation of pathogen growth could have resulted from the release of additional metabolites into the medium by the antagonists, as a result of autolysis, or as by-products of secondary metabolism. Similar stimulatory effects on the growth of two *Phytophthora* spp. by filtrates of 7-week-old cultures of *T. hamatum* and *G. virens* were reported by Chambers & Scott (1995).

In contrast to the culture filtrates experiments, both BNR1 and BNR2 significantly inhibited growth of *P. u. sporangiiferum* in agar media following their removal on cellophane overlays. The main purpose of the cellophane membranes was to separate physically the antagonists from the medium on which they were growing, while allowing diffusion of enzymes and nutrients. This method was applied in detection of antibiotic activity of isolates of *Trichoderma* spp. by Dennis & Wester (1971*a*), *T. viride* and *T. harzianum* by Roberti *et al.* (1993) and *Rhizoctonia* spp. by Burton & Coley-Smith (1993). The results presented here show a clear trend in the inhibition of growth of *P. u. sporangiiferum* by soluble metabolites of either BNR1 or BNR2 produced on cellophanes overlaying the three media tested. As the nutrient content of the medium underneath the cellophane membranes increased, from that of WA to 3/4 PDA, the reduction in *P. u. sporangiiferum* growth decreased. The effect of medium on the reduction of pathogen growth led to the hypothesis that cellophane

membranes were utilised by both BNR isolates as a carbon source on a nutrient deficient medium. Breakdown of cellulose requires production of extracellular enzymes, in particular cellulase, which could have been released by BNR into the WA and adversely affected the hyphae of *P. u. sporangiiferum*. As the nutrient content of the medium increased, in particular the soluble sugars such as glucose, the need for cellulose hydrolysis by the antagonists decreased. A study by Kohlmeyer (1956) appears to support the above hypothesis, in that the decomposition of cellulose by *Rhizoctonia* spp. was greatly reduced by the presence of soluble carbohydrates. In the presence of glucose the fungus utilised only 0.4 to 0.5% of the available cellulose, whereas without glucose, about 20% of the cellulose was used. If cellulase was, in fact, responsible for the reduction of growth of *P. u. sporangiiferum* on agar media with cellophane overlays, then the absence of cellulosic substrate in liquid PDB could have explained the lack of inhibitory effect of BNR culture filtrates on the pathogen. Even if a cellulase substrate was present in the medium, the high nutrient content of the liquid cultures could, perhaps, have overcome the inhibitory effect produced by BNR.

Pronounced changes in colony morphology of *P. u. sporangiiferum* were apparently caused by inhibitory substances produced by the antagonists on cellophane membranes overlaying WA. They included sparse mycelial growth of the pathogen, with hyphae being less branched than the control grown on WA without prior inoculation of BNR, and showing an increased vacuolation of the cytoplasm as well as lysis. Walther & Gindrat (1987) also observed intense vacuolation, disappearance of cytoplasm and apparent lysis of *P. ultimum*, *P. oligandrum* and *Aphanomyces cochlioides* hyphae in the presence of either multinucleate *R. solani*, binucleate *R. cerealis* or binucleate *R. fragariae*, in corn-meal agar. They concluded that cellulase produced by the antagonists was apparently responsible for the damage caused to pathogen hyphae by the *Rhizoctonia* spp. Dennis & Webster (1971*a*) also observed increased vacuolation in hyphae of *R. solani* and *Heterobasidion annosum* that apparently resulted from

antibiotic activity of *Trichoderma* spp. on 2% malt extract agar with cellophane overlays. In their study, however, the inhibited fungal colonies were usually more dense than those of controls, with hyphae being generally more branched and thicker than untreated hyphae.

Antibiosis against *P. u. sporangiiferum* was also mediated by volatile substances of unknown nature produced by both BNR1 and BNR2 in agar media as well as in sterile potting mix. Although the inhibitory effect of these volatile compounds was significant, the reduction in growth of the pathogen was actually slight. Moreover, no definite morphological changes in the hyphae of *P. u. sporangiiferum* were observed after the fungus was grown in the presence of volatile metabolites produced by BNR in agar media and in sterile potting mix. Dennis & Webster (1971*b*), however, showed that certain *Trichoderma* spp. produced volatile metabolites that were responsible for stunted mycelial growth of *P. ultimum* in agar media. Liberation of volatile compound(s) by either BNR1 or BNR2 in sterile potting mix is believed to result from the decomposition of organic matter, for example the rice hulls, during the saprophytic phase of growth. Thus, volatile metabolite(s) could contribute to the biological control of *P. u. sporangiiferum* by the two antagonists, even though, their inhibitory effect is not as great as that of soluble metabolites.

In light of the results discussed earlier, subsequent studies investigated the production of extracellular enzymes by BNR1 and BNR2, and their possible role in the biological control of *P. u. sporangiiferum*. The choice of enzymes was based on the report by Bartnicki-Garcia (1967) that the principal component of the cell walls of Oomycetes was an alkali-insoluble and cuprammonium-insoluble glucan(s) containing β-1,3- and β-1,6-linkages. Cellulose was apparently not a major cell wall component, as had been assumed previously, and chitin was found in non-significant amounts (usually less than 1%) in some fungi only. Consequently, β-1,3 glucanase and cellulase were chosen as lytic enzymes with the potential to affect the structure of *P. u. sporangiiferum* cell

walls, as well as the overall fitness of the pathogen hyphae. BNR1 and BNR2 were found to produce β-1,3 glucanase and cellulase on various substrates. Production of the two enzymes on the mycelial preparation of *P. u. sporangiiferum*, as a sole carbon source, provides additional evidence for the involvement of β-1,3 glucanase and cellulase in parasitism of *P. u. sporangiiferum* by the two antagonists (see Chapter 4). Other potential mycoparasities have been shown to produce extracellular enzymes, such as β-1,3 glucanase, cellulase and chitinase, while growing on cell walls of their target pathogens. Elad *et al.* (1982) reported that *Trichoderma harzianum* excreted β-1,3 glucanase and chitinase into the medium when grown on either cell walls or homogenised sclerotia of *Sclerotium rolfsii*. Culture filtrates of *Pythium nunn* had high activity of cellulase and β-1,3 glucanase when the fungus was grown on cell walls of *Pythium* spp. β-1,3 glucanase and chitinase were also produced by *P. nunn* when the antagonist was grown on cell walls of *Rhizoctonia solani* or *Sclerotium rolfsii* in liquid SM (Elad *et al.*, 1985). Similarly, production of β-1,3 glucanase and protease by *Pythium oligandrum* was induced by cell walls of target pathogens, including *P. ultimum* and *R. solani* (Lewis *et al.*, 1989).

Investigation into the nature of inhibitory substances produced by BNR1 and BNR2 on cellophane membranes overlaying agar media, revealed a positive relationship between the percentage reduction in *P. u. sporangiiferum* radial growth and the amount of enzyme units of either β-1,3 glucanase or cellulase produced by the two antagonists. Thus, inhibition of *P. u. sporangiiferum* is believed to occur through enzyme-mediated antibiosis, at least in this particular system. Furthermore, stunted mycelial growth, increased cytoplasmic vacuolation, lysis and possibly changes in cytoplasmic streaming, as reported in Chapter 5, are thought to result from the action of lytic enzymes. In addition, the inhibitory role of other extracellular enzymes, such as chitinase, lipases and proteases requires investigation.

β-1,3 glucanase and cellulase were also produced by BNR1 and BNR2 in liquid culture in full strength PDB, but the inhibitory effect on the growth of *P. u. sporangiiferum* was not detected after incorporating the filtrates into WA medium. The catalytic activity of enzymes, however, is known to be dependent on the maintenance of their native structure such that any slight variation may lead to significant changes in this activity. Parameters which most significantly affect the production, activity and stability of enzymes are temperature and pH (Holme & Peck, 1983). In addition, detection of metabolite production and its activity can be influenced by factors such as agar depth, age of cultures and amount of inoculum (Vidaver *et al.*, 1972), as well as soil osmotic potential (Wong & Griffin, 1974) and nutrition of the microorganism (Weinhold & Bowman 1968). For BNR, the procedures involved in the preparation of cell-free culture filtrates could have been responsible for the failure to detect any inhibitory effects, apparently caused by the lytic enzymes, on the growth of *P. u. sporangiiferum*. Centrifugation of the culture filtrates, filtration through Millipore filters as well as mixing with warm WA, could have reduced enzyme activity. Furthermore, mixing of culture filtrates with WA in Petri dishes resulted in relatively uniform distribution of the enzymes throughout the gelled medium, reducing the possibility of hyphae of *P. u. sporangiiferum* encountering any regions of high enzyme concentration. In contrast, enzymes produced on cellophane membranes in agar media were under the same conditions throughout the experiment, and would diffuse directly on to the surface of the medium, such that expanding pathogen colonies were in direct contact with the released enzymes. Thus, the sole use of culture-filtrates for demonstrating antibiosis is believed to be inappropriate, especially when relatively unstable compounds are involved, like the extracellular enzymes. Consequently, it is important that other means of testing antibiotic production are considered concurrently with the culture-filtrate method, including the use of cellophane overlays, enzyme assays and microscopic examination of the pathogen structures prior to or upon contact with biocontrol agent(s).

Reese & Mandels (1959) showed that β-1,3 glucanase was a constitutive enzyme in fungi, and was produced on a variety of carbon sources including glucose, maltose, cellobiose, laminarin, starch and cellulose. Cellulase, on the other hand, is an adaptive enzyme in fungi, and has been formed by *Trichoderma viride* while growing on cellulose, lactose, glucose or cellobiose (Mandels & Reese, 1957). Cellulose and lactose were found to be direct inducers of cellulase, whereas glucose was not. In addition, many compounds metabolised through glucose, such as starch, maltose, trehalose and β-methyl glucoside, also did not induce cellulase directly. Mandels & Reese (1957) concluded that glucose had to be metabolised into an inducer first, possibly β-glycoside, before cellulase induction could take place. Consequently, the presence of glucose-derived inducer, starch, cellulose or other carbohydrates originating from potato tissue which is the main constituent of PDB, could explain the production of lytic enzymes by BNR in the absence of added laminarin or CMC.

BNR1 and BNR2 produced β-1,3 glucanase and cellulase when grown saprophytically in sterile potting mix. Rice hulls, which constitute about 15% of the total volume of Falg's potting mix, are believed to be the main carbon substrate for enzymes produced by the antagonists. The optimum conditions for lytic enzyme production and activity include a temperature of 30 to 40^{o}C, and pH in the range of 3.0 to 5.0 for β-1,3 glucanase and 4.0 to 5.0 for cellulase. In addition, maximal stability of β-1,3 glucanase and cellulase at 30^{o}C is between pH 5.0 to 9.0 and pH 3.0 to 8.0, respectively (Reese & Mandels, 1959; Bateman, 1964; Elad *et al.*, 1982). In this study, Falg's potting mix was incubated at 25^{o}C and had a natural pH of approximately 5.6, which made it a suitable environment for production of these enzymes by BNR.

Production of extracellular enzymes on *Capsicum* seeds or roots colonised by the BNR was not investigated in this study. However, production of antibiotic substances on plant tissues by antagonistic microorganisms is well documented. For example, Wright (1956*a,b*) demonstrated that antibiotic production occurred in wheat straw or

seeds buried in untreated soil. Inoculation of pea seeds with *Trichoderma viride*, *Penicillium frequentans* and *P. gladioli* resulted in production of gliotoxin, frequentin and gladiolic acid, respectively, in the seed coat. In addition, *T. viride* growing naturally in soil apparently infected pea seeds and produced gliotoxin in the seed coat. Wright (1956*b*) thus concluded that ecologically significant concentrations of antibiotics were not common in bulk soil, and that their production was limited to microniches relatively rich in nutrients, such as seed coats, organic matter (crop residues) and, possibly, root exudates. From the literature presented in this discussion, it can be supposed that lytic enzymes would also be present in such nutrient-rich sites. However, the type and quantity of the lytic enzymes would be determined by the amount of soluble sugars present in these sites, as well as complex carbohydrates, the conversion of which into simple sugars requires the action of extracellular enzymes.

Results presented in this chapter suggest a role for lytic enzymes, such as β-1,3 glucanase and cellulase, in biological control of *P. u. sporangiiferum* by the antagonistic BNR. First, lytic enzymes are believed to be directly involved in hyperparasitism during degradation of *P. u. sporangiiferum* cell walls. Second, they have been shown to inhibit the growth of *P. u. sporangiiferum*, causing adverse cytoplasmic changes, including increased vacuolation and lysis, in pathogen hyphae. Third, these extracellular enzymes, which are products of BNR primary metabolism, may inhibit *P. u. sporangiiferum* while the antagonists and the pathogen are growing saprophytically in the potting mix.

CHAPTER 6 PROTECTION OF PLANT SURFACES BY BINUCLEATE *RHIZOCTONIA*

6.1 INTRODUCTION

Seeds, seedling roots, seedling hypocotyls and wounds are subject to infection by a number of damping-off fungi, especially *Pythium* and *Rhizoctonia* spp., whereas *Phytophthora*, *Fusarium* and *Sclerotinia* spp. cause less widespread problems. The damping-off fungi are unspecialised pathogens which use host exudates for saprophytic growth before attacking young seedlings that have not yet developed effective mechanical or chemical barriers to infection (Campbell, 1989*b*). As the young tissues are susceptible to infection for a relatively short time, protection against the pathogens is required also for a short time only. Once seedlings become well established, their resistance mechanisms become fully functional. Similarly, protection of wounds is necessary until callus tissue is formed, restricting colonisation by microorganisms (Gindrat, 1979).

Pathogenic or non-pathogenic microorganisms penetrate the host plant through infection sites, which may include stomates, lenticels, cuticle and epidermis of various organs. The inoculum density of a potential coloniser and the location of infection sites in relation to the host's exudation pools determine the number of these infection sites colonised by fungi (Sneh, 1990).

Protection of plant surfaces with antagonistic microorganisms may be achieved by elimination, displacement or exclusion of a target pathogen in sites where infection normally occurs. Although the pathogen population may consequently decline, such decline is not essential for biological control of this kind to be successful.

Mechanisms by which antagonists may protect the plant surfaces include competitive displacement, antibiosis and prior establishment of the biocontrol agent in the host tissues (Cook, 1981). An example of biocontrol system in which these three mechanisms are apparently involved is the protection of pine roots by the ectomycorrhizal fungus *Leucopaxillus cerealis* var. *piceina* against root rot caused by *Phytopthora cinnamomi* (Marx, 1975). The ectomycorrhizal fungus produces an antibiotic in culture media, and possibly in nature, which is strongly inhibitory to mycelial growth and zoospore germination in *P. cinnamomi.* In addition, the fungal mantle on pine roots protects the root tissues from infection by providing a mechanical barrier to invasion by *P. cinnamomi.* Furthermore, this ectomycorrhizal fungus apparently reduces the amount of nutrients available for the pathogen to initiate successful infection, by competing with *P. cinnamomi* for host exudates at the root surface. Whipps (1987*b*) has also shown that application of *Trichoderma harzianum* or *Gliocladium roseum* to plant tissue segments 24 hours before, or at the same time as, application of *Sclerotinia sclerotiorum,* resulted in significantly greater inhibition of sclerotium formation than when the antagonist was applied 24 hours after application of the pathogen. Apparently, competition for active sites and physical exclusion of *S. sclerotiorum* from the plant tissues were responsible for the reduction in sclerotium formation.

According to Cook & Baker (1983), prevention of pathogen inoculum formation and subsequent disease development may be achieved by means of cross-protection, when the diseased tissue is colonised by an antagonist of the target pathogen. Antagonists that displace the pathogen in lesions in an infected host plant, with the aid of parasitism or antibiosis, can slow or even stop disease development (Cook, 1981). Most studies, to date, on the displacement of pathogens by non-pathogens in infected host tissues have concerned trees. Gibbs & Smith (1978) suggested that *Trichoderma viride*, which is often associated with woody material in nature, shows considerable antagonistic activity in dual culture with *Heterobasidion annosum* and can readily

replace the pathogen in infected host tissues. Similarly, *Trichothecium roseum*, which produces the antibiotic trichothecin, and *Gliocladium roseum*, a mycoparasite with antibiotic capabilities, may have the potential to displace or pre-empt the Dutch elm pathogen *Ceratocystis ulmi* in host bark (Gibbs & Smith, 1978).

Plants which are tolerant to a particular fungal pathogen, i.e. they are colonised by the pathogen but show no disease symptoms, commonly respond to attempted pathogenesis by activation of multiple defence mechanisms which, in susceptible plants, are latent or are expressed too late to control the disease (Heath, 1980). Such mechanisms of induced host resistance may include: 1) the accumulation of anti-microbial low molecular weight chemicals such as phytoalexins and leaf surface diterpenes; 2) strengthening of tissues via accumulation of lignin, callose and glycoproteins; 3) increase in production of chitinases, β-1,3 glucanases, peroxidases and other pathogenesis-related proteins (Tuzun & Kloepper, 1994). Systemic or local resistance to infection by the pathogen can be induced by prior challenge of the host with avirulent strains or races related to the pathogen, or organisms that are pathogens themselves but on other hosts (Cook, 1981). For example, Biles & Martyn (1989) reported that inoculation of watermelon seedlings with an avirulent race of *Fusarium oxysporum* f. sp. *niveum* or the incompatible pathogen *F. o. cucumerinum* 24 or 72 hours prior to challenge with a virulent race of *F. o. niveum*, significantly reduced disease symptoms in infected seedlings. In addition, when roots of the wilt-susceptible watermelon cultivar were inoculated with *F. o. cucumerinum*, and the leaves challenged with pathogenic *Colletotrichum lagenarium* 24 or 72 hours later, 50% fewer lesions developed on leaves of induced plants than on non-induced inoculated controls. Biles & Martyn (1989) concluded that induced resistance to *F. o. niveum* was both local and systemic, as well as pathogen-nonspecific.

In this chapter, experiments are presented which investigated: 1) the nature of seed and seedling colonisation by BNR1, BNR2 and *P. u. sporangiiferum* in pasteurised potting mix in glasshouse conditions; and 2) the mechanisms of protection of *Capsicum* tissues by the two isolates of BNR in *in vitro* and *in vivo* bioassays, including induced host resistance and displacement of *P. u. sporangiiferum* from lesions *in planta*.

6.2 MATERIALS AND METHODS

6.2.1 The host plant

The morphology of a typical 10-day-old *Capsicum* seedling, with anatomical terms used throughout this chapter, is presented in Fig. 6.1.

6.2.2 Potting mix

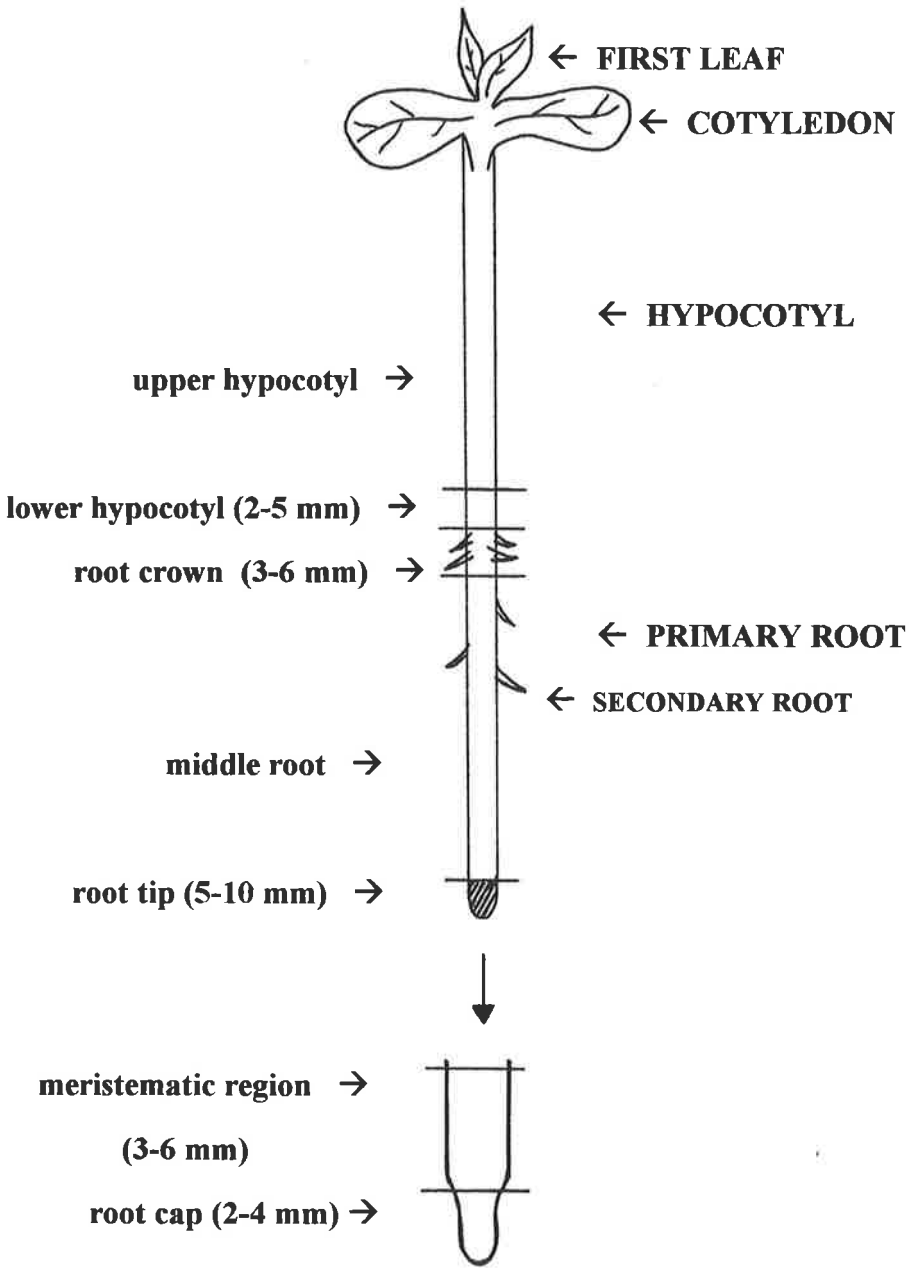
The moisture content of the potting mix used in the *in vitro* experiments was adjusted to *ca* -0.03 bars (see Section 3.3.5). The moisture content of the pasteurised potting mix in glasshouse punnets was determined daily (see Section 3.3.3) during the first 3 days of an experiment, before and after each watering. The moisture content ranged from *ca* -0.2 bars before watering to *ca* -0.3 after watering. The pH of the potting mix in the *in vitro* and *in vivo* experiments was between 5.4 and 5.6 (see Section 3.3.2).

