INTERACTIONS BETWEEN ARBUSCULAR MYCORRHIZAL FUNGI AND OTHER ROOT-INFECTING FUNGI

Rina Sri Kasiamdari

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Faculty of Agricultural and Natural Resource Sciences
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Department of Soil and Water
Waite Campus
The University of Adelaide

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ABSTRACT

Rina Sri Kasiamdari. **Interactions between arbuscular mycorrhizal fungi and other root-infecting fungi.** PhD thesis, Department of Soil and Water, The University of Adelaide, Australia

Pot cultures, which are a system to multiply propagules of arbuscular mycorrhizal (AM) fungi, often have a risk of fungal contaminations. A common contaminant fungus of pot cultures was isolated, and identified and characterised as binucleate *Rhizoctonia* sp. (BNR) using traditional and molecular techniques. The BNR isolate CFM1 had septate hyphae and monilioid cells, and two nuclei inside each cell. The isolate belonged to anastomosis group (AG)-Bo. A molecular approach based on the polymerase chain reaction (PCR) confirmed the identity of BNR isolate CFM1. The ITS sequences had high similarity with isolates of BNR AG-Bo and AG-A, but not with other sequences of isolates of different AGs of BNR available in GenBank.

BNR specific-primers were designed from the ITS sequences with the aim of providing tools for detection of this fungus in pot cultures. The BNR-specific primers were specific against various non-AM and AM fungi, mycorrhizal roots and uninfected roots. Although they amplified DNA from mycelium of BNR AG-A, due to similarity of ITS rDNA sequences, the BNR-specific primers were useful for monitoring the presence of BNR AG-Bo or AG-A in roots and in soils. From 16 pot cultures tested, seven mycorrhizal root samples of different hosts and four soils gave positive signals with those primers, and were contaminated with BNR. The labelled BNR-specific primers could be used for quantification of fungal DNA in infected roots using a DNA hybridisation assay.

The pathogenicity of BNR isolate CFM1 on mung bean was tested *in vitro* and in the glasshouse and compared to isolates of *Rhizoctonia solani* of different AGs. BNR isolate CFM1 rapidly infected roots, and caused disease symptoms; however, it was less pathogenic than most isolates of *R. solani*. BNR isolate CFM1 had negative effects on root growth of mung bean in a long-term inoculation experiment in soil.

Interactions between the AM fungus, *Glomus coronatum*, and BNR isolate CFM1 or *R. solani* were studied in the root systems of mung bean. In soil without phosphorus (P), *G. coronatum* reduced the infection by BNR isolate CFM1, but not by *R. solani*. Improved P nutrition appeared to enhance plant growth, and had little effect on disease severity. During interactions between *G. coronatum* and BNR isolate CFM1 or *R. solani*, defence-related compounds are produced. Cytochemical reactions showed that inoculation with BNR or *R. solani* in non-mycorrhizal and mycorrhizal roots induced qualitative changes in the accumulation of defence-related compounds at sites of BNR or *R. solani* penetration.

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STATEMENT

I declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Rina Sri Kasiamdari
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Dedicated to the memory of my mother

CHAPTER 1. INTRODUCTION

1.1. OVERVIEW OF PROBLEM

Arbuscular mycorrhizas (AM) occur very commonly in a wide range of plants. The roots of the host plants and the AM fungi live in a balanced or symbiotic relationship in which both partners usually gain benefit from the association (Smith and Gianinazzi-Pearson, 1988). As AM fungi are obligate symbionts, their propagules must be grown on roots of an appropriate host plant (Williams, 1991). Producing clean, pathogen-free and high quality inoculum of AM fungi is a priority for research and commercial scale use of mycorrhizas. Although attempts have been made to produce propagules in *in vitro* culture, the use of open pot cultures of AM fungi on host plants has been the most commonly used technique for increasing propagule numbers (Menge, 1984). Mixed inoculum of spores and root segments from open pot cultures of mycorrhizal plants has been the usual source of inoculum for research purposes.

Microorganisms, usually saprophytes and parasites, including many bacterial and fungal species, frequently infect pot cultures, and can be transferred to new pot cultures with or within spores (Menge, 1984; Brundrett, 1991). Plant pathogens can also be found in, and recovered from, pot cultures (Menge, 1984). It has not yet been proven conclusively that plant pathogens in general can reduce the inoculum potential of AM fungi, affect mycorrhizal symbiosis or reduce plant growth responses to AM fungi. Therefore, further understanding of the biology of contaminant fungi is needed. The lack of such information has hampered the development of effective quality control of other organisms in pot cultures.

Several techniques such as heat treatments (Menge *et al.*, 1979) or pesticides (Menge *et al.*, 1979; Seymour *et al.*, 1994, Bakhtiar *et al.*, 2001) have been used, and shown to reduce contamination by unwanted organisms and eliminate parasites and plant pathogens. Careful preparation of initial inoculum, sterilisation of growth media and good sanitary control in greenhouse conditions have also been employed as preventative measures to eliminate or reduce contaminants (Menge, 1984). Methods to assess contamination have relied on the observation of morphological characteristics under the microscope, which is often laborious and time-consuming for a large number of samples. Furthermore, the identification of fungi that infect roots is often difficult, due to similarities in morphology of hyphae between fungal species. Therefore, it is essential to develop a quick and reliable method to identify and detect contaminants in pot cultures, which is also directly applicable to infected plants.

In recent years, the polymerase chain reaction (PCR) has been employed as an important technique for fungal detection and identification, offering higher sensitivity and specificity than many traditional methods (Henson and French, 1993). This method can be used for identification and detection of certain fungi in soil and roots if specific primers are designed (Kageyama *et al.*, 1997). Species-specific primers developed from sequences within the ITS region of the rDNA have the potential to be used in diagnostic studies (Mazzola *et al.*, 1996). The simplicity of this technique and its potential to detect very small numbers or amounts of target organisms, make it a suitable method for monitoring pathogens (Vollosiouk *et al.*, 1995), and for plant disease detection (Henson and French, 1993). However, the potential use of PCR to detect fungal contaminants in pot cultures has never been explored. Clearly, an efficient and sensitive method, which specifically detects and differentiates the fungal contaminant from AM fungi in plant tissues, would be extremely beneficial.

In Australia and world wide, binucleate *Rhizoctonia* spp. (BNR), which are common in roots and soil (Cubeta *et al.*, 1991), have been found in roots used to culture AM fungi (S. Smith, personal communication). The presence in roots of non-pathogenic fungi introduces a confounding factor into experiments on plant growth and nutrition and is a serious problem in studies of gene expression. A concurrent study of BNR and AM fungi would be useful, because they may influence each other within plant root systems and affect physiological processes of the plant. Little is known about the effect of the interactions between AM fungi and BNR on plant growth. Comparison with a pathogenic *Rhizoctonia* species, i.e. *R. solani.* would be an advantage since this fungus is a related, morphologically similar plant pathogen that causes major diseases in various crops and is commonly associated with members of BNR in nature (Anderson, 1982; Sneh *et al.*, 1996). Attempts to control *Rhizoctonia* diseases chemically are not always effective (Frisina and Benson, 1988; Carling *et al.*, 1990); therefore attention has been directed towards alternative methods such as the use of AM fungi to control disease (Caron, 1989).

Interactions between AM fungi and other root-infecting fungi have been studied (see Chapter 2). The benefits of AM fungi have received increased attention not only in terms of improved nutrition, but because of the resistance they may confer on host plants to disease caused by root pathogens (Harley and Smith, 1983; Caron, 1989; Linderman, 1994). Studies have shown that the protective effects of AM fungi against pathogens and disease development can sometimes be attributed to better plant nutrition, enhanced growth and physiological stimulation in mycorrhizal plants (Dehne, 1982). The mechanisms involved in mycorrhiza-pathogen interactions are still poorly understood. One of the more common explanations for protection of roots by AM fungi is based on improved P uptake by the mycorrhizal plant (Dehne, 1982). However, further studies are needed since results on the effect of improvement of P nutrition are still conflicting, with reports stating that disease might be increased, decreased or

not affected (Perrin, 1990). Infection by AM fungi might induce a slight and transient activation of metabolic pathways related to disease resistance mechanisms (Harrison and Dixon, 1993; Volpin *et al.*, 1994). Several compounds and enzymes are likely to be involved in mycorrhizal symbioses, and could predispose the plant roots to a more rapid response to infection by a pathogen (Benhamou *et al.*, 1994; Gianinazzi-Pearson *et al.*, 1996). However, little attention has been focused on this specific topic.

1.2. AIMS OF THE PROJECT

The general aim of the project was to elucidate the biology and pathogenicity of BNR as a fungal contaminant in mycorrhizal pot cultures, as well as develop a method for effective detection of BNR in pot cultures. The interactions between BNR and the AM fungus, *Glomus coronatum* and *R. solani* were also investigated. This project was divided into four sections:

1. Characterisation and identification of BNR isolated from a mycorrhizal pot culture.
2. Design of BNR-specific primers for PCR detection of the presence of BNR in mycorrhizal root tissues and soil of pot cultures, and for quantification of fungal infection in roots.
3. Tests of the pathogenicity of the BNR isolate to the host in comparison with pathogenic *R. solani* isolates.
4. Studies of interactions between the AM fungus, *G. coronatum*, and BNR or *R. solani* in relation to improved phosphorus nutrition and detection of defence-related compounds produced during the interactions.

CHAPTER 2. LITERATURE REVIEW

2.1. INTRODUCTION

The roots of most plant species live in symbiosis with certain fungi, referred to as mycorrhizal fungi. The arbuscular mycorrhizal (AM) fungi have been recognised to play an important role in improving plant growth, enhancing nutrient uptake and protecting plants against pathogen attack (Linderman, 1994). Due to current inability to grow AM fungi in the absence of the host, inoculum must be produced in pot culture (Menge, 1984; Williams, 1991). Maintaining clean pot cultures is not easy due to problems of fungal contamination. Microorganisms, including plant pathogenic fungi such as *Rhizoctonia* sp. and binucleate *Rhizoctonia* sp. (BNR), are often found in pot cultures and there is currently limited information on the detection of fungi causing contamination in pot cultures.

Numerous studies have been done on the interactions between AM fungi and plant pathogenic fungi. AM fungi have been reported to have suppressive effects on diseases caused by a number of plant pathogenic fungi (see Table 2.1). Although conflicting results are still being obtained from studies on mycorrhiza-pathogen interactions, several mechanisms are likely to be involved in the interactions between AM fungi and root pathogenic fungi.

This chapter covers the main characteristics of AM fungi, how they develop in root systems, their benefits, especially in improving P uptake and enhanced plant growth, and problems in growing them in the pot cultures. The interactions between AM fungi and plant pathogenic fungi are reviewed. The genus *Rhizoctonia,* particularly BNR and *R. solani,* is reviewed with respect to infection, disease symptoms, anastomosis groups and

pathogenicity, with emphasis on BNR. The development of molecular techniques for detection and identification of this fungus is discussed.

2.2. ARBUSCULAR MYCORRHIZAL SYMBIOSIS

2.2.1. Occurrence and characteristics

Arbuscular mycorrhiza (AM) is the most common type of mycorrhizal association, and is a mutualistic symbiosis between fungi in the order Glomales and the roots of most higher plants (Schenck and Perez, 1990). So far, more than 80% of plant species examined can form mycorrhizal associations (Smith and Read, 1997). The symbiosis occurs in the majority of plant families, but most members of *Caryophyllaceae, Brassicaceae, Juncaceae, Proteaceae, Cyperaceae,* and *Chenopodiaceae,* and the genus *Lupinus* (Leguminosae), rarely form mycorrhizas (Brundrett, 1991).

AM fungi are obligate symbionts that depend completely on plant roots for extended growth and completion of their life cycle (Williams, 1991). During their life cycle, AM fungi are characterised by developing spores or sporocarps, intercellular and intracellular growth inside the root of infected plants, including hyphal coils, and highly branched arbuscules. In some cases vesicles are also formed (Harrison, 1997). AM fungi infect only specific root tissues, such as the epidermal and cortical tissues, but do not usually enter meristems and vascular tissues (Bonfante-Fasolo and Perotto, 1992). In symbiotic interactions of plant roots with AM fungi, the plant benefits from an enhanced supply of mineral nutrients, especially P, while the fungus, in turn, receives reduced carbon from the plant (Smith and Gianinazzi-Pearson, 1988).

The fungi are placed in the class Zygomycetes, the order Glomales, and representative genera include *Acaulospora, Entrophospora, Gigaspora, Glomus, Sclerocystis* and *Scutellospora* (Morton and Benny, 1990). *Glomus,*

Acaulospora and *Sclerocystis* are characterised by the intraradical production of both vesicles and arbuscules, and are frequently referred to as vesicular-arbuscular mycorrhizal (VAM) fungi, whereas *Gigaspora* and *Scutellospora* produce only arbuscules and inter- and intracellular hyphae. They do not produce vesicles in infected roots, but do form auxiliary cells on extraradical hyphae (Gerdemann, 1968; Smith and Read, 1997).

2.2.2. Infection and development of AM fungi

Infection of roots by AM fungi involves several steps: (i) spore germination, (ii) hyphal growth around roots, (iii) appressorium formation, (iv) penetration by intraradical hyphae, (v) arbuscule formation (Harley and Smith, 1983). The first indication of recognition between the fungus and the plant takes place when hyphae growing from spores in the soil or from adjacent plant roots contact the root surface and then differentiate to form an appressorium (Abbott *et al.*, 1992; Bonfante-Fasolo and Perotto, 1992). Penetration of the roots by hyphae occurs via the appressoria, partially by mechanical pressure (Carling and Brown, 1982), or by enzymatic activity (Garcia-Romera *et al.*, 1990).

Once penetration of the epidermis has been achieved, the fungus produces intracellular coils or intercellular hyphae, which grow into the inner cortex of the roots. Development within the root cells continues, where hyphal branches penetrate the cortical cell walls and produce highly branched, terminal structures called arbuscules (Harrison, 1997). By transplanting leek (*Allium porrum* L.) seedlings into a pot culture containing an established *G. versiforme* mycelium, Brundrett *et al.* (1985) found that the first arbuscules were initiated between days 3 and 4, and started as a trunk with a few branching hyphae. The development of arbuscules was completed in 4 to 5 days. These findings were confirmed by Rosewarne *et al.* (1997) by using a nurse pot system with *G. intraradices* and tomato (*Lycopersicon esculentum* L.)

to monitor infection and arbuscule development. Arbuscules are believed to play a role in the transfer of nutrients and are the sites for movement of soil-derived nutrients, such as P and Zn, to the plant (Smith and Read, 1997).

Following the formation of arbuscules, some species of AM fungi, except *Scutellospora* and *Gigaspora,* form thick-walled vesicles (Gerdemann, 1968; Smith and Read, 1997). Vesicles may be formed intercellularly or intracellularly in the root cortex (Abbott, 1982; Buwalda *et al.*, 1984). Vesicles contain lipids, have numerous nuclei, and are presumed to be storage organs. They sometimes function as survival propagules when the infected root dies and disintegrates (Biermann and Linderman, 1983; Harrison, 1997; Smith and Read, 1997). In addition to internal growth, the fungus also develops a network of extraradical hyphae that extends from the root into the soil, and is responsible for the acquisition of mineral nutrients (Smith and Gianianazzi-Pearson, 1988; Smith *et al.*, 1992).

The spread of infection in the roots normally follows a sigmoid form. An initial lag phase, where the percentage of mycorrhizal infection remains low, is followed by a rapid increase in infection, in which fungal spread exceeds the rate of root growth, and is then followed by a plateau phase where spread of the fungus and the growth of root are constant with respect to each other. Rapid infection (short lag phase and rapid spread), which is determined by root compatibility, propagule density, growth rate of the roots and competition between roots of different species (Hayman, 1983), have been shown to be important in influencing nutrient absorption and growth (Smith and Walker, 1981). Nutrient status in the soil, especially P, may affect plateau levels of mycorrhizal infection (Sanders and Sheikh, 1983; Bolan *et al.*, 1984; Smith and Read, 1997; Dickson *et al.*, 1999a). Environmental factors, such as propagule density (Allen, 1989; Abbott and Robson, 1991), temperature (Bowen, 1987; Haugen and Smith, 1992), and light (Smith and Gianinazzi-Pearson, 1990) influence the percentage infection of roots by AM fungi and development of arbuscules.

2.2.3. Phosphorus nutrition and growth

The effects of AM fungi on plant growth and nutrition are well documented. Increased growth has been demonstrated in a variety of host plants where mycorrhizal plants generally have higher dry weights of roots and shoots, lower root:shoot ratio, and higher tissue P concentration than non-mycorrhizal plants (Smith and Read, 1997). Mycorrhizal plants often show better growth than non-mycorrhizal plants in soils low in plant-available P, as a direct result of increased absorption of P and other mineral nutrients (Hayman, 1983; Schwab *et al.*, 1983; Smith and Gianinazzi-Pearson, 1988). Nutrient uptake by mycorrhizal roots is often more efficient and greater than in the uninfected control plants. This is because the mycorrhizal plants possess a network of extraradical hyphae that gives a larger and better absorbing surface (Sanders and Sheikh, 1983). The extraradical hyphae of AM fungi, which extend throughout the soil and beyond the zone of mineral depletion surrounding the plant root, can absorb P from the soil solution and translocate it to the root (Pearson and Tinker, 1975; Gianinazzi-Pearson and Gianinazzi, 1983; Smith and Gianinazzi-Pearson, 1988; Jakobsen *et al.*, 1992).

Increasing soil P results in reduced infection of plant roots by many AM fungi, which may be a direct result of lower carbohydrate concentrations within the roots (Same *et al.*, 1983; Smith and Gianinazzi-Pearson, 1990; Thomson *et al.*, 1991; Amijee *et al.*, 1993). Pearson *et al.* (1994) found that when the soil P increased, the percent of total infection by *Glomus* sp. in subterranean clover (*Trifolium subterraneum* L.) was reduced, whilst infection by *S. calospora* was largely unaffected by increasing amounts of P applied to the soil. The authors suggested that these differences were caused by differences in the ability of each isolate to take up and transfer P to the host. *S. calospora* has been shown to have a poor ability to increase the P content of some hosts compared to *Glomus* sp. (Jakobsen *et al.*, 1992; Pearson and Jakobsen, 1993). The effect of P supply is partly mediated by the rate of

growth of the root system (Smith and Walker, 1981). Bruce *et al.* (1994) found that an increase in the rate of growth in length of roots contributed to the low values of percent infection, and addition of P reduced the number of arbuscules and vesicles.

Direct evidence about the efficiency of P absorption has been achieved by measuring the inflows of P in mycorrhizal roots, and these were consistently higher in mycorrhizal plants than in non-mycorrhizal plants (Sanders *et al.*, 1977; Smith *et al.*, 1986; Smith *et al.*, 1994; Dickson *et al.*, 1999b). For example, Dickson *et al.* (1999a) investigated the effect of two AM fungi, *G. coronatum* (WUM16) and *S. calospora* in *Allium porrum* L. and found that P concentrations in roots and shoots of mycorrhizal plants inoculated with either *G. coronatum* or *S. calospora* were higher than in non-mycorrhizal plants, especially at later harvests. In some studies, the enhanced P nutrition and improvement of growth by AM fungi have been shown to reduce the severity of disease caused by plant pathogenic fungi (see section 2.2.5.1 for more details).

2.2.4. AM inoculum and the problem of contaminant microorganisms

As with other soil-borne and most root-inhabiting fungi, AM fungi have a variety of propagules, such as spores, living hyphae, isolated vesicles, mycorrhizal root segments, and external hyphae in the soil, which are capable of initiating infection and can be used as inoculum (Brundrett, 1991). Inoculum used in experiments usually consists of a bulky mixture of infected roots and spores in soil, termed 'mixed inoculum' (Menge and Timmer, 1982). Hyphae in soil originating from an established hyphal network can be effective sources of inoculum (Abbott *et al.*, 1992). Root pieces infected by AM fungi are good sources of inoculum because they contain active hyphae with intraradical vesicles (Menge and Timmer, 1982). Spores can be used as sources of inoculum, especially for establishing pot cultures for experimental

or identification purposes (Smith and Read, 1997). However, they are slower to infect the roots, and their survival in storage and ability to infect the roots are not as consistent as that of mixed inoculum (Daniels and Menge, 1981).

Since AM fungi are obligate symbionts, all inoculum must be grown on roots of an appropriate host plant, usually in pot cultures (Menge, 1984; (Williams, 1991). It is difficult to maintain pot cultures free from the risk of pathogen contamination (Schüßler, 1999 and see Chapter 1.). AM fungi may be parasitised by actinomycetes, chytrids, and other fungi, which can be transferred to new pot cultures within spores (Brundrett, 1991). Chytridiaceous fungi such as *Phlyctochytrium* sp. and *Rhizidiomycopsis stomatosa* have been observed to produce sporangia on spores of *Glomus*, *Gigaspora* and *Acaulospora* (Ross and Ruttencutter, 1977; Schenck and Nicolson, 1977; Daniels and Menge, 1980). *Pyronema, Trichoderma, Peziza ostracoderma* and many bacterial species frequently infest sterilised soil, although they do not appear to affect plant growth or mycorrhizal symbiosis (Menge, 1984). Bacteria-like organisms (BLOs) were reported to be present inside the spores of AM fungi (Mosse, 1970), and *Burkholderia* is commonly present in high numbers during life stages of *Gi. margarita* (Scannerini and Bonfante-Fasolo, 1991; Bianciotto *et al.*, 1996). Plant pathogens with saprophytic phases, such *as Fusarium, Pythium, Alternaria, Papulospora* spp. (Menge, 1984) and *Rhizoctonia* (Williams, 1985), can contaminate pot cultures, and care should be taken to eliminate them.

It has not yet been studied in detail how fungal contaminants or parasites can affect inoculum potential of AM fungi, affect mycorrhizal symbiosis or reduce plant growth responses. However, several methods have been used to prevent such fungal contaminants or parasites from becoming established in pot cultures. These include preparation of initial inoculum, sterilisation of soil with steam, fumigation, irradiation, pasteurization or heat treatment prior to establishing pot cultures, and good sanitary control in

greenhouses (Menge, 1984; Douds *et al.*, 2000). The fungicide metalaxyl was effective in preventing contamination by *Pythium myriotylum* of a *Glomus* culture on maize (Seymour *et al.*, 1994), and has been recommended for routine application in AM fungus pot cultures since it did not affect mycorrhizal development. Metalaxyl was also effective in controlling plant pathogens belonging to the order Peronosporales (Buchenauer, 1990). The fungicide ethazole was especially effective in preventing parasitism of AM fungi by *Phlyctochytrium* (Ross and Ruttencutter, 1977). Benlate (benomyl) has been shown to interfere with mitosis in cells of *Aspergillus nidulans* (Davidse, 1986) and to affect the microtubules of *Fusarium acuminatum* (Howard and Aist, 1977; 1980). However, benlate has been shown to reduce mycorrhizal plant growth and percentage of infection by AM fungi (Nemec, 1980; Kough *et al.*, 1987; Fitter and Nichols, 1988; Sukarno *et al.*, 1993). Aliette (fosetyl-Al) can control downy mildew as well as *Phytophthora* diseases in various crops (Buchenauer, 1990), and this fungicide was shown to reduce the root length and the length of infected roots of onion, but did not influence the number of living intercellular hyphae (Sukarno *et al.*, 1993).

Recently, 'glomalean' contamination has been demonstrated following phylogenetic analyses of nucleotide sequences in databases. Sequences of SSU rRNA obtained from a culture thought to be *Scutellospora castanea* clustered with sequences characteristic of the Ascomycetes, not the Glomales, suggesting that the spores examined in the study were contaminated (Schüßler, 1999). Redecker *et al.* (1999) reported high numbers of contaminating sequences in studies of 'glomalean' spores. The authors suggested that contamination was likely to have arisen from organisms such as algae, bryophytes, fungal spores, and mycoparasites, or micoorganisms that attached to the spore wall of 'glomalean' fungi, and are difficult to remove completely.

Observation of morphological characteristics of fungal contaminants in root samples has been used to assess the presence of contaminants in pot cultures. However, this method is laborious and time consuming for a large number of samples. Generally, if the populations of fungal contaminants are high in pot cultures, the inoculum must be discarded. Since very little is known about the effect of fungal contaminants on AM fungi, research is needed to study the interactions between fungal contaminants and AM fungi and their effects on plant growth, disease severity and root infection. Previous studies on the interactions between AM fungi and plant pathogenic fungi are reviewed in the next section.

2.2.5. Interactions between AM fungi and plant pathogenic fungi

Besides the benefits of improving plant growth, AM fungi can increase resistance or tolerance of plants to plant pathogenic fungi (Linderman, 1994). Reports have indicated that AM fungi can reduce, have no effect on, or increase the severity of disease caused by pathogens (for reviews see Dehne, 1982; Perin, 1990). Table 2.1 summaries some of studies of interactions between AM fungi and soil-borne pathogens on different host plants. Variable results were obtained due to the many factors which may influence the development of disease, such as environmental conditions, differences in AM fungi, pathogens and host plants used in the experiments, differences in experimental design or methods for assessing disease severity.

Although there have been suggestions that AM fungi are possible agents of biological control, the mechanisms by which AM fungi may act in this regard are not well understood (Hooker *et al.*, 1994). However, recent literature suggests that the contribution of AM fungi to controlling disease may involve mechanisms such as (i) improvement of plant nutrition (Hooker *et al.*, 1994; Linderman, 1994), (ii) competition for infection and infection sites (Dehne, 1982; Linderman, 1994; Cordier *et al.*, 1996), (iii) reduction of physical

stresses (Linderman, 1994; Azcón-Aguilar and Barea, 1996), (iv) anatomical and morphological changes in the root systems (Atkinson *et al.*, 1994; Linderman, 1994), (v) microbial population changes in the mycorrhizosphere (Citernesi *et al.*, 1996; Linderman, 1994), and (vi) stimulation of plant defence mechanisms (Linderman, 1994; Azcón-Aguilar and Barea, 1996). Previous work on the mechanisms of the interactions between AM fungi and plant pathogenic fungi are reviewed in the next section, focusing on the improvement in plant nutrition (P) and accumulation of defence responses. So far, there are no data available on the effect of P nutrition on the interaction between AM fungi and BNR or *R. solani*, the subject of this study. Research is needed to determine the effect of P nutrition on the interaction between the AM fungi and BNR or *R. solani* on the infection and disease of the host.