6.2.3 Study of colonisation of *Capsicum in vivo*, and plant responses to the presence of BNR or *P. u. sporangiiferum*

6.2.3.1 Growth and inoculation of *Capsicum* seedlings in potting mix

Experiments were conducted in undivided, plastic punnets (*ca* 13.9 × 8.5 cm; 350 ml capacity; Masrac Pots, Adelaide), filled with approximately 125 ml of pasteurised potting mix, which was inoculated with six millet seeds (two rows by three seeds) colonised with BNR1, BNR2 or *P. u. sporangiiferum*.

Fig. 6.1 Morphology of a typical 10-day-old *Capsicum* seedling



The inoculum was covered by another 125 ml of potting mix, and 24 *Capsicum* seeds were sown on top in two rows of four seeds. Seeds were gently pressed into the potting mix and covered with *ca* 100 ml of sterilised sand. For controls, potting mix in punnets was inoculated with six sterile millet seeds instead of the fungi. Plants were grown in a glasshouse at 25°C (±5°C), with relative humidity of *ca* 60%, and were watered daily until draining (*ca* -0.3 bar) with deionised water. There were eight replicate punnets for each fungal treatment and the control, arranged in a randomised block design.

Six *Capsicum* seeds or seedlings growing with BNR1, BNR2 or *P. u. sporangiiferum*, or without fungi, were sampled from each punnet at 3, 7, 10 and 21 days, and prepared for light-microscopy examination (see Section 6.2.3.2).

In addition, survival and growth responses of *Capsicum* seedlings in the presence of BNR1, BNR2 or *P. u. sporangiiferum* were determined on day 21. An additional 12 punnets were established for each fungal treatment at the same time as the above experiment. Punnets were prepared and inoculated as described above. After 21 days of growth the number of surviving seedlings was determined for each treatment, and twelve standing plants were then cut at soil level, their tops dried at 60°C for 3 days and then weighed.

The experiments were repeated once and the results subjected to analysis of variance. The Tukey's HSD test (Zar, 1984) was used to determine pairwise-mean-differences between the treatments.

6.2.3.2 Microscopic examination of the colonisation process

Seeds or seedlings were removed from each punnet taking care not to disrupt neighbouring seedlings and potting mix 3, 7, 10 and 21 days after inoculation, and gently washed in sterile deionised water using a small paint brush.

Tissues were cleared in 10% KOH for 1.5 hours at *ca* 25°C, rinsed in 10% HCl, and soaked in a wetting solution (see Appendix) for 1 hour before being stained with trypan blue in lactoglycerol (see Appendix). Excess stain was removed by rinsing in sterile deionised water. Intact *Capsicum* seeds were mounted in lactoglycerol (see Appendix) in plastic Petri dishes (90 mm diam.) and observed at various magnifications using a Leitz Wild M3Z stereomicroscope. These seeds were later dissected and the seed coats, endosperm and embryos mounted in lactoglycerol on a microscope slide. Whole seedlings were mounted in lactoglycerol on a microscope slide and gently squashed with a coverslip. In addition, hypocotyl and root tissues of seedlings not used as whole (see Appendix) and cut into transverse sections (*ca* 30 μm thick) using a freezing microtome (Frigistor Instruments, Ltd.). Sections were stained with trypan blue in lactoglycerol on a microscope slide. Whole seedling-mounts and the transverse sections were viewed at various magnifications using a Leitz Laborlux S compound microscope.

6.2.4 Long-term survival of BNR on *Capsicum* seedlings

Capsicum seedlings were grown in the presence of either BNR1 or BNR2 in a glasshouse in pasteurised potting mix, as described in Section 6.2.3.1. Plants were grown for 5 weeks at 25°C (±5°C) and relative humidity of *ca* 60%, and were watered until draining (*ca* -0.03 bar) twice a week with a nutrient solution (see Appendix), and on other days with deionised water. There were 15 replicate punnets for each BNR treatment, and punnets were arranged in a glasshouse in a randomised block design. After 5 weeks, five seedlings were taken at random from each punnet and their roots prepared as whole mounts as described in Section 6.2.3.2. The presence of either BNR1 or BNR2 in the plant tissues was determined at ×400 using a Leitz Laborlux S compound microscope. The remaining seedlings were transplanted from each of the above punnets into 1.5 litre plastic pots, six seedlings per pot, containing freshly pasteurised potting mix and grown for a further 10 weeks, until plants began senescence. Three plants were then taken at random from each pot,

and potting mix adhering to the roots gently shaken off into Petri dishes (90 mm diam.) containing 1/4 PDA and incubated at 25°C for 3 days. Main and secondary roots were washed in sterile deionised water and cut into 2 cm long pieces from the crown, middle root and the tip and prepared as whole mounts, as described in Section 6.2.3.2. The whole root mounts as well as Petri dishes containing the rhizosphere potting mix, were examined for the presence of BNR1 or BNR2 at various magnifications using a Leitz Laborlux S compound microscope.

6.2.5 Induced systemic resistance in *Capsicum* seedlings against infection by *P. u. sporangiiferum*

6.2.5.1 Induced systemic resistance - *in vivo* bioassay

Experiment 1

Undivided, plastic punnets were filled with approximately 125 ml of pasteurised potting mix and inoculated with six millet seeds (in two rows of three seeds) colonised with either BNR1 or BNR2. The inoculum was covered by another 125 ml of the potting mix, on top of which eight *Capsicum* seeds (in two rows of four seeds) were sown and covered with *ca* 65 ml of sterilised sand. In controls potting mix in punnets was inoculated with six sterile millet seeds instead of BNR1 or BNR2. Plants were grown in a glasshouse at 25°C (±5°C) and relative humidity of *ca* 60%, and were watered until draining (*ca* -0.3 bar) twice a week with a nutrient solution, and on other days with deionised water. Two days after seedling emergence (12 days from sowing), all eight seedlings in each punnet were challenged with *P. u. sporangiiferum*. Plugs (8 mm diam.) of *P. u. sporangiiferum* were taken from the inner regions of 3-day-old colonies on 1/4 PDA, and placed on sterile pieces of Whatman No. 2 filter paper (5 mm^2), such that the aerial hyphae of the pathogen were uppermost. The sand next to each seedling was gently pushed aside, making a small hollow around the stem and a single plug of the pathogen on the filter paper was applied to each hypocotyl,

such that the aerial hyphae were in contact with the plant tissue. The filter paper protected the plug from drying-out and kept it in place during subsequent waterings. Plants were grown for a further 14 days during which time survival was recorded at days 7 and 14. There were three control treatments, consisting of: 1) *Capsicum* seedlings challenged with plugs of sterile 1/4 PDA in the absence of either BNR1 or BNR2; 2) *Capsicum* seedlings challenged with plugs of sterile 1/4 PDA in the presence of either BNR1 or BNR2; and 3) *Capsicum* seedlings challenged with *P. u. sporangiiferum* in the absence of BNR. There were 10 replicate punnets for each antagonist × pathogen combination and for the controls, arranged in a randomised block design. The experiment was repeated once and the results subjected to analysis of variance. The Tukey's HSD test was used to determine pairwise-mean-differences between the treatments.

Experiment 2

In this experiment, which was prepared exactly as for Experiment 1, formation of lesions by *P. u. sporangiiferum* on *Capsicum* hypocotyls was investigated. In this experiment, however, three living seedlings from each punnet, from each treatment or control, were destructively sampled at 7, 14 and 21 days after inoculation with the pathogen, and lesions on the hypocotyls were measured. Whole seedling-mounts, and transverse sections (*ca* 30 μm thick) of diseased hypocotyls were prepared as described in Section 6.2.3.2, stained with trypan blue in lactoglycerol on a microscope slide and viewed at various magnifications using a Leitz Laborlux S compound microscope. There were a total of 12 replicate punnets for each treatment and the controls. The experiment was repeated once and the results subjected to analysis of variance. The Tukey's HSD test was used to determine pairwise-mean-differences between the treatments.

Experiment 3

A preliminary histochemical study was undertaken to determine if colonisation of *Capsicum* seedlings by either BNR1 or BNR2, in the absence of *P. u. sporangiiferum*, led to alterations in cell walls in the main root and hypocotyl through deposition of substances such as cellulose, lignin, callose or polyphenolic compounds. *Capsicum* seedlings were grown in undivided, plastic punnets (eight seedlings per punnet) in pasteurised potting mix in the presence of either BNR1 or BNR2, as described in Experiment 1. For controls, punnets were inoculated with six sterile millet seeds instead of BNR1 or BNR2. Plants were grown in a glasshouse at 25°C (±5°C) and relative humidity of *ca* 60% for 14 days. Three seedlings were taken at random from each punnet, and the adhering potting mix gently washed off in sterile deionised water with a small paint brush. The crowns, root tips and hypocotyls (first 1.0 cm above the crown) were cut into 3 mm pieces, placed in gelatine blocks and cut into transverse sections (*ca* 30 µm thick) using freezing microtome (Frigistor Instruments). Sections were stained immediately using methods described by Jensen (1962), and examined at various magnifications with a Leitz Laborlux S compound microscope, for the following compounds:

(a) Lignin: sections were mounted in a drop of 1% phloroglucinol in 70% ethanol with a drop of concentrated hydrochloric acid. A red-violet colour indicates lignified tissue.

(b) Cellulose: sections were mounted in one quarter-strength zinc-chloriodide solution which stains the cellulose blue and lignin yellow to orange.

(c) Callose: sections were stained in a 0.005% solution of aniline blue in 50% ethanol for 6 hours. Cells walls containing callose stain blue.

(d) Polyphenols: transverse sections and intact root tips (*ca* 0.5 cm long) were mounted in equal volumes of 10% sodium nitrate, 20% urea and 10% acetic acid. After 3 min, two volumes of 2 N sodium hydroxide were added and the sections examined immediately for development of a cherry-red/brown colour indicating the presence of polyphenolic substances.

There were 10 replicate punnets for each BNR treatment and the controls, arranged in a randomised block design. The experiment was repeated once.

6.2.5.2 Induced systemic resistance - *in vitro* bioassay

Based on the observations from the *in vivo* bioassay (see Section 6.3.3.1), the possibility of occurrence of induced systemic resistance was tested *in vitro* using different approach. Thirty *Capsicum* seeds were germinated in glass Petri dishes (180 mm diam.) containing 40 g of sterilised potting mix, in the presence of either four 1/4 PDA plugs (8 mm diam) of BNR1 or BNR2, or four plugs of sterile 1/4 PDA (8 mm diam). Seedlings were incubated for 6 days at 25°C in the dark until the hypocotyls were about 1 cm long with two cotyledons. Seedlings of uniform size, with adhering potting mix, were then transferred aseptically to pre-prepared experimental plates. These were 90 mm diam. sterile, plastic, divided Petri dishes (Disposable Products, South Australia) prepared such that one half of each plate contained a sterile Whatman No. 2 filter paper (semi-circle of radius 45 mm) and the other half contained 10 g of freshly prepared, sterile potting mix. Three grooves were made in the divider of each Petri dish with a hot spatula. For each treatment, i.e. *Capsicum* seedlings grown with either BNR1 or BNR2, or without added fungi, three replicate seedlings were placed in each plate, one seedling per groove such that the roots and lower hypocotyl (*ca* 1-3 mm above the crown) were in direct contact with potting mix, whereas the upper hypocotyl and cotyledons rested on the filter paper. Plugs of *P. u. sporangiiferum* (5 mm diam.) taken from 2-day-old colonies growing on 1/4 PDA, were placed on the filter papers underneath each upper hypocotyl such that the plant tissue was in direct contact with the pathogen inoculum. The filter paper was moistened with sterile deionised water to stop the inoculum plugs from drying out. A thick layer of soft white paraffin was applied from a plastic 10 ml syringe along each divider, including the grooves, to prevent the growth of BNR from roots, along the plant surface, to the pathogen-inoculated hypocotyls. There were two control treatments, each with *Capsicum* seedlings germinated in the absence of BNR.

In the first control treatment, *Capsicum* hypocotyls were inoculated with *P. u. sporangiiferum* in 1/4 PDA plugs (5 mm diam.), and in the second control treatment, seedling hypocotyls were inoculated with sterile 1/4 PDA plugs (5 mm diam.). Plates were incubated at 25°C in the dark for 5 days during which time hypocotyls were examined daily for symptoms of damping-off. After 5 days the number of diseased *Capsicum* plants was assessed. There were six replicate plates for each antagonist × pathogen combination, and for control. The experiment was repeated once and the results analysed with one-way analysis of variance. The Tukey's HSD test was used to determine pairwise-mean-differences between the treatments.

6.3 RESULTS

6.3.1 Study of colonisation of *Capsicum in vivo*, and plant responses to the presence of BNR or *P. u. sporangiiferum*

6.3.1.1 Microscopic examination of colonisation by BNR

Germinating *Capsicum* seeds harvested on days 3 and 7 were densely colonised by both BNR1 and BNR2. The two fungi formed dense hyphal mats on the outer seed coat (Plate 6.1.A), inner seed coat and the site of emergence of the radicle (Plate 6.1.B). The seed endosperm was seldom colonised by either BNR1 or BNR2, and the embryo and the emerging radicle were free from BNR while inside the seed. However, once outside the seed coat, the radicle was colonised at least partially by BNR hyphae which extended from the outer seed coat (Plate 6.1.C). Control seeds showed no fungal infection (Plate 6.1.D).

Colonisation of the 10-day-old *Capsicum* seedlings was similar for both BNR1 and BNR2. In general, neither BNR1 nor BNR2 colonised seedling hypocotyls, although in some plants hyphae were visible on the lower hypocotyl just below or at the surface of

Plate 6.1 Colonisation of *Capsicum* seeds by BNR in pasteurised potting mix.

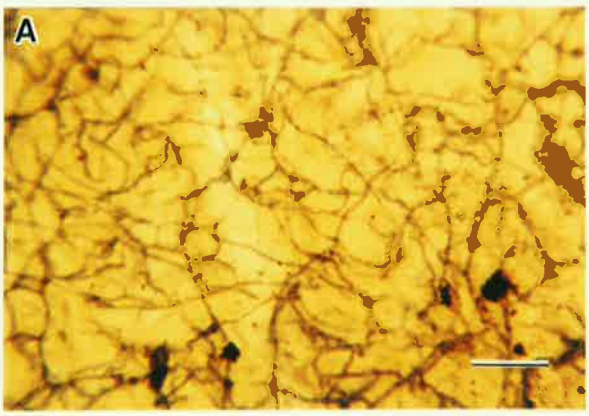
Seeds were harvested 3 and 7 days after sowing in pasteurised potting mix that had been inoculated with either BNR1 or BNR2, or with sterile millet seeds. Seeds were gently washed in sterile deionised water, cleared in 10% KOH for 1.5 hours at *ca* 25°C, rinsed in 10% HCl, and soaked in a wetting solution for 1 hour before being stained with trypan blue in lactoglycerol. Intact seeds were mounted in lactoglycerol in plastic Petri dishes and examined using a Leitz Wild M3Z stereomicroscope. Also seed coats were dissected out and mounted in lactoglycerol on a microscope slide and examined using a Leitz Laborlux S compound microscope.

A: BNR1 hyphae on the outer surface of a seed coat 3 days after sowing. Bar = 25 μm.

B: Hyphal mat of BNR2 inside the seed at the point of emergence of the radicle 3 days after sowing. Bar = 250 μm.

C: BNR2 hyphae (arrowheads) on the radicle emerging from a seed coat 7 days after sowing. Bar = 250 μm

D: Control seed showing the point of emergence of the radicle 3 days after sowing. The seed is free from hyphae. Bar = 250 μm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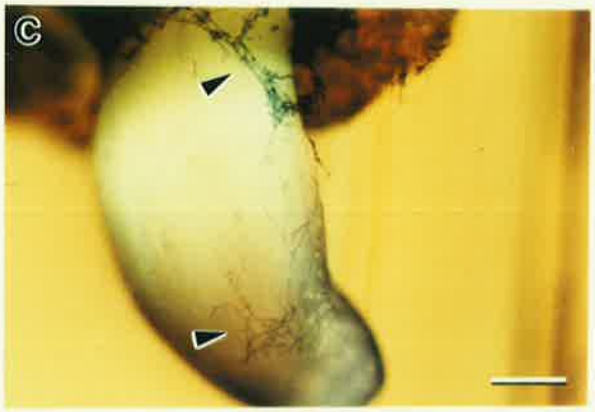
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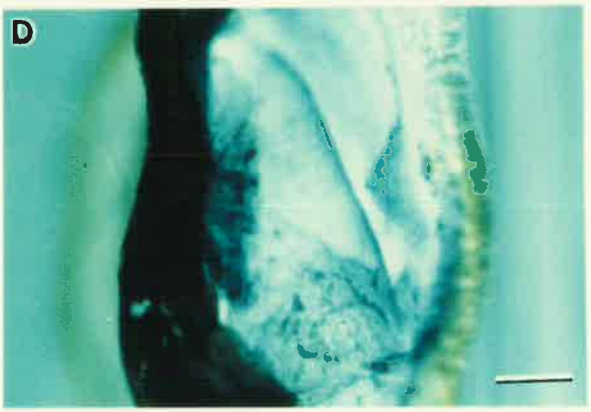
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the potting mix. *Capsicum* roots, however, were colonised by both antagonists. BNR1 and BNR2 densely colonised the crown (Plate 6.2.A), with epidermal cells being colonised externally and internally by relatively short, branched BNR hyphae. The middle root was poorly colonised by both BNR isolates, with long, slim and less branched hyphae that were mainly intercellular (Plate 6.2.B & C). The root tips were densely colonised by BNR which produced masses of short, swollen cells that formed hyphal mats on the surface of the root tip (Plate 6.2.D & E) and inside the epidermal cells (Plate 6.2.F). The root tips of seedlings which had not yet emerged from the potting mix were not colonised by either BNR1 or BNR2. Browning of the root tips was frequently observed in seedlings colonised by BNR, and very sporadically in the control plants. Control seedlings showed no fungal infection.

Colonisation of 21-day-old *Capsicum* seedlings by both BNR1 and BNR2 closely resembled that of the 10-day-old seedlings. The crown was densely colonised by BNR hyphae growing on the root surface, along the cell junctions, and inside the epidermal cells. Transverse sections of the crown region revealed that, in addition to epidermal cells, the outermost layers of the root cortex were occasionally invaded by both BNR isolates (Plate 6.3.A). Monilioid cells of the two BNR isolates were observed inside the epidermal cells of the crown region (Plate 6.3.B). Colonisation of the middle root was less then that of the crown region. In addition to long and narrow superficial hyphae, short, swollen BNR hyphae were occasionally visible inside the plant cells. The root tip area, however, was most densely colonised by both BNR1 and BNR2, with the meristematic region and the root cap covered by dense hyphal mats of short, swollen BNR cells. Furthermore, in some plants, BNR hyphae could be seen among the loose root cap cells and often forming uniform hyphal network among them (Plate 6.3.C & D). Colonisation of the secondary roots was similar to that of the main roots, with the crown and tips being densely colonised by both BNR isolates (Plate 6.3.E & F). Control seedlings showed no fungal infection.

Plate 6.2 Colonisation of 10-day-old *Capsicum* seedlings by BNR in pasteurised potting mix.

Seedlings were harvested 10 days after sowing in pasteurised potting mix that had been inoculated with either BNR1 or BNR2, or with sterile millet seeds. Seedlings were gently washed in sterile deionised water, cleared in 10% KOH for 1.5 hours at *ca* 25°C, rinsed in 10% HCl, and soaked in a wetting solution for 1 hour before being stained with trypan blue in lactoglycerol. Whole seedlings were mounted in lactoglycerol on a microscope slide, gently squashed with a coverslip and primary roots examined using a Leitz Laborlux S compound microscope.

A: BNR1 hyphae (arrowhead) growing on the root crown region. Bar = 50 μm.

B: BNR1 hyphae (arrowhead) growing among root hairs (double arrowhead) on the middle root region. Bar = 50 μm.

C: BNR1 hypha (arrowhead) growing between the epidermal cells of the middle root. Bar = 10 μm.

D: Swollen BNR1 hyphae growing on the surface of the meristematic region of the root tip. Bar = 10 μm.

E: BNR2 hyphae growing internally (arrowhead) and externally (double arrowhead) just above the meristematic region of the root tip. Bar = 25 μm.

F: Swollen BNR1 hyphae (arrowheads) growing inside the epidermal cells in the meristematic region of the root tip. Bar = 10 μm.

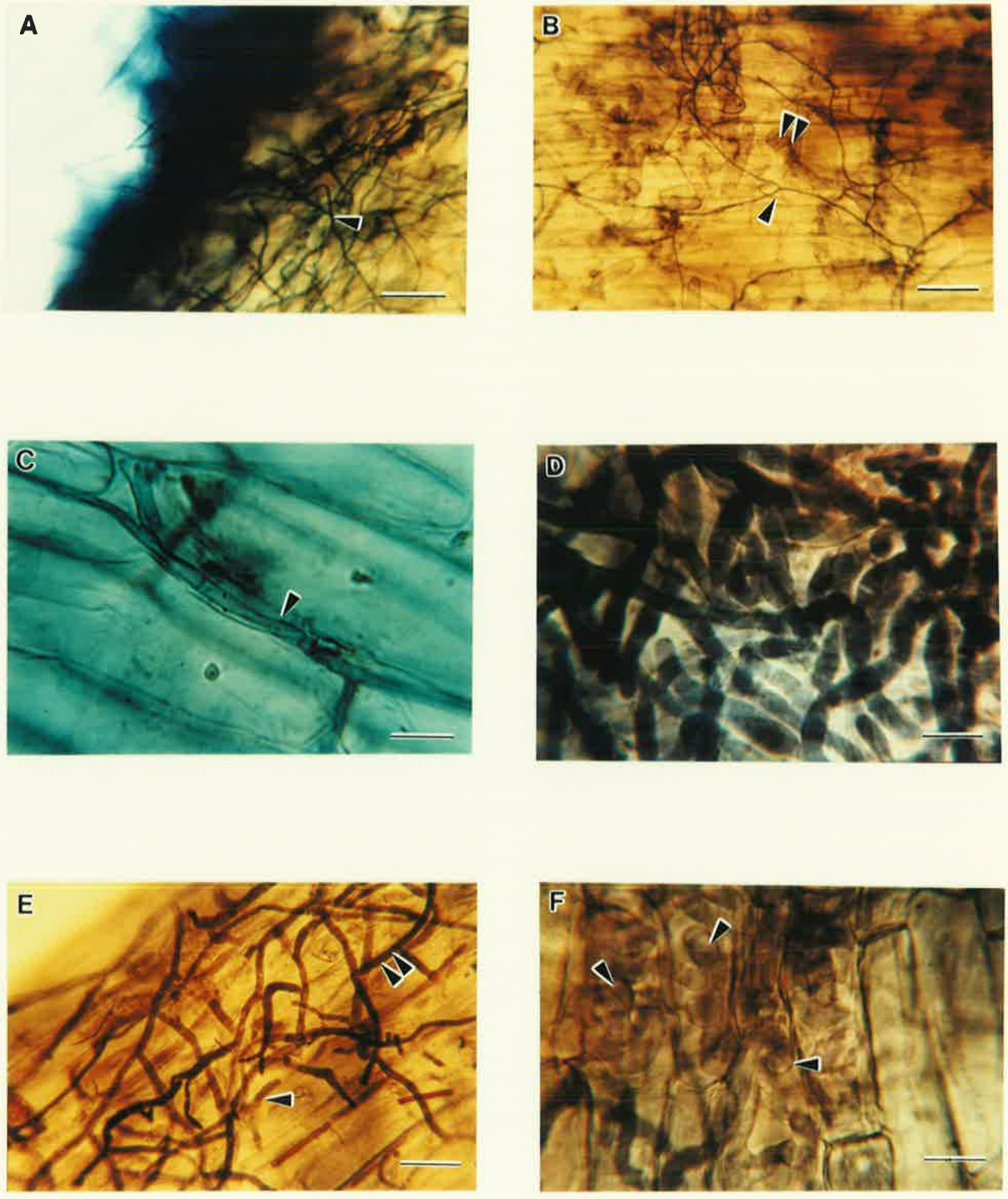
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Plate 6.3 Colonisation of 21-day-old *Capsicum* seedlings by BNR in pasteurised potting mix.

Seedlings were harvested 21 days after sowing in pasteurised potting mix that was inoculated with either BNR1 or BNR2, or with sterile millet seeds. Seedlings were gently washed in sterile deionised water, cleared in 10% KOH for 1.5 hours at *ca* 25°C, rinsed in 10% HCl, and soaked in a wetting solution for 1 hour before being stained with trypan blue in lactoglycerol. Whole seedlings were mounted in lactoglycerol on a microscope slide, gently squashed with a coverslip and primary and secondary roots examined using a Leitz Laborlux S compound microscope.

A: BNR1 hypha (arrowhead) growing in a cortical cell (c) in the crown region of a primary root. Bar = 10 μm.

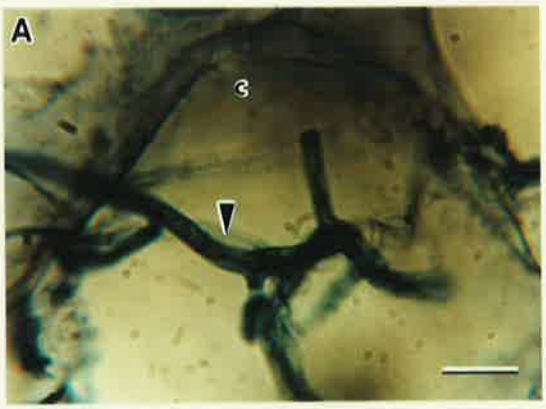
B: Monilioid cells of BNR2 (arrowheads) inside the epidermal cells in the middle region of a primary root. Bar = 25 μm.

C: BNR1 hyphae between the root cap cells of a primary root. Bar = 25 μm.

D: BNR2 in an uniform hyphal network (arrowheads) on the root cap of a primary root. Bar = 50 μm.

E: BNR2 hyphae on the surface of a secondary root in the crown region. Bar = 31 μm.

F: BNR1 hyphae growing internally (arrowhead) and externally (double arrowhead) just above the meristematic region in the tip of a secondary root. Bar = 25 μm.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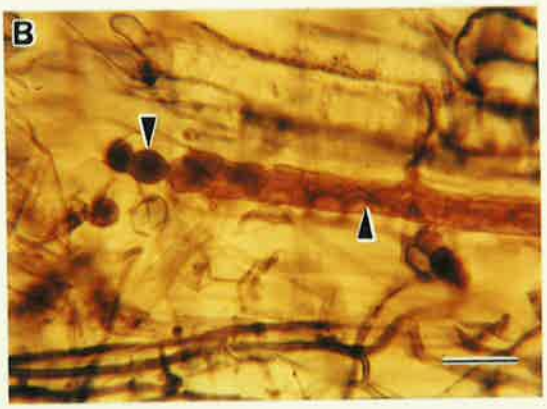
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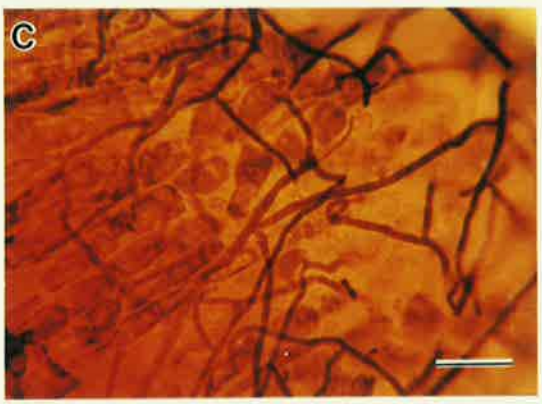
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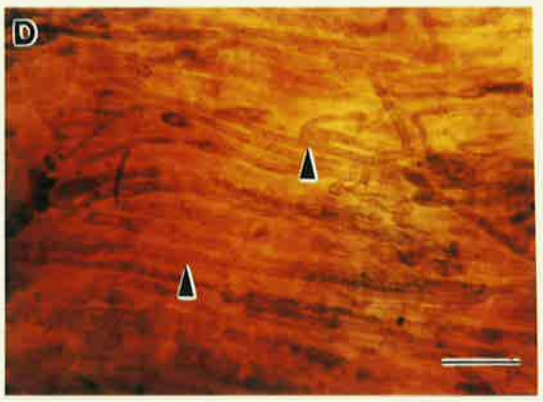
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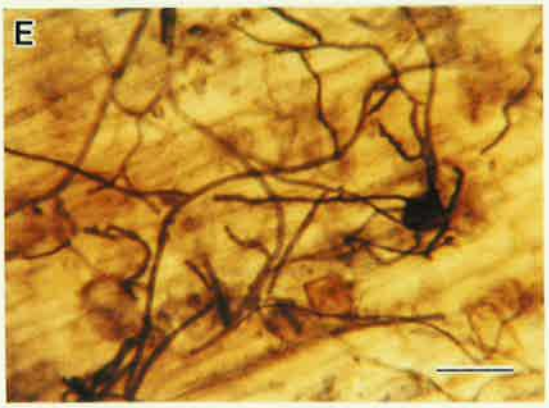
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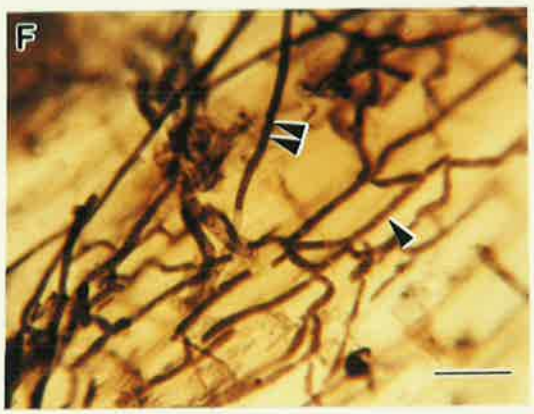
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6.3.1.2 Microscopic examination of colonisation by *P. u. sporangiiferum*

Although hyphae of *P. u. sporangiiferum* could be seen across the entire seed surface, the site of greatest colonisation by the fungus was between and inside the grooves of *Capsicum* seed coats (Plate 6.4.A). The site of emergence of the radicle was colonised by the pathogen but hyphal mats were not formed inside it. Only in few instances were hyphae of *P. u. sporangiiferum* found in the endosperm or in the embryo. Infection of most *Capsicum* seeds by *P. u. sporangiiferum* was apparently initiated from the emerged radicle rather than the embryo. Control seeds showed no fungal infection.

The 10-day-old *Capsicum* seedlings examined for the presence of *P. u. sporangiiferum* apparently escaped pre-emergence damping-off. *P. u. sporangiiferum* was absent from some of these seedlings, while in others, either the roots but not the hypocotyls were colonised by the pathogen, or both the hypocotyl and the roots were colonised by the fungus. Colonisation of the primary root by *P. u. sporangiiferum* was mainly confined to the crown region, in which relatively few hyphae were observed among the root hairs (Plate 6.4.B) and no symptoms of disease. Formation of lesions by *P. u. sporangiiferum,* indicated by the presence of yellowish-brown spots on seedling hypocotyls, was observed on a number of *Capsicum* seedlings. Penetration of hypocotyl tissues was via distinct appressoria or slight hyphal swellings (Plate 6.4.C). Transverse sections of infected hypocotyls revealed that the fungus colonised the epidermal cells densely, with fewer hyphae being present in the cortical cells. Occasionally, sporangia and oospores were observed inside the colonised tissues. Control seedlings showed no fungal infection.

Seedlings harvested on day 21 apparently escaped post-emergence damping-off during the first 3 weeks of growth. On closer examination, however, about half of the surviving seedlings had hypocotyls infected with *P. u. sporangiiferum*. Two types of pathogen hyphae were found in the lesions. The first one consisted of elongated, regularly-shaped hyphae spreading within the host, and intracellular more often than

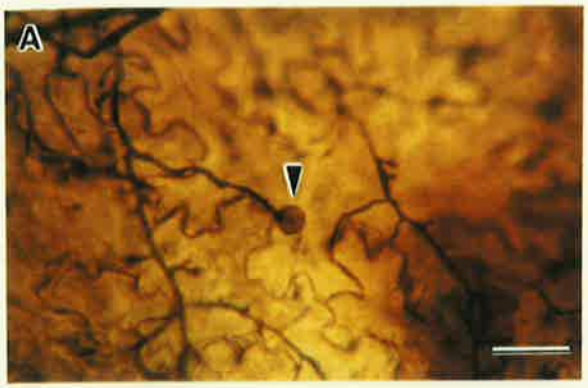
Plate 6.4 Colonisation of *Capsicum* seeds and seedlings by *P. u. sporangiiferum* in pasteurised potting mix.

Seeds or seedlings harvested 3, 7 and 10 days after sowing in pasteurised potting mix infested with BNR1 or BNR2, or with sterile millet seeds, were gently washed in sterile deionised water, cleared in 10% KOH for 1.5 hours at *ca* 25°C, rinsed in 10% HCl, and soaked in a wetting solution for 1 hour before being stained with trypan blue in lactoglycerol. Whole seedlings were mounted in lactoglycerol on a microscope slide, gently squashed with a coverslip and primary roots examined using a Leitz Laborlux S compound microscope.

A: *P. u. sporangiiferum* hyphae on the outer surface of a seed coat 3 days after sowing. Hyphal branch with a terminal sporangium (arrowhead) can be seen.
Bar = 25 μm.

B: *P. u. sporangiiferum* hyphae on the surface of a primary root in the crown region 10 days after sowing. Bar = 25 μm.

C: *P. u. sporangiiferum* appears to penetrate the epidermal cells of the hypocotyl 10 days after sowing. Appressoria or hyphal swellings (arrowheads) can be seen on the surface of the hypocotyl. Bar = 25 μm.

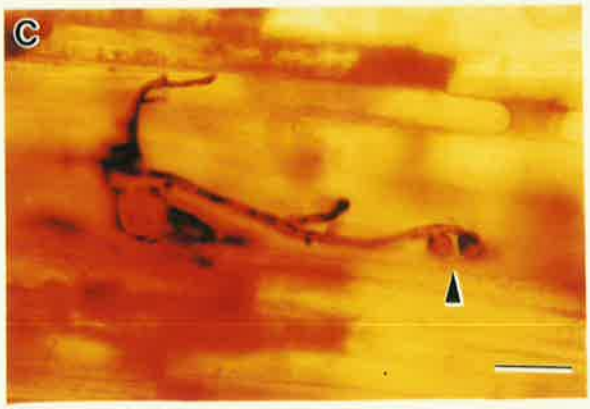

A


B


C


A


B


C


D

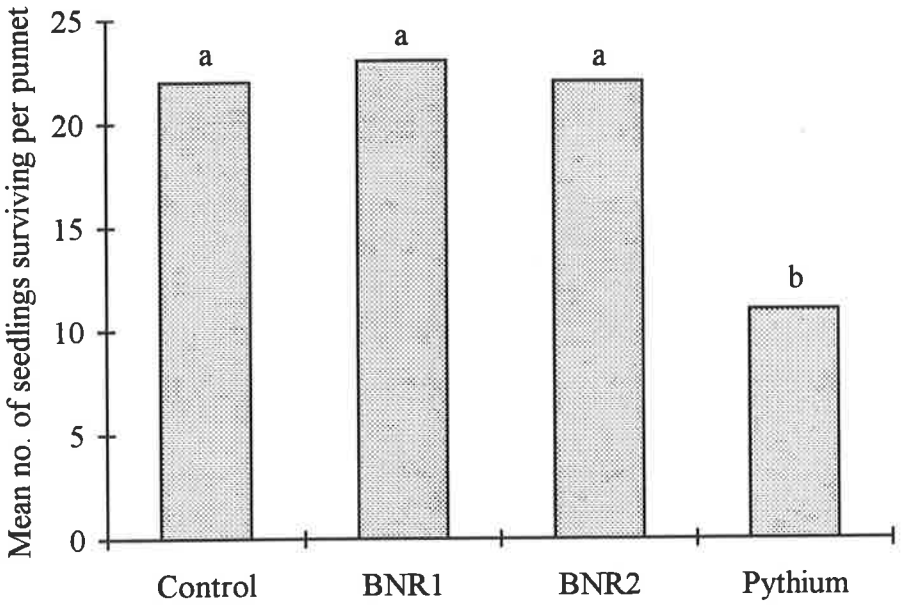
Fig. 6.2 Survival and growth of *Capsicum* seedlings in pasteurised potting mix in the presence of BNR or *P. u. sporangiiferum*.

Capsicum seedlings were grown in undivided, plastic punnets in pasteurised potting mix, 24 seedlings per punnet, inoculated with either BNR1, BNR2 or *P. u. sporangiiferum* (Pythium). For controls, punnets were "inoculated" with sterile millet seeds. Plants were grown in a glasshouse at 25°C (± 5°C) for 21 days. The surviving seedlings were counted, and the shoots excised at soil level, dried for 3 days at 60°C and weighed. Data are means of 12 replicate punnets for each fungal treatment, and the control. Values for each treatment followed by the same letter are not significantly different ($P > 0.05$) by Tukey's HSD test.

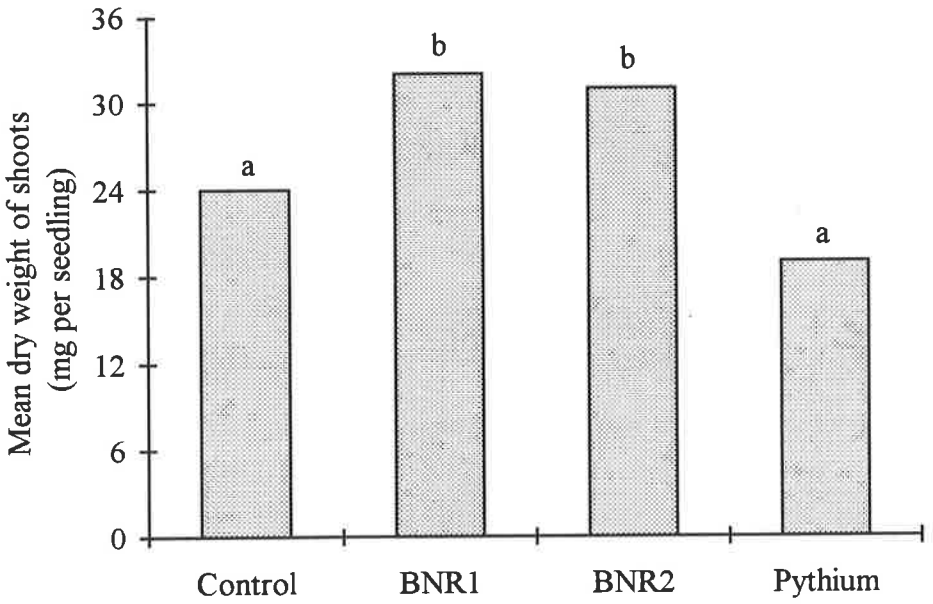
Fig. 6.2.A Survival of *Capsicum* seedlings in pasteurised potting mix in the presence of either BNR1, BNR2 or *P. u. sporangiiferum*.

Fig. 6.2.B Shoot dry weight of *Capsicum* seedlings in pasteurised potting mix in the presence of either BNR1, BNR2 or *P. u. sporangiiferum*.

(A)


Mean no. of seedlings surviving per punnet
25
20
15
10
5
0
a
a
a
b
Control
BNR1
BNR2
Pythium

(B)


Mean dry weight of shoots (mg per seedling)
36
30
24
18
12
6
0
a
b
b
a
Control
BNR1
BNR2
Pythium

In addition, both BNR1 and BNR2 promoted significant ($P \leq 0.05$) increases in shoot dry weights of 21-day-old *Capsicum* seedlings in pasteurised potting mix in the absence of *P. u. sporangiiferum* (Fig. 6.2B). The shoot dry weight of seedlings was increased by 27% in the presence of BNR1 and by 25% with BNR2 when compared to that in the uninoculated control. The shoot dry weight of seedlings growing in the presence of *P. u. sporangiiferum* alone did not differ significantly from that of control seedlings (Fig. 6.2B).

6.3.2 Long-term survival of BNR on *Capsicum* seedlings in pasteurised potting mix

Colonisation of re-potted *Capsicum* plants by either BNR1 or BNR2 after 15 weeks of growth in pasteurised potting mix resembled that of 21-day-old seedlings, as described in Section 6.3.1. Transplanting of seedlings from punnets to larger pots after 5 weeks did not affect the survival of either of the two BNR isolates in the plant tissues, or in the rhizosphere. However, the overall density of either BNR1 or BNR2 appeared to be lower throughout the plant roots than in younger seedlings. The crowns and tips of primary and secondary roots were the sites of greatest internal and external colonisation by both BNR isolates.

6.3.3 Induced systemic resistance in *Capsicum* seedlings against infection by *P. u. sporangiiferum*

6.3.3.1 Induced systemic resistance - *in vivo* bioassay

Both BNR1 and BNR2 protected *Capsicum* seedlings against damping-off caused by *P. u. sporangiiferum* in pasteurised potting mix at the two sampling times (Fig. 6.3A and B). The incidence of damping-off was greatest ($P \leq 0.05$) in *Capsicum* seedlings growing in the absence of either BNR1 or BNR2 on days 7 and 14 after inoculation with the pathogen.

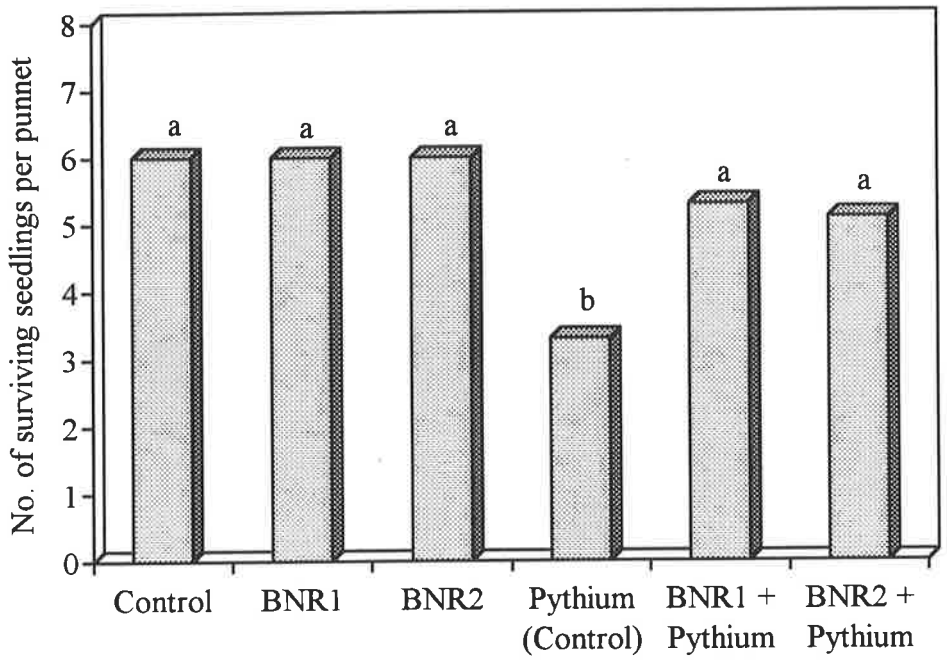
Fig. 6.3. Survival of *Capsicum* seedlings in *in vivo* bioassay.

Capsicum seedlings were grown with either BNR1 or BNR2 in pasteurised potting mix, eight seedlings per punnet, in a glasshouse for 10 days at 25°C (±5°C). Seedling hypocotyls were challenged with plugs of *P. u. sporangiiferum* in 1/4 PDA for 14 days, after which time the number of surviving plants was recorded for each treatment on days 7 and 14. Control treatments consisted of: 1) seedlings challenged with plugs of sterile 1/4 PDA in the absence of BNR; 2) seedlings challenged with plugs of sterile 1/4 PDA in the presence of BNR; and 3) seedlings challenged with *P. u. sporangiiferum* in the absence of BNR. Data are means of 10 replicate punnets for each antagonist × pathogen combination and controls. Values for each treatment followed by the same letter are not significantly different ($P > 0.05$) by Tukey's HSD test.