2.2.5.1. Effect of phosphorus nutrition on the interaction

Several reports indicate that the effect of AM fungi on disease development might be related to enhanced P nutrition (Linderman, 1994). This improvement in plant nutrition can enhance plant development and, therefore, may make the plant more resistant to, or compensate for, the effect of disease (Dehne, 1982). A well-established mycorrhizal infection has been reported to reduce disease severity caused by a number of pathogenic fungi such as *Phytophthora* (Cordier *et al.*, 1996; Trotta *et al.*, 1996), *Verticillium* (Hwang *et al.*, 1992; Liu, 1995), *Fusarium* (Hwang *et al.*, 1992; Dugassa *et al.*, 1996) and *Sclerotium* species (Krishna and Bagyaraj, 1983). However, other studies have shown that infection of roots by AM fungi did not reduce diseases (Davis, 1980; Bääth and Hayman, 1984). In addition, soil P and *G. fasciculatum* increased the severity of *Verticillium* wilt in cotton (Davis *et al.* 1979).

Table 2.1. Summary of selected studies on the interactions between AM fungi and plant pathogenic fungi

AM fungi	Pathogens	Host plant	Effect of interactions on AM infected plants*	Effect of interactions on pathogens**	References
Glomus mosseae	*Phytophthora nicotianae* var. *parasitica*	Tomato	No effect on SDW and RDW	Less disease (necrosis)	Trotta *et al.*, 1996
G. mosseae	*Fusarium oxysporum*	Pea	SDW and infection increased; no effect on RDW	No effect on CFU	Fracchia *et al.*, 2000
G. fasciculatum	*F. oxysporum*	Pea	No effect on SDW, RDW and infection	No effect on CFU	
G. intraradices	*F. oxysporum*	Pea	No effect on SDW, RDW and infection	No effect on CFU	
G. deserticola	*F. oxysporum*	Pea	SDW and infection increased; no effect on RDW	No effect on CFU	
G. clarum	*F. oxysporum*	Pea	No effect on SDW, RDW and infection	No effect on CFU	
G. intraradices	*Pythium ultimum*	Onion	No effect on infection; root length reduced	Not reported	Afek *et al.*, 1990
G. intraradices	*Aphanomyces eutiches*	Pea	Not reported	Fewer oospores; no effect on disease severity on roots and epicotyl	Bødker *et al.*, 1998
G. intraradices	*F. oxysporum* f.sp. *radicis lycopersici*	Tomato	No effect on total dry weight	Less disease (necrosis), fewer propagules	Caron *et al.*, 1986a
Glomus sp. or *G. etunicatum*	*P. ultimum*	Cucumber	Leaf area of plants with *Gomus* sp. reduced; no effect for *G. etunicatum*	Damping-off reduced	Rosendahl and Rosendahl, 1990

Table 2.1. continued

AM fungi	Pathogens	Host plant	Effect of interactions on AM infected plants*	Effect of interactions on pathogens**	References
G. fasciculatum	*Sclerotium rolfsii*	Peanut	No effect or reduction on RDW[1]; no effect on spore number; SDW, infection, root P content, shoot P content reduced	No effect on sclerotial bodies	Krishna and Bagyaraj, 1983
G. fasciculatum	*Verticillium dahliae*	Cotton	SDW, RDW, plant height reduced; no effect on infection	No effect or increase in vascular discoloration index and number of propagules[2]	Davis *et al.*, 1979
G. fasciculatum	*P. ultimum*	Poinsettia	Infection increased	No effect on CFU	Kaye *et al.*, 1984
Glomus spp., *G. fasciculatum*, *G. mosseae*	*V. arbo-atrum* or *F. oxysporum* f.sp. *medicaginis*	Alfalfa	Infection and number of vesicles reduced	*Verticillium* wilt, *Fusarium* wilt and propagule number of both fungi reduced	Hwang *et al.*, 1992
G. mosseae + *G. caledonium*	*V. arbo-atrum*	Tomato	SDW reduced; no effect or reduction on RDW[3]	No effect on CFU	Bääth and Hayman, 1983
G. fasciculatum	*P. parasitica*	Citrus	SFW, RFW, spore production, plant height, % healthy roots reduced	Not reported	Davis *et al.*, 1978

Table 2.1. continued

AM fungi	Pathogens	Host plant	Effect of interactions on AM infected plants*	Effect of interactions on pathogens***	References
G. fasciculatum	*P. cinnamomi*	Avocado	SFW, RFW, plant height, % healthy roots reduced	Not reported	Davis *et al.*, 1978
G. fasciculatum	*P. megasperma*	Alfalfa	Total plant weight reduced		
G. mosseae	*Macrophomina phaseolina*	Soybean	Shoot weight, root weight, plant height, infection and seed weight reduced	No effect on infection and number of propagules	Zambolin and Schenck, 1983
G. mosseae	*Rhizoctonia solani*	Soybean	No effect or reduction on shoot weight, root weight, plant height[3]; infection and seed weight reduced	No effect on disease index	
G. mosseae	*F. solani*	Soybean	Shoot weight, root weight, infection reduced; no effect on plant height and seed weight	No effect on infection	
G. mosseae	*Alternaria alternata* and *F. equiseti*	Maize or lettuce	No effect or reduction on SDW and infection[4]; no effect on RDW	No effect on CFU	McAllister *et al.*, 1997

Results are in comparison with plants inoculated with *AM fungi alone or **pathogen alone.
Results based on [1]strain types; [2]amount of P applied to the soil; [3]harvest time; [4]time of mycorrhizal inoculation.
Notes of abbreviation: SDW=shoot dry weight, RDW=root dry weight, CFU=colony forming unit.

Reports on the role of enhanced P nutrition by AM fungi in host resistance are conflicting. The interaction between *G. intraradices* and *Fusarium oxysporum* f.sp. *radicis-lycopersici* in tomato showed that a higher concentration of P in mycorrhizal plants was not responsible for lower disease development and lower populations of *F. oxysporum* f.sp. *radicis-lycopersici* (Caron *et al.*, 1986b). Studies on the interaction between *G. mosseae* and *Phytophthora nicotianae* var. *parasitica* on tomato plants (Trotta *et al.*, 1996), and the interactions between *Pythium ultimum* and *G. intraradices* on marigold (St-Arnaud *et al.*, 1994) showed that the protective effect of mycorrhiza was not related to enhanced nutritional status of the host. Similarly, the reduction of root necrosis in tomato caused by *F. oxysporum* f.sp. *radicis-lycopersici* could not be explained by improved P nutrition, but could be attributed to the presence of *G. intraradices* within roots (Caron *et al.*, 1985). In other studies, significant reduction by *G. fasciculatum* of take-all disease caused by *Gaeumannomyces graminis* was associated with improved P nutrition (Graham and Menge, 1982). Plant defence responses following mycorrhizal infection are believed to play a role in the increased disease resistance of mycorrhizal plants (Benhamou *et al.*, 1994; Volpin *et al.*, 1994,1995).

2.2.5.2. *Induction of defence-related compounds*

Studies indicate that increased disease resistance of mycorrhizal roots may be associated in part with marked metabolic changes in the host plant. There are three kinds of mechanisms involved in plant defence that may be influenced by mycorrhizal formation, and which would be likely to inhibit or at least restrict pathogen invasion. These include: (i) enhanced production of secondary metabolites, such as phenolics (Spanu and Bonfante-Fasolo, 1988) and phytoalexins (Morandi *et al.*, 1984); (ii) production of enzymes such as chitinases and β-1,3-glucanases (Dumas-Gaudot *et al.*, 1984; Spanu *et al.*, 1989), and peroxidases (Spanu and Bonfante-Fasolo, 1988); (iii) deposition of structural defence barriers, such as lignin (Schönbeck, 1979), callose

(Gianinazzi-Pearson *et al.*, 1996) and hydroxyproline-rich glycoproteins (Mazau and Esquerré-Tugayé, 1986). The induction of such defence mechanisms by AM fungi would be likely to inhibit or at least restrict pathogen invasion.

Increased deposition of phenolic compounds in plant roots has been suggested as part of the mechanism involved in the protection of plants from pathogens by AM fungi (Grandmaison *et al.*, 1993). An increase in total soluble phenols in mycorrhizal roots of peanut has been reported (Krishna and Bagyaraj, 1984). Grandmaison *et al.* (1993) found that *Allium cepa* L. roots infected by *G. intraradices* showed a higher concentration of wall-bound phenolic compounds, but there were no qualitative differences in the soluble and bound phenolics between non-mycorrhizal and mycorrhizal roots. However, Codignola *et al.* (1989) observed no differences in concentration or localisation of wall-bound phenols in non-mycorrhizal and mycorrhizal roots of leek.

It is considered that the increase activity of enzymes, such as peroxidases, chitinases and β-1,3-glucanases, may be of key importance in the resistance of mycorrhizal roots to pathogenic fungi (Dalisay and Kùc, 1995). Peroxidases are involved in cell wall reinforcement during plant reactions to pathogens (Collinge *et al.*, 1994). However, Spanu and Bonfante-Fasolo (1988) reported only a transient or early peak of peroxidase activity following mycorrhizal infection, and they concluded that mycorrhizal establishment does not significantly induce this enzymatic activity. Expression of chitinase has been detected at early stages of mycorrhizal infection in roots of leek (Spanu *et al.*, 1989), bean (Lambais and Mehdy, 1993) and alfalfa (Volpin *et al.*, 1994), and interpreted as a host defence response. Vierheilig *et al.* (1994) investigated chitinase and β-1,3-glucanase activities in various host and non-host plants inoculated with *G. mosseae*. In contrast to work reported above, they did not find an early increase in chitinase activity in tomato roots, but

they observed an overall decrease in enzyme activities at a later stage of mycorhizal development. Additional chitinase isoforms were detected in tomato roots after *P. parasitica* infection, but not in mycorrhizal roots subsequently infected with the pathogen (Pozo *et al.*, 1996).

Lignification is a part of disease resistance expression. Lignin and callose are known to inhibit or retard the enzymatic digestion of host cell walls, thereby hindering fungal growth through the host tissue (Vance *et al.*, 1980; Hinch and Clarke, 1982; Lewis and Yamamoto, 1990). As lignin strengthens the cell wall, it may give protection against attack by pathogens. However, it is unclear whether lignification is rapid enough to play a role in resistance of plants to disease (Vance *et al.*, 1980). AM fungi have been shown to stimulate lignification of endodermal cell walls and vascular tissues (Schönbeck, 1979). Daft and Okusanya (1973) found that the lignification of the xylem was greater in mycorrhizal tomato and petunia plants and more vascular bundles were produced in mycorrhizal maize plants than in non-mycorrhizal plants. Thickening of cell walls through lignification and production of other polysaccharides in mycorrhizal plants have been shown to prevent the penetration by, and growth of, *F. oxysporum* (Dehne and Schönbeck, 1979). Callose is a polysacharide composed of β-1,3-glucans, which is produced by plants in response to physical or physiological stimuli (Hinch and Clarke, 1982). The synthesis of β-1,3-glucans has been found in mycorrhizal pea, tobacco and leek (Gianinazzi-Pearson *et al.*, 1996), but not in maize (Balestrini *et al.*, 1994). β-1,3-glucans have been detected within the structural host wall material around the point of penetration of mycorrhizal hyphae into the plant cell, but later disappear when the fungus branches to form an arbuscule (Gianinazzi-Pearson *et al.*, 1996).

In general, plant defence-related genes are only weakly or transiently expressed in response to infection by AM fungi (Harrison and Dixon, 1993, 1994; Bonfante-Fasolo and Perroto, 1995; Gianinazzi-Pearson *et al.*, 1996).

Mycorrhizal symbioses were reported to elicit some defence reactions at early stages of root infection, which were then suppressed or maintained at low levels at later stages of mycorrhizal development (Spanu *et al.*, 1989; Lambais and Mehdy, 1993; Volpin *et al.*, 1994, 1995). However, even this low accumulation of compounds or enzymes potentially protects the mycorrhizal roots, to some extent, from pathogen attacks. The resistance induced against pathogens in mycorrhizal roots may result from both localised defence responses in arbuscule-containing cells and systemic defence responses in non-mycorrhizal parts of mycorrhizal roots (Cordier *et al.*, 1998). The resistance induced by AM fungi against pathogenic fungi is mainly restricted to the root segments that are mycorrhizal, and the degree of resistance is influenced by the level of root infection (Dehne, 1982).

Few studies have been done on the interaction between AM fungi and *R. solani* in relation to defence mechanisms. For example, in tobacco, chitinase activity in transgenic plants expressing a chitinase gene, was found to enhance resistance to *R. solani*, a chitin-containing root pathogen (Broglie *et al.*, 1991; Vierheilig *et al.*, 1993), but had no effect on infection by *G. mosseae* (Vierheilig *et al.*, 1993), which also has chitin in its cell walls. Xue *et al.* (1998) reported that there were higher concentrations of peroxidases, chitinases and β-1,3-glucanases in bean plants treated with BNR than in control plants. Cytological data obtained from young bean seedlings inoculated with BNR showed accumulation of an electron dense barrier rich in suberin, phenolic compounds and pectic substances, which were not seen in *R. solani*-infected bean seedlings (Jabaji-Hare *et al.*, 1999). Phytoalexins, which are anti-microbial phenolics induced by pathogens, have also been studied in association with mycorrhizal symbiosis. Morandi *et al.* (1984) found that the absolute concentration of phytoalexins (glyceollin) in mycorrhizal tissues remained low compared with tissues of soybean infected by pathogens. Similarly, Wyss *et al.* (1989) found that the level of glyceollin remained low at

20 days after inoculation by *G. mosseae*, whereas rapid accumulation was found in roots infected by *R. solani*.

2.3. THE GENUS *RHIZOCTONIA*

Rhizoctonia is a genus of basidiomycetous imperfect fungi, and has considerable economic and ecological significance since it includes a number of important pathogens of crop plants (Mordue *et al.*, 1989). *Rhizoctonias* are worldwide in distribution, with an extremely wide host range, and vary in morphology, pathology and physiology among species, as well as in the diseases they cause on different hosts. Groups are generally characterised by hyphal anastomosis, and differences in morphology, pathogenicity, physiology, cultural appearance, and ecology (Ogoshi, 1987; Sneh *et al.*, 1991; Burpee and Martin, 1992).

The genus *Rhizoctonia* is characterised by the following characters: (i) branching near the distal septum in young vegetative hyphae; (ii) formation of a septum in the branch near the point of origin; (iii) constriction of branch hyphae at the point of origin; (iv) presence of the dolipore septal apparatus; (v) absence of clamp connections, conidia and rhizomorphs; (vi) sclerotial tissues when formed, not differentiated into rind and medulla (Parmeter and Whitney, 1970; Ogoshi, 1987; and also Figure 2.1). Hyphae of non-pathogenic isolates are frequently hyaline (Sneh *et al.*, 1989b), whereas the pathogenic isolates are generally brownish to greyish (Sneh *et al.*, 1991). The colours are determined by the deposits of melanin in their hyphal cell wall, and might be essential for their pathogenicity.

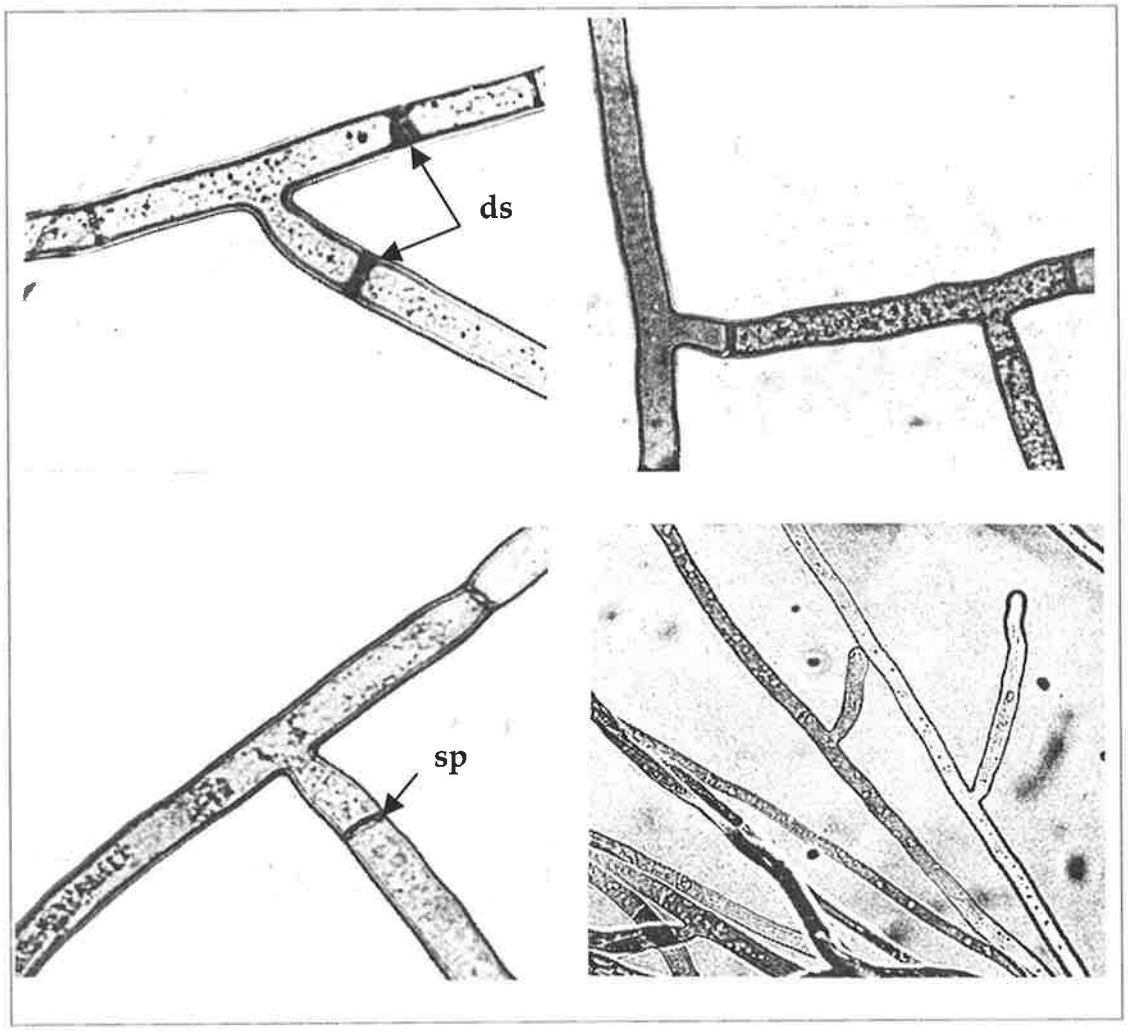


Figure 2.1. Hyphae of colony of *Rhizoctonia* on PDA showing characteristic branching. sp = septum proximal, ds = dolipore septa. From Butler and Bracker (1970).

Based on the characteristics of their sexual structures, Ogoshi (1987) divided *Rhizoctonia* isolates into three major groups: (i) multinucleate *Rhizoctonia*, for example *R. solani*, which have three or more nuclei per cell, hyphae 6-10 μm in diameter, and a teleomorph in the genus *Thanatephorus* Donk; (ii) binucleate *Rhizoctonia*, which have two nuclei per cell (rarely one or three), hyphae 4-7 μm in diameter, and a teleomorph (sexual or perfect stage) in the genus *Ceratobasidium* Rogers; (iii) *R. oryzae* and *R. zeae*, which are multinucleate, and have the teleomorphs of the genus *Waitea* Warcup & Talbot.

The following review covers only *R. solani* and binucleate *Rhizoctonia* (BNR), which are the subject of this study.

Both *R. solani* and BNR can be arranged into several anastomosis groups (AGs) that often show different host preferences and levels of pathogenicity (Ogoshi, 1987). Isolates belonging to the same AG are capable of recognising each other and can perform hyphal fusion (Vilgalys and Cubeta, 1994). *R. solani* isolates have been divided into at least 12 AGs (AG-1 to AG-11 and AG-BI) (Sneh *et al.*, 1996). AG-BI stands for 'bridging isolates', because members have the ability to anastomose with members of other AGs (Carling, 1996; Neate and Warcup, 1985). For some AGs, subgroups or intraspecific groups (ISGs) have been defined, based on nutritional requirements, host specificity, frequency of hyphal fusion, cultural morphology and genetic relatedness (Ogoshi, 1987). BNR isolates have also been divided into several anastomosis groups. Burpee *et al.* (1980a) used the *Ceratobasidium* anastomosis groups (CAG) system to separate BNR isolates, and identified seven anastomosis groups (CAG1 to CAG7) among BNR isolates obtained in the United States, whereas Ogoshi *et al.* (1979) separated BNR isolates obtained in Japan into at least 15 anastomosis groups (AG-A to AG-Q).

2.3.1. *Rhizoctonia solani*

2.3.1.1. Root infection and disease symptoms

Infection of plant roots by *R. solani* usually involves four major steps: (i) attachment of hyphae to the surface of the plant roots; (ii) formation of infection structures which are generally described as either lobate appressoria or dome-shaped infection cushions; (iii) penetration; (iv) infection of host plant tissues (Dodman and Flentje, 1970; Armentrout and Downer, 1987). Infection is initiated when hyphae make contact with the root surface. After attachment, the hyphae align along the junction between epidermal cell walls, followed by lateral branching. Hyphae may branch to form simple appressorium-like structures with swollen hyphal tips, or if the branching process continues, a more complex structure is produced such as a tightly dome-shaped infection cushion with swollen hyphal tips (Keijer, 1996; Weinhold and Sinclair, 1996). An infection cushion may originate from one or many hyphae, or sometimes arises by anastomosis of hyphae from which multiple infection pegs are produced for penetration of the plant surface (Murray, 1982). Different types of infection cushions are produced depending upon the resistance of cultivars (Marshall and Rush, 1980a, 1980b), and the number of infection cushions formed is influenced by temperature (Kousik and Snow, 1991).

Penetration of the epidermal cell wall by *R. solani* is followed by hyphal proliferation within the root cortical cells, resulting in browning. The hyphae do not enter the xylem, but severely infected roots are truncated as the cortical cells collapse and the exposed stele breaks off to leave a typical 'spear point' (Weinhold and Sinclair, 1996). Hofman and Jongebloed (1988) found that infection of potato tissue by *R. solani* AG-3 was initially restricted to one or two cell layers underneath the infection cushion. Girdling was observed on young seedlings and caused death. In another study, Yang *et al.* (1992) found that infection of a susceptible canola cultivar by *R. solani* AG2-1

was completed after 84 hours, by which time the entire cortex was infected; however it appeared that the fungus was not able to infect cells with lignified walls (xylem).

Isolates of *R. solani* can cause seed decays, damping-off of seedlings, stem cankers, root rots, fruit decays and foliage diseases (Baker, 1970; Ogoshi, 1985). Engelkes and Windels (1996) observed that the first above-ground symptom of *Rhizoctonia* root and stem rot of beans was wilting, and it occurred within 1 week after inoculation. A few days after wilting began, leaves became necrotic. On basal stems or slightly below the soil line, reddish brown lesions were observed to girdle the stem. Infected roots showed a light discoloration in association with pruned taproots, secondary roots and/or root hairs.

2.3.1.2. Anastomosis groups and pathogenicity

Studies have shown a level of host specificity in relation to AG. For example, isolates of AG-1 have mainly been obtained from the *Leguminosae* and the *Gramineae*. AG-1 has been divided into three subgroups, AG1-1A, AG1-1B and AG1-1C, based on pathogenicity and morphology in culture (Ogoshi, 1987; Yang *et al.*, 1990). A number of isolates of AG-2 are from the *Cruciferae* and *Brassicaceae* (Anderson, 1982). AG-2 has been divided into two subgroups, AG2-1 and AG2-2, based on cultural morphology, thiamine requirement and frequency of anastomosis between subgroups (Ogoshi, 1987). Isolates of AG2-2 are further divided into AG 2-2IIIB, comprising isolates infecting mainly shoots and leaves of the *Poaceae*, and AG2-2IV, comprising isolates causing root rots of *Chenopodiaceae* (Ogoshi, 1987; Yang *et al.*, 1990). Most isolates of AG-3 are pathogenic to members of the *Solanaceae*, AG-4 isolates cause numerous diseases of the *Chenopodiaceae, Leguminosae* and *Solanaceae*, and AG-5 is pathogenic to the *Leguminosae* (Ogoshi, 1987). AG-6 and AG-7 are considered to be non-pathogenic (Sneh *et al.*, 1991). Isolates of AG-8 cause

disease on the *Gramineae* and *Leguminosae* (Sweetingham *et al.*, 1986), while AG-10 has been isolated from barley (Ogoshi *et al.*, 1990). There is no specific information about the host ranges of AG-6, AG-7, AG-9, AG-11 and AG-BI isolates.