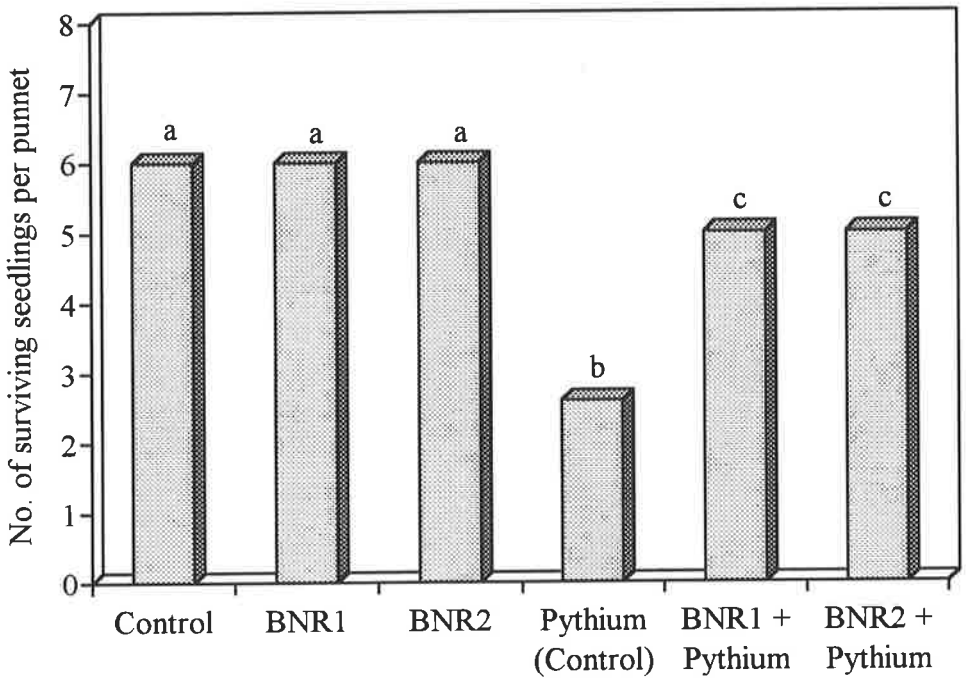
Fig. 6.3.A Mean number of surviving *Capsicum* seedlings 7 days after inoculation with *P. u. sporangiiferum.*

Fig. 6.3.B Mean number of surviving *Capsicum* seedlings 14 days after inoculation with *P. u. sporangiiferum.*

(A)


No. of surviving seedlings per punnet
0
1
2
3
4
5
6
7
8
a
a
a
b
a
a
Control
BNR1
BNR2
Pythium (Control)
BNR1 + Pythium
BNR2 + Pythium

(B)


No. of surviving seedlings per punnet
0
1
2
3
4
5
6
7
8
a
a
a
b
c
c
Control
BNR1
BNR2
Pythium (Control)
BNR1 + Pythium
BNR2 + Pythium

Survival of *Capsicum* seedlings colonised by either BNR1 or BNR2 in the presence of *P. u. sporangiiferum* was not significantly different from that of control seedlings, or seedlings growing with BNR only for 7 days after inoculation with the pathogen (Fig. 6.3A). However, 14 days after the hypocotyls were challenged with *P. u. sporangiiferum* the number of seedlings in the treatments inoculated with either BNR1 or BNR2, which remained alive, decreased slightly ($P \leq 0.05$) compared to controls and to survival of seedlings grown in the presence of BNR alone (Fig. 6.3.B). However, seedling survival was still significantly higher ($P \leq 0.05$) in the above treatments than in controls inoculated with *P. u. sporangiiferum* alone.

Development of lesions on *Capsicum* hypocotyls was observed 7 and 14 days after inoculation with *P. u. sporangiiferum* (Fig. 6.4.A and B). Lesions were formed on the upper hypocotyl where the pathogen inoculum was applied. The length of lesions at the two sampling times was greatest ($P \leq 0.05$) in *Capsicum* seedlings inoculated with *P. u. sporangiiferum* alone compared to controls and to seedlings grown in the presence of BNR only. Furthermore, the mean length of lesions in the absence of BNR increased by about 37% between days 7 and 14. Seedlings with roots colonised by either BNR1 or BNR2 did develop lesions as a result of infection by *P. u. sporangiiferum*, however, these lesions were significantly smaller ($P \leq 0.05$) than those observed in seedlings grown in the absence of BNR. In addition, mean lesion length did not increase significantly ($P > 0.05$) between day 7 and 14 in contrast to that in seedlings inoculated with *P. u. sporangiiferum* only. Lesions were observed on control *Capsicum* seedlings grown without any added fungi, as well as on seedlings colonised by either BNR1 or BNR2 alone. These lesions were significantly smaller ($P \leq 0.05$) than those observed in seedlings inoculated with the pathogen, and were found mainly on the lower hypocotyl. Examination of these lesions by light-microscopy failed to reveal *P. u. sporangiiferum* or any other fungus. No new lesions were found on *Capsicum* hypocotyls at day 21, in treatments or controls, and wound healing was observed in many of the seedlings.

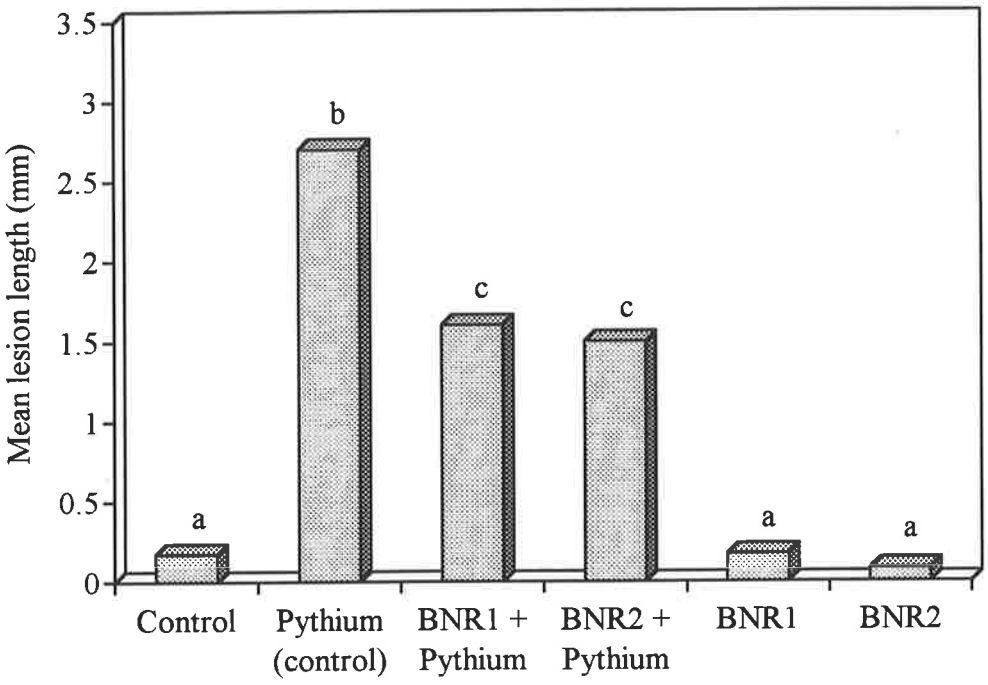
Fig. 6.4 Development of lesions on *Capsicum* hypocotyls in *in vivo* bioassay.

Capsicum seedlings were grown with either BNR1 or BNR2 in pasteurised potting mix for 10 days in a glasshouse at 25°C (±5°C). Seedling hypocotyls were challenged with plugs of *P. u. sporangiiferum* in 1/4 PDA and grown for a further 14 days. Three seedlings from each punnet, from each treatment or control, were destructively sampled on days 7, 14 and 21, and the mean lesion length was determined. Control treatments consisted of: 1) seedlings challenged with plugs of sterile 1/4 PDA in the absence of BNR; 2) seedlings challenged with plugs of sterile 1/4 PDA in the presence of BNR; and 3) seedlings challenged with *P. u. sporangiiferum* in the absence of BNR. Data are means of 12 replicate punnets for each antagonist × pathogen combination and controls. Values for each treatment followed by the same letter are not significantly different ($P > 0.05$) by Tukey's HSD test.

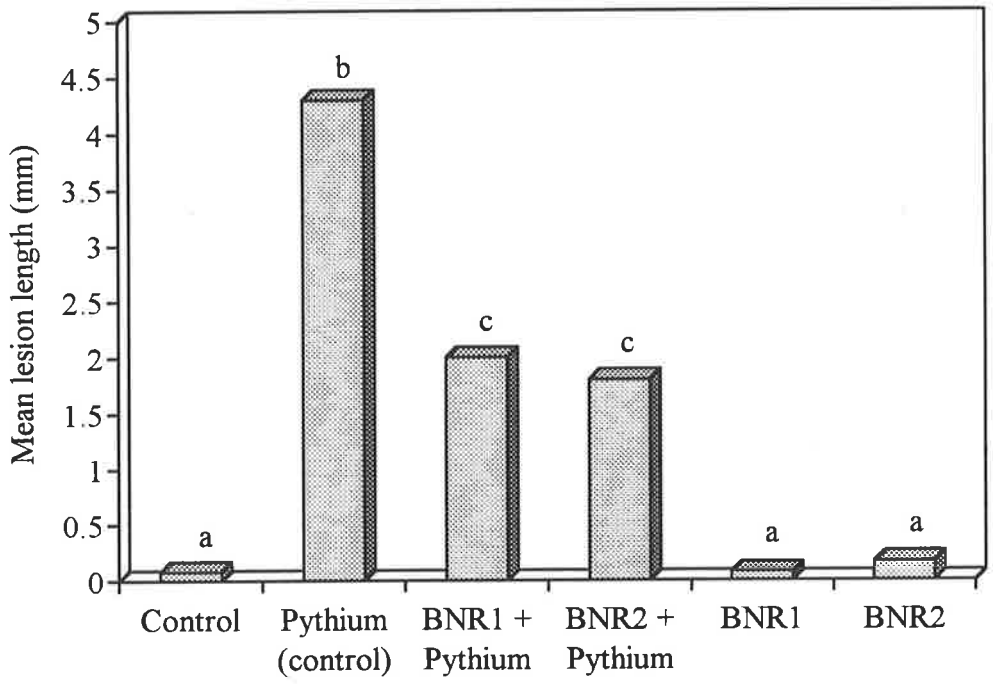
Fig. 6.4.A Mean lesion length on *Capsicum* hypocotyls 7 days after inoculation with *P. u. sporangiiferum*.

Fig. 6.4.B Mean lesion length on *Capsicum* hypocotyls 14 days after inoculation with *P. u. sporangiiferum*.

(A)


Mean lesion length (mm)
3.5
3
2.5
2
1.5
1
0.5
0
a
b
c
c
a
a
Control
Pythium (control)
BNR1 + Pythium
BNR2 + Pythium
BNR1
BNR2

(B)


Mean lesion length (mm)
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
a
b
c
c
a
a
Control
Pythium (control)
BNR1 + Pythium
BNR2 + Pythium
BNR1
BNR2

Light-microscopic examination of lesions caused by *P. u. sporangiiferum* on *Capsicum* hypocotyls (Plate 6.6.A) revealed the presence of both the pathogen and BNR on and inside the diseased tissues. Although BNR1 and BNR2 were rarely present on healthy *Capsicum* hypocotyls, their presence inside and next to lesions on diseased hypocotyls was quite conspicuous (Plate 6.6.B & C). In some seedlings, BNR1 or BNR2 were observed growing from the crown towards the lesions formed by the pathogen in the upper hypocotyl (Plate 6.6.D). Inside the lesions, both BNR1 and BNR2 were observed in close contact with *P. u. sporangiiferum* hyphae (Plate 6.6.E), or less frequently with sporangia (Plate 6.6.F). However, no definite mycoparasitic activity by either BNR towards the pathogen was observed inside the lesions. The three-dimensional nature of lesions as well as yellowing/browning of the diseased tissues, prevented the assessment of hyphal interactions between *P. u. sporangiiferum* and either BNR1 or BNR2 inside the lesions.

Histochemical study did not reveal any obvious differences between *Capsicum* seedlings colonised by either BNR1 or BNR2 in the absence of *P. u. sporangiiferum,* and the control seedlings, with respect to the presence of lignin, cellulose, or callose. Lignin and callose were detected in xylem vessels, the latter in the phloem also, of main roots and hypocotyls in both BNR treatments and the control. Cellulose was present in cell walls of the epidermis, cortex and the vascular bundle. No polyphenolic substances were detected in hypocotyls or roots of *Capsicum* seedlings colonised by either BNR1 or BNR2, or in control plants. In addition, the presence of polyphenolic substances in *Capsicum* roots with browning tips could not be determined, because the brownish-red discolouration of the root tip cells interfered with the detection of the cherry-red or brown colour, which would identify polyphenolic substances.

6.3.3.2 Induced systemic resistance - *in vitro* bioassay

Capsicum hypocotyls were not protected by either BNR1 or BNR2 against damping-off caused by *P. u. sporangiiferum* in divided Petri dishes (Fig. 6.5).

Plate 6.6 Colonisation of lesions caused by *P. u. sporangiiferum* in *Capsicum* hypocotyls by BNR in pasteurised potting mix.

Seedlings harvested 7 and 14 days after inoculation of the hypocotyl with *P. u. sporangiiferum* were gently washed in sterile deionised water, cleared in 10% KOH for 1.5 hours at *ca* 25°C, rinsed in 10% HCl, and soaked in a wetting solution for 1 hour before being stained with trypan blue in lactoglycerol. Whole seedlings, and transverse sections (*ca* 30 µm thick) of diseased hypocotyls were mounted in lactoglycerol on a microscope slide and examined using a Leitz Wild M3Z stereomicroscope or a Leitz Laborlux S compound microscope.

A: Lesion caused by *P. u. sporangiiferum* 7 days after the hypocotyl was inoculated with the pathogen. Bar = 1.5 mm.

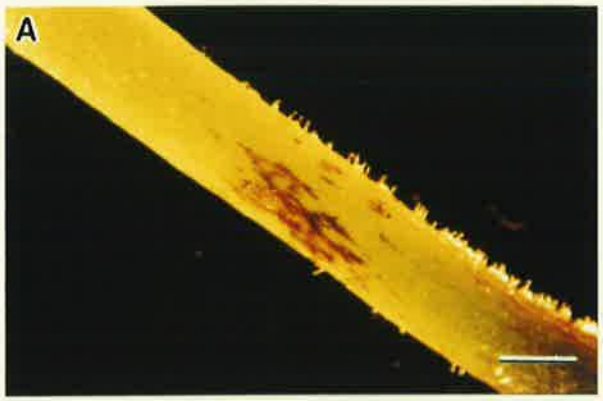
B: BNR1 hyphae (arrowhead) on a lesion 7 days after inoculation with *P. u. sporangiiferum*. Bar = 50 µm.

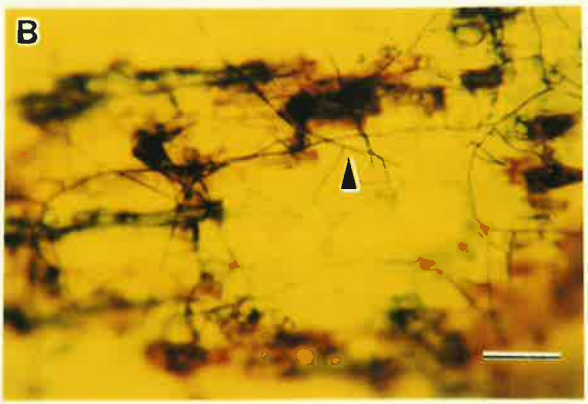
C: BNR2 hyphae on a lesion 14 days after inoculation with *P. u. sporangiiferum*. BNR hyphae (arrowheads) densely colonised diseased epidermal cells.
Bar = 50 µm.

D: BNR1 hyphae (arrowhead) growing from the crown towards a lesion (L) on the hypocotyl 14 days after inoculation with *P. u. sporangiiferum*. Bar = 100 µm.

E: Transverse section through a lesion colonised by BNR1 and *P. u. sporangiiferum* 7 days after the hypocotyl was inoculated with the pathogen. Hyphae of BNR1 (arrowheads) in close contact with pathogen hyphae (double arrowhead) inside a diseased cortical cell (c). BNR1 hyphae (arrow) also visible in the epidermal cell (e).
Bar = 10 µm.

F: BNR2 hyphae (arrowhead) in close contact with a sporangium of *P. u. sporangiiferum* (double arrowhead) in diseased epidermal cell 14 days after the hypocotyl was inoculated with the pathogen. BNR2 hyphae (arrow) visible nearby.
Bar = 25 µm.


A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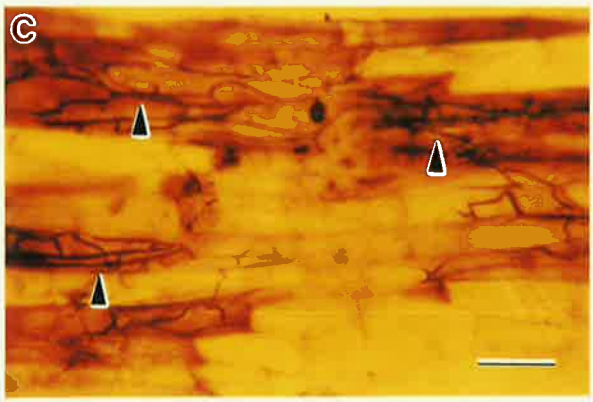
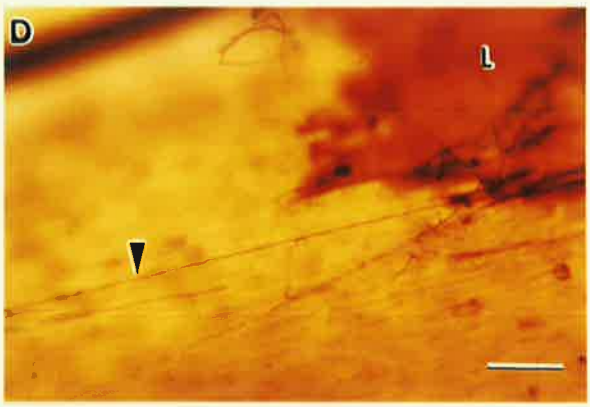
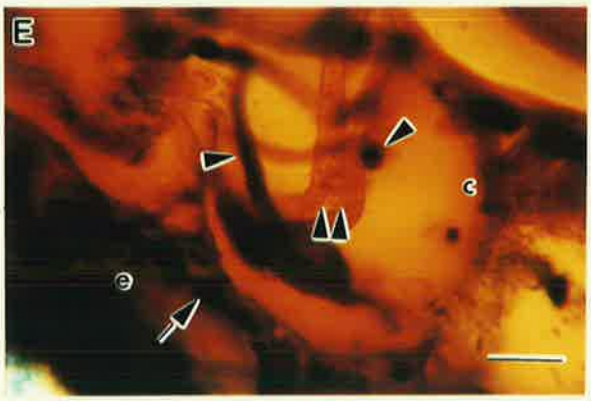

C


D
L


E
c
e

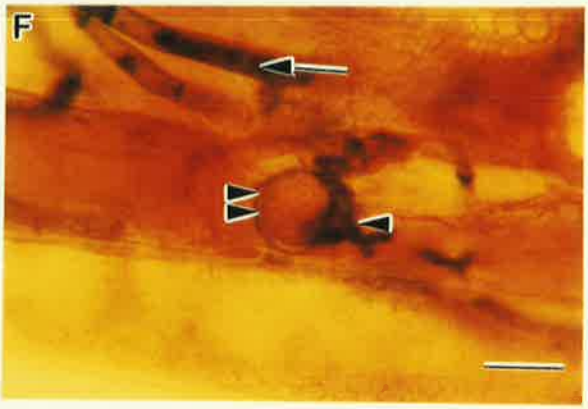
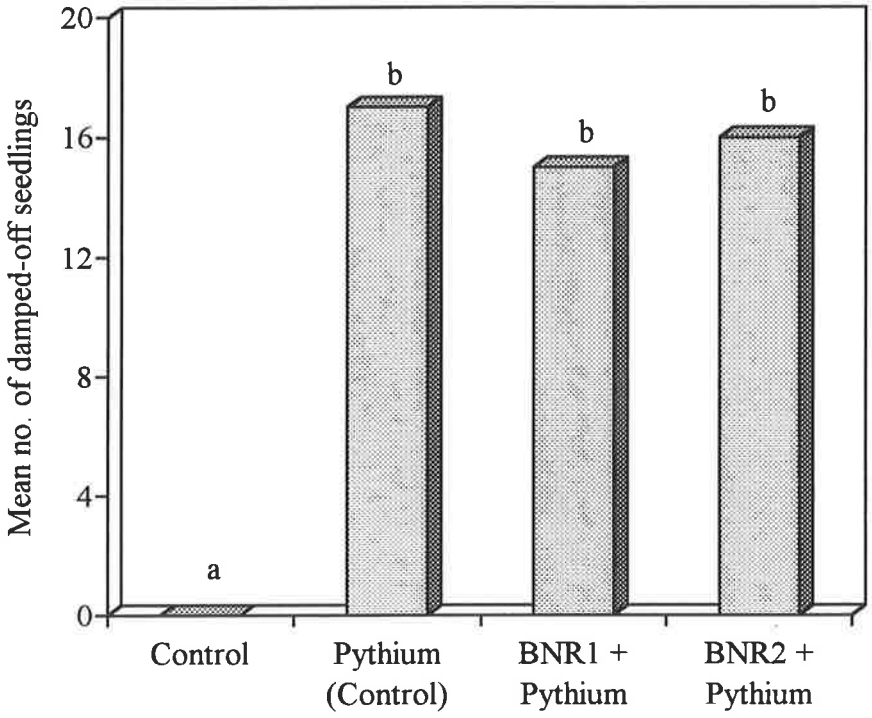

F

Fig. 6.5 Damping-off of *Capsicum* seedlings in *in vitro* bioassay.

Seven-day-old *Capsicum* seedlings colonised by either BNR1 or BNR2 were placed in divided, plastic Petri dishes, three seedlings per dish, such that the roots were "separated" from the hypocotyls. The latter were inoculated with plugs of *P. u. sporangiiferum* (Pythium) in 1/4 PDA. Control treatments consisted of *Capsicum* seedlings challenged with *P. u. sporangiiferum* or sterile 1/4 PDA plugs, in the absence of either BNR1 or BNR2. Plates were incubated at 25°C for 5 days and the number of damped-off (i.e. dead) seedlings counted. Data are means of six replicate plates for each antagonist × pathogen combination and controls. Values for each treatment followed by the same letter are not significantly different ($P > 0.05$) by Tukey's HSD test.


Mean no. of damped-off seedlings
20
16
12
8
4
0
a
b
b
b
Control
Pythium (Control)
BNR1 + Pythium
BNR2 + Pythium

Formation of lesions and subsequent rotting of the whole hypocotyl by the pathogen was observed in seedlings with roots colonised by either BNR1 or BNR2. Similar symptoms were observed in *Capsicum* seedlings colonised by *P. u. sporangiiferum* without BNR. Control plants showed no fungal infection, and did not appear affected by the experimental protocol.

6.4 DISCUSSION

Both BNR1 and BNR2 extensively colonised *Capsicum* seeds, forming dense, uniform mycelial mats over the entire seed surface, including the site of radicle emergence. Colonisation of the emerging radicle by each of the two BNR isolates apparently originated from hyphae on the seed coat or spermosphere, as it was not colonised by the antagonists while inside the seed. The pattern of colonisation of *Capsicum* seeds by *P. u. sporangiiferum* was similar to that of both BNR isolates, suggesting that the pathogen can potentially compete with BNR for the same infection sites while co-colonising the seed. Consequently, complete displacement or suppression of the pathogen by BNR from the seed surface or the spermosphere could be accomplished by one or both of the following mechanisms: 1) "interference competition", whereby one organism increases its share of the limiting resource via antibiosis and/or mycoparasitism; 2) "exploitation competition", where co-utilisation of a resource by the more efficient competitor leads to the inhibition of the other organism without any direct interaction between the two (Lockwood, 1981; Wicklow, 1981). Moreover, the dense hyphal mats formed by BNR prior to establishment of *P. u. sporangiiferum* on *Capsicum* seeds could block or alter infection sites, or pre-empt the nutrient supply needed by the pathogen for infection, thus excluding the pathogen from the seed surface or even the spermosphere. For example, *Trichoderma hamatum*, when applied as a coating of spores on seeds of pea and radish, protected the seedlings against pre-emergence damping-off caused by *Pythium* spp. and *R. solani*

(Harman *et al.*, 1980, 1981). The mechanisms of seed protection by *T. hamatum* are thought to involve the pre-emption of seed exudates at the seed surface and, in some instances, mycoparasitism or lytic enzyme production (Cook & Baker, 1983).