Although some AGs show host specificity, isolates are generally able to infect host plants other than the one from which they were originally obtained. For example, *R. solani* AG2-2 from pinto bean gave a higher root rot rating on sugar beet than did an isolate from sugar beet (Engelkes and Windels, 1994). Liu and Sinclair (1991) found that AG2-2IIIB isolates from soybean produced crown and root rot on inoculated plants in the *Chenopodiaceae, Fabaceae* and *Poaceae*. Nelson *et al.* (1996) further reported that the AG2-2IIIB isolates from soybean also were pathogenic to dry bean, mustard, flax, sunflower and corn. Isolates of *R. solani* AG2-2IIIB obtained from pinto bean, soybean and table beet, and AG2-2IV obtained from sugar beet and broad bean, were pathogenic to sugar beet, navy bean, pinto bean, soybean and broad bean (Engelkes and Windels, 1996). Although AG2-1 is a common pathogen on *Brassica* spp., it was also reported to be highly pathogenic on cowpea and moderately virulent on snap bean and lima bean (Sumner, 1985).

From the above points of view, it is clear that most isolates of *R. solani* have a broad range of host plants. Although the pathogenicity of *R. solani* on a variety of beans has been investigated, few studies (e.g. Kataria and Grover, 1987; Ehteshamul-Haque and Ghaffar, 1993) have used mung bean as a host plant. Therefore, pathogenicity of different AGs of *R. solani* (commonly associated with beans) on mung bean was included in the investigation.

2.3.2. Binucleate *Rhizoctonia*

2.3.2.1. Isolation, characterisation and identification

BNR can be found in soil and plant debris as saprophytes or may form parasitic relationships with plants or mycorrhizal associations with orchids (Cubeta *et al.*, 1991). For example, approximately 8% of 107 fungal isolates collected from diseased table beet over a period of more than 2 years in New York belonged to BNR (Olaya and Abawi, 1994a). Those BNR isolates had low virulence to both foliar and root tissue of beet. In another study, Nelson *et al.* (1996) found that BNR isolates comprised less than 5% of the total population of *Rhizoctonia* recovered from roots and stems of soybean. Of 41 isolates of BNR studied by Ogoshi *et al.* (1990), 24 were from roots of wheat and barley, and 17 were from soils.

BNR possess the same general characteristics as the genus *Rhizoctonia.* In addition, many isolates of BNR produce monilioid cells, which are simple and branched chain cells, hyaline or brown in colour, in various shapes (Sneh *et al.*, 1991), and ranging from 10-20 to 25-40 μm in length (Saksena and Vaartaja, 1961). The monilioid cells can be found on or above the surface of a host or a substrate, or within host tissue (Sneh *et al.*, 1991), and they are thought to be important in survival of the fungus (Ferriss *et al.*, 1984).

Various techniques involving culturing and observation of morphological characteristics on semi-selective media (Ko and Hora, 1971; Trujillo *et al.*, 1987), and baiting techniques (Papavisaz *et al.*, 1975; Sneh *et al.*, 1991) have been used for isolation of BNR from soil, plant debris or plant tissues. Since the semi-selective media also support the growth of multinucleate *Rhizoctonia*, nuclear staining is normally used to differentiate between the binucleate and multinucleate *Rhizoctonia* (Sneh *et al.*, 1991). BNR possess two nuclei per hyphal cell which can be visualised using several staining methods, such as HCl-Giemsa, 0.5% aniline blue, 0.05% trypan blue

in lactophenol or the fluorescent stain 4′, 6′-diamidino-2-phenyl-indole (DAPI) (Herr, 1979; Sneh *et al.*, 1991).

Reliable characterisation and identification of isolates of BNR are often difficult due to similarity in the morphological characteristics of the anamorphs and teleomorphs of BNR. The teleomorphs of most AGs of BNR isolates have been assigned to *Ceratobasidium* sp. (AG-E, -F, -G, -H, -J to -M, --O, -R, and -S), or *C. cornigerum* (AG-A, -Bo, -C, -P, and -Q). The AG-Ba, -Bb and -D have been assigned as teleomophs *C. setariae, C. oryzae-sativae* and *C. graminearum*, respectively, whereas the teleomorphs of AG-I and AG-N are unknown (Sneh *et al.*, 1991). As the teleomorph of BNR is difficult to induce in culture, hyphal anastomosis has been used for identifying BNR isolates. However, characterisation and identification of BNR are often complicated by the ability of some isolates to anastomose with more than one tester isolate, or by loss of ability to anastomose. Recently Cubeta *et al.* (1991) used the polymerase chain reaction (PCR) to distinguish AGs of BNR isolates. Cubeta *et al.* (1991) found that by using restriction analysis of PCR-amplified rDNA, 13 isolates of the 21 AGs of BNR previously identified by Burpee *et al.* (1980a) and Ogoshi *et al.* (1979) could be differentiated.

2.3.2.2. *Pathogenicity*

Although AGs and pathogenicity are related to some extent, evidence from several studies indicates considerable pathogenic variation within an AG. Most isolates of AG-C, -H, -J, -L, -M, -N, and -O were reported to be non-pathogenic (Sneh *et al.*, 1991). Ogoshi (1985) reported that AG-A caused diseases, such as damping-off of sugar beet seedlings, strawberry root rot, tortoise shell-like symptoms of potato tubers, and browning of peanut pods, but were non-pathogenic to cucumber (Villajuan-Abgona *et al.*, 1996a). Isolates of AG-Bo were pathogenic on rice, causing sheath blight-like lesions (Ogoshi *et al.*, 1979; Ogoshi, 1985), but non-pathogenic on corn and wheat

(Herr, 1989). CAG-4 (AG-F) and CAG-5 (AG-R) caused damping-off of bean, pea and tomato seedlings (Burpee *et al.*, 1980b). An isolate of AG-G was non-pathogenic on potato (Escande and Echandi, 1991), but pathogenic to strawberry and peanut, causing browning of peanut pods (Ogoshi, 1985). Isolates within AG-K were reported by Ichielevich-Auster *et al.* (1985a) to be pathogenic to seedlings of radish, tomato, carrot, onion, lettuce, cucumber and cantaloupe, but Schisler *et al.* (1993) found that AG-K had only low virulence on brussel sprouts, bell pepper and wheat.

Some isolates of BNR AG-A (CAG-2), AG-D (CAG-1) and *Ceratobasidium* spp. were reported to form mycorrhizal associations with orchids (Ogoshi *et al.*, 1983; Sneh *et al.*, 1991). In an *in vitro* experiment, Masuhara *et al.* (1993) reported that BNR isolates obtained from non-orchid sources induced symbiotic germination of seeds of *Spiranthes sinensis* var. *amoena*, whereas Ogoshi *et al.* (1983) found some isolates of BNR also induced symbiotic germination of Australian orchid seeds.

2.3.2.3. Occurrence in mycorrhizal pot cultures and root infection

The presence of fungal contaminants in mycorrhizal roots was first reported in the early nineteen century by Peyronel (1923, 1924). He reported that sterile, septate fungi with the hyphal morphology and appearance of the septate endophytes similar to those of the orchid mycorrhizal fungus, *R. repens* Bernard, regularly associated with AM fungi. These fungi were associated with over 100 species of flowering mycorrhizal plants and were regarded as quasi-parasites or saprophytes.The infection of the fungus was restricted to the epidermis and outer cortex of the roots, and characterised by the monilioid mycelium, formed in the outer layer of the cortex. Williams (1985) found that sterile, septate fungi (*Rhizoctonia*), attributed to the orchid mycorrhizal fungi, commonly occurred in pot cultures of AM fungi. The *Rhizoctonia* isolates were obtained from spore-like cells in intraradical

vesicles, from extraradical hyphae, and chlamydospores of roots of pot culture plants. In culture, they were characterised by producing irregular septate hyphae, monilioid hyphae and terminal or intercalary chlamydospores, and had pale to yellow-brown colonies. Inoculation by this fungus affected the plant growth only when AM fungi were also present, but did not have an effect on plant growth in the absence of AM fungi.

The infection of plants by BNR obtained from pot cultures has not been studied in detail. However, work with other BNR in roots showed that infection does vary between isolates. The hyphal tips of BNR may penetrate between the epidermal cells (i.e. grow intercellularly) and do not grow intracellularly (Cardoso and Echandi, 1987; Eayre and Echandi, 1988; Sneh *et al.*, 1989b), or can penetrate epidermal cells and be visible within these cells (Poromarto *et al.*, 1998). The hyphae do not form appressoria or infection cushions on the host tissue, in contrast to *R. solani* (Cardoso and Echandi, 1987; Poromarto *et al.*, 1998). Histological studies of seedlings of *Phaseolus vulgaris* L. (bean) inoculated with BNR showed that, during penetration, the host tissues were not damaged and layers of cells below the epidermis were not penetrated (Cardoso and Echandi, 1987).

Observation of three isolates of BNR on soybean roots showed that the hyphae began penetrating the epidermal cells within 5.5 h following inoculation and were visible within cells within 8 h. Branched hyphae were formed 24 h after inoculation and the network of hyphae was established within 72 h. After 144 h, there was still no indication that BNR had penetrated the cortical cells. Cell necrosis was observed in the infected epidermal cells and in adjacent epidermal cells, where there was no evidence of the presence of BNR hyphae, suggesting that enzymatic action or a toxin was involved in the infection process (Poromarto *et al.*, 1998). BNR are capable of infecting up to 96% of the root system of a plant, and isolates capable of infecting 60-96%

of roots are considered to have potential as biological control agents (Herr, 1988).

In Australia and worldwide, BNR isolates have been found infecting the roots used to culture AM fungi. BNR infect the epidermal cells of a number of species, such as cabbage, leek, clover, tomato, and to a lesser extent lupin roots, and do not appear to cause disease or kill the plants (S. Smith, personal communication). There is little information about the origin of BNR in the pot cultures, details of the cultural morphology or the anastomosis group of these BNR, and, so far, there have been no investigations on the effect of the interaction between BNR and AM fungi on plant growth. Therefore, investigation of the interactions between BNR and AM fungi, and their effects on plant growth and infection is necessary.

2.3.2.4. Role in biological control

Recently, non-pathogenic BNR isolates have been investigated as biological agents for the control of diseases caused by pathogenic *Pythium* spp. (Harris *et al.*, 1993), *Phytophthora parasitica* var. *nicotianae* (Cartwright and Spurr, 1998) and *R. solani* (Harris *et al.*, 1994; Villajuan-Abgona *et al.*, 1996a). Compared to other fungi, BNR isolates have several advantages as biological control agents. They have an ubiquitous occurrence, infect roots rapidly and can tolerate a wide range of environmental conditions (Burpee and Goulty, 1984; Cardoso and Echandi, 1987; Cartwright and Spurr, 1998). Isolates belonging to CAG-2 (AG-A) (Bell *et al.*, 1984) and CAG-5 (AG-R) (Cardoso and Echandi, 1987) were highly effective in controlling bean root-rot. CAG-4 (AG-F) was effective in controlling *R. solani* AG2-2 on corn (Bell *et al.*, 1984). Escande and Echandi (1991) found that eight isolates of BNR, including an isolate of AG-G, reduced disease severity and incidence of *Rhizoctonia* stem and stolon canker by an average of 78 and 85%, respectively. Three isolates of AG-K, which were non-pathogenic to soybean, were reported to decrease the

severity of *R. solani* AG-2-2 damping-off and stem canker on soybean (Khan *et al.*, 1992).

Control of plant disease by BNR is likely to involve several mechanisms. Cardoso and Echandi (1987) suggested that the most likely mechanisms of biological control were competition for infection sites or induction of defence mechanisms in the host. There was an indication that BNR may compete with other fungi physically to recognise and occupy infection sites (Sneh *et al.*, 1989a, 1989b), or may compete for nutrients. The inhibition of infection cushion formation, hyphal growth and sclerotial germination of *R. solani* by BNR supports the competition mechanism (Cardoso and Echandi, 1987). Changes of the composition of root exudates caused by BNR may lead to a shortage of essential nutrients for *R. solani* or production and release of compounds toxic to *R. solani*. In addition, BNR isolates may induce systemic resistance in plants, which could lengthen the duration of protection of plants from pathogen attacks (Burpee and Goulty, 1984; Cardoso and Echandi, 1987). Biochemical investigations showed that inoculation by a BNR isolate increased the activity of several defence-related enzymes (glucanases, chitinases and peroxidase), which may provide physical and chemical barriers to invasion by other fungi (Xue *et al.*, 1998). There was no evidence of mycoparasitism in several studies of different BNR isolates (Ichielevich-Auster *et al.*, 1985b, Cardoso and Echandi, 1987; Harris *et al.*, 1993), but research by Siwek *et al.* (1995) suggested mycoparasitism of *P. ultimum* by non-pathogenic BNR.

2.4. MOLECULAR APPROACHES TO STUDY BINUCLEATE *RHIZOCTONIA* SP. (BNR)

Molecular approaches have been widely used in the study of *Rhizoctonia* spp. because of the difficulties in distinguishing isolates or groups using conventional methods involving observation of morphology.

Compared to molecular studies that have been done on *Rhizoctonia* spp., particularly *R. solani*, very limited studies have been carried out on BNR. Most studies on BNR have supported the AG concept, and restriction fragment length polymorphisms (RFLPs) have been used in the identification of isolates within specific AGs. For example, Cubeta *et al.* (1991) tested seven US and 16 Japanese BNR anastomosis tester isolates, representing 21 different AGs, and found that CAG-2, AG-A and AG-Bo produced identical RFLP patterns with the eight restriction enzymes tested, whereas CAG-6, CAG-7 and AG-E also had identical or nearly identical RFLP patterns to each other but not to the other AGs. CAG-1 and AG-D also produced similar patterns with all enzymes tested, but were distinct from all other AGs. Mazzola *et al.* (1996) used Cubeta's technique to confirm the AG of BNR isolates recovered from apple roots and orchard soils. Some isolates produced the same restriction pattern as the tester isolates, whereas others gave different patterns. The authors suggested that genetic diversity occurred within the AG of isolates of *Rhizoctonia* spp., and this can cause problems for consistent identification.

Most molecular research for the detection and identification of plant pathogenic fungi has used techniques based on PCR. Such techniques have been widely used to study plant pathogenic fungi, such as *Verticillium* (Moukhamedov *et al.*, 1994), *Fusarium* (Schilling *et al.*, 1996) and *Pythium* (Kageyama *et al.*, 1997), *Gaeumannomyces* (Schesser *et al.*, 1991), and *Rhizoctonia* species (Johanson *et al.*, 1998; Salazar *et al.*, 2000), as well as AM fungi (Lanfranco *et al.*, 1995; Sanders *et al.*, 1995). Primers designed from specific sequences of the ITS regions, have been used to detect and identify plant pathogenic fungi (White *et al.*, 1990). These methods can be used to identify the fungus accurately but cannot be directly used for quantifying biomass. As an alternative, competitive PCR based on the relative degree of the amount of fungal DNA ('target') with known standard DNA ('competitor') was used for quantifying biomass from plant tissues and soil

(Mahuku *et al.*, 1995; Nicholson *et al.*, 1996, 1997; Heinz and Platt, 2000). DNA-based methods to quantify fungi in soil have been developed for *R. solani* AG-8 (Whisson *et al.*, 1995) and *G. graminis* var. *tritici* (Herdina *et al.*, 1996; 1997) using a DNA hybridisation assay, but this has never been explored for BNR. The design of specific primers would be valuable for quantifying BNR in infected roots, and the DNA hybridisation assay could be applied as a rapid and reliable method to quantify fungal DNA of BNR in infected roots.

2.4.1. Design of specific-primers for detection of BNR

Common methods used for detection and identification of BNR involve culturing the isolates on nutrient media followed by examination of the morphology. These procedures are time consuming and not specific. BNR may produce symptoms on plants similar to those caused by *R. solani*, which makes identification by symptoms difficult. The PCR offers a sensitive method to identify and detect fungi rapidly in plant tissue and soil by amplification of specific DNA sequences. This technique is powerful because of its simplicity, selectivity and speed. Besides, PCR requires only very small amounts of target DNA and sometimes it does not require culturing of the fungi (Vollosiouk *et al.*, 1995).

For BNR, the use of PCR has been combined with other molecular techniques, such as randomly amplified polymorphic DNA (RAPD). Selected products from RAPD assays have been cloned and sequenced to produce specific primers (Leclerc-Povtin *et al.*, 1999). Leclerc-Povtin *et al.* (1999) developed two primer pairs (BR1a F/R and BR1b F/R) from RAPD-PCR, which are specific to the non-pathogenic BNR AG-G. Amplification with those primers was obtained from DNA of isolates of AG-G, and from bean seedlings, peat and field soil inoculated with AG-G or AG-G + *R. solani*. The

two primer pairs did not amplify DNA of other AGs of BNR, *R. solani* or other pathogens commonly found infecting beans.

2.4.2. Internal transcribed spacers (ITS) of the rDNA region

The ITS region consists of two non-coding variable regions (ITS1 and ITS2) which are located within the ribosomal DNA (rDNA) repeats between the highly conserved small subunit, the 5.8 subunit, and the large subunit rRNA genes (White *et al.*, 1990). The ITS region is relatively short (500-800bp) and is easily amplified by universal primers pairs (White *et al.*, 1990). Many researchers have used sequences selected from the ITS regions to develop species-specific primers, because the sequences tend to be similar within fungal species, but variable between fungal species (Sreenivasaprasad *et al.*, 1996).

Analysis of the ITS regions has been used to construct phylogenetic trees of *R. solani* (Liu and Sinclair, 1992, 1993; Boysen *et al.*, 1996; Salazar *et al.*, 1999). The specific sequences of the ITS regions have been used to design primers specific of AG-2 (Salazar *et al.*, 2000). Johanson *et al.* (1998) designed primers from unique regions within the ITS which are specific to *R. oryzae sativae*, *R. solani* and *R. oryzae* obtained from the leaf sheaths of infected rice plants or from soil from a rice-based cropping system, to overcome problems in identification.

The ITS regions of the rDNA have also been used to generate specific primers capable of differentiating many closely related fungal species. Two primer pairs, Rhsp1-ITS4B and Rhsp2-ITS1F, have been designed by Salazar *et al.* (2000) from the specific sequences of the ITS regions in *R. solani*. Those primers were specific at the genus level, and could detect BNR (AG not mentioned) and AG-1, 2, 3, 4, 5 of *R. solani*, but did not amplify DNA of other fungal species tested or DNA of tomato, radish and cauliflower. So far, no

information is available on the design of specific primers from the sequences of the ITS region of BNR; therefore the design of BNR-specific primers from sequences of the ITS region needs to be considered.

2.5. CONCLUSIONS

AM fungi are characterised by their spores, hyphae, arbuscules and vesicles, and benefit to the host plant by improving nutrient uptake, especially P. Production of mycorrhizal inoculum has relied on the use of pot cultures, which often have a risk of contamination, including the fungi *Rhizoctonia* and BNR. The effects of BNR on the mycorrhizal symbiosis and plant growth responses are not well understood; therefore further research on these topics is needed.

Research on the BNR isolates responsible for this contamination, and the design of specific primers are important. Detection of a fungal contaminant has up to now relied on microscopic examination, which is time consuming. Recent developments in molecular techniques, such as PCR, facilitate detection and identification of fungi because of their simplicity, sensitivity and speed. Specific primers developed from the ITS regions of rDNA have been useful in detecting fungi in plant tissues and in soil. However, this approach has not yet been applied to BNR. Characterisation and identification of BNR isolated from pot cultures are necessary for improve better understanding of the biology of BNR as a contaminant in pot cultures. Furthermore, the development of BNR-specific primers will facilitate the rapid detection of BNR in plant roots and among propagules in pot cultures.

Interactions between AM fungi and pathogenic fungi have been studied. However, conflicting results are still being obtained in some studies, especially with respect to the effects of P on the interactions. AM fungi may

play a role in reducing disease severity and may induce defence responses that may protect the plant from pathogen attack. However, few studies have been done on the interactions between AM fungi and *R. solani*. Currently there is no information available on the interactions between AM fungi and BNR. Research on the interactions between AM fungi and BNR or *R. solani* is necessary to give a better understanding of the significance of AM fungi in reducing the effects of BNR or *R. solani* on plant growth.

CHAPTER 3. GENERAL MATERIALS AND METHODS

This chapter describes methods frequently used in the investigations, including the choice of the host plant, the fungi used in the study, measurement of plant growth, assessment of infection and disease severity, and methods used in the molecular study. Details of the individual experiments are given in the appropriate chapters with additional methods specific to these experiments.

3.1. HOST AND FUNGI

The host used in all investigations was mung bean (*Vigna radiata* L. Wilzeck cv. Emerald). Mung bean was chosen because it is responsive to *Rhizoctonia* (Anderson, 1985) and mycorrhizal fungi (Vogelzang *et al.*, 1993), and has been the subject of study in mycorrhizal research (e.g. Mathew and Johri, 1989; Vogelzang *et al.*, 1993). *Glomus coronatum* Giovannetti was chosen and used in most investigations because it has big spores, which are easy to pick out and count in preparation of standard inoculum. A preliminary study showed that pot cultures infected with *G. coronatum* were clean and contained no binucleate *Rhizoctonia* sp. (BNR). A BNR isolate from a mycorrhizal pot culture of *G. mosseae* on *Trifolium subterraneum* L. cv Mt. Barker was used to represent BNR. *R. solani* AG1-1C, AG2-1, and AG2-2IIIB were used because they have been shown to attack a variety of bean plants (Sumner, 1985; Ogoshi, 1987; Engelkes and Windels, 1996). Various other AM and non-AM fungi, and infected roots were used for DNA extraction to test the specificity of PCR. Roots and soils of pot cultures potentially contaminated by BNR were used to detect BNR in pot cultures by PCR. Details of the isolates and their origins used in this study are given in Table 3.1.

Table 3.1. List of isolates of AM and non-AM fungi used in this study

Species	Isolate	Source/host	Origin
AM fungi*			
Glomus mosseae (Nicol. & Gerd.) Gerdemann & Trappe	NBR 1-2	Pot culture-clover (*Trifolium subterraneum* L. cv Mt. Barker)	Narabrai, New South Wales
G. mosseae	NBR 4-1	Pot culture-clover	Narabrai, New South Wales
G. mosseae	NBR 4-2	Pot culture-clover	Narabrai, New South Wales
G. mosseae		Pot culture-clover	Dijon, France
G. etunicatum Becker & Gerdemann	JT316A-1	Pot culture-clover	INVAM (International Culture Collection of Arbuscular and Vesicular-Arbuscular Mycorrhizal Fungi)
G. etunicatum	MD107-1	Pot culture-clover	INVAM
G. intraradices Schenck & Smith	DAOM 181602	Pot culture-clover	Canada
G. versiforme (Karsten) Berch		Pot culture-clover	Italy
G. fasciculatum (Thaxter) Gerd. & Trappe emend. Walker & Koske	BEG 5	Pot culture-clover	France
G. coronatum Giovannetti	WUM 16	Pot culture-clover/leek (*Allium porrum* L. cv. Vertina)	University of Western Australia
Gigaspora margarita Becker & Hall	–	Pot culture-clover/leek	Dijon, France
Gi. rosea Nicolson & Schenck	–	Pot culture-plantago (*Plantago lanceolata* L.)	Waite Campus, South Australia
Acaulospora laevis Gerdemann & Trappe	–	Pot culture-plantago	Waite Campus, South Australia
Scutellospora calospora (Nicol. & Gerd.) Walker & Sanders	WUM 12(2)	Pot culture-clover	New South Wales

Table 3.1. continued

Species	Isolate	Source/host	Origin
Non-AM fungi			
Binucleate *Rhizoctonia* (BNR)			
AG-Bo*	CFM1	Pot culture *G. mosseae*	Dept. of Soil and Water, The University of Adelaide, South Australia
AG-A**	C-517	Strawberry (*Fragaria* sp. L.)	Hokkaido University, Japan
AG-Bo**	SIR-2	Sweet potato (*Ipomoea batatas* (L.) Lam.)	Hokkaido University, Japan
AG-C**	OR-706	Orchid (*Gymnadenia conopsea)*	Hokkaido University, Japan
AG-G**	AHC-9	Peanut (*Arachis* sp. L.)	Hokkaido University, Japan
AG-O**	FKO6-2	Soil	Hokkaido University, Japan
R. solani Kuhn			
AG1-1C***	01R01	Sugar beet (*Beta vulgaris* L.)	Hokkaido University, Japan
AG2-1***	21RM03	Alfalfa (*Medicago* sp. L.)	Mallee, South Australia
AG2-2 IIIB***	22R02	Mat rush (*Juncus effusus* L. var. *decipiens*	Hokkaido University, Japan
AG-8***	NS21	Barley (*Hordeum vulgare* L.)	South Australia
Pythium echinulatum V.D. Matthews****	BH3	Soil	South Australia
Gaeumannomyces graminis var. *tritici* (Sacc) Arx & D.L. Oliver & Walker****	Ggt 800	Wheat (*Triticum aestivum* L.)	South Australia
Fusarium sp.*	_	Soil-pot cultures	South Australia
Rhizopus sp.*	_	Soil-pot cultures	South Australia

*Pot cultures were from the collection of Dept. of Soil and Water, The University of Adelaide, and kindly supplied by Prof. Sally Smith and Ms Debbie Miller; Fungal materials were kindly supplied by **Prof. Shigeo Naito, Graduate School of Agriculture, Hokkaido University, Japan, ***Dr Stephen Neate, CSIRO Land and Water, Adelaide, and ****Dr Kathy Ophel-Keller, SARDI, Adelaide.

3.2. FUNGAL CULTURES

3.2.1. AM fungi

3.2.1.1. Production of G. coronatum *inoculum*

G. coronatum inoculum was derived from pot cultures. These were prepared by growing clover (*Trifolium subterraneum* L. cv. Mt Barker) in a Mallala soil/sand mix (1:9) amended with 10% (by weight) of inoculum of *G. coronatum* (WUM 16) from previous pot cultures (see section 3.3). The plants were grown in a glasshouse and watered to 12% gravimetric water content with reverse osmosis (RO) water three times per week and given 10 ml of modified Long Ashton solution minus P weekly (Smith and Smith, 1981, see section 3.5 for details). After approximately 4 months, the watering was stopped, and plants and soil were allowed to dry out. Spores of *G. coronatum* from pot cultures were used as inoculum.

3.2.1.2. Separation of spores of AM fungi from soil

Separation of AM spores from soil of pot cultures was conducted using a modification of a wet sieving method (Gerdemann and Nicholson, 1963), and followed by separation by sucrose density gradient centrifugation (Brundrett *et al.*, 1996). Approximately 100 g soil from pot cultures were put in a 250 ml beaker. Water was then added in the beaker, stirred and left at room temperature for 1 h. The suspension was passed through 20 cm diameter soil sieves with 500 µm and 38 µm wide mesh. More water was added to the beaker, the sand allowed to settle for a few minutes and then poured off again. This process was continued until the water was clear. The content of the 38 µm wide mesh sieve was poured into the centrifuge tubes, water was added, and tubes were centrifuged at approximately 1,600 *g* for 5 min. Then, the surface scum was poured from the centrifuge tube carefully

using a circular motion. Then a half volume of sucrose solution (60%, w/v) was added to the tube. The tubes were centrifuged at approximately 2,000 *g* for 20 sec. The spores caught at the interface between two layers were poured into a clean 38 μm sieve, and washed thoroughly to remove traces of sucrose solution. The contents of each sieve were transferred to a nematode dish. Spores with similar size and maturity were selected for inoculation and were counted using a hand tally counter under a dissecting microscope. In all investigations, 250 spores of *G. coronatum* were used for inoculation, placed directly under the seed in each pot.

3.2.2. Non-AM fungi

3.2.2.1. Maintenance and production of inoculum

Isolates of BNR, *R. solani* and other fungi were grown on potato dextrose agar (PDA Difco). A 3-mm diameter plug of mycelium from a previous culture was placed on the centre of a Petri dish containing approximately 20 ml of PDA and incubated at 25°C. The fungal mycelium was sub-cultured every 2 weeks to maintain viability. Inoculum of BNR or *R. solani* was prepared according to a modified method of McDonald and Rovira (1985). Autoclaved millet seeds (*Panicum miliaceum* L.) were spread evenly on the surface of PDA layers, and then a plug of mycelium (5 mm-diameter taken from a 7-day-old culture on PDA) of either BNR or *R. solani* was placed in the centre of the Petri dish. The cultures were incubated at 20-25°C in darkness for 7 days, and infected millet seeds were used as inoculum. Six millet seeds were placed evenly at a depth of 5 cm around each plant in the potting mix. Controls received six sterile millet seeds, non-inoculated.

3.3. PLANT MATERIAL, SOIL AND NUTRIENTS

Seeds of mung bean were surface-sterilised by rinsing with 70% ethanol followed by soaking for 15 min in 3% sodium hypochlorite plus three drops of Tween-20, and then rinsed three times with sterile RO water. The seeds were pre-germinated for 2 days at 25°C between two layers of Whatman filter paper soaked in sterile distilled water. One germinated seed was planted per pot.

White, plastic pots (diameter 11 cm, height 13 cm) were filled with 1.4 kg of soil:sand mixture (1:9 w/w). Soil was collected from Mallala, South Australia, had pH 7.4 (1:5 soil measured in 0.01 M $CaCl_2$) and contained 14.7 mg $NaHCO_3$-extractable P kg^{-1} as determined by the method of Colwell (1963). Soil and steamed sand were autoclaved separately at 121°C and 240 kPa pressure for 1 h on two occasions before mixing. One seed was sown per pot at 2 cm depth. Before sowing the seed, a few drops of *Rhizobium* solution were poured onto the soil directly under the seed. The plants were grown in a glasshouse with some temperature control aimed to achieve 22°C (night)-25°C (day) temperatures and watered to 12% gravimetric water content with RO water three times per week. A modified Long Ashton solution minus P (Smith and Smith, 1981) was applied at a rate of 10 ml per pot per week. The nutrient solution contained macronutrients 2 mM K_2SO_4, 1.5 mM $MgSO_4$, 4 mM $CaCl_2$, 8 mM $NaNO_3$ and micronutrients 2.86 mg L^{-1} H_3BO_3, 1.81 mg L^{-1} $MnCl_2.4H_2O$, 0.22 mg L^{-1} $ZnSO_4.7H_2O$, 0.08 mg L^{-1} $CuSO_4.5H_2O$, 0.025 mg L^{-1} $Na_2MoO_4.2H_2O$ and Fe-EDTA to give 5 mg Fe L^{-1}.

3.4. PLANT HARVEST

At harvest, plants were washed free of soil, and shoots and roots were separated. Fresh weight of shoots and roots were recorded and disease severity and disease index determined (see section 3.5). Shoot material and sub-samples of roots for dry weights were oven-dried at 80°C overnight, and weighed. Sub-samples of roots required for DNA extraction were frozen in liquid nitrogen immediately after washing and blotting dry, and kept at –80°C. Sub samples of roots for determining fungal infection were weighed, cleared, stained and stored as described in section 3.5.

3.5. ASSESSMENT OF DISEASE RATING AND ROOT INFECTION

To assess the disease severity, the roots were rated visually using a 0-4 scale in which 0 = no lesions, 1 = small lesions, each 1-3 mm, 2 = more extensive lesions, up to 5 mm, 3 = lesions longer than 5 mm and with necrotic areas, 4 = lesions over 50% of the root area and necrosis (Liu and Sinclair, 1991). A Disease Index (DI) was calculated using the following equation: % DI = (Σ (n x Sn) x 100/4N) x 100, where n = number of plants with a given disease rating, Sn = disease rating, and N = total number of plants rated (Liu and Sinclair, 1991).

For assessment of fungal infection, sub-samples of root material were cut into approximately 1 cm segments. They were cleared in 10% KOH for four days or longer at room temperature, and then were washed twice with distilled water and rinsed once with 1 N HCl. The root samples were then stained in trypan blue (1.3 g trypan blue, 650 ml lactic acid, 600 ml glycerol, 800 ml RO water) for 30 min, a modification of the method of Phillips and Hayman (1970), omitting phenol from the reagents. Roots were rinsed several times with distilled water to remove excess stain and stored in lactoglycerol (50% lactic acid:50% glycerol).

The roots were then spread in a Petri dish, with a grid consisting of squares with length of 0.8 cm, stuck to the underside. The entire grid was examined under 20X magnification using a dissecting microscope and the intersections between roots and gridlines counted. The percent root infection was calculated by dividing infected root intersects by total root intersects, x 100 (Giovannetti and Mosse, 1980). Root infection was measured based on the presence of fungal structures in the roots. If two fungi were present together in the same roots (for example in *G. coronatum* + BNR or *G. coronatum* + *R. solani* treatments), infection by each fungus was estimated separately. Total root length of the root sub-samples was measured and calculated using the equation of Tennant (1975): R=N x L(grid) x 11/14, where R is the root length (cm), L(grid) is the length of grid (0.8 cm), and N is the number of intersects between roots and gridlines.

3.6. STATISTICAL ANALYSIS

Data were analysed using the GENSTAT 5 programme (GENSTAT 5 Committee, 1987). Analysis of variance (ANOVA) was carried out to determine the significance differences. Means were separated using the LSD test at 5% level of probability. Percentage data for fungal infection were arcsin transformed prior to analysis.

3.7. MOLECULAR TECHNIQUES

3.7.1. Extraction and measurement of DNA

3.7.1.1. DNA extraction from mycelium of non-AM fungi

The DNA from mycelium of non-AM fungi was extracted using a method modified from Raeder and Broda (1985). Mycelium was scraped from a 5-7 day-old culture on PDA, and then blotted dry with No. 3 Whatman filter

paper. The mycelium was frozen and ground in liquid nitrogen to a fine powder using a mortar and pestle. Approximately 350 mg of ground mycelium was placed in a 1.5 ml Eppendorf tube and 500 µl extraction buffer was added. The composition of extraction buffer was modified from the original method (0.1 M NaCl, 0.1 M Tris-HCl, 0.01 M EDTA, 0.1 M Na_2SO_3 and 1% N-Lauroylsarcosine, pH 8.5 [Guidet *et al.*, 1991]). The mixture was incubated at 65°C for 30 min prior to addition of 500 µl phenol:chloroform:isoamyl-alcohol (25:24:1) and centrifugation at 13,000 *g* for 20 min. The upper aqueous layer was collected and incubated with 25 µl ribonuclease solution (10 mg ml^{-1} RNase A [70 U mg^{-1}] in TE buffer (10 mM Tris-HCl, 1 mM EDTA) for 1 min. An equal volume of chloroform:isoamyl-alcohol (24:1) was added, followed by centrifugation at 13,000 *g* for 10 min. The upper aqueous layer was collected and DNA was precipitated with 0.5 volume of cold isopropanol. The DNA pellet was washed in 1 ml of 70% ethanol, dried and resuspended in 50 µl sterile deionised water. Dissolved DNA samples was kept at –20°C until used.

3.7.1.2. DNA extraction from spores of mycorrhizal fungi

Groups of spores with visible contents, and apparently viable, were selected for DNA extraction. Spores were placed in 1.5 ml Eppendorf tubes containing of 50 µl extraction buffer as described in section 3.7.1.1. (Guidet *et al.*,1991). The spores were crushed in the extraction buffer, heated for 10-15 min, kept on ice for 2-3 min, and then centrifuged at 2,900 *g* for 3 min at room temperature. The supernatant containing DNA was transferred into a clean Eppendorf tube and stored at –20°C until used.

3.7.1.3. DNA extraction from roots

Extraction of DNA from roots was based on the method of Rogers and Bendich (1985). Root samples (approximately 1 g of fresh tissue) were frozen in liquid nitrogen and ground to a fine powder using a mortar and pestle. Ground tissue was suspended in 1 ml of 2x CTAB (100 mM Tris-HCl [pH 8.0], 1.4 mM NaCl, 2% cetylmethylammonium bromide-CTAB, and 1% polyvinyl polypyrrolidone) at 65°C for 3 min. An equal volume of chloroform:isoamyl alcohol (24:1) was added, mixed and vortexed before centrifugation at 11,600 *g* for 2 min. The supernatant was transferred to a fresh 1.5 ml Eppendorf tube, one volume of CTAB precipitation buffer (1% CTAB, 50 mM Tris-HCl (pH 8.0), 10 mM EDTA) was added and the mixture centrifuged at 11,600 *g* for 1 min. The pellet was resuspended in 200 µl TEN buffer (10 mM Tris-HCl [pH 7.0], 10 mM EDTA, 1 M NaCl) at 65°C for 10 min, 10 mg ml^{-1} RNase A was added and the sample was incubated for a further 30 min at 37°C. The DNA was then precipitated by adding two volumes of cold ethanol (95%) and 1 µl 2 M ammonium acetate (CH_3COONH_4) and centrifuged at 13,600 *g* for 20 min. The DNA pellet was washed with 70% ethanol, dried and resuspended in 50 µl of sterile deionised water. When dried root samples were used, the DNA was extracted using the DNeasy Plant Mini Kit (QIAGEN) following the manufacturer's instructions.

3.7.1.4. DNA extraction from soils

Extraction of DNA from soils was carried out by using the Ultra Clean Soil DNA Kit (Mobio Laboratories) following to the manufacturer's instructions.

3.7.1.5. Measurement of DNA samples

The amount of DNA was determined by measuring absorbance at 260 nm by UV spectrophotometry and confirmed with a known amount of DNA after running an aliquot on a 1% agarose gel against a known amount of *Hind* III digested lambda DNA (Sambrook *et al.*, 1989).

3.7.2. Polymerase Chain Reaction (PCR)

3.7.2.1. Primers

Primers used in the study were:

Primer	Size (mer)	Sequences (5′ > 3′)
ITS1	19	TCC GTA GGT GAA CCT GCG G
ITS4	20	TCC TCC GCT TAT TGA TAT GC
ITS1F	22	CTT GGT CAT TTA GAG GAA GTA A
ITS4B	23	CAG GAG ACT TGT ACA CGG TCC AG
NS3	21	GCA AGT CTG GTG CCA GCA GCC
NS4	20	CTT CCG TCA ATT CCT TTA AG

The primers ITS1 and ITS4, are universal primers used to amplify fungal DNA (White *et al.*, 1990), primer pairs ITS1F and ITS4B are specific for amplification of Basidiomycetes (Gardes and Bruns, 1993), and the universal primers NS3 and NS4 target a region in the 18S ribosomal gene, and amplify DNA in all plant samples (White *et al.*, 1990; Gardes and Bruns, 1993). The location of the annealing sites of the primers is presented in Figure 3.1.

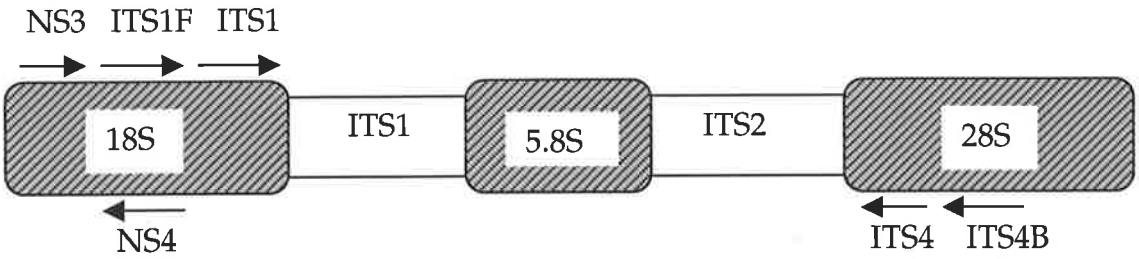


Figure 3.1. Schematic representation of the rDNA region, showing ITS1 and ITS2 regions. The shadowed boxes represent the ribosomal subunits. Annealing sites of the primers listed are indicated.

3.7.2.2. PCR amplification

The amplification reactions were performed in a 25 µl volume containing 1 µl of diluted DNA, 250 µM 10x buffer (20 mM Tris-HCl [pH 8.0 at 25°C], 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 50% glycerol, 1% Triton X100), 1.5 mM $MgCl_2$, and 0.5 unit of *Taq* DNA polymerase (Promega), 250 µM dNTPs, and 25 pmol ITS1 and ITS4 primers, 28 pmol ITS1F and ITS4B primers, or 15 pmol NS3 and NS4 primers. Reactions were carried out in a Programmable Thermal Cycler (MJ Research Inc., PTC-60 Thermal Cycler, Watertown, Mass.) with following parameters: initial denaturation at 94°C for 2 min 30 sec, followed by 40 cycles consisting of denaturation at 94°C 35 sec, annealing 58°C 30 sec (when ITS1 and ITS4, or NS3 and NS4 primers were used) or 55°C for 30 sec (when ITS1F and ITS4B primers were used), and extension at 72°C 1 min 30 sec. There was a final extension step at 72°C for 10 min followed by cooling to 4°C until samples were recovered. Each reaction was overlaid with a drop of mineral oil (Sigma). A negative control that did not contain DNA was included in every experiment. Amplification reactions were performed at least three times to check the consistency of the method.

3.7.3. Gel electrophoresis

Aliquots (5 µl) of each PCR product were separated by electrophoresis on 1.2% agarose gel in Tris-acetate-EDTA buffer (100 mM Tris, 125 mM sodium acetate, 1 mM EDTA, pH 8.0). Gels were stained with ethidium bromide and visualised over a UV transilluminator. In all electrophoresis experiments, a 1 kb DNA marker (Gibco BRL) was used as a molecular size standard or otherwise stated.

CHAPTER 4. MORPHOLOGICAL AND MOLECULAR CHARACTERISATION OF AN ISOLATE OF BINUCLEATE *RHIZOCTONIA* SP. (BNR) FROM A MYCORRHIZAL POT CULTURE

4.1. INTRODUCTION

Pot cultures, which are used to multiply propagules of AM fungi, often have a risk of contaminant microorganisms. The contaminant microorganisms, such as non-pathogenic bacteria, actinomycetes, chytrids or plant pathogens could be present in soil together with AM propagules, attached to the spores or infecting the roots (Mosse, 1970; Menge, 1984; Williams, 1985; Scannerini and Bonfante-Fasolo, 1991; Biancioto *et al.*, 1996). Their presence becomes a problem in experiments where clean root materials or spores are needed. Studies on how they affect inoculum potential of AM fungi, influence mycorrhizal symbioses or plant growth responses are limited. The only known publication concerning fungal contamination in pot cultures was from Williams (1985). He found that sterile, septate fungi (*Rhizoctonia*), attributed to orchid mycorrhizal fungi occurred, in pot cultures of AM fungi. Inoculation of plants with this fungus affected the plant growth only when the AM fungi were present, but did not have an effect on plant growth in aseptic culture (without AM fungi).

Binucleate *Rhizoctonia* sp. (BNR) have been found infecting roots of a number of plants used as hosts in pot cultures (S. Smith, personal communication). The characteristics and identification of the BNR isolated from mycorrhizal pot cultures have yet to be studied. The lack of information on the identity and character of BNR isolates in pot cultures causes difficulties in developing an effective control of this fungus. Currently there are 21 anastomosis groups (AGs) of BNR, but there is no information on which AG of BNR was responsible for causing contamination in pot cultures.

Since the AGs often show differences in host preferences and levels of pathogenicity (Ogoshi, 1987), investigations on the AG of the isolate of BNR from pot cultures were needed.

Molecular techniques have been widely used in the identification of plant pathogenic fungi and shown to be more sensitive and specific than many traditional methods (Henson and French, 1993). The PCR assay is one approach that allows detection of extremely small quantity of specific DNA. The use of PCR has been successfully applied for BNR isolated from soil or infected plants (Cubeta *et al.*, 1991; Kuninaga *et al.*, 1997; Gonzales *et al.*, unpublished; MacNish and O'Brien, unpublished), but has never been applied for identification and detection of BNR isolates from pot cultures.

This study aimed to identify and characterise a previously unknown isolate of BNR from a mycorrhizal pot culture using traditional morphological techniques and molecular techniques. The morphological characteristics of the BNR were observed. The AG of the isolate of BNR was determined by PCR-RFLP technique and further confirmed by hyphal anastomosis grouping. For the molecular approach, identification was achieved by sequencing the rDNA of BNR. The ITS sequence was further used for assessment of percentage nucleotide similarity with reference sequences of BNR obtained from GenBank and design of specific primers. The ability of the isolate of BNR to form typical infection structures in plant roots (as observed in pot cultures is described in Chapters 6 and 7).

4.2. MATERIAL AND METHODS

4.2.1. Isolation and DNA extraction

The BNR isolate was collected from a *Glomus mosseae* pot culture of clover (*Trifolium subterraneum* L. cv. Mt Barker). Preliminary investigations showed that there was contamination by BNR in mycorrhizal roots of clover. The aim was to obtain pure infection of the BNR isolate and eliminate further infection of *G. mosseae*. Therefore, a soil and sand mixture of this pot culture was used as a growth medium for cabbage (*Brassica oleracea* L.), which is a non-host of AM fungi. Using this method, mycorrhizal infection was excluded and BNR infection was obtained. The plant was grown from surface-sterilised seed in a 600 g of potting mix in a white plastic pot for 6 weeks.

At harvest, small sections of roots of cabbage were surface sterilised for 10 min with 3% (v/v) sodium hypochlorite, rinsed in three changes of sterile distilled water, plated on 2% water agar, and incubated at 25°C. After 48 h, fungal colonies from the tissue pieces were examined with a light microscope at x100 magnification. Colonies with mycelial characteristics of BNR were transferred to fresh PDA and incubated at 25°C. Mycelium of BNR on PDA was used for morphological identification and DNA extraction.

DNA was extracted from mycelium of 5-7 day-old cultures of the isolate of BNR grown on PDA. The mycelial mat was blotted dry with No. 3 Whatman filter paper. The method for DNA extraction from fungal mycelium was modified from Raeder and Broda (1985) and described in Chapter 3 section 3.7.1.1.

4.2.2. Morphological identification and culture characteristics

4.2.2.1. Fungal characteristics

Morphological characteristics of the BNR isolate were described following growth for 7 days on PDA in 9-cm diameter Petri dishes at 25°C in the dark. Mycelium was observed under a light microscope. The identification of the BNR isolate was based on the characteristics of the hyphal branching and septation pattern (Sneh *et al.*, 1991).

4.2.2.2. Colour and growth rates

The colour of the mycelium was defined from colonies grown on PDA for 7-14 d at 25°C. To measure the colony growth rate, an agar disk was excised from the margin of an actively growing culture of BNR, and placed at the edge of fresh PDA plates. Cultures were incubated at 20 and 25°C with three replicate plates at each temperature. Hyphal growth rate on PDA was determined every 24 h until the colony reached the edge of the petri dish.

4.2.2.3. Hyphal width and length

The width and length of hyphae and monilioid cells were measured in 48-72 h-old cultures grown on 6% water agar on sterile microscope slides at 20°C in humid conditions.

4.2.2.4. Nuclear number

Nuclei were stained by treating 7-day-old hyphae of BNR grown on PDA with a solution of 1µg/ml of 4′, 6′-diamidino-2-phenylindole (DAPI) (Sneh *et al.*, 1991). Fluorescent nuclei were observed at 400X magnification using a Zeiss photomicroscope equipped with fluorescence using an excitation filter G365, dichromatic beam splitter FT 395, and barrier filter LP 240 (Olaya and Abawi, 1994a).

4.2.2.5. Determination of anastomosis group

AG determination was carried out by PCR-RFLP (Cubeta *et al.*, 1991) and confirmed by hyphal anastomosis grouping (Carling *et al.*, 1990).

4.2.2.5.1. PCR-RFLP

PCR amplification

The PCR-RFLP method was based on the work of Cubeta *et al.* (1991). Two oligonucleotide primers LR0R (5′-ACC CGC TGA ACT TAA GC-3′) and LR7 (5′-TAC TAC CAC CAA GAT CT-3′) were used to amplify a portion of the 25S rRNA gene (rDNA). PCR reaction was set up in 20 µl volume using buffer conditions as described in Chapter 3 section 3.7.2.2. Reactions were carried out in an Automated Thermal Cycler (Perkin-Elmer) with the following parameters: initial denaturation at 94°C for 4 min, followed by 30 cycles consisting of denaturation at 94°C for 1 min, annealing at 50°C for 45 sec, followed by 50-72°C gradual increase for 1 min, and extension at 72°C for 1 min. The reaction was followed by final extension at 72°C for 7 min. A negative control without DNA template was used in each amplification. After amplification, a 5 µl aliquot from each sample was resolved by electrophoresis in 1.2% agarose gel and visualised over a UV transilluminator following ethidium bromide staining.

Restriction digestion of PCR products

Amplified PCR products were extracted once with 1 volume of chloroform/isoamyl alcohol (24:1). Then the DNA was precipitated by addition of 1/10 volume of 3 M sodium acetate (pH 5.0) followed by 3 volumes of 95% ethanol. Ample time was given for precipitation (>1 h at –20°C or 24 h at room temperature). The precipitates were collected by centrifugation for 15 min in a microfuge, washed in 80% ethanol, dried under vacuum, and resuspended in 50 µl TE buffer. For restriction analysis, 8 µl of

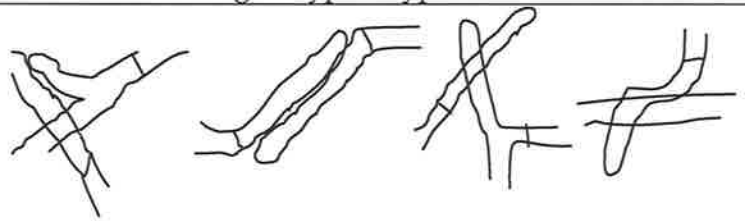
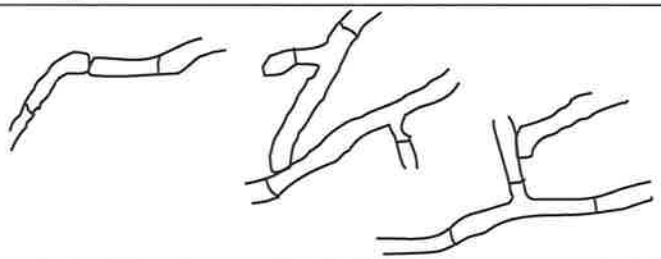
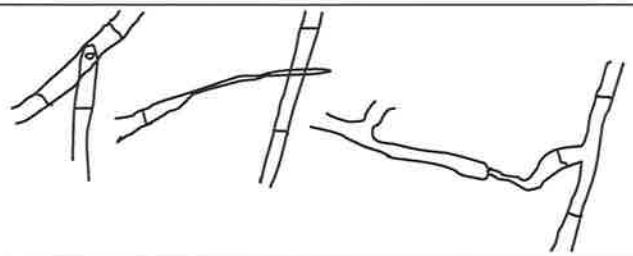
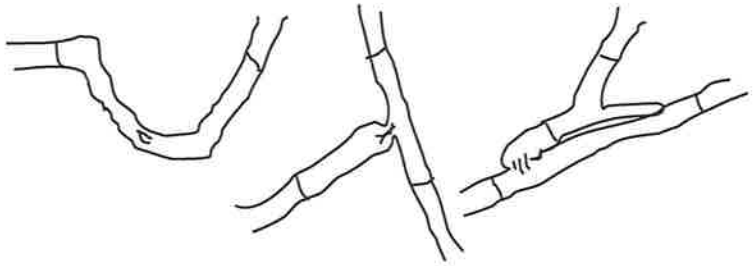
PCR product (approximately 300 ng) was digested with either *Taq* 1, *Hha*1, *Sau*3AI, *Hae*III, or *Hin*fI for 3 h following the manufacturer's instructions (Promega). These 4 enzymes have been useful in separating the different AGs of BNR (Cubeta *et al.*, 1991). After digestion, samples were subjected to electrophoresis in a 3% agarose gel. Bacteriophage (Φ-X174) DNA digested with *Hae*III and 1 kb DNA marker (Gibco-BRL) were used as a molecular size standards to determine the size of restriction fragments. Gels were stained with ethidium bromide and visualized over a UV transilluminator. The experiments were conducted twice.