Colonisation of *Capsicum* seedlings by BNR1 and BNR2 was confined mainly to the root system, with fungal growth occasionally observed on the lower hypocotyl. Furthermore, both BNR isolates colonised the host epidermal cells and invaded the outermost layers of the root cortex. Similarly, Cardoso & Echandi (1987) reported, in their greenhouse studies, that a non-pathogenic BNR-like fungus extensively colonised the rhizosphere and rhizoplane of bean seedlings and invaded the epidermal cells of hypocotyls and roots. However, in an earlier greenhouse study, Cardoso & Echandi (1986) observed that non-pathogenic BNR-like fungi extensively colonised the root and hypocotyl surface of snapbean seedlings but did not penetrate the primary roots or the hypocotyl tissues. Jabaji-Hare *et al.* (1993) also observed that only epidermal cells of bean roots were colonised by non-pathogenic BNR, with no evidence of invasion of cortical cells.

The pattern of colonisation of primary and secondary roots of *Capsicum* seedlings by BNR1 and BNR2 was irregular. Regions densely colonised by BNR included the crown with emerging lateral roots, and the root tips. The meristematic region of the root tips and the root cap were the main sites of formation of dense hyphal mats by the two BNR isolates. Drozdowski & Manning (1988), likewise, reported that the root cap and meristematic region of asparagus seedlings, growing aseptically in liquid cultures, were heavily colonised by BNR. One likely explanation for the preferential colonisation of root tissues by BNR is the localised distribution of exudation sites along the root surface. According to Hale *et al.* (1978) and Schumann (1991), these exudation sites on a growing root include: 1) the root cap, with a mucilaginous material and sloughed root cap cells; 2) the areas of cell division and elongation, where

nutrients leak out of the continuously differentiating cells; and 3) regions of exit of lateral roots, where the cells are disrupted at the point of root emergence.

Vancura & Hanzlikowa (1972) postulated that the extent of root exudation is governed by many plant properties, including the number and size of cotyledons and their photosynthetic efficiency. The photosynthates may appear as exudates in the rhizosphere either in their original form, usually sucrose, or they may be altered metabolically between the sites of synthesis and exudation (Hale *et al.*, 1978). The fact that root tips of *Capsicum* seedlings were not colonised by BNR while the cotyledons were still below the soil surface, and thus were not photosynthetically active, supports the hypothesis that root exudates were important in determining the pattern of colonisation of *Capsicum* roots by the two antagonists. The occasional presence of BNR on lower hypocotyls of *Capsicum* seedlings might also be the result of increased exudation in this area. Magyarosy & Hancock (1974) reported that, in squash seedlings, the greatest amount of exudation, in particular of amino acids and sugars, was observed in the zone of transition between the hypocotyl and the root.

For *P. u. sporangiiferum,* the extent of colonisation of *Capsicum* was apparently determined by the susceptibility of seedlings to infection. Apparently tolerant and susceptible seedlings have been reported to be colonised by the pathogen, however, disease development differed between the two. Mellano *et al.* (1970*a*) suggested that tolerance is directly related to inherent factors, other than lignin, in mature tissues of roots. They proposed that sterols in older root tissues may affect the virulence of *P. ultimum* by reducing its production of macerating pectic enzymes and stimulating the fungus to form oospores instead of mycelium. Hence, conversion of the fungus to a relatively nonvirulent parasite is achieved. This could explain the presence, in this study, of oospores and sparse hyphae of *P. u. sporangiiferum* on the crowns of 21-day-old *Capsicum* seedlings which showed no sign of root- or hypocotyl-rotting. Only in some secondary roots were small lesions formed by the pathogen near the root

tips. Similarly, in tolerant roots of 25- and 30-day-old *Antirrrhinum majus, P. ultimum* colonised only root tips of tertiary and quaternary roots, causing small necrotic lesions, but was unable to colonise the mature portions of primary and secondary roots (Mellano *et al.*, 1970*b*). In susceptible *Capsicum* seedlings, both the hypocotyl and the root tips were infected with *P. u. sporangiiferum* hyphae, which caused extensive lesion formation. The pathology of hypocotyl and root infection of *Capsicum* seedlings by *P. u. sporangiiferum* resembled that of other *Pythium* spp., as described by Endo & Colt (1974).

When comparing the overall pattern of colonisation of *Capsicum* seedlings by *P. u. sporangiiferum* and by either BNR1 or BNR2, it became apparent that the crown and the root tips were the main colonisation sites for all three fungi. Thus, competition for these exudate-rich sites, between *P. u. sporangiiferum* and BNR, could have resulted in the protection of susceptible *Capsicum* roots against root rot caused by the pathogen. Furthermore, formation of dense hyphal mats by BNR prior to root colonisation by *P. u. sporangiiferum* could lead to the exclusion of the pathogen from these infection sites. Cintas *et al.* (1995) reported that competition for infection sites is a primary factor responsible for limiting the development of stem rot of rice, caused by *Sclerotium oryzae*, by binucleate *Rhizoctonia oryzae-sativae*. They suggested that *R. oryzae-sativae* germinated faster and attached more quickly to the host surface thus limiting the availability of infection sites to *S. oryzae.*

BNR1 and BNR2 promoted significant increases in shoot dry weight of 21-day-old *Capsicum* seedlings in pasteurised potting mix in the absence of *P. u. sporangiiferum.* Plant growth promotion by BNR isolates in pasteurised potting medium has also been reported, for *Capsicum* and *Celosia* seedlings, by Harris *et al.* (1993*b*, 1994) and Harris (1994). The mechanism(s) of plant growth enhancement although not investigated here, could have included: 1) production of growth regulators by BNR in the rhizosphere or induction within the plant; 2) solubilisation of certain unavailable

minerals in the rhizosphere, or excretion of substances that enhance seed germination, root growth and production of lateral roots; 3) dense colonisation of root surfaces that could increase the absorption rate of water and minerals from the soil by increasing the effective root surface and/or changing the electrochemical properties of the roots; 4) changing the chemical composition of certain nutrients, such as transforming nitrate into ammonium; and 5) protection of plants from minor pathogens (Elad & Misaghi, 1985). Windham *et al.* (1986) studied the mechanisms of growth promotion induced by *Trichoderma* spp. They reported that the addition of *T. harzianum* or *T. koningii* to autoclaved soil increased the rate of emergence of tomato and tobacco seedlings compared to that of the controls, and significantly increased the root and shoot dry weights of the 8-week-old seedlings. In addition, they found that population densities of soil microflora (other than *Trichoderma* spp.) did not differ between soils infested with *Trichoderma* spp. and the controls. They concluded that these growth-promoting *Trichoderma* spp. had a direct effect on the host plant by producing a growth-regulating factor which increased the rate of seed germination and dry weight of shoots. It was also suggested that the increased plant growth was not a result of decreased activity of minor plant pathogens by the action of the *Trichoderma* spp. Masuhara *et al.* (1993) reported that 59 of 67 isolates of non-orchidaceous BNR induced symbiotic germination of seeds of the orchid *Spiranthes sinensis* var. *amoena in vitro*. Consequently, the possibility of a symbiotic association of either BNR1 or BNR2 with *Capsicum* seedlings should not be ruled out as a mechanism of plant growth promotion.

Cubeta *et al.* (1991) investigated the survival of BNR in soil and plant debris under field conditions. They reported that the fungi survived in field soils in snapbean stems for 11 months and in ground oat kernels for up to 9 months. In the present study, the ability of BNR1 and BNR2 to survive in *Capsicum* until the plants began senescence was investigated. The two antagonists were found in mature plant tissues as well as in the rhizosphere after 15 weeks of growth in pasteurised potting mix.

The ability of BNR to survive in soil and in mature plant tissues for long periods of time may be an important factor in determining their biocontrol potential in field conditions against a variety of other plant pathogens, including *R. solani*.

Protection of *Capsicum* seedlings by BNR against the post-emergence damping-off *in vivo* is believed to be the result of the displacement or suppression of *P. u. sporangiiferum* in the pathogen-induced lesions via cross protection rather than by induced systemic resistance. Lesions formed by the pathogen on the hypocotyls are thought to result from the action of pectinolytic and proteolytic enzymes, which cause maceration of cells and tissues (Krupa & Dommergues, 1979). Such changes in the chemical composition evidently alter the quality and quantity of exudates that leak out from the lesions onto the external surfaces of the host. Exudates released from wounds may stimulate other pathogenic or non-pathogenic fungi to colonise these newly created or modified infection courts (Dickinson, 1979). This could explain the presence of BNR on *Capsicum* hypocotyls infected by *P. u. sporangiiferum*, since colonisation of healthy, unwounded seedlings by the antagonists was confined mainly to the root system. Once inside the lesion, inhibition of *P. u. sporangiiferum* by BNR is believed to have taken place, most probably by means of interference competition. BNR1 and BNR2 were found also inside lesions apparently caused by mechanical injury during seedling emergence from the potting mix in the absence of *P. u. sporangiiferum*. This observation further supports the hypothesis that wound exudates stimulated the two BNR isolates to colonise the injured hypocotyl tissues. Furthermore, increased survival of seedlings infected with *P. u. sporangiiferum* apparently resulted from the suppression of further lesion development by BNR by means of cross protection. Competitive displacement or suppression of the pathogen in lesions or man-made wounds, by biocontrol agents, have been reported. For example, Buczacki (1973) observed that the fungus *Trichoscyphella willkommii*, responsible for larch canker, was competitively displaced by several sequentially invading saprophytic fungi, such as *Cryptosporiopsis abietina*, *Zalerion arboricola*, *Tympanis laricina*, and

a *Phialophora* sp. Extension of the cankers colonised by *T. willkommii* was also reduced by artificial inoculation of these sites with antagonists. Corke & Hunter (1978) also reported that inoculation of pruning wounds on apple tree shoots with *Trichoderma viride* and *Bacillus subtilis*, resulted in fewer infections from subsequent inoculations with *Nectria galligena*. Moreover, the lesion length on diseased apple shoots was significantly reduced in the presence of the antagonists.

In vitro pre-inoculation of *Capsicum* seedlings with BNR did not protect young hypocotyls against subsequent infection by *P. u. sporangiiferum*. In addition, there were no obvious changes in the cell walls of primary root or hypocotyl tissues in *Capsicum* seedlings pre-colonised *in vivo* with either BNR1 or BNR2 in the absence of the pathogen. In the *in vitro* bioassay, BNR was apparently prevented from interacting with *P. u. sporangiiferum* on the host surface, thus the role of induced systemic resistance is believed to have been examined without the involvement of cross protection. It, therefore, seems unlikely that systemic induced resistance is a mechanism by which BNR protects *Capsicum* seedlings from damping-off caused by *P. u. sporangiiferum*, although the effect of the *in vitro* system on the outcome of this investigation can not be ruled out. In contrast, Jabaji-Hare *et al.* (1993) reported that non-pathogenic BNR induced increased production of an electron-dense material associated with the cell walls of bean seedlings colonised by the fungi. In adjacent non-colonised cortical cells, this material was less abundant. They suggested that the amorphous material may act as a protective barrier against infection by *R. solani*. Similarly, Cardoso & Echandi (1986 & 1987) suggested that BNR-like fungi induced metabolic responses in snapbean and bean seedlings that suppressed the pathogenic *R. solani* at the infection site.

In summary, protection of *Capsicum* seedlings by BNR1 or BNR2 against pre- and post-emergence damping-off caused by *P. u. sporangiiferum*, is believed to be attained by: 1) the exclusion of the pathogen from plant surfaces pre-colonised by BNR;

or 2) by competition for infection sites between the co-occurring fungi; or 3) by competitive displacement or suppression of the pathogen by BNR in infected plant tissues. Furthermore, depending on the pathogen inoculum potential versus that of the two antagonists, as well as the host's predisposition to infection, one or a combination of the above mechanisms might be required to operate simultaneously to achieve optimal disease control.

CHAPTER 7 COMPETITIVE SAPROPHYTIC ACTIVITIES OF BINUCLEATE *RHIZOCTONIA* AND *P. U. SPORANGIIFERUM*

7.1 INTRODUCTION

Essentially all plant pathogenic fungi which are capable of saprophytic and parasitic growth spend an important part of their life cycle in the residue of their host. Such pathogens are of two groups: those that are entirely dependent on the residue for survival, and those that form resistant structures, such as oospores, sclerotia or chlamydospores and thus are capable of survival after the decomposition of the host residue (Cook *et al.*, 1978). Pathogens which are also good saprophytes, i.e. are able to colonise the host residue after the plant is dead, can increase their biomass or enhance their longevity in soil, increasing the chance of infection of a new host (Cook & Baker, 1983). For example, *Sclerotium rolfsii* colonises dead leaves or other debris of a previous crop on the soil surface and then uses this as a food base while attacking the current crop (Boyle, 1961). Increased germination of *P. ultimum* oospores in soil amended with organic residue was also reported by Agnihotri & Vaartaja (1967*b*).

According to Garrett (1981), the success of competitive colonisation of a substrate by a given fungus is directly determined by its competitive saprophytic ability and its inoculum potential at the surface of a substrate, and is inversely determined by the inoculum potential of competing fungi. Competitive saprophytic ability was defined by Garrett (1970) as the summation of physiological characteristics that make for success in competitive colonisation of dead organic substrates. The four characteristics that favour competitive saprophytic ability include 1) high growth rate and rapid

germination of spores; 2) production of appropriate enzymes, such as cellulose and lignin, for substrate decomposition; 3) production of antibiotics; and 4) tolerance to fungistatic substances produced by other soil microorganisms. Inoculum potential was defined by Garrett (1970) as "the energy of growth of a fungus available for colonisation of a substrate", and is determined by: 1) an area of the fungus in contact with an area of the host plant; 2) the relative vigour of the hyphae attempting to invade the host; and 3) the effect of environmental conditions that vary from optimal to completely inhibitory, and determine the actual or realised energy for fungal growth. Some studies have shown a high correlation between the competitive saprophytic ability and the pathogenicity of unspecialised root-infecting fungi, such as *R. solani* and *Pythium* spp. (Sneh *et al.*, 1966; Papavizas *et al.*, 1975; Bouhot, 1979). For example, Walker (1991) observed that damping-off of carrot seedlings by *P. ultimum* at 12°C was highly correlated with the relatively fast growth rate (*ca* 0.52 mm/h) of the fungus at this temperature, and this observation was consistent with previous reports (Thomson *et al.*, 1971; Hancock, 1977).

An important strategy for biological control is to prevent the pathogen population from building up to damaging levels, or otherwise to reduce its inoculum potential by manipulating its microbial and physical environment (Jarvis, 1992). Crop residues (or organic amendments) can affect the development of plant diseases by 1) providing the pathogen with nutrients and space required for its growth and reproduction; 2) affecting the physical environment occupied by the host and pathogen, thus stimulating or inhibiting disease development; and 3) intensify soil microbial activity, and together with decomposition products (some fungitoxic or phytotoxic) affecting the pathogen, susceptibility of the host plant, or both (Cook *et al.*, 1978). Increased soil microbial activity as a response to organic residues may result in biological control of pathogen inoculum which, according to Cook & Baker (1983), involves: 1) destruction of pathogen propagules or biomass; 2) prevention of inoculum

formation; 3) weakening or displacement of the pathogen from infested residue (the food base); and 4) reduction of vigour or virulence of the pathogen.

Simon & Sivasithamparam (1989) argued that suppression of the saprophytic phase of growth of soilborne plant pathogens, by biotic or abiotic factors, should be given greater emphasis in the biological control of root diseases, since it is in this phase that the pathogen is most vulnerable to antagonism. In addition, organic residues can exert an important influence on plant diseases, therefore, they can be used to examine interactions between the pathogen, its biocontrol agents and the host plant (Cook *et al.*, 1978). Hence, the principal aim of experiments presented here was to elucidate the saprophytic capabilities of BNR1 and BNR2 in comparison with those of *P. u. sporangiiferum* in potting mix, in terms of: 1) their saprophytic growth rates; 2) competitive colonisation of organic residue; and 3) the outcome of interactions in the organic residue between the pathogen and BNR in relation to the saprophytic and pathogenic activities of *P. u. sporangiiferum*.

7.2 MATERIALS AND METHODS

7.2.1 Potting mix

The moisture content and pH of the potting mix used in the *in vitro* and glasshouse experiments were determined as described in Section 6.2.2.

7.2.2 Saprophytic growth of BNR1, BNR2 and *P. u. sporangiiferum*

Experiment 1 Growth in sterilised potting mix - *in vitro* study

Approximately 100 g of autoclaved potting mix (see Section 3.3) were placed aseptically in glass Petri dishes (120 mm diam.) and inoculated with three millet seeds colonised with BNR1, BNR2 or *P. u. sporangiiferum* (Fig. 7.1.A).

These millet seed inocula were "germinated" on 1/4 PDA in plastic Petri dishes (90 mm diam.) at 25°C in the dark for 24 hours, as it had been observed in preliminary experiments that the growth in potting mix of *P. u. sporangiiferum* from "non-germinated" millet seed inocula was very sporadic and inconsistent. In contrast, the growth of the two BNR isolates in the potting mix was the same whether the millet seed inocula were "germinated" first or not. Toothpicks used to detect the growing hyphae (M. Ryder, 1994, pers. comm.) were first boiled in deionised water for 20 min to remove chemical residues, cut into *ca* 5 mm pieces, and autoclaved in glass Petri dishes (90 mm diam.) for 20 min at 121°C. Sterile toothpick segments were arranged vertically in five rows of five toothpicks, and a letter was assigned to each toothpick to mark its position with reference to the fungal inoculum (Fig. 7.1.A). Plates were sealed with plastic film and incubated at 25°C in the dark until each fungus reached the opposite side of the plate. During this time the cumulative distance covered by each fungus (i.e. the distance from the inoculum to the point measured at a given time), was determined daily, by removing from each plate the two rows of toothpick segments closest to a row previously colonised by the fungus. Toothpick segments removed from the plates were placed aseptically on 1/4 PDA in plastic Petri dishes (90 mm diam.), six segments evenly distributed per plate, and the plates incubated at 25°C in the dark for about 24 hours. The identity of each fungus, growing out of the colonised toothpick segments into the underlying medium, was confirmed *in situ* at ×320 with Leitz Laborlux S compound microscope. Removed toothpick segments were replaced with sterile ones after each sampling to maintain a constant number of toothpicks in each plate during the experiment. The cumulative linear growth rate of each fungus, at each sampling time, was calculated using the formula:

$$\text{Growth rate}_x = \frac{\text{Distance}_x}{\text{Time}_x}$$

where:

"x" denotes the sampling time

$Distance_x$ is the distance from the point of inoculation to that at $Time_x$ (mm);

$Time_x$ is the time taken by the fungus to cover the $Distance_x$ (h);

There were eight replicate plates for each fungal treatment and the experiment was repeated twice. The results were analysed with one-way analysis of variance and Tukey's HSD (Zar, 1984) test to determine pairwise-mean-differences between the treatments.

Experiment 2 Growth in pasteurised potting mix - *in vitro* study

In this experiment, which was prepared exactly as for Experiment 1 (Section 7.2.2), growth of BNR1, BNR2 or *P. u. sporangiiferum* from "germinated" millet seed inocula in pasteurised potting mix (see Section 3.3) was investigated. The cumulative distance grown by each fungus was measured daily, and the linear growth rate calculated using the formula shown in Experiment 1 (Section 7.2.2). There were 10 replicate plates for each fungal treatment and the experiment was repeated twice. The results were analysed with one-way analysis of variance and Tukey's HSD test to determine pairwise-mean-differences between the treatments.

Experiment 3 Growth on 1/4 PDA

Fungal growth was examined on 1/4 PDA in glass Petri dishes (120 mm diam.). Approximately 40 ml of 1/4 PDA per plate was inoculated with three "germinated" millet seed inocula of either BNR1, BNR2 or *P. u. sporangiiferum*. Plates were incubated at 25^{o}C in the dark until the fungi reached the opposite side of the plate. The cumulative distance grown by each fungus was measured daily, and the linear growth rate calculated using the formula shown in Experiment 1 (Section 7.2.2). There were 10 replicate plates for each fungus, the experiment was repeated twice, and the

results were analysed with one-way analysis of variance and Tukey's HSD test to determine pairwise-mean-differences between the treatments.

Experiment 4 Growth in pasteurised potting mix - *in vivo* study

Saprophytic growth of the three fungi was determined in pasteurised potting mix in glasshouse conditions. Undivided, plastic punnets (*ca* 13.9 × 8.5 cm; 350 ml capacity; Masrac Pots) were filled with *ca* 300 ml of pasteurised potting mix. Three "germinated" millet seed inocula colonised with BNR1, BNR2 or *P. u. sporangiiferum* were placed in a small hole (5 mm deep) in the potting mix at one end of each punnet, and the whole surface was covered with *ca* 100 ml of sterilised sand. Whole toothpicks (60 mm long) were prepared as described in Experiment 1 (Section 7.2.2) and arranged vertically in each punnet in three rows of either five or six toothpicks (Fig. 7.1.B). A letter was assigned to each toothpick to mark its position in the potting mix with reference to the fungal inoculum. One toothpick was placed vertically next to the fungal inoculum to serve as the initial reference point in determining the cumulative distance covered by each fungus. Punnets were kept in the glasshouse at 25°C (±5°C), with the average relative humidity of *ca* 60%, and were watered daily until draining (*ca* -0.3 bar) with deionised water. The cumulative distance covered by each fungus was determined daily by removing from each punnet four toothpicks that were in closest proximity to a toothpick colonised by the fungus at a previous sampling time. Toothpicks removed were placed aseptically on 1/4 PDA in plastic Petri dishes (90 mm diam.), four toothpicks evenly distributed per plate, and the plates incubated at 25°C in the dark for about 24 hours. The identity of each fungus, growing out of the colonised toothpicks into the underlying medium, was confirmed *in situ* at ×320 with a Leitz Laborlux S compound microscope. Removed toothpicks were replaced with sterile ones after each sampling as before. The linear growth rate of each fungus, at each sampling time, was calculated using the formula in Experiment 1 (Section 7.2.2). There were 12 replicate punnets for each fungus, arranged in a randomised block design. The experiment was repeated twice and the results analysed with one-way

analysis of variance and Tukey's HSD test to determine pairwise-mean-differences between the treatments.