4.2.2.5.2. Hyphal anastomosis grouping

Discs (5 mm) of the isolate of BNR and each tester isolate (details in Chapter 3 Table 3.1) were placed in pairs, 2-3 cm apart, on 2% water agar-coated slides, and placed on moist filter paper in Petri dishes which served as moisture chambers. The Petri dishes were incubated at 25°C in the dark and the growth was monitored every 24 h. As soon as hyphae of the two isolates made contact, slides were removed from the Petri dish, and examined under the microscope at x100 magnification.

In all isolate pairings, the three main reaction categories were detected and scored as follows: no hyphal attraction or anastomosis ('C0'), contact between hyphae without evidence of wall penetration ('C1'), hyphal anastomosis followed by a cellular killing reaction at the hyphal fusion junctions ('C2'), and 'perfect' fusion involving no killing reaction and maintenance of cytoplasmic continuity ('C3') (see Table 4.1). All pairings were made on at least three separate occasions. AG determination was scored positively (confirmed at 400x magnification) when five or more hyphal anastomosis points were observed (Carling *et al.*, 1987; Carling, 1996).

Table 4.1. Categorisation of anastomosis between hyphae in *Rhizoctonia* (Carling *et al.*, 1990)

Category	Schematic drawing of hypha-hypha reaction*	Description of interaction	Relatedness
'C0'		No interaction	Not related (different anastomosis groups)
'C1'		Contact between hyphae Apparent attachment of wall but no evidence of wall penetration or membrane-membrane contact Occasionally one or both anastomosing cells and adjacent cells may die	Distantly related (different anastomosis groups or same anastomosis groups)
'C2'		Wall penetration obvious Membrane contact uncertain, location of reaction site obvious Pore at point of penetration less than hyphal diameter Anastomosing and adjacent cells frequently die	Related (same anastomosis groups but different clones)
'C3'		Walls fuse Membranes fuse Point of fusion not obvious Pore at point of connection equal or nearly equal to hyphal diameter Anastomosing cells may die but generally do not	Closely related (same anastomosis groups, clone or isolate)

*redrawn from Sweetingham and MacNish, 1994

4.2.3. Molecular identification

4.2.3.1. PCR amplification

Amplification of the fungal DNA was performed in a total of volume of 25 µl by mixing 1 µl of the template with 10 x buffer, $MgCl_2$, dNTPs, *Taq* DNA polymerase and the primers ITS1F and ITS4B. Details of the buffer and primers were given in Chapter 3 section 3.7.2.2. A negative control that did not contain DNA was included in every reaction. Amplification was performed in a Programmable Thermal Cycler (MJ Research Inc.) with conditions described in Chapter 3 section 3.7.2.2. After amplification, a 5 µl aliquot from each sample was resolved by electrophoresis in 1.2% agarose gel and visualised over a UV transilluminator following ethidium bromide staining.

4.2.3.2. Sequencing of the ITS regions, sequence analysis and design of specific primers

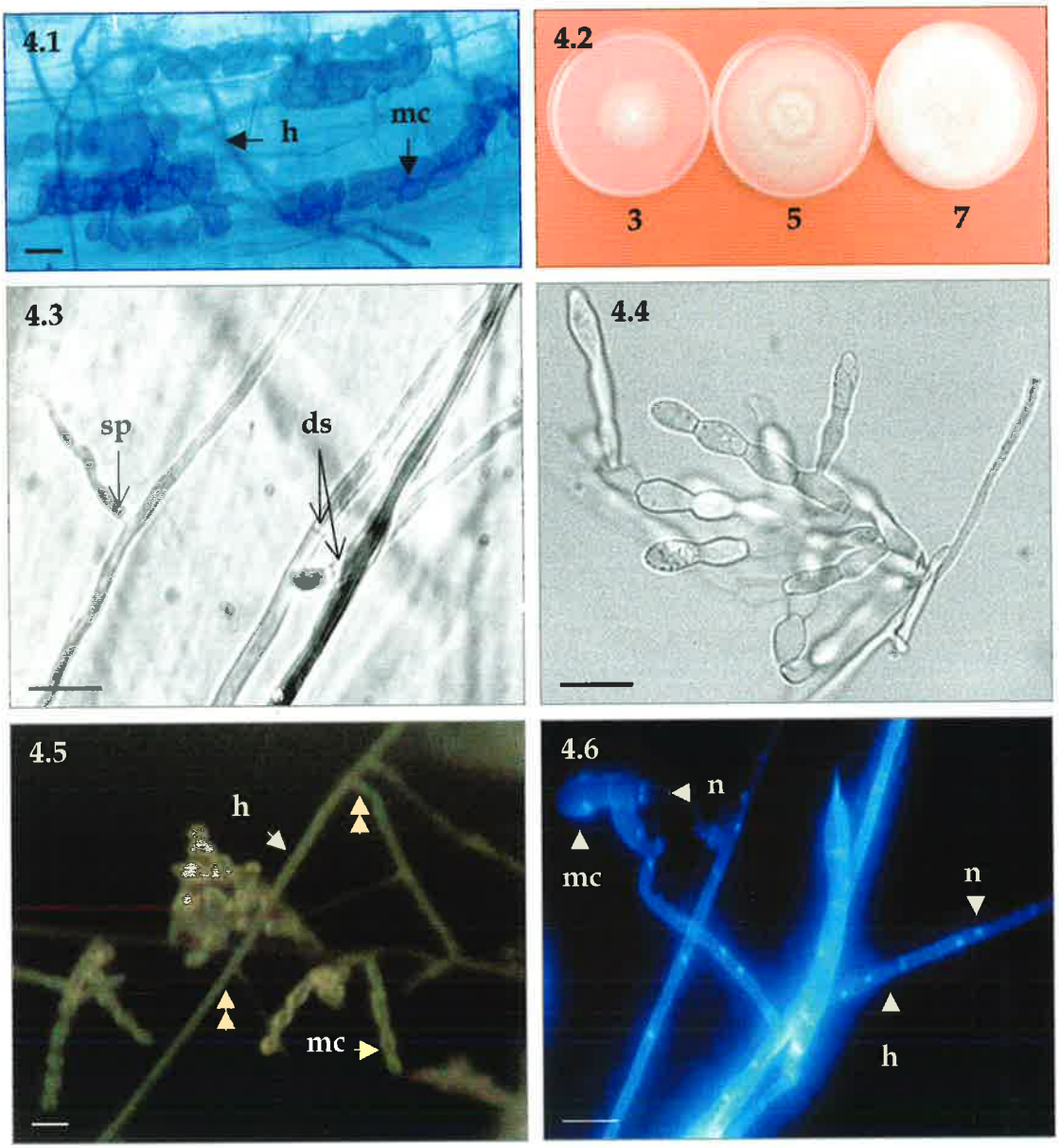
Amplification products were purified with the QIAquick Gel Extraction Kit (Qiagen). Purified DNA was sequenced with ITS1F and ITS4B primers using an Applied Biosystems automatic sequencer, conducted at the Nucleic Acid Sequencing Unit, Department of Plant Science, The University of Adelaide. The sequence data were analysed using the DNASTAR software. BLAST was used to compare the ITS sequences obtained in this study with DNA sequences in GenBank (Altschul *et al.*, 1990). Percent similarity in the ITS sequences was determined after multiple sequence alignment with the Clustal method omitting all sites with gaps using the MegAlign program (DNASTAR). Specific primers were designed from the ITS region of the rDNA, and checked for complementary sequences within a primer and between primers using the Primer Select, program (DNASTAR).

4.3. RESULTS

4.3.1. Colony morphology, nuclear condition and growth rates

Typical infection of BNR in roots was recognised by the appearance of chains of cells (monilioid cells) and thin hyphae (Figure. 4.1). The isolate was able to infect the roots; however, the hyphae and monilioid cells were only observed in the epidermal cells and never penetrated the cortical cells. Isolation from roots of cabbage yielded one isolate of BNR, named as CFM1, after incubation at 25°C on water agar. The hyphae of the isolate were observed within 2 days of incubation of small root fragments, which did not show any indication of necrosis. BNR isolate CFM1 showed the mycelial characteristics of *Rhizoctonia* (Sneh *et al.*, 1991). On PDA, the colony colour was uniformly white and concentric zonation was commonly observed (Figure 4.2.). The colony growth rate of BNR isolate CFM1 on PDA was usually radial and moderately rapid (3.5 to 7.2 mm/day at 20°C, or 6.5 to 9.4 mm/day at 25°C).

BNR isolate CFM1 produced septate and branched hyphae (Figures 4.3 and 4.6). Branch hyphae had a septum proximal to the hypha from which they originated, characteristic of *Rhizoctonia* (Figure 4.3). Branches arose at right angles (90°) from the main hypha or at acute angles (45°) to the main hypha (Figure 4.5). The width of the hyphae was 2.4-4.9 µm and the length was 12.1 to 92.3 µm. As hyphae matured (3-7 days in culture), hyaline chains of cells (monilioid cells) (Figure 4.4), which were sometimes simple or branched, were produced. The monilioid cells were lobate, 6.1-12.1 µm wide and 12.1-29.1 µm long. Fluorescence of nuclei after DAPI staining of the hyphae and monilioid cells confirmed that each 'cell' of the hyphae and monilioid cells contained two nuclei (binucleate) (Figure 4.6).



Figures 4.1-4.6. Morphological characteristics of the BNR isolate obtained from a mycorrhizal pot culture. **Figure 4.1.** Typical infection of BNR in roots after staining with trypan blue. **Figure 4.2.** White colony colour of BNR isolate CFM1 at 3, 5 and 7 days after incubation at 25°C on PDA. **Figure 4.3.** Septum proximal (sp) in the branch hyphae from the main hyphae, and presence of dolipore septa (ds) of BNR isolate CFM1. **Figure 4.4.** Monilioid cells of BNR isolate CFM1. **Figure 4.5.** Growth of mycelium of BNR isolate CFM1 on PDA shows branches arise at 90° (double arrows) from main hyphae (h), and simple or branched monilioid cells (mc). **Figure 4.6.** Fluorescence of nuclei after DAPI staining shows 2 nuclei (binucleate) inside each hyphal cell (h) and monilioid cell (mc) of BNR isolate CFM1. *Bar* = 25 μm.

4.3.2. Determination of anastomosis group

AG determination of BNR isolate CFM1 was first carried out using PCR-RFLP. Result showed that one band (1.4 kb) of rDNA product was obtained following amplification with PCR using LR0R and LR7 primers (Figure 4.7). PCR products digested with four restriction enzymes showed differences in restriction fragment size. When the results were compared with the results of Cubeta *et al.* (1991), the restriction patterns of the isolate were similar to the restriction pattern of the isolates of BNR AG-A and AG-Bo. This indicates that BNR isolate CFM1 could be either AG-A or AG-Bo, and further techniques to determine AG of BNR were used.

The traditional method of hyphal anastomosis grouping based on the method of Carling *et al.* (1990) was used to determine the AG of BNR. The hyphae of BNR isolate CFM1 were tested only against the hyphae of the tester isolates of BNR AG-A (C-517) and AG-Bo (SIR-2). Results showed that perfect fusions (C3), involving cytoplasmic fusion and continuity at hyphal tip-tip contacts and killing reaction (C2), were detected between BNR isolate CFM1 and the tester isolate of BNR AG-Bo (SIR-2). This indicated that BNR isolate CFM1 was AG-Bo (Figures 4.8, 4.9). There was a C0 reaction (no interaction or anastomosis of hyphae) when the hyphae of BNR isolate CFM1 were tested against the hyphae of the tester isolate of BNR AG-A (C-517) (Figure 4.10). This indicated that BNR isolate CFM1 did not belong to AG-A. Repeated experiments showed the same results.

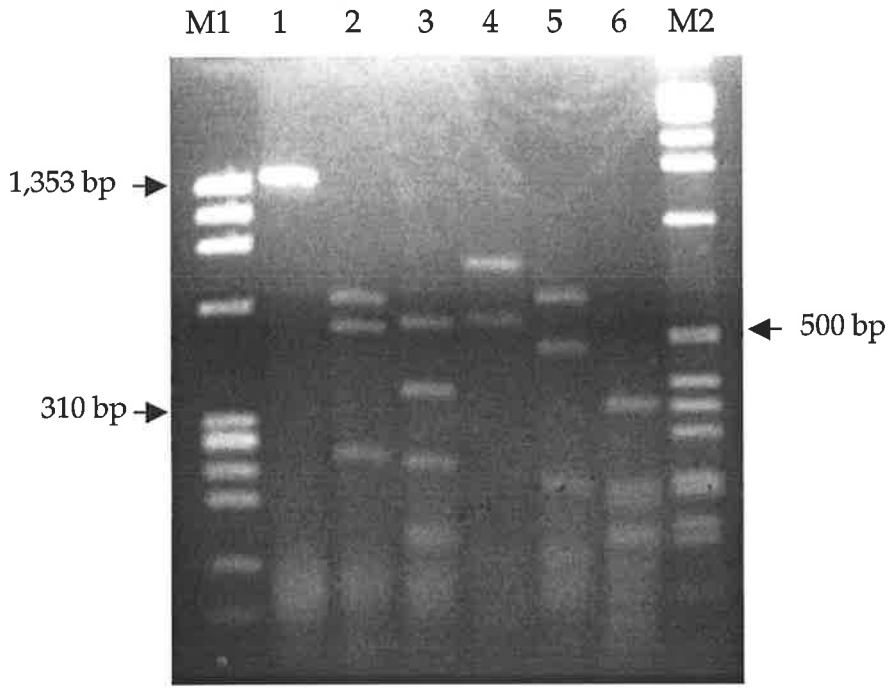
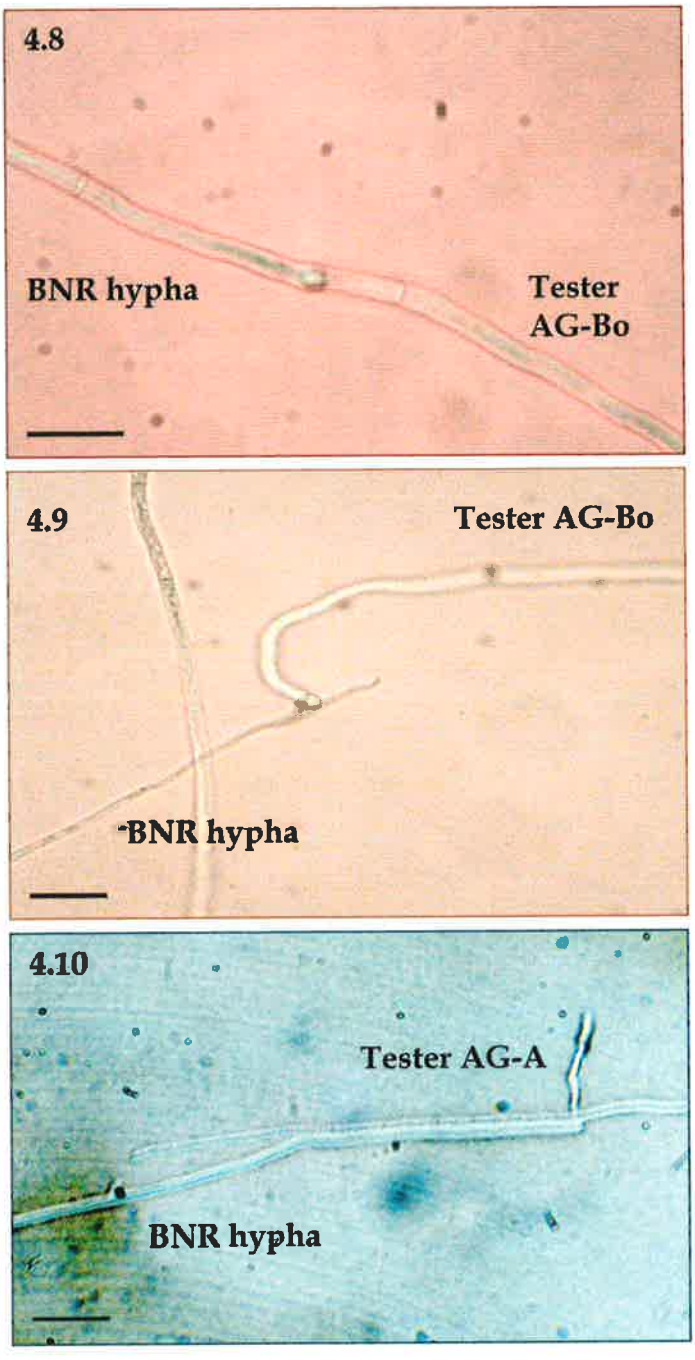


Figure 4.7. Amplified rDNA of BNR isolate CFM1 digested with four different restriction enzymes after electrophoresis in 3% agarose at 100V for 3 h and staining with ethidium bromide. Amplified rDNA with LR0R and LR7 primers before digestion produced a 1.4 kb fragment (lane 1). Lanes 2-6. Digestion with *Taq*1 (2), *Hha*I (3), *Sau*3AI (4), *Hae*III (5) and *Hin*fI (6) produced different restriction patterns. M1 = Φ-X174 marker digested with *Hae*III, M2 = 1 kb molecular size marker (Gibco BRL).



Figures 4.8-4.10. Hypha-hypha reactions between BNR isolate CFM1 from a mycorrhizal pot culture and tester isolates of BNR, AG-Bo or AG-A, on water agar-coated slides. **Figure 4.8.** Perfect fusion involving membrane and wall fusions (C3 reaction) between hyphae of BNR isolate CFM1 and the tester isolate of BNR AG-Bo (SIR-2). **Figure 4.9.** Killing reaction (C2) showing cell death and reduction of the diameter hypha of BNR isolate CFM1 when paired with hypha of the tester isolate of BNR AG-Bo (SIR-2). **Figure 4.10.** No interaction and anastomosis (C0 reaction) between hyphae of BNR isolate CFM1 and the tester isolate of BNR AG-A (C-517). *Bar* = 25 μm.

4.3.3. Molecular identification

4.3.3.1. ITS region amplification and sequencing

PCR amplification of BNR isolate CFM1 genomic DNA with the ITS1F and ITS4B resulted in a single amplification fragment (Figure 4.11). Direct sequencing of the PCR product showed a total length of 667 bp (Figure. 4.12), which included the ITS1 primer site, ITS1 region, 18S, 5.8S, and 28S genes, ITS2 region and the ITS4 primer site. The ITS1 region (180 bp) started at 38 base position from the 5′ end and the ITS 2 (274 bp) started at position 373 from the 5′ end. The complete sequence was deposited in the GenBank database, accession number AF446088.

4.3.3.2. Design of BNR-specific primers

From the results of the ITS sequence, specific primers were designed with the aim of obtaining specific amplification of BNR AG-Bo for identifying this fungus in plant roots and in soil (see Chapter 5). The Primer Select (DNASTAR) found conserved stretches of bases from the ITS region between 109 to 128 bp (CF1f) and from the ITS2 region between 567 to 587 bp (CF2r). The primers CF1f (5′- TGT GCA CTT GTG AGA CTG GA -3′) and CF2r (5′- GAA TGG ACT ATT AGA AGC GG -3′) would amplify a specific 438 bp fragment (Figure 4.12).

4.3.3.3. Sequence similarity

Database searches using BLAST of the ITS sequence obtained in the present study produced a significant alignment with the ITS sequence of *Ceratobasidium* sp. AG-Bo, anamorph: *Rhizoctonia* sp. (AF354091; Gonzales *et al.*, unpublished, result not shown).

Figure 4.11. PCR amplification of mycelium DNA of BNR AG-Bo isolate CFM1 with ITS1F and ITS4B primers shows a single amplification product (line 1). M = 1 kb molecular size marker (Gibco BRL).

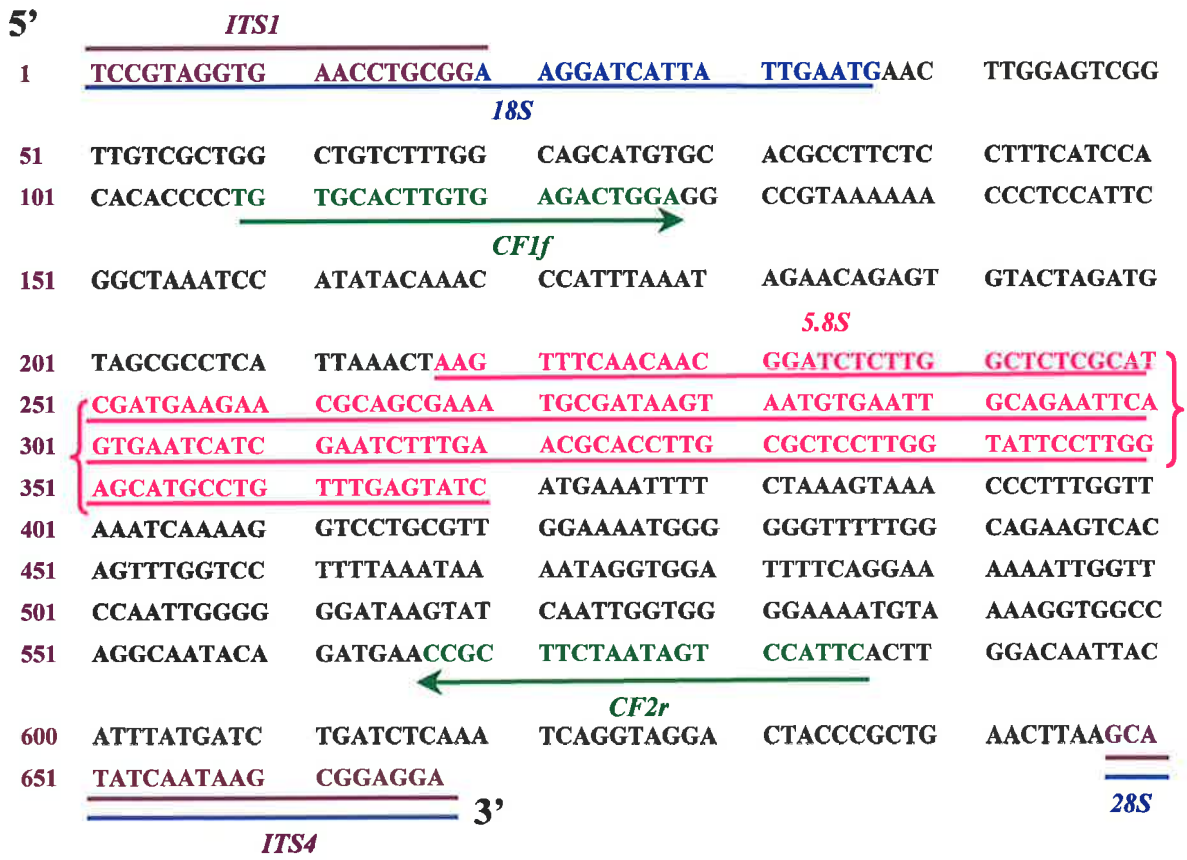


Figure 4.12. Sequence of ribosomal DNA region (667 bp) (5'-3') amplified with ITS1F and ITS4B primers from BNR AG-Bo isolate CFM1 (accession number AF446088 in GenBank). Sequence of rDNA shows the position of ITS1 primer and ITS4 primer, 18S, 5.8S, and 28S genes, and the BNR specific primers, CF1f (forward primer) and CF2r (reverse primer).

Table 4.2. Percent similarity between ITS sequence (ITS region and 5.8S gene) of BNR AG-Bo isolate CFM1 (AF446088) with available sequences of BNR in GenBank after multiple sequence alignment with the Clustal method using the MegAlign program (DNASTAR).

Acession number	Isolate	AG/CAG	Origin	Sequence length	ITS sequence similarity (%)
AF354091	SIR-2	AG-Bo	Japan	599 bp	90.2
AB000040	NS-2	AG-A	Japan	615 bp	88.5
AF354092	C-662	AG-A	Japan	589 bp	88.1
AF354087	C-455	AG-Bb	Japan	621 bp	68.6
AF354088	C-460	AG-Ba	Japan	631 bp	64.8
AF354090	C-610	AG-D	Japan	629 bp	70.9
AF354085	SIR-1	AG-F	Japan	623 bp	77.2
AF354089	STC-9	AG-H	Japan	597 bp	79.2
AF354093	FK02-1	AG-L	Japan	583 bp	83.1
AF354094	FK06-2	AG-O	Japan	592 bp	83.7
AF354095	C-620	AG-Q	Japan	636 bp	72.9
AF354086	BN1	CAG-1	USA	627 bp	71.1
AF354081	BN31	CAG-3	USA	651 bp	76.0
AF354081	BN38	CAG-4	USA	620 bp	76.5
AF354082	BN37	CAG-5	USA	620 bp	77.9
AF354083	BN74	CAG-6	USA	615 bp	76.9
AF354084	BN22	CAG-7	USA	625 bp	76.7
AF407005	TBR37	nd	Australia	581 bp	59.1
AF407006	TBR39	nd	Australia	607 bp	57.2
AF407007	TBR40	nd	Australia	504 bp	59.5
AF407008	TBR41	nd	Australia	609 bp	59.0
AF407009	TBR48	nd	Australia	587 bp	58.8

nd = not determined

The ITS sequence of BNR isolate CFM1 (AG-Bo) obtained in the present study was compared to available ITS sequences of BNR in GenBank (Table 4.2). About 90 % of the ITS sequence of BNR isolate CFM1 (AG-Bo) obtained in the present study aligned with the published ITS sequence of BNR AG-Bo isolate SIR-2 (Gonzales *et al.*, unpublished). The ITS sequence of BNR isolate CFM1 had about 88% similarity with the sequences of AG-A (Kuninaga *et al.*, 1997; Gonzales *et al.*, unpublished), and about 65-83% with other isolates of BNR of different AGs from Japan. BNR isolate CFM1 sequence had 61-68% similarity with the isolates of BNR of different AGs from the USA. There was less similarity (57-59%) of the ITS sequence between BNR isolate CFM1 and different isolates of BNR from Australia (AG not determined) (MacNish and O'Brien, unpublished).

4.4. DISCUSSION

This morphological characterisation in conjunction with molecular identification provides a firm basis to recognise the isolate of BNR obtained from a pot culture of AM fungi.Many species of fungi belonging to BNR have been isolated from roots of many cultivated plants or from soil (Cubeta *et al.*, 1991; Sneh *et al.*, 1991). The present study reported for the first time an isolate of BNR (CFM1) recovered from a pot culture of mycorrhizal fungi belonged to AG-Bo. Identification was based on morphological characteristics and number of nuclei per hypal cell, which are the most useful techniques for identification of *Rhizoctonia* spp. The morphological characteristics of the isolate were matched with the general features of described species of *Rhizoctonia* species, given by Sneh *et al.* (1991), which are: branching near the distal septum of cells in young vegetative hyphae, constriction of hyphae and formation of septa a short distance from the point of origin of hyphal branches, the presence of dolipore septa, and the absence of clamp connections, conidia, rhizomorphs. Although colonies of BNR isolate CFM1 in pure culture were not always easy to distinguish from other typical

Rhizoctonia spp., it was further determined that there were two nuclei inside each hyphal cell, confirming that it was a binucleate *Rhizoctonia*.

A molecular technique based on PCR-RFLP for determination of AG described by Cubeta *et al.* (1991) was first chosen because it is rapid and more efficient than the traditional hyphal anastomosis grouping method. This technique has been successfully used to differentiate AGs of BNR (Cubeta *et al.*, 1991). Using the same primers and four restriction enzymes described by Cubeta *et al.* (1991), BNR isolate CFM1, obtained from a pot culture of AM fungi, was tested. It produced the same restriction patterns as BNR AG-Bo or AG-A. Further analysis of anastomosis groupings was needed to confirm the AG of BNR isolate CFM1. The results suggest that the isolate used in this study was closely related to isolates from other parts of the world, and might have very closely related DNA sequences (Neuvéglise *et al.*, 1994). Although this technique can be used without the need of systematic sequencing, making the PCR approach efficient, molecular tools for the analysis of AGs have limitations because some isolates belonging to different AGs produced similar banding patterns (Cubeta *et al.*, 1991). PCR amplification products which have identical RFLP patterns with different restriction enzymes have already been shown to have very closely related DNA sequences (Neuvéglise *et al.*, 1994).

A second approach was made to determine the anastomosis group of BNR isolate CFM1. Anastomosis groupings were carried out by pairing BNR isolate CFM1 with the tester isolates of BNR belonging to AG-A and AG-Bo. Although this method was time consuming, it was useful to confirm that the AG of BNR isolate CFM1 belonged to AG-Bo. The AG analysis further indicated that BNR isolate CFM1 belonged to the AG-Bo grouping known to contain members that are both pathogenic or non-pathogenic. Previous study showed that isolates of AG-Bo could be pathogenic on rice, causing sheath blight-like lesions (Ogoshi *et al.*, 1979; Ogoshi, 1985), or non-pathogenic on

corn and wheat (Herr, 1989). Previous study reported that the teleomorph of BNR AG-Bo is *Ceratobasidium cornigerum*, which involves non-pathogenic root associations with orchids (Ogoshi *et al.*, 1983; Cubeta *et al.*, 1991).

Due to similarity in morphology, it has been a problem to differentiate hyphae of BNR and *R. solani*. The introduction of molecular techniques has provided powerful tools to be used for rapid and accurate identification of fungi. PCR has been widely used as a method for rapid identification of pathogenic fungi (e.g. Moukhamedov *et al.*, 1994; Schilling *et al.*, 1996; Kageyama *et al.*, 1997; Schesser *et al.*, 1991; Johanson *et al.*, 1998), but limited study has been done with BNR. In the present study, the rDNA sequence chosen for this purpose was the ITS, which is a particularly useful region for molecular characterisation studies in fungi (Bridge and Arora, 1998). A single amplification product (667 bp) was obtained after PCR amplification with ITS1F and ITS4B primers using DNA template of the BNR AG-Bo isolate CFM1. The complete sequence was submitted to the GenBank database, accession number AF446088. The identity of the sequence was similar to *Ceratobasidium/Rhizoctonia* sp. AG-Bo (AF354091) (Gonzales *et al.*, unpublished). This molecular identification confirmed the identification of BNR isolate CFM1 by morphological characteristics and anastomosis group determination. The sequence derived from BNR isolate CFM1 had high similarity to sequences of isolates of AG-A (88%), 65-83% similarity to other sequences of BNR from different AGs from Japan and the USA, but produced low similarity to isolates from Australia. It has been reported that these BNR isolates from Australia (AF407005-407009) represented a new group, characterised by narrow hyphae (2-4 μm) and causing root diseases in lupin (MacLeod and Sweetingham, 1997). This finding showed that the ITS sequence was highly variable between isolates of the same species, as has been observed by other researchers (Gardes and Bruns, 1993; Boysen *et al.*, 1996; Kuninaga *et al.*, 1997).

The ITS regions have been used to design specific primers of *R. solani* (Salazar *et al.*, 2000), but there was no information available on the design of specific primers from sequences of the ITS region of BNR AG-Bo. Therefore, the sequencing of amplified rDNA products specific to BNR AG-Bo was further used to design specific primers that would discriminate this particular AG of BNR from other plant-associated fungi, including AM fungi. The primers might then be employed to amplify BNR DNA, thereby distinguishing BNR from other *Rhizoctonias*, which have similar characteristics and type of disease. Specific amplification of DNA of BNR AG-Bo could facilitate the identification of BNR AG-Bo at an early stage of infection, which would be useful for monitoring the contamination by this isolate of pot cultures.

4.5. CONCLUSIONS

An isolate of BNR (CFM1) was isolated from a pot culture of AM fungi and characterised by traditional and molecular techniques. Morphological identification of BNR isolate CFM1 provided information on the general characteristics of the isolate. This technique is useful to recognise typical characteristics of BNR in pure culture and distinguish it from other fungi, which might also be present in pot cultures. The ability of BNR isolate CFM1 to form typical infection patterns observed in pot cultures was tested to appropriate hosts to further investigate the effects of infection by BNR on disease and plant growth (see Chapter 6 and 7). BNR isolate CFM1 belonged to AG-Bo. Up to now, there has been limited publication on this particular AG. This present study reported for the first time that BNR AG-Bo was present as a contaminant in mycorrhizal pot cultures.

Molecular approaches for identification of BNR isolate CFM1 were carried out by PCR-based methods. The ITS sequence from mycelium DNA of BNR isolate CFM1 (AG-Bo) was obtained after amplification with ITS1F and

ITS4B primers. Although there are only a limited number of sequences of the isolates of BNR in GenBank, the ITS sequence of BNR AG-Bo isolate CFM1 (AF446088) matched its identity with *Rhizoctonia/Ceratobasidium* sp. AG-Bo (AF354091) published in GenBank. The ITS sequences of BNR isolate CFM1 had high similarity with the ITS sequences of BNR AG-Bo and AG-A, but not with other BNR sequences. From the ITS sequences, BNR-specific-primers (CF1f and CF2r) were designed for use in detection of BNR AG-Bo in pot cultures. Further study is needed to test the specificity and sensitivity of these primers and the usefulness of the BNR-specific-primers for detecting the presence of BNR in roots and in soils (see Chapter 5).

CHAPTER 5. DETECTION OF BNR IN MYCORRHIZAL ROOTS AND SOILS OF POT CULTURES USING PCR WITH BNR-SPECIFIC PRIMERS AND CONVENTIONAL METHODS

5.1. INTRODUCTION

Binucleate *Rhizoctonia* sp. (BNR) AG-Bo isolate CFM1 has been found in roots of plants used to culture AM fungi (see Chapter 4). BNR AG-Bo infects the roots of most plants used as hosts in pot cultures, and is present together with AM fungi in the same roots. The presence of BNR AG-Bo becomes a problem when roots infected by AM fungi only are needed, especially in molecular-related studies. Current diagnosis of BNR AG-Bo in pot cultures has relied on the microscopic observation of roots. This method is not practical for routine testing because it is laborious and time consuming, and difficulties are encountered due to the similar morphological characteristics of BNR and other *Rhizoctonia* species.

Most BNR live as saprophytes in various types of soil (Cubeta *et al.*, 1991; Sneh *et al.*, 1991). Isolation of BNR from soil has been carried out using a plant debris particles isolation method (Boosalis and Scharen, 1959; Ichielevich-Auster *et al.*, 1985a, Villajuan-Abgona *et al.*, 1996b), a colonisation method using stem segments (Papavisas and Davey, 1962, Villajuan-Abgona *et al.*, 1996b), and a soil-clump plating method (Ko and Hora, 1971; Villajuan-Abgona *et al.*, 1996b). However, these techniques are not specific because they also result in the isolation of other *Rhizoctonia* species such as *R. solani* and *R. zeae* (Ichielevich-Auster *et al.*, 1985a, Villajuan-Abgona *et al.*, 1996b). Therefore, it is desirable to develop a reliable, rapid, sensitive and specific method for detecting BNR AG-Bo in pot cultures. It would be particularly advantageous if the method could be applied directly to both infected roots and soil.

A new approach using molecular techniques could offer several advantages and provide specific and sensitive detection of the target organism in the host tissues and in soil. PCR is a highly sensitive method for amplification of diagnostic markers, and can be used for identification, detection and quantification if species-specific primers are available (e.g. Nicholson *et al.*, 1996, 1997, 1998; Edel *et al.*, 2000; Heinz and Platt, 2000). Primers designed from the ITS regions have been used for detection of fungi causing diseases (e.g. Nicholson and Parry, 1996; Schilling *et al.*, 1996; Salazar *et al.*, 2000), but this approach has never been explored for detection of fungal contaminants in pot cultures of mycorrhizal fungi.

The aims of the present study were to test the specificity and sensitivity of the BNR-specific primers (CF1f and CF2r) designed from the ITS region of BNR AG-Bo described in Chapter 4, and to use the BNR-specific primers for a rapid detection of BNR AG-Bo in roots and soils of pot cultures. DNA extracted from other root-infecting fungi and AM fungi was used to test the specificity of the BNR-specific primers. The specific primers were also used in a dot-blot hybridisation assay for quantification of BNR AG-Bo in infected roots. The detection of BNR AG-Bo using CF1f and CF1r primers was carried out using roots and soils of pot cultures of different AM fungi, hosts and sources.

5.2. MATERIALS AND METHODS

5.2.1. Fungal material

BNR AG-Bo isolate CFM1 (Chapter 4), other isolates of BNR of different AGs, and other root-infecting fungi, including AM fungi used in the present study are listed in Chapter 3 Table 3.1. All except the AM fungi were maintained on PDA at 25°C. The spores of AM fungi were obtained from pot cultures after separation from soil as described in Chapter 3 section 3.2.1.2.

5.2.2. Plant material and pot cultures

Various plants were grown to obtain root material to test the specificity of the BNR-specific primers. Seeds of tomato, clover, barley and mung bean were surface sterilised, germinated, and one seedling was planted into a pot containing 1.4 kg of sterilised soil/sand mix as described in Chapter 3 section 3.3. The plants were grown in the glasshouse for 6 weeks and pots were given 10 ml of modified Long Ashton solution minus P weekly (see Chapter 3 section 3.3).

Infected mung bean roots were used to test the specificity of the primers. Mung bean plants were inoculated with BNR AG-Bo isolate CFM1, BNR AG-Bo (CFM1) + the AM fungus *Glomus coronatum*, *R. solani* AG2-2IIIB, and *R. solani* AG2-2IIIB + *G. coronatum*. Methods for preparation of inoculum and inoculation were described in Chapter 3 section 3.2. The plants were grown for 6 weeks as described above. At harvest, roots were separated from shoots, thoroughly washed and frozen in liquid nitrogen for DNA extraction.

Pot cultures used in the present study were kindly supplied by Ms Debbie Miller, Soil Biology Group, Department of Soil and Water, The University of Adelaide. Samples of roots and soils of pot cultures of different AM fungi, hosts and sources were tested and are listed in Chapter 3 Table 3.1.

5.2.3. DNA extraction

The method for extraction of DNA from mycelium, spores, infected and uninfected roots was described in detail in Chapter 3 section 3.7.1. DNA was extracted from mycelium of non-mycorrhizal fungi using a method modified from Raeder and Broda (1985). The extraction of DNA from fresh roots was based on the method of Rogers and Bendich (1985). The extraction of DNA from dried roots of pot cultures was carried out using the DNeasy

Plant Mini Kit (QIAGEN) following the manufacturer's instructions. The DNA from soils of pot cultures was extracted using the Ultra Clean Soil DNA Kit (MoBio Laboratories) following the manufacturer's instructions.

5.2.4. PCR amplification

PCR amplification of genomic DNA was performed in a total volume of 25 µl by mixing 1 µl of the template with 250 µM 10 x Buffer, 1.5 mM $MgCl_2$, 250 µM dNTPs, 0.5 unit of *Taq* DNA polymerase and 25 pmol for ITS1 and ITS4 primers or 15 pmol for NS3 and NS4 primers. The primers ITS1 and ITS4 are universal primers used to amplify fungal DNA (White *et al.*, 1990), whereas the universal primers NS3 and NS4 target a region in the 18S ribosomal gene, and amplify DNA from all plant material (White *et al.*, 1990; Gardes and Bruns, 1993). The annealing site of the primers is schematically presented in Figure 5.1. Details of the buffer and primers used in the present study were given in Chapter 3 section 3.7.2.2. A negative control that did not contain DNA was included in every reaction. Each reaction was overlaid with a drop of mineral oil (Sigma). Amplification was performed in a Programmable Thermal Cycler (MJ Research Inc.) at an annealing temperature 58°C and 40 cycles using conditions described in Chapter 3 section 3.7.2.2. The amplification products were resolved by electrophoresis in 1.2% agarose gel and visualised over a UV transilluminator following ethidium bromide staining.

5.2.5. Detection of BNR by PCR-specific primers

BNR-specific primers for detection of BNR AG-Bo were designed by using the Primer Select (DNASTAR software program) on a sequence within the ITS region of rDNA of BNR AG-Bo isolate CFM1 (see Chapter 4). The forward primer CF1f (5′- TGT GCA CTT GTG AGA CTG GA -3′) and the reverse primer CF2r (5′- GAA TGG ACT ATT AGA AGC GG-3′) amplified a

fragment of 438 bp. The annealing site of CF1f and CF2r primers in the ITS region is schematically presented in Figure 5.1 below.

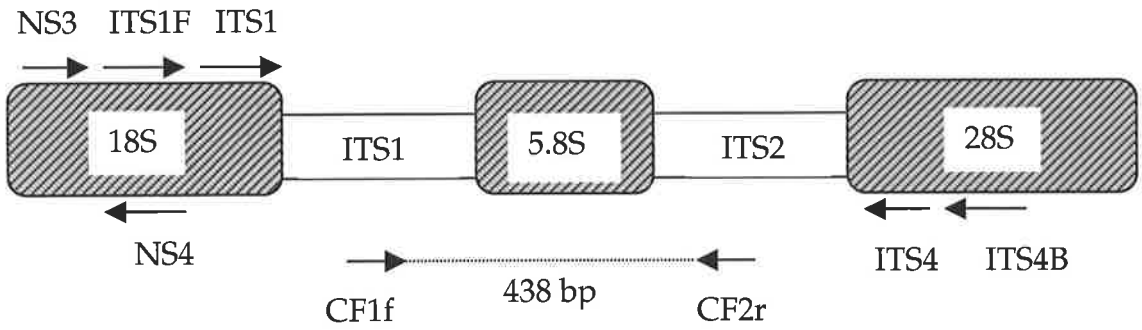


Figure 5.1. Schematic representation of the rDNA region of fungi. The shadowed boxes represent the ribosomal genes. The arrows represent the positions of the primers previously reported and the new BNR-specific primers (CF1f and CF2r) designed within the ITS1 and ITS2 regions. The BNR-specific primers (CF1f and CF2r) amplify a fragment 438 bp in length.

5.2.5.1. PCR amplification with BNR-specific primers

PCR conditions were optimised by testing different annealing temperatures, and the following procedure was adopted for routine amplifications (see Results). PCR was performed at an annealing temperature of 62°C and 35 cycles using a Programmable Thermal Cycler (MJ Research Inc.). The amplification reactions were performed in a 25 µl volume containing 1 µl of diluted template DNA mixed with buffers and enzymes as described in section 5.2.4, but using 28 pmol of each of the CF1f and CF2r primers. A negative control that did not contain DNA was included in every reaction. Each reaction was overlaid with a drop of mineral oil (Sigma). The amplification reactions were conducted with the following parameters: initial denaturation at 94°C for 4 min, followed by 35 cycles at 94°C for 1 min, annealing at 62°C for1 min,extension at 72°C for 2 min, and a final extension cycle at 72°C for 5 min. Amplification reactions were performed at least three times

on separate occasions to check the reproducibility of the method. The amplification products were resolved by electrophoresis in 1.2% agarose gel and visualised over a UV transilluminator following ethidium bromide staining.

5.2.5.2. Sensitivity and specificity of BNR-specific primers

Amplification reactions were conducted with decreasing amounts of genomic DNA from mycelium of BNR AG-Bo isolate CFM1 and from mung bean roots infected by BNR AG-Bo isolate CFM1 to determine the minimum amount of template DNA required to produce a detectable product using the BNR-specific primers (CF1f and CF2r). DNA from mycelium of non-mycorrhizal fungi, spores of AM fungi, infected roots of BNR AG-Bo isolate CFM1, *G. coronatum* or *R. solani* AG2-2IIIB, and uninfected roots was used to test the specificity of the primers.

5.2.5.3. Detection of BNR by BNR-specific primers

Dried root samples and soils of 16 pot cultures of different AM fungi, hosts and sources as listed in Chapter 3 Table 3.1 were used to test the utility of the primers in detecting contamination by BNR AG-Bo. These pot cultures were chosen because they have been routinely used for experimental purposes in the Department of Soil and Water, The University of Adelaide.

5.2.6. Detection of BNR by conventional methods

5.2.6.1. Detection of BNR by fungal infection in roots

About 0.2 g of dried mycorrhizal root samples obtained from pot cultures (section 5.2.5.3) were cut into approximately 1 cm segments. Root samples were cleared and then stained with trypan blue (Phillips and

Hayman, 1970), and stored in lactoglycerol. Details of this procedure were given in Chapter 3 section 3.5. The roots were then spread on a Petri dish with a grid attached (Giovannetti and Mosse, 1980), and infection of roots by fungi with morphology typical of BNR was estimated (see Chapter 4).

5.2.6.2. Detection of BNR by soil dilution plate technique

The monilioid cells of BNR function as asexual spores in soil.

Soil dilutions were performed using a modification of the procedure described by Johnston *et al.* (1994). Five grams of soil from each of 16 pot cultures were added to 100 ml autoclaved 0.2% acidified water agar and shaken for 20 min. One milliliter of this sample was added to 9 ml of 0.2% acidified water agar and shaken for 5 min. The sample was further diluted from 1 to 50 in 0.2% acidified water agar and shaken for 5 min. Aliquots of 1 mlwerepoured on PDA plates and incubated at 23°C in the dark.

5.2.7. Dot-blot assay for quantification

A vacuum dot blot micro-filtration apparatus (Bio-Rad) with a nylon N membrane was assembled according to the manufacturer's recommendations (Bio-Rad). The DNA samples from mycelium of BNR AG-Bo, roots infected by BNR AG-Bo (56%) and uninfected roots were amplified by PCR using the ITS1 and ITS4 primers as described in section 5.2.4, except that reactions were conducted for 30 cycles. DNA extracted from mycelium of BNR AG-Bo was loaded on the membrane as a standard. The concentration of DNA was determined by running the samples in 1% agarose gel against a known amount of molecular marker. The DNA samples with different concentrations were denatured with 25 µl of 2 M NaOH for 20 min, neutralised with an equal volume of 2 M NH_4OAc and loaded into the wells of the dot-blot apparatus. The DNA was fixed to the membrane by baking at 80°C for 30 min.

5.2.8. DNA labelling and hybridisation

The oligonucleotide primer CF1f was concentrated to $10x10^{-12}$ moles in 50 µl and was labelled with [α-^{32}P]dCTP using a 3'-end labelling kit (Promega). The oligonucleotide primer and DNA fragments were labelled to a specific activity of about 1.8 x 108 cpm μg^{-1} and separated from unincoporated nucleotides through a Sephadex G-100 column.

Hybridisation of the membranes was performed at 45°C overnight in a hybridisation buffer containing 2 ml of sterile water, 3 ml 5 x HSB (3 M NaCl, 100 mM PIPES and 25 mM Na_2EDTA, pH 6.8), 1 ml 10 x Denhardts III solution (2% gelatin, 2% Ficoll, 2% polyvinyl pyrollidone, 10% SDS and 5% tetrasodium pyrophosphate), 4 ml 25% dextran sulphate and 0.2 ml denatured salmon sperm DNA (5 µg ml^{-1}). After hybridisation, the membranes were washed under high stringency conditions at 60°C using sequential washes of 2 x SSC and 0.1% SDS, 1 x SSC and 0.1% SDS, and 0.5 SSC and 0.1% SDS for 15 min each. The washed membranes were exposed to a phosphor screen overnight and quantified using Storm Phosphor Image software (Molecular Dynamics).

5.3. RESULTS

5.3.1. Sensitivity and specificity of BNR-specific primers

The primers CF1f and CF2r used in the present study were designed from the ITS region of BNR AG-Bo obtained in Chapter 4. The length of each primer was 20 bp. All amplification involving different annealing temperatures ranging from 45°C to 70°C, with DNA template from mycelium of BNR AG-Bo, produced strong bands (Figure 5.2), and an annealing temperature at 62°C was chosen for routine PCR.

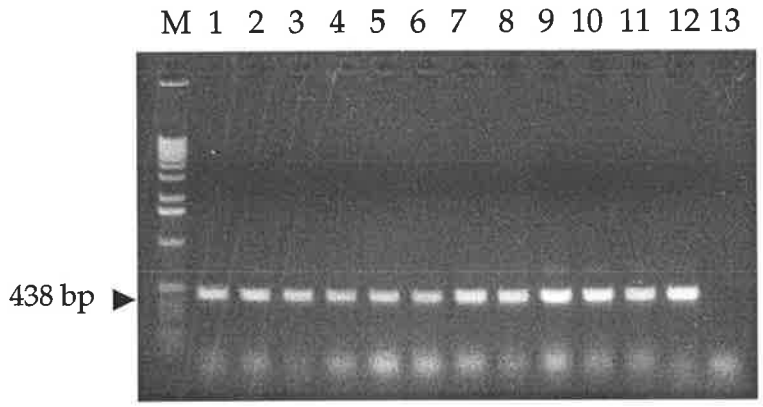


Figure 5.2. PCR amplification of mycelial DNA of BNR AG-Bo isolate CFM1 using BNR-specific primers (CF1f and CF2r) at different annealing temperatures (45-70°C) produced a 438 bp fragment. Lanes 1-12. Annealing temperatures of 45°C (1), 47°C (2), 49°C (3), 51°C (4), 54°C (5), 57°C (6), 59°C (7), 62°C (8), 63°C (9), 65°C (10), 67°C (11), 70°C (12). Lane 13. No DNA template. M = 1 kb molecular size marker (Gibco BRL).

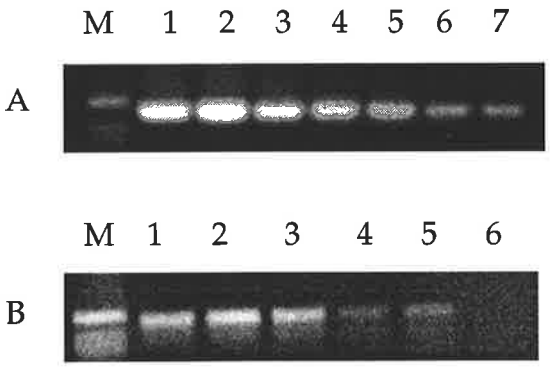


Figure 5.3. Sensitivity tests show the detection limit of BNR-specific primers (CF1f and CF2r) on mycelial DNA of BNR AG-Bo isolate CFM1 (1 pg) (A), and roots infected by BNR AG-Bo isolate CFM1 (5 pg) (B). Lanes 1-7A. Mycelium DNA at 100 ng, 50 ng, 5 ng, 1 ng, 50 pg, 5 pg, and 1 pg. Lanes 1-6B. Root infected by BNR AG-Bo isolate CFM1 at 50 ng, 5 ng, 1 ng, 50 pg, 5 pg, 1 pg. M = 1 kb molecular size marker (Gibco BRL).

In sensitivity tests with a serial dilution of BNR total genomic DNA, the limit of detection (the lowest concentration of DNA from the target fragment which was amplified) for the BNR-specific primers was 1 pg for DNA extracted from mycelium, and 5 pg for DNA extracted from roots infected by BNR AG-Bo (CFM1) (Figure 5.3A,B). PCR amplification from all fungal DNA samples was first performed with ITS1 and ITS4 primers to show that the DNA could be amplified from all the samples. All fungal DNA samples listed in Table 5.1 were amplified using ITS1 and ITS4 primers, but fragments of different sizes were produced depending on the fungus tested (Figure 5.4). Direct amplification from BNR with the BNR-specific primers showed the expected DNA band of 438 bp (Figure 5.5). The BNR-specific primers did not amplify DNA from plant pathogenic fungi and soil-inhabiting fungi such as *Rhizoctonia, Pythium, Fusarium, Gaeumannomyces, Rhizopus,* and the AM fungi *G. coronatum* and *Gi. margarita* (Figure 5.5).