7.2.3 Saprophytic activities of *P. u. sporangiiferum* and BNR in the presence of seed residue

7.2.3.1 Competitive colonisation of seed residue by *P. u. sporangiiferum* and BNR

*C*apsicum seeds were mixed with 500 g of autoclaved potting mix (7.5 g of seed per 100 g of potting mix) in metal trays, and autoclaved for 20 min at 121°C. Twenty grams of the seeded potting mix were placed aseptically in plastic Petri dishes (90 mm diam.) and inoculated with 1/4 PDA plugs (*ca* 6 mm diam.) of *P. u. sporangiiferum* and either BNR1 or BNR2. To evaluate colonisation of autoclaved *Capsicum* seeds (i.e. the seed residue) under different inoculum density regimes, three inoculum ratios of the pathogen to each of the two antagonists were used. These were: (a) ratio 1:1, six plugs of *P. u. sporangiiferum* to six plugs of either BNR1 or BNR2; (b) ratio 1:2, three plugs of *P. u. sporangiiferum* to six plugs of either BNR1 or BNR2; and (c) ratio 2:1, six plugs of *P. u. sporangiiferum* to three plugs of either BNR1 or BNR2. Plugs of each fungus were evenly arranged on the surface of the potting mix. For controls, seeded potting mix in plates was inoculated with 1/4 PDA plugs (6 mm diam.) of *P. u. sporangiiferum*, BNR1 or BNR2, together with sterile 1/4 PDA plugs (6 mm diam.) in ratios of 1:1 and 2:1, six plugs of a given fungus to either six or three sterile plugs. Plates were sealed with plastic film and incubated at 25°C in the dark for 7 or 14 days. Ten seeds were removed from each plate from each treatment and control at each sampling time, and gently washed in sterile, deionised water using a small paint brush to dislodge any potting mix. Seeds were dried by blotting with sterile tissues, which had been autoclaved for 15 min at 121°C then dried for 20 min in the autoclave, and placed on WA in plastic Petri dishes (90 mm diam.), four seeds evenly distributed per plate. Plates were incubated at 25°C in the dark for *ca* 24 hours, and examined

at ×320 and ×400 with a Leitz Laborlux S compound microscope for the presence and identity of the fungi growing out of the seeds. The percentage of seeds colonised by each of the three fungi alone, or by *P. u. sporangiiferum* and either BNR1 or BNR2 (i.e. dually colonised seeds), was determined from 100 seeds tested from each treatment and controls at each sampling time. There were 10 replicate plates for each pathogen × antagonist × inoculum ratio combination, and each control × inoculum ratio combination for the two incubation times (i.e. 7 and 14 days). The experiment was repeated once and the results analysed with one-way analysis of variance. The Tukey's HSD test was used to determine pairwise-mean-differences between the treatments.

7.2.3.2 Saprophytic growth of *P. u. sporangiiferum* and BNR from colonised seed residue

Seeds which had been colonised by *P. u. sporangiiferum* and either BNR1 or BNR2, at the three inoculum ratios of pathogen to antagonist during 7 or 14 days of incubation, were removed from WA plates (see Section 7.2.3.1) and placed near the edge of plastic Petri dishes (90 mm diam.), three seeds per plate, containing *ca* 20 g of autoclaved potting mix. Sterile toothpick segments (*ca* 5 mm long), prepared as described in Section 7.2.2 (Experiment 1), were arranged in five rows of five toothpick segments (Fig. 7.1.C) with a letter assigned to each toothpick segment to mark its position with reference to the fungal inoculum. Plates were sealed with plastic film and incubated at 25°C in the dark until the fungi reached the opposite side of the plate. During this time the cumulative distance covered by each fungus was determined, and the linear growth rate of each fungus, at each sampling time, calculated as described in Section 7.2.2 (Experiment 1). There were 12 replicate plates for each pathogen × antagonist × inoculum ratio combination, as well as each control × inoculum ratio combination for the two incubation periods (i.e. 7 and 14 days). The experiment was repeated once and the results analysed with one-way analysis of variance.

Fig. 7.1 Arrangement of toothpicks in Petri dishes and in punnets used to detect growing hyphae of BNR and *P. u. sporangiiferum* in potting mix.

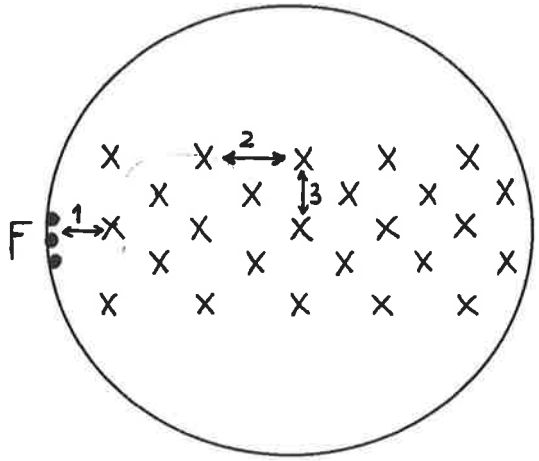
Toothpicks were boiled in deionised water for 20 min and then autoclaved for 20 min at 121°C before being used. Whole toothpicks were used to detect fungal growth in pasteurised potting mix in punnets. Toothpick segments (*ca* 5 mm) were used to detect fungal growth in 1) sterilised and pasteurised potting mix in glass Petri dishes (120 mm diam.), and 2) sterilised potting mix in plastic Petri dishes (90 mm diam.). Numbers next to double-headed arrows, drawn between two toothpicks, represent the distance (cm) between them. **F** is a fungal inoculum; × is a toothpick.

Fig. 7.1.A Arrangement of toothpick segments in sterilised or pasteurised potting mix in glass Petri dishes.

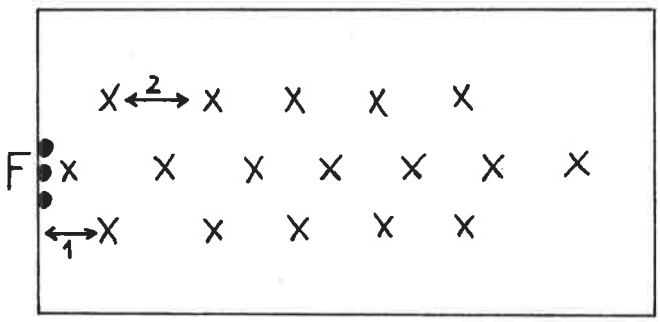
Fig. 7.1.B Arrangement of whole toothpicks in pasteurised potting mix in punnets.

Fig. 7.1.C Arrangement of toothpick segments in sterilised potting mix in plastic Petri dishes.

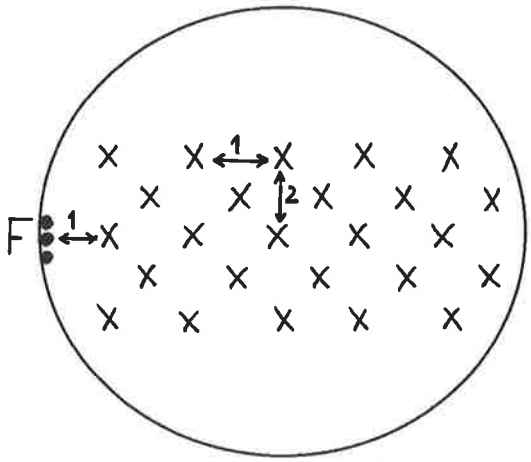
(A)



(B)



(C)



The Tukey's HSD test was used to determine pairwise-mean-differences between the treatments. When there were no significant differences between treatments at the three inoculum ratios, the results for each fungus were pooled to provide a mean of the data from the three inoculum ratios.

7.2.3.3 Pathogenic activity of *P. u. sporangiiferum* in the presence of BNR in the seed residue - *in vivo* study

A total of 120 seeds was taken randomly from each treatment and controls, from the experiment described in Section 7.2.3.1, placed on WA in plastic Petri dishes (90 mm diam.), six seeds evenly distributed per plate, and incubated at 25°C for *ca* 24 hours. Seeds which were dually colonised by both *P. u. sporangiiferum* and either BNR1 or BNR2 (at the three inoculum ratios), and seeds colonised by the pathogen, BNR1 or BNR2 alone (at the ratio 1:1 only), were then used as fungal inocula in the glasshouse study. In addition, *ca* 100 fresh *Capsicum* seeds were autoclaved for 20 min at 121°C in glass Petri dishes (90 mm diam.) on Whatman No. 2 filter papers and used as sterile inocula. Undivided, plastic punnets (*ca* 13.9 × 8.5 cm; 350 ml capacity; Masrac Pots) were filled with approximately 125 ml of pasteurised potting mix and six colonised seeds were arranged in two rows of three seeds on the surface of the potting mix. The colonised seeds were covered by another 125 ml of the potting mix on top of which 16 untreated *Capsicum* seeds (in four rows of four seeds) were sown and covered with *ca* 100 ml of sterilised sand. For controls, potting mix in punnets was inoculated with six colonised seeds of BNR1, BNR2 or *P. u. sporangiiferum*, or with six sterile seeds, before sowing untreated *Capsicum* seeds as described above. Plants were grown in a glasshouse at 25°C (±5°C), with the average relative humidity of *ca* 60%, and were watered until draining (*ca* -0.3 bar) twice a week with a nutrient solution, and on other days with deionised water. After 4 weeks the number of surviving plants in each fungal treatment was compared with that in controls. There were 10 replicate punnets for each pathogen × antagonist × inoculum ratio × sampling time combination, and for controls. The experiment was repeated once and the results analysed with one-way

analysis of variance. Tukey's HSD test was used to determine pairwise-mean-differences between the treatments. When there were no significant differences between treatments at the three inoculum ratios, the results for each fungus were pooled together to provide a mean of the data from the three inoculum ratios.

7.3 RESULTS

7.3.1 Saprophytic growth of BNR1, BNR2 and *P. u. sporangiiferum*

Mean growth rate of BNR1 in sterilised potting mix was not significantly different ($P > 0.05$) from that of BNR2 (Fig. 7.2.A). Mean growth rate of *P. u. sporangiiferum* was significantly ($P \leq 0.05$) faster than that of BNR1 or BNR2 during the first 95 hours (Fig. 7.2.A). However, at 125 hours, the growth of the pathogen was not significantly ($P > 0.05$) different from that of each BNR. The growth rate of *P. u. sporangiiferum* decreased from 0.67 mm/h at 49 hours to 0.52 and 0.47 mm/h at 95 and 125 hours, respectively.

The mean growth rate of BNR1 in pasteurised potting mix *in vitro* was not significantly different ($P > 0.05$) from that of BNR2 during the first 41 hours (Fig. 7.2.B). The growth of BNR2 was significantly faster ($P \leq 0.05$) than that of BNR1 by *ca* 0.08 mm/h and 0.15 mm/h at 65 and 114 hours, respectively. *P. u. sporangiiferum* grew faster ($P \leq 0.05$) than each of the two BNRs during the first 41 hours. The mean growth rate of the pathogen was not significantly ($P > 0.05$) different from that of BNR2, but it was significantly ($P \leq 0.05$) different from that of BNR1 at 65 and 114 hours (Fig. 7.2.B).

On 1/4 PDA, the growth of *P. u. sporangiiferum* was not significantly ($P > 0.05$) different from that of BNR1 or BNR2 during the first 24 hours (Fig. 7.2.C).

Fig. 7.2 Growth rates of *P. u. sporangiiferum*, BNR1 and BNR2.

Linear growth rate of *P. u. sporangiiferum* (Pythium), BNR1 and BNR2 was investigated in *in vitro* experiments in glass Petri dishes (120 mm diam.) containing 100 g of sterile or pasteurised potting mix, or 20 ml 1/4 PDA, at 25°C in the dark. In the *in vivo* experiments, fungi were grown in glasshouse conditions at 25°C (±5°C) in plastic punnets filled with *ca* 300 ml of pasteurised potting mix. Fungal growth was measured daily, and sterile toothpicks were used to detect the presence of fungi in experiments involving potting mix. An asterisk * next to a mean indicates that the value is significantly different ($P \leq 0.05$) from other means shown in that figure, at a given time, by Tukey's HSD test.

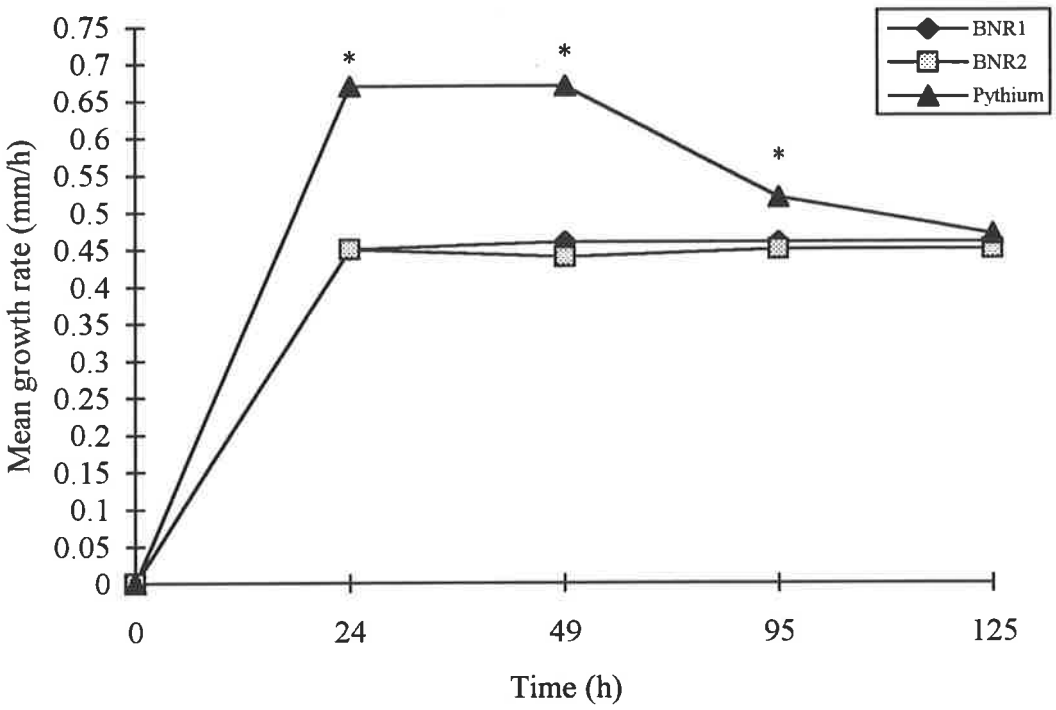
Fig. 7.2.A *In vitro* growth rates of *P. u. sporangiiferum*, BNR1 and BNR2 in sterilised potting mix. Data are means of eight replicate plates.

Fig. 7.2.B *In vitro* growth rates of *P. u. sporangiiferum*, BNR1 and BNR2 in pasteurised potting mix. Data are means of 10 replicate plates.

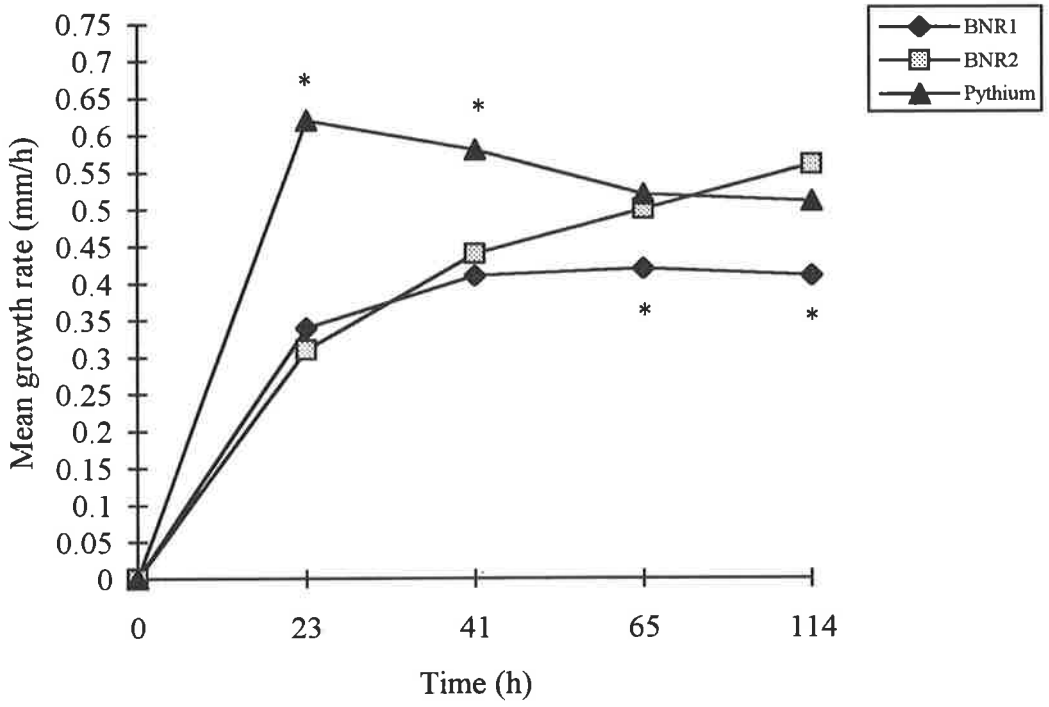
Fig. 7.2.C *In vitro* growth rates of *P. u. sporangiiferum*, BNR1 and BNR2 in 1/4 PDA. Data are means of 10 replicate plates.

Fig. 7.2.D *In vivo* growth rates of *P. u. sporangiiferum*, BNR1 and BNR2 in pasteurised potting mix. Data are means of 12 replicate plates.

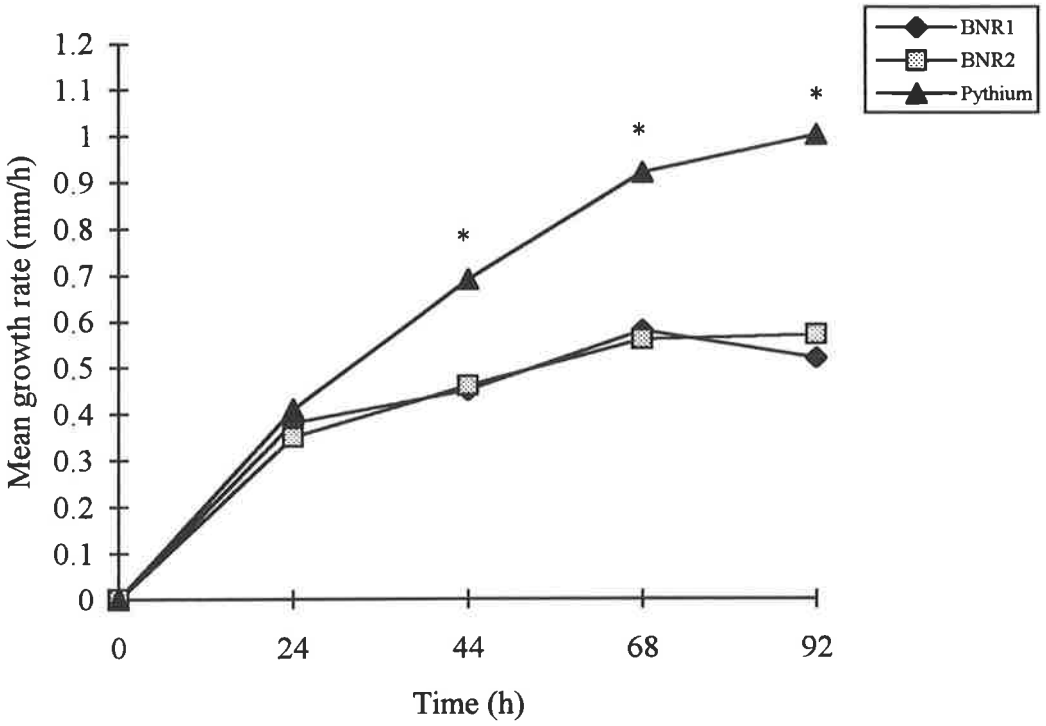
(A)


BNR1
BNR2
Pythium
Mean growth rate (mm/h)
0.75
0.7
0.65
0.6
0.55
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
*
*
*
0
24
49
95
125
Time (h)

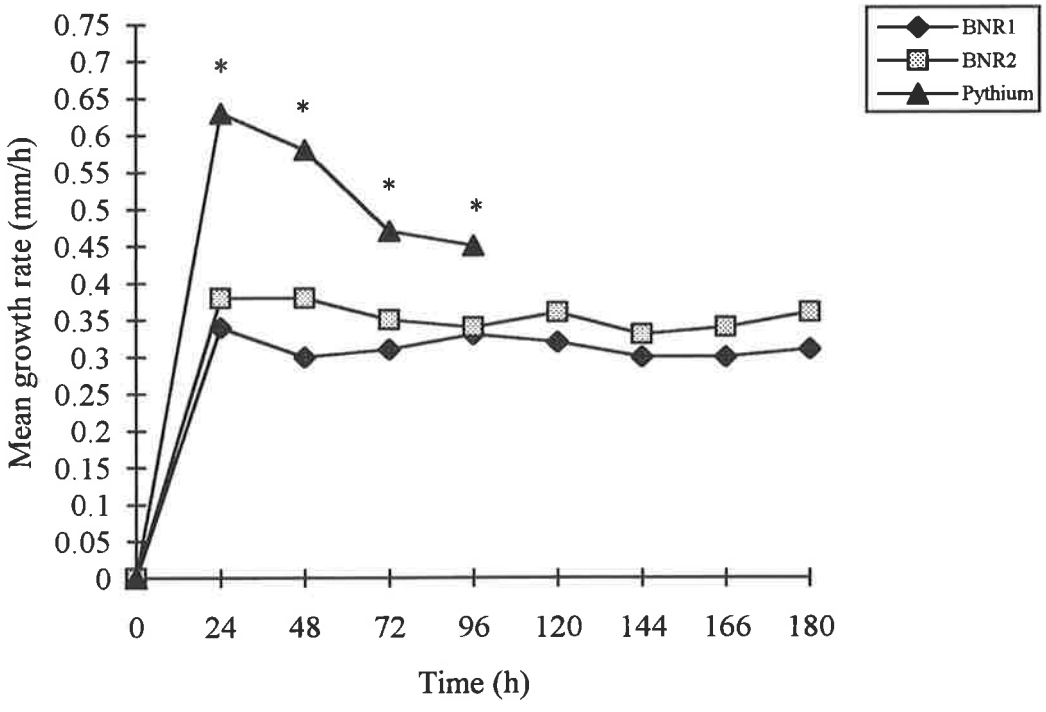
(B)


BNR1
BNR2
Pythium
Mean growth rate (mm/h)
0.75
0.7
0.65
0.6
0.55
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
*
*
*
*
0
23
41
65
114
Time (h)

(C)


BNR1
BNR2
Pythium
Mean growth rate (mm/h)
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
*
*
0
24
44
68
92
Time (h)

(D)


BNR1
BNR2
Pythium
Mean growth rate (mm/h)
0.75
0.7
0.65
0.6
0.55
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
*
*
*
*
0
24
48
72
96
120
144
166
180
Time (h)

The mean growth rate of the pathogen was significantly ($P \leq 0.05$) faster than that of BNR1 and BNR2 by *ca* 0.23, 0.34 and 0.43 mm/h at 44, 68 and 92 hours, respectively. BNR1 and BNR2 did not differ ($P > 0.05$) in their mean growth rates on 1/4 PDA.

In pasteurised potting mix in the *in vivo* experiment, the mean growth rate of *P. u. sporangiiferum* was significantly faster ($P \leq 0.05$) than that of BNR1 or BNR2 (Fig. 7.2.D).

However, the growth rate of the pathogen gradually decreased from 0.62 to 0.51 mm/h at 24 and 96 hours, respectively, and the fungus was not detected in the potting mix at 120 hours. BNR1 and BNR2 did not differ ($P > 0.05$) in their mean growth rates in pasteurised potting mix *in vivo*.

7.3.2 Saprophytic activities of *P. u. sporangiiferum* and BNR in the presence of seed residue

7.3.2.1 Competitive colonisation of seed residue by *P. u. sporangiiferum* and BNR

When grown alone in sterilised potting mix, *P. u. sporangiiferum*, BNR1 and BNR2 were recovered from 99 to 100% of the autoclaved *Capsicum* seeds tested at 7 or 14 days (Fig. 7.3.A & B), irrespective of the initial inoculum ratio of each fungus to sterile 1/4 PDA plugs (data not shown).

The recovery of the pathogen from the seed residue was, however, significantly ($P \leq 0.05$) reduced when in combination with either BNR1 or BNR2 and incubated for 7 and 14 days in the inoculum ratios of 1:1, 1:2 and 2:1 of pathogen to antagonist (Fig. 7.3.A & B).

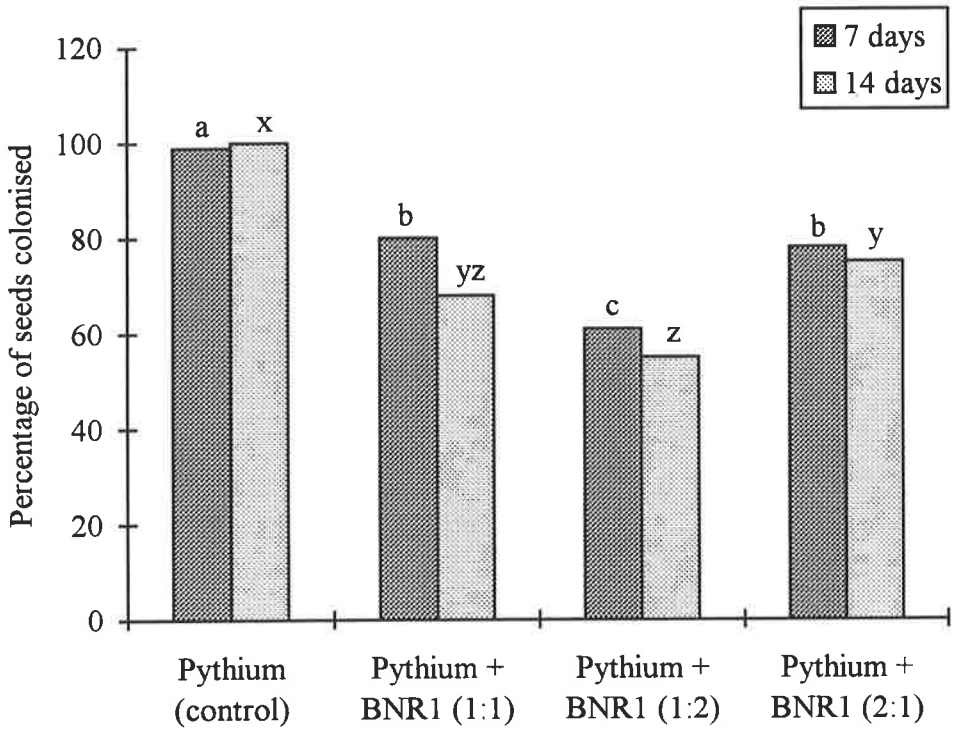
Fig. 7.3 Competitive colonisation of the seed residue by *P. u. sporangiiferum* in the presence of BNR.

Colonisation of autoclaved *Capsicum* seeds by *P. u. sporangiiferum* (Pythium) in combination with either BNR1 or BNR2 at the inoculum ratios of 1:1, 1:2, and 2:1 of pathogen to antagonist, was investigated in sterilised potting mix in plastic Petri dishes (90 mm diam.). Control plates were inoculated with sterile 1/4 PDA plugs and *P. u. sporangiiferum*, BNR1 or BNR2 in the ratios 1:1 and 1:2. Plates were incubated at 25°C in the dark for 7 or 14 days, and the percentage of seeds colonised by the pathogen determined. Data are means of 10 replicate plates for each pathogen × antagonist × inoculum ratio combination, and each control × inoculum ratio combination. Results for *P. u. sporangiiferum* controls are means of combined data from the ratios 1:1 and 2:1. Values for each incubation period followed by the same letter are not significantly different ($P > 0.05$) by Tukey's HSD test.

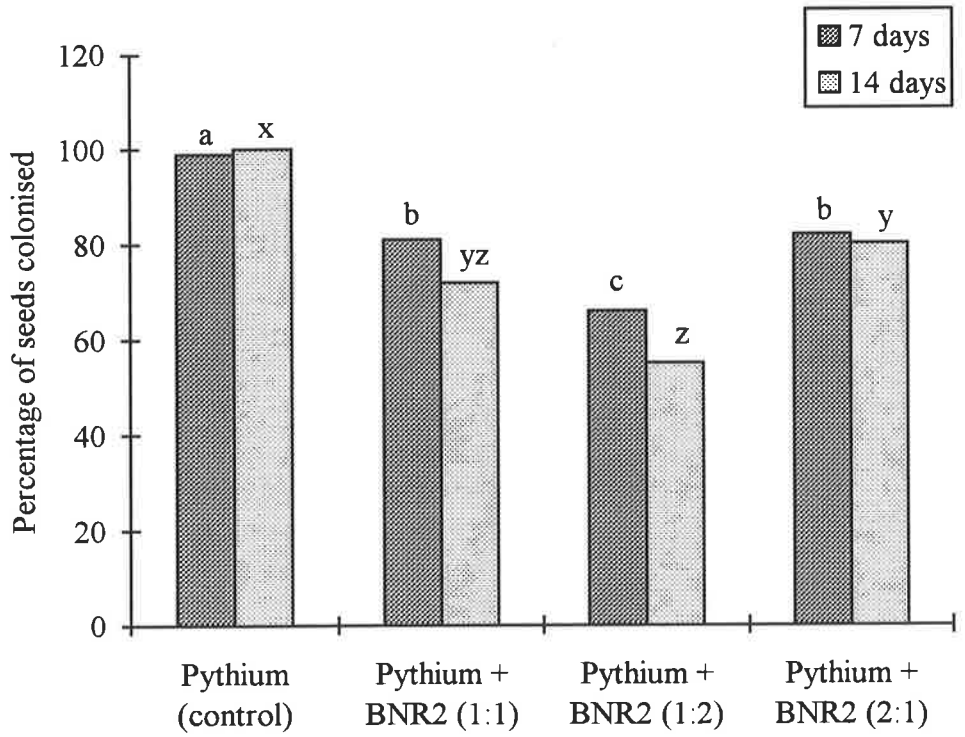
Fig. 7.3.A Competitive colonisation of seed residue by *P. u. sporangiiferum* in the presence of BNR1 at 7 and 14 days.

Fig. 7.3.B Competitive colonisation of seed residue by *P. u. sporangiiferum* in the presence of BNR2 at 7 and 14 days.

(A)


7 days
14 days
Percentage of seeds colonised
120
100
80
60
40
20
0
a
x
b
yz
c
z
b
y
Pythium (control)
Pythium + BNR1 (1:1)
Pythium + BNR1 (1:2)
Pythium + BNR1 (2:1)

(B)


7 days
14 days
Percentage of seeds colonised
120
100
80
60
40
20
0
a
x
b
yz
c
z
b
y
Pythium (control)
Pythium + BNR2 (1:1)
Pythium + BNR2 (1:2)
Pythium + BNR2 (2:1)

P. u. sporangiiferum was recovered from significantly fewer ($P \leq 0.05$) seeds when grown for 7 days with either BNR1 or BNR2 in the ratio 1:2 compared with that in the ratios 1:1 and 2:1 (Fig. 7.3.A & B). When *P. u. sporangiiferum*, however, was grown in combination with each of the two BNRs for 14 days, the number of seeds colonised by the pathogen at the inoculum ratio 1:2 was not significantly different ($P > 0.05$) from that at 1:1, but it was different ($P \leq 0.05$) from that at the ratio 2:1 (Fig. 7.3.A & B).

7.3.2.2 Saprophytic growth of *P. u. sporangiiferum* and BNR from colonised seed residue

Growth rates of *P. u. sporangiiferum*, BNR1 and BNR2 were not significantly different ($P > 0.05$) whether the inoculum ratio of pathogen to antagonist, prior to incubation for 7 and 14 days, was 1:1, 1:2 or 2:1 (data not shown).

The mean growth rate of *P. u. sporangiiferum* from the seed residue incubated for 7 or 14 days without BNR1 or BNR2, was significantly slower ($P \leq 0.05$) than that of either of the two BNR isolates grown in the absence of the pathogen (Fig. 7.4.A & B).

Growth of *P. u. sporangiiferum* from the seed residue in the presence of either BNR1 or BNR2 was significantly slower ($P \leq 0.05$) than that in the absence of the two antagonists (Fig. 7.4.A & B). In comparison with controls, the growth rate of the pathogen from the seed residue incubated for 7 and 14 days in the presence of BNR1 was lower by 42% and 27%, respectively, and in the presence of BNR2 by 27% and 29%, respectively.

The mean growth rate of BNR1 was not significantly affected ($P > 0.05$) by the presence of *P. u. sporangiiferum* in the same seed residue colonised for 7 or 14 days (Fig. 7.4.A). The mean growth rate of BNR2, however, was slightly lower ($P \leq 0.05$) in the presence of *P. u. sporangiiferum* in the seed residue which had been colonised

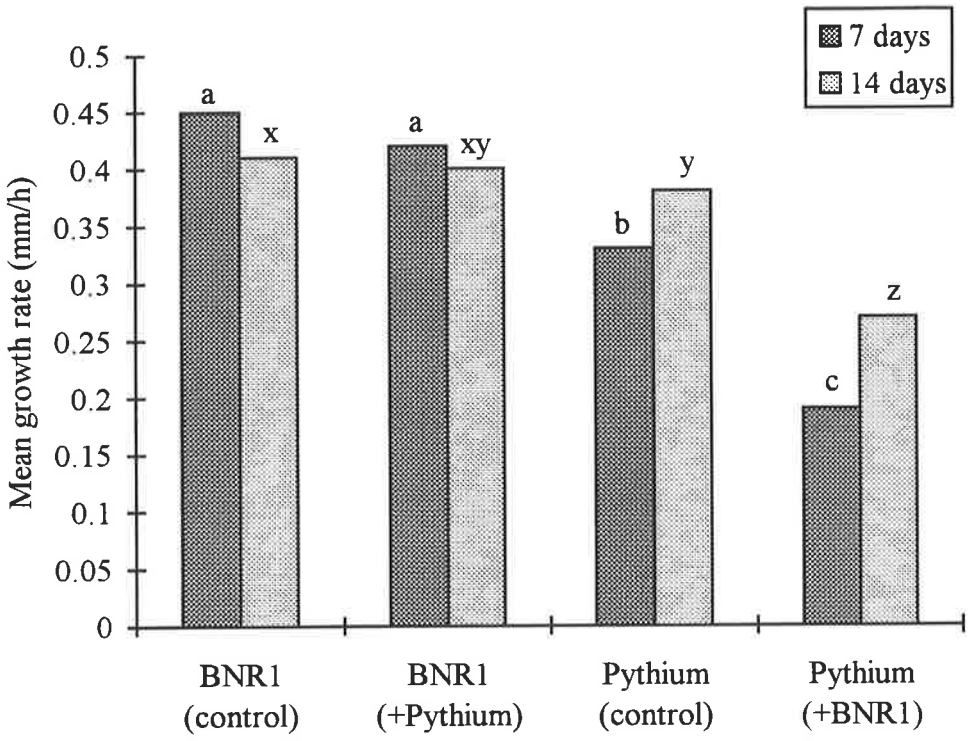
Fig. 7.4 Growth rates of *P. u. sporangiiferum* and BNR from colonised seed residue.

Linear growth rates of *P. u. sporangiiferum* (Pythium), BNR1 or BNR2 from control or dually colonised (+) autoclaved *Capsicum* seeds for 7 or 14 days in the inoculum ratios of 1:1, 1:2 and 2:1 of pathogen to antagonist, were determined over 91 hours in sterilised potting mix in plastic Petri dishes (90 mm diam.). Results for each fungus are means of combined data from ratios 1:1, 1:2 and 2:1. Data are means of 12 replicate plates for each pathogen × antagonist × inoculum ratio combination, and each control × inoculum ratio combination. Values for each incubation period followed by the same letter are not significantly different ($P > 0.05$) by Tukey's HSD test.

Fig. 7.4.A Mean linear growth rates of *P. u. sporangiiferum* and BNR1 from seed residue colonised by the fungi during 7 and 14 days of incubation in sterilised potting mix.

Fig. 7.4.B Mean linear growth rates of *P. u. sporangiiferum* and BNR2 from seed residue colonised by the fungi during 7 and 14 days of incubation in sterilised potting mix.

(A)


7 days
14 days
Mean growth rate (mm/h)
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
a
x
a
xy
b
y
c
z
BNR1 (control)
BNR1 (+Pythium)
Pythium (control)
Pythium (+BNR1)

(B)

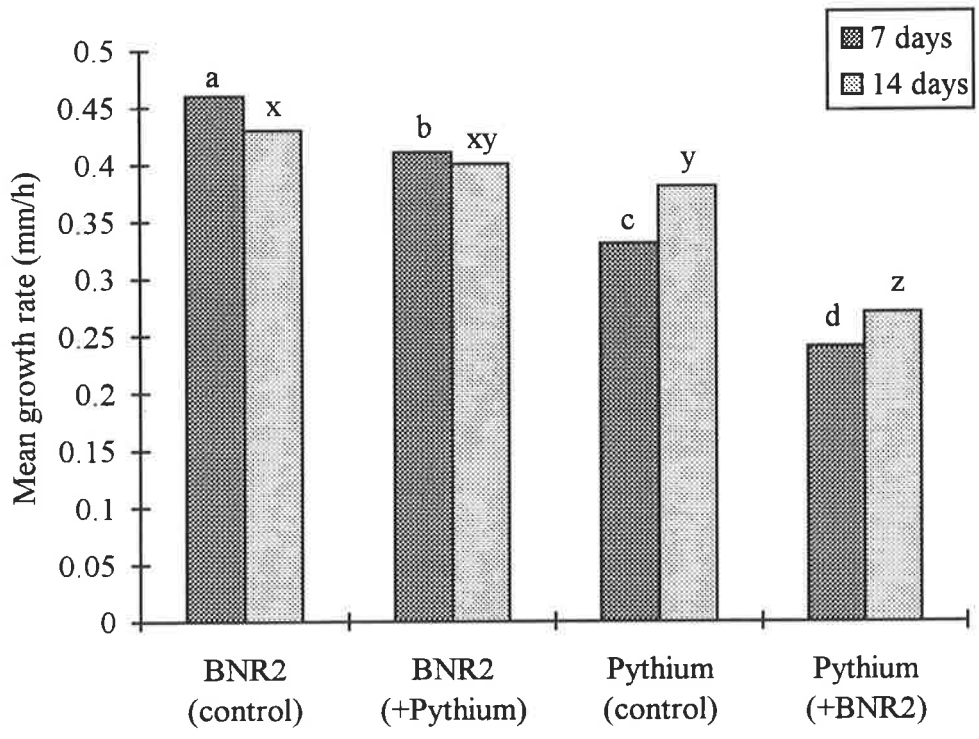

7 days
14 days
Mean growth rate (mm/h)
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
a
x
b
xy
c
y
d
z
BNR2 (control)
BNR2 (+Pythium)
Pythium (control)
Pythium (+BNR2)

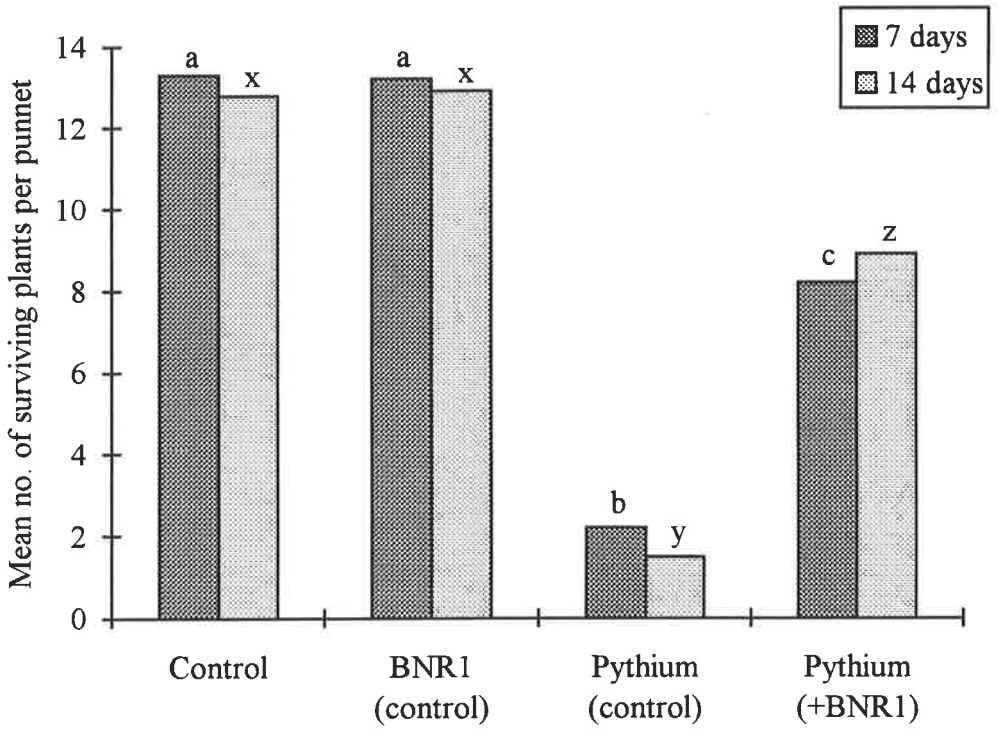
Fig. 7.5 Pathogenic activity of *P. u. sporangiiferum* as affected by the presence of BNR in the seed residue in pasteurised potting mix.

Autoclaved *Capsicum* seeds colonised by *P. u. sporangiiferum* and either BNR1 or BNR2, for 7 and 14 days at the inoculum ratios of 1:1, 1:2 and 2:1 of pathogen to antagonist, served as the fungal inocula (six seeds per punnet) in experimental punnets sown with 16 untreated *Capsicum* seeds. For controls, potting mix in punnets was inoculated with BNR1, BNR2 or *P. u. sporangiiferum* alone (using seeds colonised in ratio 1:1 only), or with sterile seeds. After 4 weeks at 25^{o}C (±5^{o}C) the number of surviving plants was compared with that in controls. Results for each treatment and controls are means of combined data from the ratios 1:1, 1:2 and 2:1. Data are means of 10 replicate punnets for each pathogen antagonist × inoculum ratio × incubation time combination, and for controls. Values for each incubation period followed by the same letter are not significantly different ($P > 0.05$) by Tukey's HSD test.

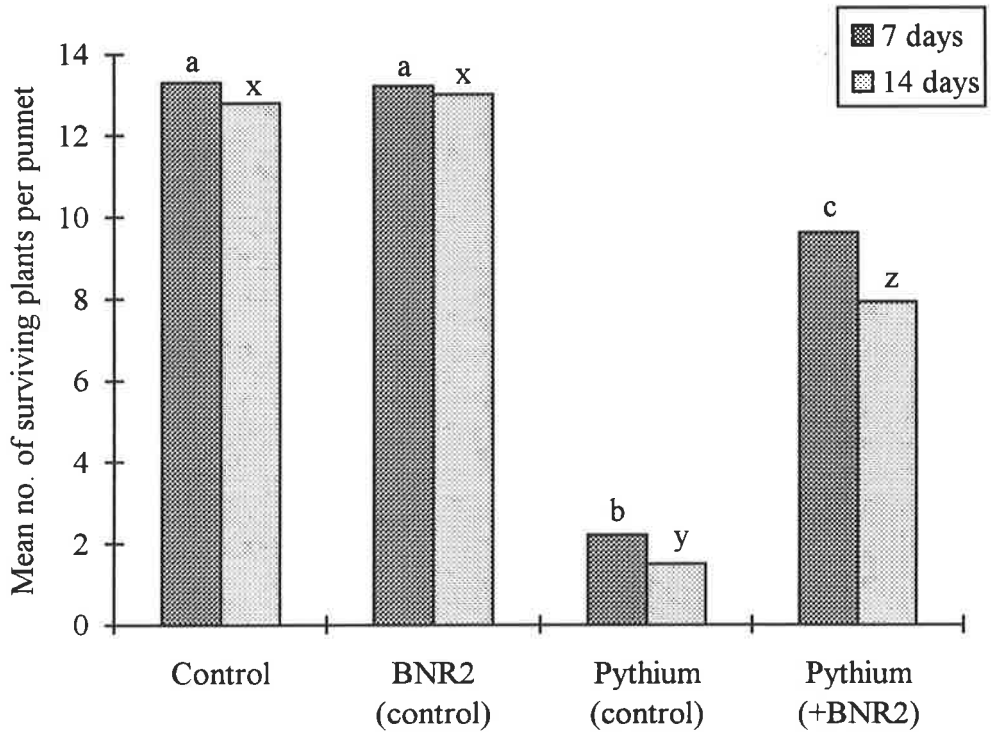
Fig. 7.5.A Pathogenic activity of *P. u. sporangiiferum* as affected by the presence of BNR1 in the seed residue.

Fig. 7.5.B Pathogenic activity of *P. u. sporangiiferum* as affected by the presence of BNR2 in the seed residue.