Amplification with ITS 1 and ITS 4 primers using mycelial DNA of BNR AG-A, AG-Bo, AG-C, AG-G, and AG-O resulted in similar fragment sizes (Figure 5.6). The BNR-specific primers did not amplify mycelial DNA of AG-C, AG-G and AG-O. However, amplification was obtained from the tester isolates of BNR AG-Bo (SIR-2) and BNR AG-A (C-517) (Table 5.1, Figure 5.7). This result was unexpected. The positive reaction with DNA from AG-A might be due to the high similarity (88%) in the ITS sequences (see Chapter 4). Also BNR AG-Bo and AG-A had similar banding patterns with PCR-RFLP (Cubeta *et al.*, 1991), indicating that they are closely related.

Table 5.1. Sources and codes of isolates of fungi used to assess specificity of BNR-specific primers (CF1f and CF2r). ITS1 and ITS4 primers amplified DNA from all fungal DNA and infected roots, but not from uninfected roots. CF1f and CF2r primers amplified DNA from mycelium of AG-Bo and AG-A, and roots infected by BNR AG-Bo.

Species	Isolate	Source/host	ITS1/ ITS4	CF1f/CF2r (Direct PCR)
Non-AM fungi				
Binucleate *Rhizoctonia*				
AG-Bo	CFM1	Pot culture *G. mosseae*	+	+
AG-Bo	SIR-2	Sweet potato	+	+
AG-A	C-517	Strawberry	+	+
AG-C	OR-706	Orchid	+	–
AG-G	AHC-9	Peanut	+	–
AG-O	FKO6-2	Soil	+	–
R. solani				
AG1-1C	01R01	Sugar beet	+	–
AG2-1	21RM03	Alfalfa	+	–
AG2-2 IIIB	22R02	Mat rush	+	–
AG-8	NS21	Barley	+	–
Pythium echinulatum	BH3	Soil	+	–
Gaeumannomyces graminis var. *tritici*	Ggt 800	Wheat	+	–
Fusarium sp.	–	Soil-pot cultures	+	–
Rhizopus sp.	–	Soil-pot cultures	+	–
AM fungi				
Glomus coronatum	WUM 16	Soil-pot cultures	+	–
Gigaspora margarita	–	Soil-pot cultures	+	–
Infected roots				
BNR AG-Bo	CFM1	Pot culture- *G. mosseae*	+*	+
BNR AG-Bo + *G. coronatum*	CFM1 + WUM 16	Pot cultures	+*	+
R. solani AG2-2IIIB	22R02	Mat rush	+*	–
R. solani AG2-2IIIB + *G. coronatum*	22R02 + WUM 16	Mat rush + pot cultures	+*	–
Uninfected roots				
Mung bean	–	–	–*	–
Clover	–	–	–*	–
Tomato	–	–	–*	–
Barley	–	–	–*	–

* Amplification obtained with NS3 and NS4 primers (see Figure 5.8A).

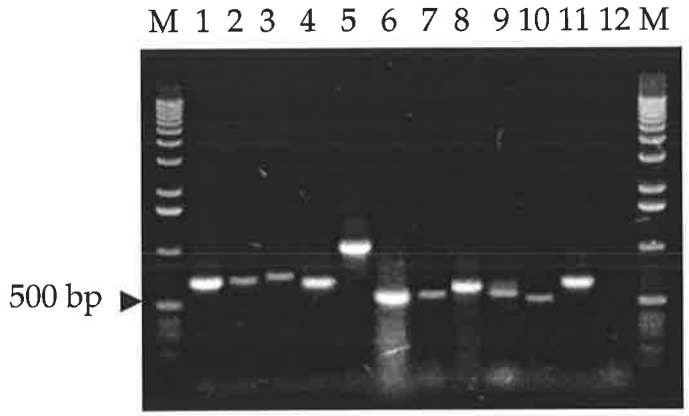


Figure 5.4. PCR amplification of DNA from a range of fungal species using the universal primers ITS1 and ITS4. Lanes 1-4. Mycelium of *R. solani* AG1-1C, AG2-1, AG2-2IIIB and AG-8, respectively; *Pythium echinulatum* (5); *Gaeumannomyces graminis* var. *tritici* (6); *Fusarium* sp. (7); *Rhizopus* sp. (8); spores of *Glomus coronatum* (9); spores of *Gigaspora margarita* (10); mycelium of BNR AG-Bo isolate CFM1 (11); no DNA template (12); M = 1 kb molecular size marker (Gibco BRL).

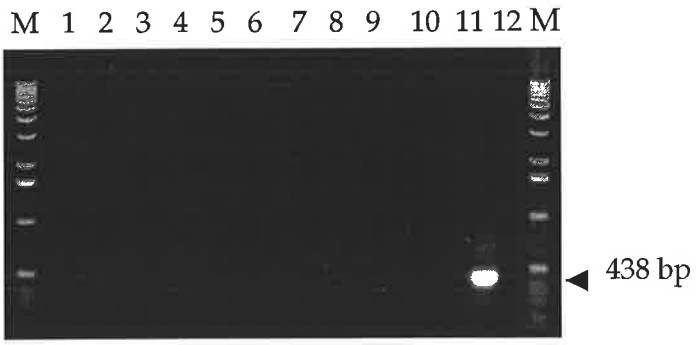


Figure 5.5. BNR-specific primers CF1f and CF2r amplify DNA from mycelium of BNR AG-Bo isolate CFM1 (lane 11), but not from other fungal species. Lanes 1-10 and 12 are as described in Figure 5.4. M = 1 kb molecular size marker (Gibco BRL).

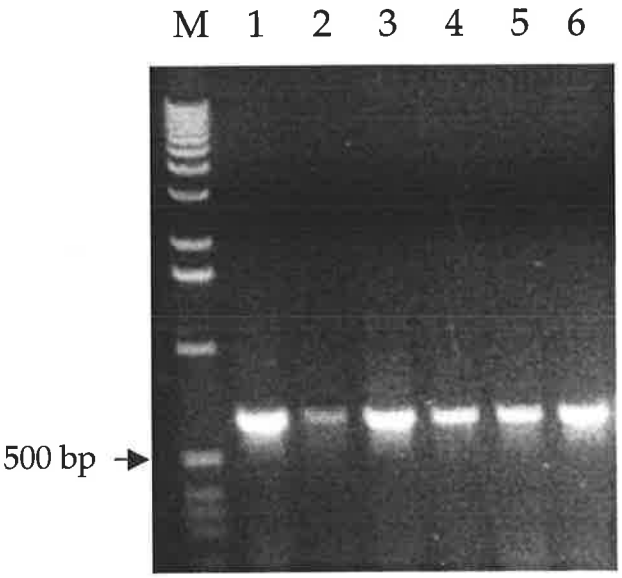


Figure 5.6. ITS1 and ITS 4 primers amplify mycelial DNA of different AGs of BNR. Lanes 1-6. BNR AG-A (isolate C-517 from strawberry-lane 1); BNR AG-Bo (isolate SIR-2 from sweet potato-lane 2); BNR AG-C (isolate OR-706 from orchid-lane 3); AG-G (isolate AHC-9 from peanut- lane 4); AG-O (isolate FKO6-2 from soil-lane 5); BNR AG-Bo (isolate CFM1 from pot culture-lane 6). M = 1 kb molecular size marker (Gibco BRL).

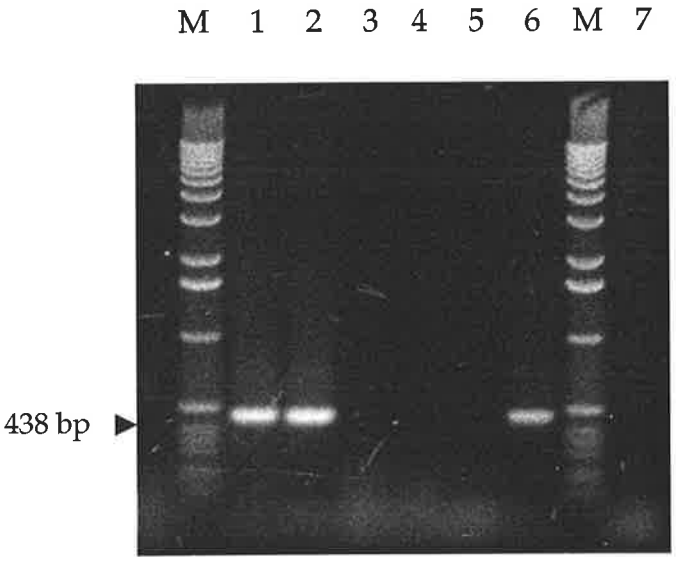


Figure 5.7. BNR-specific primers amplify mycelial DNA of BNR AG-A (isolate C-517 from strawberry-lane 1) and AG-Bo (isolate SIR-2 from sweet potato-lane 2). Amplification was not obtained from BNR AG-C, AG-G and AG-O (lanes 3-5). Lanes 1-7. BNR AG-A (1); BNR AG-Bo (2); BNR AG-C (isolate OR-706 from orchid-lane 3); AG-G (isolate AHC-9 from peanut-lane 4); AG-O (isolate FKO6-2 from soil-lane 5); BNR AG-Bo (isolate CFM1 from pot culture-lane 6); no DNA template (7). M = 1 kb molecular size marker (Gibco BRL).

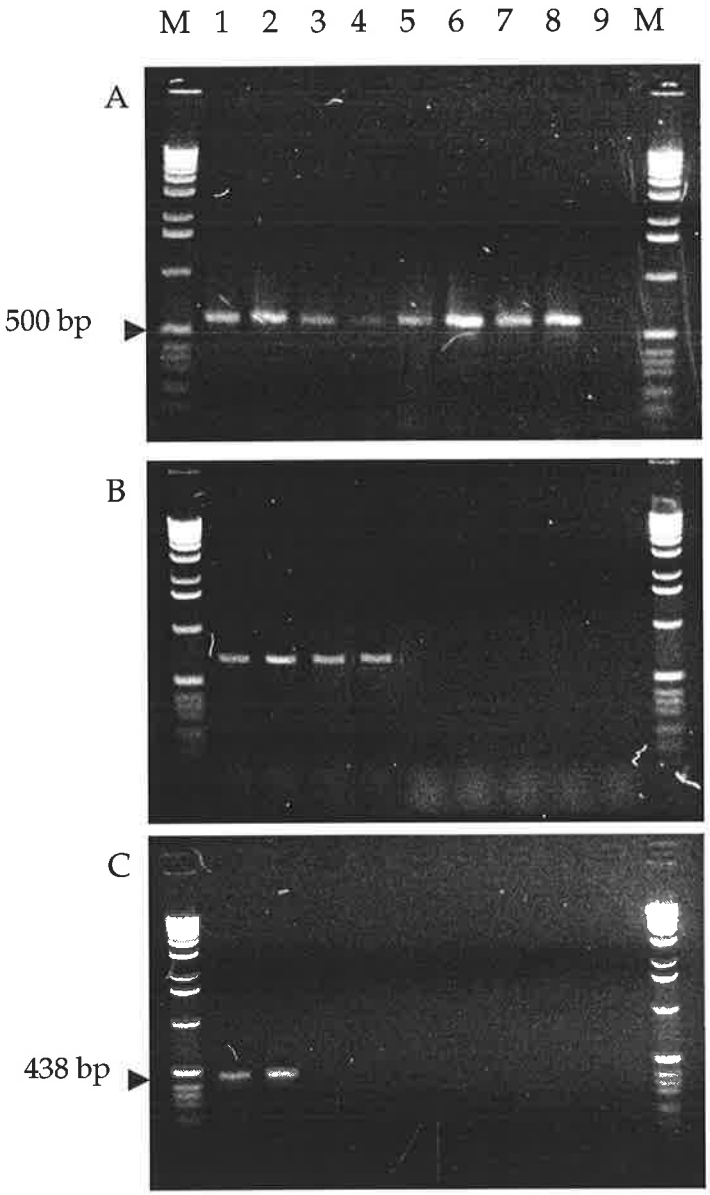


Figure 5.8. PCR amplification using NS3 and NS4 primers amplify DNA from all infected and uninfected roots (lanes 1-8A), ITS1 and ITS4 primers amplify DNA from infected mung bean roots only (lanes 1-4B), and BNR-specific primers amplify DNA from roots infected by BNR AG-Bo isolate CFM1 (lane 1C) and BNR AG Bo (CFM1) + *G. coronatum* (lane 2C). Lanes 1-4. Mung bean root infected by BNR AG-Bo isolate CFM1, BNR AG-Bo (CFM1) + *G. coronatum*, *R. solani* AG2-2IIIB, and *R. solani* AG2-2IIIB + *G. coronatum*, respectively. Remaining lanes, uninfected roots of mung bean (5), clover (6) tomato (7), barley (8), and no DNA template (9). M = 1 kb molecular size marker (Gibco BRL).

The specificity of the BNR-specific primers (CF1f and CF2r) was also tested on mung bean roots infected by BNR AG-Bo isolate CFM1, *G. coronatum* and *R. solani* AG2-2IIIB as well as uninfected roots of tomato, clover and barley. Figure 5.8A shows that all DNA templates from infected and uninfected roots were amplified using the NS3 and NS4 primers to produce a fragment of approximately 600 bp. The ITS1 and ITS4 primers amplified DNA from infected roots, but did not amplify DNA from uninfected roots of mung bean, barley, tomato or clover, suggesting that fungi were not present in these roots (Table 5.1, Figure 5.8B). A 438 bp product was amplified from roots infected by BNR AG-Bo (CFM1) or BNR AG-Bo (CFM1) + *G. coronatum*, but not from those infected by *R. solani* AG 2-2IIIB or *G. coronatum* + *R. solani* AG2-2IIIB, when CF1f and CF2r primers were used for amplification, indicating that these primers could be used for detection of BNR in infected roots either alone or in the presence of AM fungi (Figure 5.8C).

5.3.2. Detection of BNR by BNR-specific primers

5.3.2.1. Detection of BNR in roots of pot cultures

PCR amplification in all DNA samples from dried roots of pot cultures was first performed with ITS1 and ITS4 primers. Extraction of DNA from dried roots produced a low yield, and PCR amplification was weak and inconsistent (results not shown). The first attempt to amplify directly from a low concentration of fungal DNA with the BNR-specific primers (CF1f and CF2r) did not generate a specific amplification product in 16 root samples (data not shown). Therefore, detection by the BNR-specific primers was conducted by a nested PCR, in which DNA amplified in the first PCR with ITS1 and ITS4 primers, was used as template for amplification by the BNR-specific primers. An amplification product of 438 bp was produced from the nested PCR. BNR were detected in seven samples of roots of pot cultures of

G. mosseae, G. etunicatum, G. intraradices and *G. versiforme* using this method (Figure 5.9). PCR amplification for detection of BNR could be completed within 1-2 days.

5.3.2.2. Detection of BNR in soils of pot cultures

PCR amplification using ITS1 and ITS4 primers from soils of 16 pot cultures produced different sized fragments. Most PCR amplification produced products of approximately 600 bp and two out of 16 samples had a fragment of approximately 500 bp (Figure 5.10).

As for dried roots, the first round PCR amplification from soil did not amplify any fungal DNA. Detection by the BNR-specific primers using nested PCR on the 10-fold dilution of amplified ITS of DNA from the first round PCR, gave a strong signal in soils of four pot cultures of *G. mosseae, G. etunicatum, G. intraradices* and *G. versiforme* (Table 5.2, Figure 5.11). PCR amplification for detection of BNR could be completed within 1-2 days.

5.3.3. Detection of BNR by conventional methods

5.3.3.1. Detection of BNR by root infection

Infection by BNR was found in dried pot cultures of *G. mosseae, G. etunicatum, G. intraradices* and *G. versiforme,* but the amount of infection was very low (less than 5%) (results not shown). Infection by BNR was very difficult to observe, because of the small quantity of hyphae present in the roots compared to the infection by AM fungi. This method was time consuming due to the longer time needed for clearing and staining the root samples. The overall time taken for sample preparation and observation under microscope was completed in about 2 weeks.

Table 5.2. Isolate and source of pot cultures used to detect the presence of BNR AG-Bo or AG-A in plant roots and soils using BNR-specific primers (CF1f and CF2r). ITS1 and ITS4 primers amplified all fungal DNA in roots and soils of pot cultures. CF1f and CF2r primers amplified BNR fungal DNA (AG-Bo or AG-A) in 7 samples of roots and 4 samples of soils of pot cultures.

	Species	Isolate	Host	Origin*	ITS1/ ITS4		CF1f/ CF2r (Nested PCR)	
					Roots	Soils	Roots	Soils
1	*Glomus mosseae*	NBR 1-2	Clover	1	+	+	+	–
2	*G. mosseae*	NBR 4-1	Clover	1	+	+	+	–
3	*G. mosseae*	NBR 4-2	Clover	1	+	+	–	–
4	*G. mosseae*	–	Clover	2	+	+	+	+
5	*G. etunicatum*	JT316A-1	Clover	3	+	+	+	+
6	*G. etunicatum*	MD107-1	Clover	3	+	+	–	–
7	*G. intraradices*	DAOM 181602	Clover	4	+	+	+	+
8	*G. intraradices*	DAOM 181602	Clover	4	+	+	+	–
9	*G. versiforme*		Clover	5	+	+	+	+
10	*G. fasciculatum*	BEG 5	Clover	2	+	+	–	–
11	*G. coronatum*	WUM 16	Clover	6	+	+	–	–
12	*Gigaspora margarita*	–	Clover	2	+	+	–	–
13	*Gi. margarita*	–	Leek	2	+	+	–	–
14	*Gi. rosea*	–	Plantago	7	+	+	–	–
15	*Acaulospora laevis*	–	Plantago	7	+	+	–	–
16	*Scutellospora calospora*	WUM 12(2)	Clover	8	+	+	–	–

*1, Narabrai, New South Wales; 2, France; 3, INVAM; 4, Canada; 5, Italy; 6, University of Western Australia; 7, Waite Campus, South Australia; 8, New South Wales.

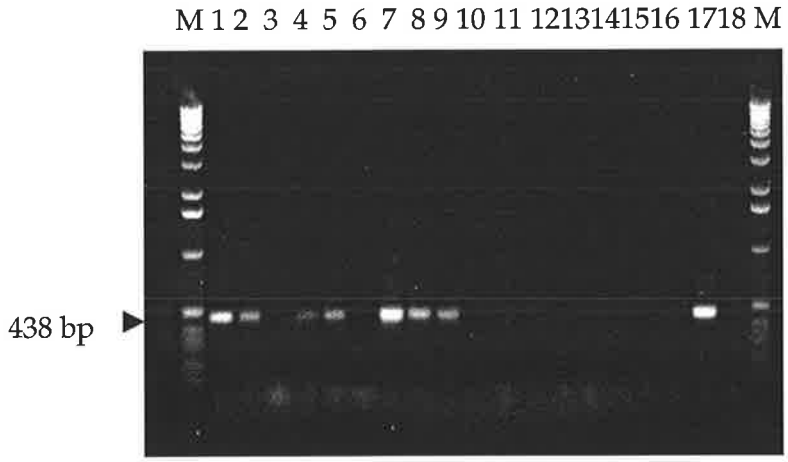


Figure 5.9. Nested-PCR amplification with BNR-specific primers (CF1f and CF2r) shows amplification products from dried roots of pot cultures of *G. mosseae* (lanes 1,2 and 4), *G. etunicatum* (lane 5), *G. intraradices* (lanes 7 and 8), and *G. versiforme* (lane 9). Lanes 1-18. Pot cultures of *Glomus mosseae* (1-4); *G. etunicatum* (5,6); *G. intraradices* (7,8); *G. versiforme* (9); *G. fasciculatum* (10), *G. coronatum* (11); *Gigaspora margarita* (12,13); *Gi. rosea* (14); *Acaulospora laevis* (15); *Scutellospora calospora* (16); mycelium of BNR AG-Bo (CFM1) (17); no DNA template (18). M = 1 kb molecular size marker (Gibco BRL).

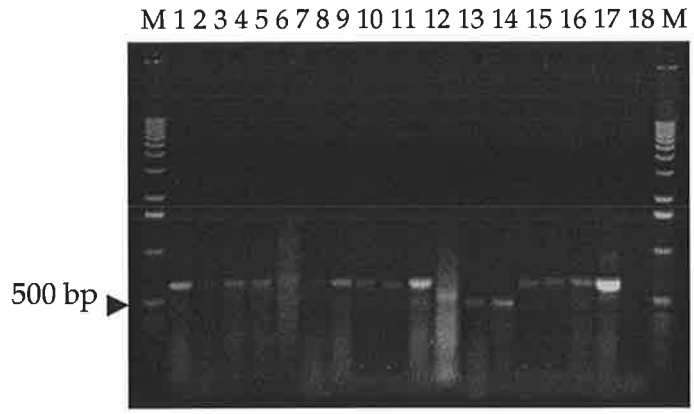


Figure 5.10. PCR amplification of fungal DNA extracted from soils of pot cultures with ITS1 and ITS4 primers shows fragments of different sizes (lanes 1-17), except for the control (lane 18). Lanes 1-18. Soils of pot cultures of *Glomus mosseae* (1-4); *G. etunicatum* (5,6); *G. intraradices* (7,8); *G. versiforme* (9); *G. fasciculatum* (10), *G. coronatum* (11); *Gigaspora margarita* (12,13); *Gi. rosea* (14); *Acaulospora laevis* (15); *Scutellospora calospora* (16); mycelium of BNR AG-Bo (CFM1) (17); no DNA template (18). M = 1 kb molecular size marker (Gibco BRL).

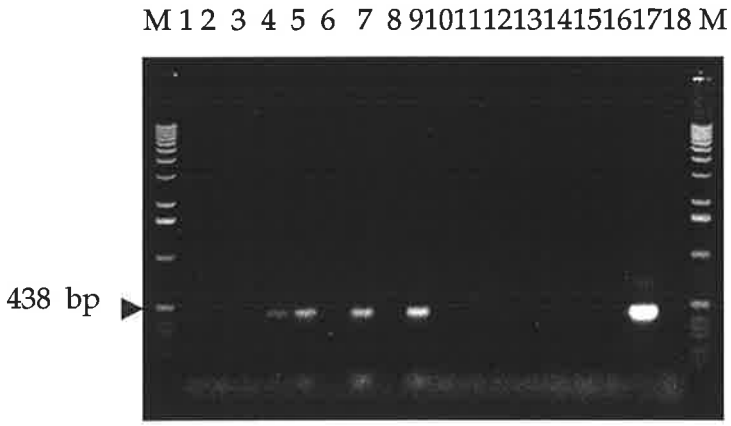


Figure 5.11. Nested-PCR amplification with BNR-specific primers (CF1f and CF2r) shows amplification of BNR AG-Bo DNA from soils of pot cultures of *G. mosseae* (lane 4), *G. etunicatum* (lane 5), *G. intraradices* (lane 7), and *G. versiforme* (lane 9). Lanes 1-18 as described in Figure 5.10. M = 1 kb molecular size marker (Gibco BRL).

5.3.3.2 Detection of BNR from soil using soil dilution plate technique

Dilution plate technique was used to enumerate colonies of BNR from 16 soil samples obtained from pot cultures. After 1-2 weeks of incubation, colonies typical of BNR could not be detected in any of the soil dilutions tested, as fast growing fungi such as *Fusarium, Aspergillus, Rhizopus* and *Trichoderma,* occupied the plates.

5.3.4. Quantification of BNR AG-Bo in infected roots by dot-blot assay

The dot-blot hybridisation assay showed a higher intensity of signal from DNA extracted from mycelium than DNA from infected roots (56% root length infected by BNR). The labelled oligonucleotide primer did not hybridise to DNA from uninfected roots, indicating that less than 5 pg of fungal DNA was present (Figure 5.12). Quantification was performed by comparison of regression analysis between the amount of fungal DNA of BNR AG-Bo in roots or mycelium versus the value obtained from phospho-imaging based on the signal detected in dot-blot hybridisation (Figure 5.12). Regression analysis based on the amount of fungal DNA extracted from mycelium of BNR AG-Bo was used as a standard to assess the amount of BNR AG-Bo DNA in roots. Figure 5.13 showed that DNA from infected roots produced a signal value of about 50,000 from the phospho-imager. This value was equivalent to 0.3 ng of mycelium DNA. This indicated that 1 ng of DNA from infected roots contained approximately 0.3 ng mycelium BNR DNA.

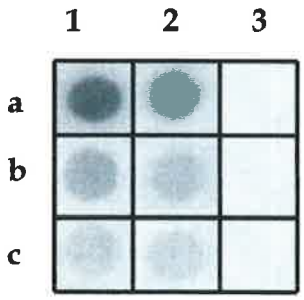


Figure 5.12. Dot-blot hybridisation of the labelled oligonucleotide primer, CF1f, with rDNA amplification products shows a stronger signal from mycelium of BNR AG-Bo isolate CFM1 (dots 1a-c) than from mung bean roots infected by BNR isolate CFM1 (dots 2a-c). There is no signal from uninfected roots (dots 3a-c). Dots 1a-c. ITS1 and ITS4 PCR product from mycelium: 3.75, 0.9, 0.2 ng sample. Dots 2a-c. ITS1 and ITS4 PCR product from roots infected by BNR AG-Bo (CFM1): 6, 1.5 and 0.7 ng sample. Dots 3a-c. ITS1 and ITS4 PCR product from uninfected roots: 6, 1.5 and 0.7 ng sample.