(A)


7 days
14 days
Mean no. of surviving plants per punnet
0
2
4
6
8
10
12
14
a
x
a
x
b
y
c
z
Control
BNR1 (control)
Pythium (control)
Pythium (+BNR1)

(B)


7 days
14 days
Mean no. of surviving plants per punnet
0
2
4
6
8
10
12
14
a
x
a
x
b
y
c
z
Control
BNR2 (control)
Pythium (control)
Pythium (+BNR2)

by the fungi for 7 days, but not for 14 days, in comparison with the BNR2 control (Fig. 7.4.B).

7.3.2.3 Pathogenic activity of *P. u. sporangiiferum* in the presence of BNR in the seed residue

Survival of *Capsicum* seedlings in the presence of *P. u. sporangiiferum* alone, applied in the seed residue colonised for 7 or 14 days, was significantly lower ($P \leq 0.05$) then that of the control seedlings grown with BNR1 or BNR2 only, or without added fungi (Fig. 7.5.A & B).

However, when *P. u. sporangiiferum* was introduced with either BNR1 or BNR2 in the seed residue colonised for 7 or 14 days, the survival of *Capsicum* seedlings increased significantly ($P \leq 0.05$) compared to that of the *P. u. sporangiiferum* control (Fig. 7.5.A & B).

Furthermore, survival of *Capsicum* seedlings in the presence of *P. u. sporangiiferum* in combination with either BNR1 or BNR2 was not significantly different ($P > 0.05$) whether the ratio of pathogen to antagonist applied to the seed residue was 1:1, 1:2 or 2:1 (data not shown).

Survival of *Capsicum* seedlings in the presence of BNR1 or BNR2 alone, applied on the seed residue colonised for 7 or 14 days, was not significantly different ($P > 0.05$) from that of the control seedlings grown without added fungi (Fig. 7.5.A & B).

7.4 DISCUSSION

Growth of *P. u. sporangiiferum* from millet seeds in sterilised or pasteurised potting mix was most rapid during the first 48 hours, with the hyphal growth slowing down

soon after, or even stopping in the case of pasteurised potting mix in punnets in the glasshouse. Hancock (1977) has suggested that mycelia of *P. ultimum* can be produced in abundance in response to exogenous nutrients, but are relatively short lived due to the susceptibility of hyphae to unfavourable environmental conditions or to antagonism from other saprophytic microorganisms. Lifshitz & Hancock (1983) also reported that *P. ultimum* has the potential to grow and reproduce in soil but that the behaviour is frequently modified in natural soil by biotic and abiotic factors.

BNR1 and BNR2 had similar patterns of growth in potting mix in the *in vitro* and *in vivo* experiments. Although generally slower than *P. u. sporangiiferum* in the potting mix and on 1/4 PDA, the two antagonists can still be considered as relatively fast growing organisms. The greatest linear growth rate of BNR1 and BNR2 after 68 hours of growth on 1/4 PDA at 25°C (Fig. 7.2.C) was 0.58 and 0.56 mm/h, respectively. Ichielevich-Auster *et al.* (1985) compared many isolates of *Rhizoctonia* spp., including *R. solani*, *R. zeae* and BNR., on the basis of their growth rates on PDA at 26°C for 3 days. A wide range of variation in mycelial growth rate was observed, ranging from 0.1 to 0.98 mm/h, with the average growth rate of the non-pathogenic isolates of about 0.4 mm/h. Similar observations were reported by Blair (1943) in his classic study on the saprophytic growth of *R. solani* in unsterilised soil. The fungus was able to grow for relatively long distances at *ca* 0.4 mm/h, utilising only those energy sources that were present in the soil, and quite independently of the inoculum from which its growth was initiated. Thus, the rapid and steady growth of BNR in glasshouse conditions is considered an indication of a good saprophytic ability.

Care must be taken in relating fungal growth in agar media to that in a more natural system. This has been clearly shown for *P. u. sporangiiferum*, where the pattern of fungal growth and the maximal growth rate on 1/4 PDA did not resemble that observed in sterilised or pasteurised potting mix in Petri dishes. In the case of BNR,

however, the rate and the pattern of growth on 1/4 PDA were similar to those observed in sterilised or pasteurised potting mix.

Colonisation of seed residue (i.e. the autoclaved *Capsicum* seeds) by *P. u. sporangiiferum* was significantly reduced, by up to 50%, in the presence of either BNR1 or BNR2 in sterilised potting mix during 7 or 14 days of incubation. The pre-emptive exclusion of the pathogen by prior establishment of each of the two antagonists in the residue, or the elimination or suppression of the pathogen after its establishment in that residue by each BNR, could have resulted in the decreased colonisation of the residue by *P. u. sporangiiferum.* Reduced colonisation of seeds by *P. u. sporangiiferum* in the presence of BNR1 or BNR2 in the inoculum ratio of 1:2, in comparison with that in inoculum ratios of 1:1 and 2:1, supports the competitive exclusion hypothesis. Thus, the increased inoculum potential of BNR next to the sterile seed residue could have been responsible for greater colonisation by BNR prior to that by the pathogen, despite the faster growth rate of *P. u. sporangiiferum* in sterilised potting mix. These observations are in agreement with the view that the colonisation of a potential substrate by a fungus is determined not only directly by its competitive saprophytic ability (with fast growth rate being one of the key factors) but also by its inoculum potential at or on the surface of that substrate. Furthermore, the share of a substrate obtained by a fungus is inversely determined by the inoculum potential of its competitors (Garrett, 1981). An example of the pre-emptive exclusion of secondary invaders of a residue by pioneer colonisers is given by Barton (1961), who reported that *Pythium mamillatum* did not colonise fragments of organic material, such as turnip and wood, when these contained pre-established sugar fungi, or cellulose- or lignin-decomposing fungi. He concluded that, in soil, *P. mamillatum* was restricted to virgin substrates only, and was not able to compete for colonised residue. Similarly, when wheat straw was allowed to stand as undisturbed stubble after harvest in the Pacific Northwest it became colonised by common air-borne fungal saprophytes, including *Alternaria*, *Aspergillus* and *Penicillium* spp. which, as the pioneer colonists

of that substrate, inhibited its colonisation by the pathogenic *Fusarium roseum* f. sp. *cerealis* 'Culmorum' (= *F. culmorum*) after burial in the soil (Cook, 1970).

Lack of growth of *P. u. sporangiiferum* from about 50% of colonised *Capsicum* seeds on WA could be the result of displacement or suppression of the pathogen in the seed residue by either BNR1 or BNR2. As the number of seeds colonised by *P. u. sporangiiferum* in combination with each of the two BNR isolates in the ratio 1:1 was not significantly different from that in the ratio 2:1, therefore, it might be appropriate to assume that at least in some instances BNR isolates were the secondary colonisers of the residue, inhibiting the pre-established *P. u. sporangiiferum*. Furthermore, the greatest reduction in the residue colonisation by *P. u. sporangiiferum* in the presence of BNRs in the ratio 1:2 may also indicate an increased antagonism by BNR against the pathogen in that residue. Lemanceau *et al.* (1993) reported that the antagonism of nonpathogenic *Fusarium oxysporum* against pathogenic *F. oxysporum* was strongly dependent on the density ratio of the antagonist to the pathogen, such that the higher the ratio, the stronger the antagonism. "Germination/lysis" may have been involved in the displacement of *P. u. sporangiiferum* from the seed residue by co-inhabiting BNR. This biological process involves germination of fungal spores, or other propagules, in response to nutrients released from the residue or, in some cases, by volatile compounds, followed by lysis and often death of the germlings (Cook *et al.*, 1978). The activities of antagonistic microorganisms were implicated by Lifshitz & Hancock (1983) as the key factors limiting the saprophytic development of *P. ultimum* in organic matter in natural soil at 21 to 30°C. Similarly, it has been reported by Qian & Johnson (1987) that soil conditions that favoured lysis of *P. ultimum* germlings, such as high pH and high P content, were also favourable for microbial growth, especially of bacteria, including actinomycetes. They suggested that germinating oospores were susceptible to degradation by antibiotics or enzymes produced in large quantities by antagonistic microorganisms under such favourable conditions. According to Stanghellini & Hancock (1971*a*), sporangia of *P. ultimum* were able to re-germinate

several times after the lysis of the first germling, although at decreasing frequencies, suggesting that "germination/lysis" can occur more than once for the same propagule, and that under reduced competitive stress some of these propagules may be able to escape the antagonistic effect. Suppressed germination of *P. u. sporangiiferum* propagules, by BNR in the seed residue, could have been responsible also for the reduced saprophytic activity of the pathogen. Suppression of the pathogen could have resulted from the energy stress imposed by the intense competition with BNR for available food sources, including exudates from the propagules themselves (Lockwood, 1981). Furthermore, changes in the chemical nature of the seed residue caused by products of BNR metabolism, including extracellular enzymes, could, to some extent, be responsible for continuous restriction of pathogen germination even under lower energy stress.

The reduction in rate of growth of *P. u. sporangiiferum* from the seed residue in the presence of either BNR1 or BNR2 was apparently the result of the competition between the fungi for limited nutrients and space (Paulitz, 1990) in the sterile potting mix in Petri Dishes. Furthermore, since the growth rate of neither BNR1 nor BNR2 was affected by the presence of *P. u. sporangiiferum*, it can be concluded that mycoparasitism and/or indirect antibiosis were assisting BNR in reducing the saprophytic growth of the pathogen. Lin & Cook (1979) concluded from their study that competition for the food base was apparently responsible for the suppression of growth of *F. roseum* 'Avenaceum' in pasteurised soil, from the oat substrate, infested with either *Trichoderma viride*, *Mucor hiemalis* or *M. plumbeus*. They suggested, however, that hyperparasitism or antibiosis were not involved in the suppression of *F. roseum*, since the two mechanisms were not observed in *in vitro* tests.

The presence of BNR1 or BNR2 in seed residue together with *P. u. sporangiiferum*, reduced the effectiveness of the pathogen in causing damping-off in *Capsicum* seedlings in pasteurised potting mix in glasshouse conditions. Reduction in growth rate

of *P. u. sporangiiferum* in the presence of BNR could have resulted in the pre-emptive exclusion of the pathogen from the host's infection sites by the faster growing antagonists, thus reducing the area of contact between the pathogen biomass and the host plant (Cook & Baker, 1983). Furthermore, increased competition between the fungi for seed or root exudates could have led to diminished vigour of pathogen hyphae, delaying or reducing the formation of lesions, and increasing the vulnerability of *P. u. sporangiiferum* to displacement in a lesion in infected host tissue by BNR (see Chapter 6). The colonisation of inoculum substrate by candidate antagonists, such as *Trichoderma viride, Mucor hiemalis* and *M. plumbeus,* discussed previously (Lin & Cook, 1979), also reduced the effectiveness of '*Fusarium roseum* 'Avenaceum' as a pathogen in pasteurised soil, without killing it or even replacing it in the substrate. This pathogen on its own in plant residue caused severe root rot and pre-emergence death of lentils, however, when co-inhabiting with antagonists in the plant residue, it was only weakly pathogenic to the host plants. Reduction in infection capacity was attributed to competition for substrates between the pathogen and the fast growing antagonists rather than antibiosis or hyperparasitism (Lin & Cook, 1979). Suppression of *P. ultimum* by antagonistic *P. nunn* was ascribed by, Paulitz & Baker (1987*b*), to reduced inoculum density and inoculum potential of the pathogen, which apparently was the primary cause of cucumber damping-off suppression in pasteurised soil. They also concluded that *P. nunn*, which was mycoparasitic on other *Pythium* spp. in *in vitro* studies, required organic substrates rather than living fungal hosts for antagonism and disease suppression to operate in pasteurised soil.

From the evidence presented in this chapter it is concluded that: 1) BNR1 and BNR2 are fast growing saprophytic fungi which can successfully compete with *P. u. sporangiiferum* for the same organic substrates; and 2) by eliminating or suppressing the inoculum potential of *P. u. sporangiiferum* in the presence of seed residue, both BNRs are able to reduce the saprophytic and pathogenic activities of the pathogen in the potting mix.

CHAPTER 8 GENERAL DISCUSSION

In this study, mechanisms of biological control of *P. u. sporangiiferum* by two BNR isolates were investigated both in *in vitro* and *in vivo* assays. The emphasis was placed on ecological attributes of the antagonists in relation to the pathogen, in conditions resembling those of the nursery environment for which biological control is intended. Based on the evidence presented in this thesis, it has been proposed that competition for resources, such as the host tissues with exudate-rich infection sites and the organic residue in the potting mix, is the principal factor influencing the interactions between *P. u. sporangiiferum* and BNR. It has also been suggested that BNR isolates employ at least three strategies to exert competitive advantage over *P. u. sporangiiferum*. These are: 1) pre-emptive exclusion of the pathogen from the host tissues by dense hyphal mats; 2) parasitism of pathogen hyphae and sporangia; and 3) reduction in pathogen growth by enzyme-mediated antibiosis. The outcome of competitive interactions between BNR and *P. u. sporangiiferum* is believed to include: 1) exclusion of the pathogen from seeds, crowns and root tips; 2) exclusion of the pathogen from organic residue; 3) displacement of the pathogen from lesions in seedling hypocotyls and roots; and 4) reduction of pathogenic and saprophytic activities of the pathogen. Accordingly, it has been postulated that the ability of BNR to capture and utilise resources, in the presence of a potential competitor, is the principal attribute of these biocontrol agents that brings about a successful control of *P. u. sporangiiferum* in nursery potting mix.

Information obtained in this study revealed for the first time the ability of BNR to parasitise another fungus, *P. u. sporangiiferum*, in agar media and on germinating *Capsicum* seeds, as well as their capacity to secrete lytic enzymes in sterilised potting mix. Cellulase and β-1,3-glucanase were shown to be inhibitory to the growth of *P. u. sporangiiferum* in agar-based tests, and their production by BNR in a synthetic

medium supplemented with mycelial preparation of the pathogen, suggests their role as cell-wall degrading enzymes. Production of cellulase by BNR has been previously suggested for *R. fragariae* (Cervone *et al.*, 1976) and *R. cerealis* (Walther & Gindrat, 1987) in agar-based assays only, however, production of β-1,3-glucanase was not considered in the above studies. Hyperparasitism and production of lytic enzymes are believed to be responsible for the reduction of inoculum potential of the pathogen both in the soil and in diseased host tissues.

Detailed study of colonisation of *Capsicum* seedlings showed the two BNR isolates to be spermosphere and rhizosphere competent, and that they densely colonised selected host tissues, such as seed coats, crowns and root tips. Pre-emptive exclusion of *P. u. sporangiiferum* from the host surfaces by dense hyphal mats of the antagonists is, therefore, considered a likely mechanism of biocontrol by BNR. Moreover, *P. u. sporangiiferum* was shown to colonise preferentially the same host tissues as did the two biocontrol agents, suggesting that the protection of susceptible plant surfaces by the two antagonists may be the result of competition between BNR and the pathogen for exudate-rich plant tissues.

BNR isolates did not appear to protect *Capsicum* seedlings against *P. u. sporangiiferum* by means of induced resistance in the host. However, cross protection has been implicated in this study, in the control of post-emergence damping-off by the two biocontrol agents. Colonisation of pathogen-induced wounds by BNR, apparently stimulated by exudates released from lesions, is believed to result in the displacement or suppression of *P. u. sporangiiferum* in diseased hypocotyls.

The results from *in vitro* and *in vivo* studies on the saprophytic behaviour of BNR support the hypothesis that the two antagonists are highly competitive saprophytic fungi. These potential biocontrol agents have been shown, in this study, to colonise organic substrates competitively and adversely affect the saprophytic and pathogenic activities of *P. u. sporangiiferum* in the potting mix.

From an industry point of view, a successful biocontrol product must have an efficient delivery system for the active organism. A sufficient quantity of the biological agent must be delivered in an appropriate state of activity and to the right place at the right time to attain economical control (Powell, 1991). As BNR isolates were screened for efficacy in controlling damping-off diseases in nursery seedlings, protection of seeds, young roots and hypocotyls by these antagonists is of primary importance. From the evidence presented in this thesis, it is clear that the two antagonists are able to protect both the subterranean and the aerial parts of the host. Thus, the two biocontrol agents could be effective in protecting a crop plant whether applied to seeds directly or introduced into potting mix at the time of sowing. However, since BNR isolates do not sporulate, formulation as dusts or powders for seed applications may not be the best method for delivery of these antagonists to potting mix. On the other hand, introduction of these biocontrol agents directly into potting mix on a food-base carrier, such as cereal grains or dried plant segments, may be the best delivery system for BNR.

An important requirement for a successful biocontrol product is that it survives after being delivered to the site of action, for as long as the plant is susceptible to disease. The ability of BNR isolates to persist in potting mix and within living host tissues for a long time fulfils that requirement and supports their potential as biocontrol agents. One way of enhancing the survival and activity of biocontrol agents at the time of delivery is to apply crop residues and/or organic matter to soil to stimulate their saprophytic growth. Such increased activity of microbial antagonists in the presence of organic amendments has been implicated previously in the suppression of damping-off and root rot pathogens in container media (Kwok *et al.*, 1987; Kuter *et al.*, 1988; Huang & Kuhlman, 1991; Hardy & Sivasithamparam, 1991). Because of the ability of BNR to compete successfully with *P. u. sporangiiferum* for organic residue, further increase in their antagonistic activities may be possible by the addition of organic substrates to nursery potting mixes. Seeds or seed by-products may be suitable (Harris *et al.*, 1993a). Incorporation of organic residues in combination with BNR

could be utilised in nurseries where longer-term rather than short-term disease control is required, or in plant growing media with low organic matter content. This approach of using organic amendments to promote biological control, however, requires further research in order to identify organic substrates that stimulate the activities of BNR rather than the pathogen.

The capacity of BNR to suppress the pathogen in a lesion, and thus prevent further disease development, could be of benefit to the nursery industry. This is because the two biocontrol agents could be employed to protect plant wounds created during nursery operations, such as trimming, grafting or defoliating, against the infection by *Pythium* spp., or even other pathogens. As only a few antagonists have been identified as being capable of displacing or suppressing their target pathogen(s) within infected host tissues (Cook & Baker, 1983), the potential of BNR as wound-inoculants could be evaluated in future work. In addition, BNR isolates have been shown to suppress Rhizoctonia damping-off in nursery seedlings (Harris *et al.*, 1991, 1994) and as such, could be tested for the ability to control foliar diseases, such as web blights of container-grown ornamental plants (Frisina & Benson, 1987) caused by pathogenic Rhizoctonias.

In order for a biocontrol product to be registered it has to fulfil the requirement of being non-toxic and non-pathogenic to crops, animals, people and the environment (Forsyth, 1990). As BNR were isolated from the environment in which biocontrol is to be applied, specifically from nursery seedlings and potting mixes, the risk to human health or the environment associated with their use is likely to be negligible. The two antagonists have been also shown, in this study and by Harris *et al.* (1993*b*, 1994), to promote the growth of nursery seedlings in the absence of *P. u. sporangiiferum.* The mechanism of plant growth promotion by these biocontrol agents, however, is not known, but it could involve the production of growth-stimulating metabolite(s) and/or suppression of minor pathogens in the rhizosphere. The use of BNR as growth-promoting agents has the potential for increasing nursery productivity without

increasing the actual growing area or the costs associated with the use of chemical pesticides. It is, therefore, recommended that a detailed study be undertaken to ascertain the mechanism of plant growth promotion by BNR, in order to explore the potential of these fungi in improving growth of seedlings in nursery potting mixes. The capacity of BNRs to establish a long-term association with their host plants could also be evaluated in terms of benefits of that association not only to the nursery industry but also to potential customers. One such benefit may be the "carry-over" protection of young seedlings against damping-off and root rot diseases caused by *P. ultimum*, and perhaps other pathogens like *R. solani*, after the seedlings are transplanted from a nursery to a garden or field.

Integration of biological control may be necessary in some cropping systems where no single component is effective in suppressing a particular disease, or where more than one pathogen must be controlled (Upadhyay & Rai, 1988). Combining more than one biocontrol agent can be advantageous to disease control; for example *Pythium nunn*, provided greater control of Pythium damping-off of cucumber seedlings when it was applied in combination with *Trichoderma harzianum* (Paulitz *et al.*, 1990). However, integrating BNR1 and BNR2 could not necessarily be appropriate, as the two antagonists share similar ecological attributes and are likely to compete for the same resources. Thus, the overall result could be that of reduced rather than enhanced disease control. However, pairing BNR isolates with other biocontrol agents that have slightly different ecological requirements to those of BNR, such as *P. nunn*, *P. oligandrum*, *Trichoderma* spp. or *Pseudomonas*, may prove to be important in widening the spectrum of activity of BNR against various pathogens of both nursery and field crops.

The economic feasibility of biological control depends on a number of factors including: 1) the market value of the crop to be protected; 2) the efficacy of the antagonists for a given disease; 3) attitudes of growers towards biological control (Reichelderfer, 1981). Damping-off diseases occur commonly in the nursery industry

and are responsible for substantial losses in bedding plants, bulb and floriculture crops, foliage plants and container-grown woody plants (Niedbalski-Cline, 1988) and, as such, require an appropriate control strategy. Because the market price of crops requiring protection varies, high value horticultural crops are considered the best targets for biocontrol inoculants (Jutsum, 1988; Lumsden & Lewis, 1988; Campbell, 1989*a*). The efficacy of BNR as potential biocontrol agents of Pythium damping-off in nursery potting mix has been shown in this study. Characteristics of BNR, such as good saprophytic ability, spermosphere and rhizosphere competence, longevity and the capacity for lytic enzyme production and parasitism, increase their potential effectiveness as biocontrol agents. In addition, the lower biological diversity and controlled glasshouse environment, provided by the nursery industry, are believed to be more favourable to the success of biocontrol using BNR than the more varied and unpredictable field environments. Finally, as the acceptance of biological control by the growers is crucial for its success, proper marketing and educational programs should be put in place after the product is developed.

In conclusion, antagonistic BNR have great potential to control Pythium damping-off in nursery crops and to act as plant growth promoting agents in the absence of the disease. If an effective BNR-based biocontrol product can be offered at a cost competitive with that of alternative control strategies then it should be commercially viable. Information on the ecology and mode of action of BNR, provided by this study, should support further efforts to develop and commercialise this potential biocontrol product.

APPENDIX

Wetting solution (Herr, 1979)

lactic acid	0.1 ml
Tween 80®	0.1 ml
distilled water	100 ml

Lactoglycerol trypan blue

lactic acid	650 ml
glycerol	600 ml
distilled water	880 ml
trypan blue	1.3 g

Lactoglycerol

lactic acid	250 ml
glycerol	500 ml
distilled water	250 ml

Gelatin block

gelatin	10 g
glycerol	2 ml
distilled water	100 ml

Nutrient solution

KNO_3	6.7 g
$Ca(NO_3)_2.4H_2O$	3.6 g
NH_4NO_3	6.0 g
H_3BO_3	120 mg
Na_2MoO_4	15 mg
deionised water	50 l

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