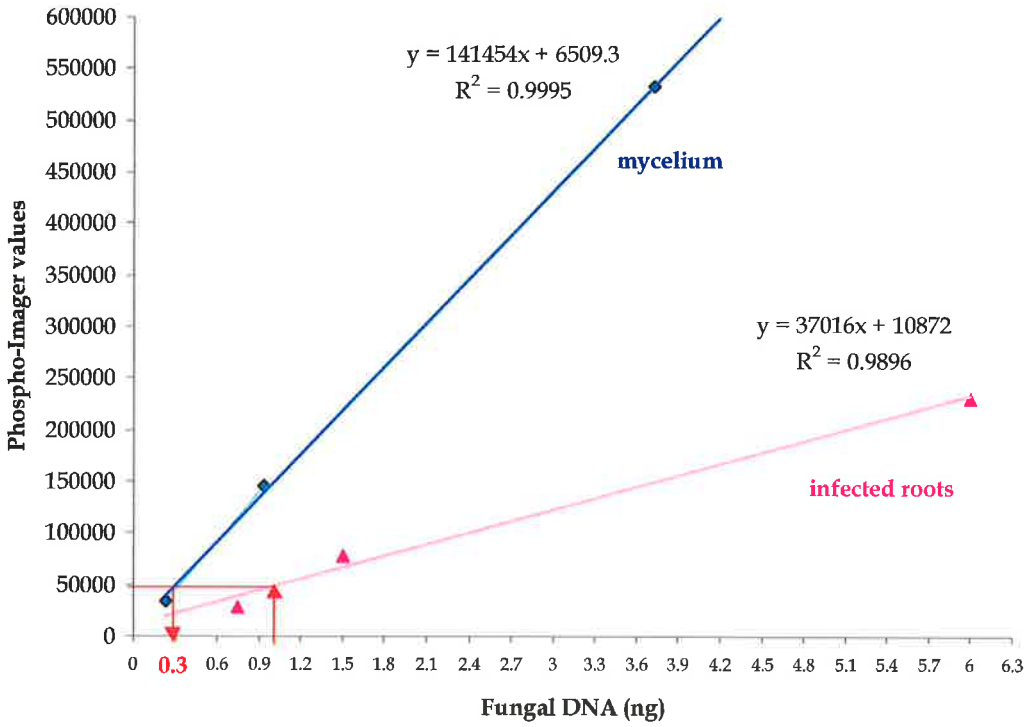


Figure 5.13. PCR quantification calculated from the regression analysis of the signal values obtained from the Phospho-imager versus fungal DNA. Values for the Phospho-imager were based on the intensity of signal shown in Figure 5.12. The graph shows that in 1 ng DNA from the infected roots contained about 0.3 ng of mycelium DNA (see arrows).

5.4. DISCUSSION

The present study illustrates the potential of the PCR method for the rapid detection of BNR from dried mycorrhizal roots and soils. PCR amplification of a fragment of 438 bp was obtained by use of BNR-specific primers (CF1f and CF2r) designed to amplify the ITS regions of BNR AG-Bo. It has been reported that the ITS is a convenient target region for molecular identification due to its variability in length and in nucleotide sequences among species (Sreenivasaprasad *et al.*, 1996). Optimisation of PCR conditions, and especially of the annealing temperatures, allowed specific amplification of BNR among the fungi analysed.

The PCR-based technique was sensitive as it successfully detected as little as 1 pg of BNR DNA template. PCR amplification of mycelial DNA was not adversely affected by the presence of large quantities of root DNA, as demonstrated by the successful amplification of template DNA as little as 5 pg from roots of inoculated mung bean. This approach was used to detect BNR in artificially inoculated mung bean plants.

The specificity of the BNR-specific primers (Cf1f and CF2r) for detection of BNR was verified by the absence of cross-reactivity with DNA from a range of plant-pathogenic fungi and soil-inhabiting fungi (*R. solani*, *Pythium echinulatum*, *Fusarium* sp., *Gaeumannomyces graminis* var. *tritici*, *Rhizopus* sp.), AM fungi (*G. coronatum* and *Gi. margarita*) as well as from various different plant species. The BNR-specific primers did not amplify DNA from AM fungi, which is valuable because it means that the BNR-specific primers are useful for detection of BNR in pot cultures. The BNR-specific primers were also valuable in detecting BNR in infected roots, since amplification always produced strong bands. However, the BNR-specific primers also recognised an isolate of BNR AG-Bo from sweet potato and of BNR AG-A from strawberry, which means that the specificity for AG-Bo was

not absolute. The primers did not amplify DNA from isolates of BNR AG-C from orchid, AG-G from peanut, and AG–O from soil. Attempts to test the CF1f and CF2r primers at different annealing temperatures, ranging from 45°C to 70°C, using a Mastercycler gradient PCR machine (Eppendorf, Hamburg, Germany) failed to differentiate between BNR AG-Bo and BNR AG-A, because all reactions amplified DNA. Annealing temperatures above 70°C failed to amplify any target DNA. The high similarity in the ITS sequence between BNR isolate CFM1 and isolates of AG-A or AG-Bo (see Chapter 4) may explain the results. BNR AG-A appears to be a saprophyte and has been isolated from soil (Kuninaga *et al.*, 1997). It is quite possible that isolates of BNR AG-A are also present in the soil of pot cultures. However, further investigations are needed to confirm this possibility. The failure of the BNR-specific primers to differentiate AG-Bo from AG-A did not detract from their potential use as tools for detection of BNR as a contaminant fungus in plant roots and soils of pot cultures. Rejection of a pot culture based on amplification of DNA with the BNR-specific primers would be a conservative decision.

Specific primers designed from the ITS regions have been used for detection of pathogenic fungi which caused diseases in a variety of plants (e.g. Nicholson and Parry, 1996; Schilling *et al.*, 1996; Salazar *et al.*, 2000) or fungi in soil (Hamelin *et al.*, 1996; Errampalli *et al.*, 2001). The present study aimed to use the BNR-specific primers for detection of the fungal contaminant in roots and soil in pot cultures. The first attempt using the method of Rogers and Bendich (1985) to extract DNA from dried roots of pot cultures was successful using fresh roots, but failed to amplify DNA from dried roots due to the low yield of DNA template. As an alternative, the DNA extraction using the DNeasy Plant Mini Kit (QIAGEN) for extraction of DNA from dried roots and the Ultra Clean Soil DNA Kit (MoBio Laboratories) for extraction of DNA from soil were used, and successfully increased the yields

of DNA, but resulted in weak amplification of fungal DNA using the universal primers ITS1 and ITS4.

Detection of BNR in roots and soils of pot cultures by BNR-specific primers was first carried out directly from DNA template. The present study failed to achieve direct amplification of the BNR-specific primers using DNA extracted from dried roots and soils, probably due to low levels of BNR in dried roots and soils, which resulted in low yield of DNA. Furthermore, various inhibitors of PCR amplification, such as humic and fulvic acids, might be present in samples of soil (Tsai and Olsen, 1992), and may have inhibited the PCR. Therefore, nested PCR was applied to ensure specificity of detection and to overcome problems caused by low DNA content or the presence of inhibiting compounds. The use of nested PCR has been shown to increase the concentration of the amplified fragments of fungal DNA, increase the amplification signal and allow the detection of small numbers of hyphae in roots and soil samples (Kreuzinger *et al.*, 1996). Using nested PCR, the BNR-specific primers amplified DNA of BNR in roots and soils of pot cultures. BNR was detected in pot cultures of *G. mosseae* from New South Wales, Australia and France, *G. intraradices* from Canada, *G. etunicatum* from the INVAM collection and *G. versiforme* from Italy. The pot cultures originated from different locations, indicating that BNR might be present in various parts of the world. An alternative explanation of the presence of BNR in pot cultures of different geographic origins is that the contaminant spread between pots during propagation in the glasshouse in Adelaide. The identity of the BNR present in the pot cultures could be AG-Bo or AG-A, but not AG-C, AG-G or AG-O.

BNR was present in less than 5% of the total root lengths of pot culture materials. The soils that indicated contamination with BNR by PCR, were also checked for the presence of BNR hyphae using a soil dilution plate technique. BNR could not be detected in any of the soils tested, due to contamination by *Fusarium, Aspergillus, Rhizopus* and *Trichoderma*. Therefore, the PCR approach proved to be well suited for the detection of BNR, which is difficult to isolate in the presence of fast growing soil fungi. This technique

was more rapid than conventional detection techniques, for example detection of BNR using the BNR-specific primers could be done by 1-2 days, whereas conventional technique took about 2-3 weeks to finish.

PCR is an extremely sensitive technique, which reveals the presence of BNR, but cannot be used directly for quantification of the fungus in roots. Techniques of plating plant parts onto selective media have been developed for quantification of fungal infection (Platt and Bollen, 1995; Mahuku *et al.*, 1999). This technique may permit relative estimation of the fungi present, but such assays are very laborious where a large number of plants are involved and more than one fungus is present. Furthermore, it is only applicable to culturable fungi. Competitive PCR has been used as a technique to quantify fungi based on the comparison of the amount of fungal DNA with standard DNA (e.g. Nicholson *et al.*, 1998; Haugland *et al.*, 1999; Moukhamedov *et al.*, 1994). In the present study, the amount of BNR ITS DNA in infected roots was successfully quantified in a dot blot assay. Similar approaches have been used for quantification of *Gaeumannomyces graminis* var. *tritici* in roots and soils (Herdina *et al.*, 1996, 1997).

Dot-blot hybridisation of radioactively labeled oligonucleotide primer CF1f to PCR products for quantification of mycelium of BNR in infected roots showed a stronger signal in DNA from mycelium than in DNA from infected roots. Using DNA from mycelium as a standard, the calculation showed that about 0.3 ng of mycelium DNA was present in 1 ng of DNA of artificial infected roots (56% infected by BNR AG-Bo). It suggests that high infection by BNR with a large number of nuclei in the hyphae and monilioid cells, might influence those results. The present study used extracted DNA from a pure culture of BNR AG-Bo as the external standard. For the future, it will be necessary to examine the PCR quantification method using a standard curve ratio of plant with a much large number of points, and the standard DNA is diluted with plant DNA rather than buffer. The quantification results were

considered preliminary, but it was shown that infection by BNR AG-Bo in roots could potentially be quantified by dot-blot hybridisation assay. The availability of a rapid and reliable method to quantify this species in the root samples will allow studies on the competitiveness with other root-infecting fungi, including AM fungi, and evaluation of the possible capacity of BNR AG-Bo to cause disease on hosts.In addition, similar approaches might be used to quantify the level of BNR inoculum in soil of pot cultures.

5.5. CONCLUSIONS

The PCR-based method using BNR-specific primers (CF1f and CF2r) developed here provided a faster method of detection of BNR AG-Bo and AG-A. This procedure allowed detection directly from infected roots and soils. The PCR-based method of diagnosis proved to be highly sensitive, detecting BNR AG-Bo or AG-A in mycorrhizal roots and soils of pot cultures. The BNR-specific primers could provide a valuable tool for testing pot cultures for contamination. The tests could be conducted from dried roots and soils by nested PCR. The BNR-specific primers provided rapid detection of fungal contaminants from dried roots and soils than the use of conventional methods such as observation by root infection and soil isolation. In addition, the BNR-specific primers could also be applied for rapid and accurate detection of diseases caused by BNR AG-Bo or AG-A in roots. The quantification of DNA using dot-blot assay in infected roots is relatively simple and efficient, and could be used for routine evaluation of infection once the standardisation has been optimised.

CHAPTER 6. PATHOGENICITY TESTS OF BNR

6.1. INTRODUCTION

Isolates of binucleate *Rhizoctonia* sp. (BNR) have been known to attack a variety of host plants, including legumes (Sneh *et al.*, 1991; Nelson *et al.*, 1996, Poromarto *et al.*, 1998). Pathogenicity of BNR has been tested on a variety of hosts (Frisina and Benson, 1989; Olaya and Abawi, 1994a; Nelson *et al.*, 1996; Mazzola, 1997). Generally, BNR isolates have been obtained from infected or diseased plants (Martin, 1988; Frisina and Benson, 1989; Green *et al.*, 1993; Olaya and Abawi, 1994a; Nelson *et al.*, 1996), from soil (Sumner and Bell, 1982; Ichielevich-Auster *et al.*, 1985a), or from potting media (Frisina and Benson, 1989), and then tested for pathogenicity on the same host species from which the isolates were obtained or on other hosts. BNR isolate CFM1 used in the present study was obtained from a mycorrhizal pot culture and belongs to AG-Bo (see Chapter 4). The isolate was capable of infecting mycorrhizal roots of clover and mung bean (see Chapter 5).

It has become apparent that particular BNR may induce diseases distinct from those induced by *Rhizoctonia solani* on certain hosts. Furthermore, isolates within AG of *R. solani* (Anderson, 1982) and BNR (Burpee *et al.*, 1980a) frequently exhibit varying degrees of host specialisation. In view of the lack of information on the ability of BNR isolate CFM1 to induce disease and affect plant growth, the experiments described in this chapter aimed to investigate the pathogenicity of this BNR isolate CFM1 on mung bean in comparison to the isolates of *R. solani*, and to assess their influence on plant growth.

Experiments were conducted in an *in vitro* culture system and in soil-grown plants in the glasshouse, and these are presented separately. Plants were inoculated with the BNR AG-Bo isolate CFM1 or with *R. solani* AG1-1C

isolate 01R01, AG2-1 isolate 21RM03 and AG2-2IIIB isolate 22R02 (see Chapter 3 Table 3.1). These AGs of *R. solani* were chosen because isolates belonging of those AGs have been shown to be pathogenic on a variety of bean species (Sumner, 1985; Ogoshi, 1987; Engelkes and Windels, 1996).

6.2. EXPERIMENT 1. *IN VITRO* EXPERIMENT

6.2.1. MATERIALS AND METHODS

6.2.1.1. Preparation of inoculum

The mycelia of BNR isolate CFM1, *R. solani* AG1-1C, AG2-1 and AG2-2IIIB, which exhibit different cultural morphology, were grown on PDA for 7 days (Figure 6.1). Plugs (3-mm square) of mycelium taken from the edge of 7 day-old cultures of BNR or *R. solani* grown on PDA were used to inoculate the plants on water agar in Petri dishes.

6.2.1.2. Bioassay

The bioassay was adapted from Keijer *et al.* (1997), and is schematically presented in Figure 6.2. A sterile Petri dish (15 cm diameter) was used as a growth chamber to allow sufficient space for six plants. Each Petri dish contained 50 ml water agar (6 g/L; Difco). Seeds of mung bean (*Vigna radiata* L. Wilczeck cv. Emerald) were surface sterilised and germinated as described in Chapter 3 section 3.3. Six seeds were placed at 2-cm intervals across the diameter of the plate and secured with a drop of water agar. Plants were inoculated with 4 plugs of mycelium of BNR or *R. solani* grown on PDA. Controls received agar plugs only. The Petri dishes were sealed with Parafilm and incubated in the dark at 23°C for 48 h to allow germination of the seeds. Then the Petri dishes were transferred to a growth chamber with a 14 h photoperiod (photon flux approximately 600 µmol $m^{-2}s^{-1}$), with temperatures

of at 23°C during the light period, and 18°C during the dark period. The Petri dishes were placed in an upright position at an angle of 60°, so that the roots grew over the agar surface, and the stem and leaves grew above the agar layer. The lower halves of the Petri dishes were wrapped in aluminium foil to protect the roots from the light. Symptom development was recorded every 2-3 days for 14 days.

6.2.1.3. Assessment of disease severity

Disease severity was assessed according to the rating schemes of Keijer *et al.* (1997) as follows:

For leaf or petiole:

0 = no symptoms.

1 = brown discolored areas or lesions on the leaf.

2 = lesions on the petiole with or without brown discolored areas on the leaf.

3 = lesions on the leaf blade and lesions on the petiole covering more than 75% of the total area.

For stem:

0 = no symptoms.

1 = small black or brown lesions less than 1 mm in diameter.

2 = lesions covering less than 75% of the stem surface.

3 = lesions covering more than 75% of the stem surface.

For roots:

0 = no symptoms.

1 = yellow or brown discoloration near the hypocotyl.

2 = yellow and brown discoloration and lesions or brown tips.

3 = a completely brown surface or lesions covering more than 75% of the root surface.

Data are presented as the means of disease severity ratings.

Figure 6.1. Cultural morphology of the isolates of BNR AG-Bo (a), *R. solani* AG1-1C (b), AG2-1 (c) and AG2-2IIIB (d) grown on PDA at 25°C for 7 days.

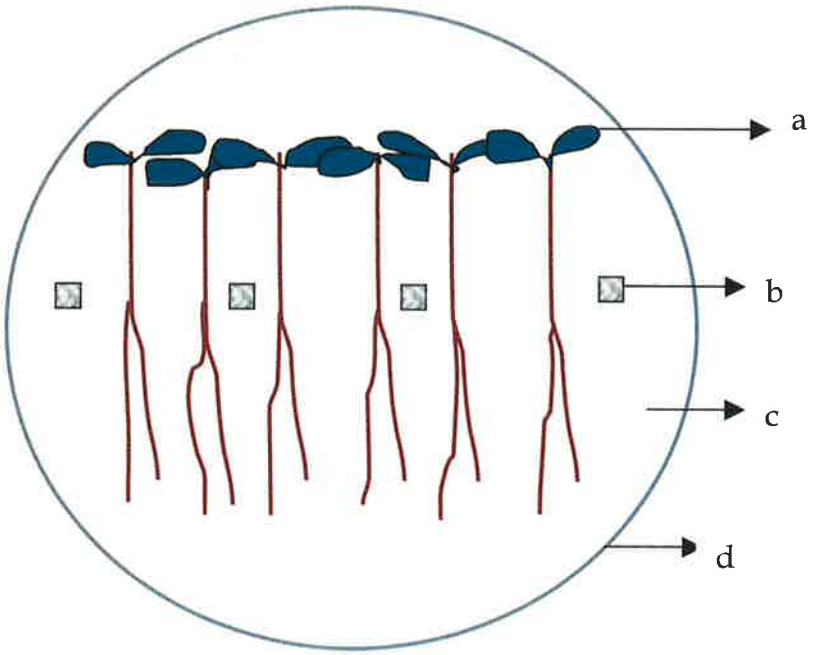


Figure 6.2. Schematic representation of the bioassay for pathogenicity. (a) plant; (b) mycelium of BNR or *R. solani;* (c) water agar medium; (d) a 15-cm diameter Petri dish.

6.2.1.4. Experimental design

The bioassay was conducted using a randomised complete block design with eight Petri dish replications. Treatment factors were control, BNR isolate CFM1, isolates of *R. solani* AG1-1C, AG2-1 and AG2-2IIIB. Each treatment had six plants per Petri dish (see Figure 6.2). At harvest (16 days), root samples were taken from three plants per plate, cleared and stained with trypan blue. The root infection was assessed microscopically by a gridline intersect method and the root length was calculated (see Chapter 3 section 3.5). ANOVA was used to determine statistical significance and data were analysed using the GENSTAT package as described in Chapter 3 section 3.6.

6.2.2. RESULTS

6.2.2.1. Symptom development and disease severity

The vegetative hyphae of BNR isolate CFM1 were white, and different from the colour of the *R. solani* isolates, which varied from light to dark brown, and covered the surface of PDA plate within 7 days (Figure 6.1). On water agar, the mycelium of BNR or *R. solani* generally grew towards the roots faster than towards the stem or leaves, and completely covered the agar surface 4-7 days after inoculation. Disease symptoms were observed at almost the same time on each plant inoculated with the same isolate. Generally, in all inoculation treatments, disease severity on leaves, stems and roots increased from day 2 to day 16, except for the isolates that did not cause any disease symptoms in particular parts of the plants or in control treatments.

Figure 6.3 shows the appearance of mung bean plants on water agar 16 days after inoculation. Control plants were healthy. Inoculation with BNR isolate CFM1 resulted in yellow discoloration on leaves. The growth of leaves was severely reduced by the isolates of *R. solani* AG1-1C and AG2-2IIIB, but

not reduced by AG2-1. The isolates of *R. solani* AG1-1C and AG2-2IIIB caused brown discoloration, with lesions covering almost 75% of the stem surface by that time, whereas the isolate of AG2-1 caused only small lesions. Symptoms on roots of mung bean infected by BNR isolate CFM1 differed from those on roots infected by *R. solani*. BNR isolate CFM1 and *R. solani* AG2-1 caused yellow to light brown discoloration, and short lesions less than 5 mm long, that did not girdle the roots. The lesions caused by the isolate of *R. solani* AG1-1C or AG2-2IIIB were generally larger than lesions caused by the isolate of *R. solani* AG2-1. Girdling of roots was also generally observed. The isolate of *R. solani* AG1-1C produced severe lesions on main roots, which inhibited the development of lateral roots, whereas the isolate of *R. solani* AG2-2IIIB caused root rot.

Figure 6.4A shows that BNR isolate CFM1 caused disease on leaves at 11 days after inoculation. The mean disease severity due to BNR isolate CFM1 on leaves was very low compared to any of the *R. solani* between 2 to 16 days after inoculation. BNR isolate CFM1 did not cause disease symptoms on stems up to 16 days after inoculation. The isolate of *R. solani* AG1-1C had the highest mean disease severity rating (2) on stems followed by the isolates of AG2-2IIIB and AG2-1 (Figure 6.4B). As shown in Figure 6.4C, the first lesions on roots inoculated with BNR isolate CFM1 were observed 4 days after inoculation, whereas root lesions on plants inoculated with the isolates of *R.solani* AG1-1C, AG2-1 and AG2-2IIIB were produced at 2, 11 and 2 days after inoculation, respectively. After 16 days, mean disease severity due to BNR isolate CFM1 on roots (1.2) was less than that due to the isolates of *R. solani* AG1-1C (2.1) and AG2-2IIIB (2.3), but greater than the isolate of AG2-1 (0.9).

6.2.2.2. Root infection, root length and total fresh weight

Root infection was measured 16 days after inoculation on material stained with trypan blue. Figure 6.5A shows that 40% of the root length was infected by BNR isolate CFM1. This was greater than the infection by the isolate of *R. solani* AG2-1 (28%), but less than the infection by the isolate of *R. solani* AG1-1C (52%) or AG2-2IIIB (97%). As shown in Figure 6.5B, BNR isolate CFM1 and the isolate of *R. solani* AG2-1 had no significant effect on the root length of mung bean, whereas the isolates of *R. solani* AG1-1C and AG2-2IIIB reduced the root lengths.

BNR isolate CFM1 and the isolate of *R. solani* AG2-1 did not reduce total fresh weights of plants, whereas the isolates of *R. solani* AG1-1C and AG2-2IIIB reduced the total fresh weights after 16 days (Figure 6.5C).

6.2.3. DISCUSSION

BNR isolate CFM1 and the isolates of *R. solani* AG1-1C, AG2-1 and AG2-2IIIB used in this study exhibited a considerable variability in morphological characteristics (see Figure 6.1). Sneh *et al.* (1989a) suggested that the colour indicates the presence of melanin in the hyphal cells. The absence of melanin in the hyphae of BNR may be a reason for their inability to penetrate and cause disease symptoms, and may, therefore, influence the pathogenicity of the isolate (Sneh *et al.*, 1989a).

The results of the *in vitro* pathogenicity test showed that BNR isolate CFM1 was weakly virulent on roots and leaves of mung bean, but did not cause any disease symptoms on stems, and overall the disease severity was lower than for the isolates of *R. solani* AG1-1C, AG2-1 and AG2-2IIIB. The highest disease rating on leaves resulted from inoculation with the isolate of *R. solani* AG1-1C, followed by AG2-2IIIB, and this was similar to AG2-1. BNR isolate CFM1 caused the lowest disease rating on leaves.

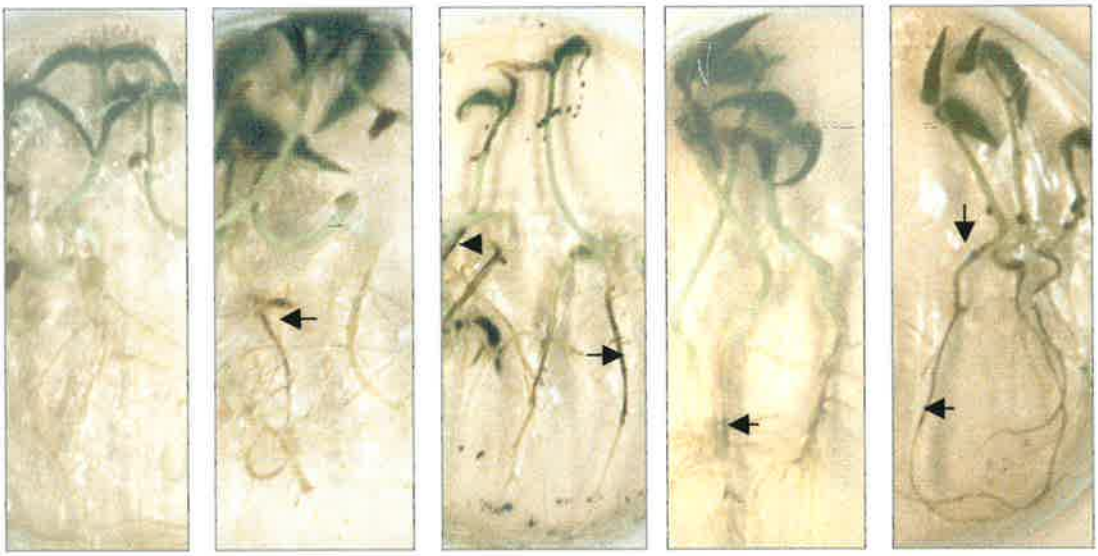


Figure 6.3. Effects of BNR isolate CFM1, *R. solani* AG1-1C, AG2-1 and AG2-2IIIB on growth of mung bean on water agar, 16 days after inoculation. Arrows indicate lesions on stem and root. Control plants did not show lesions.

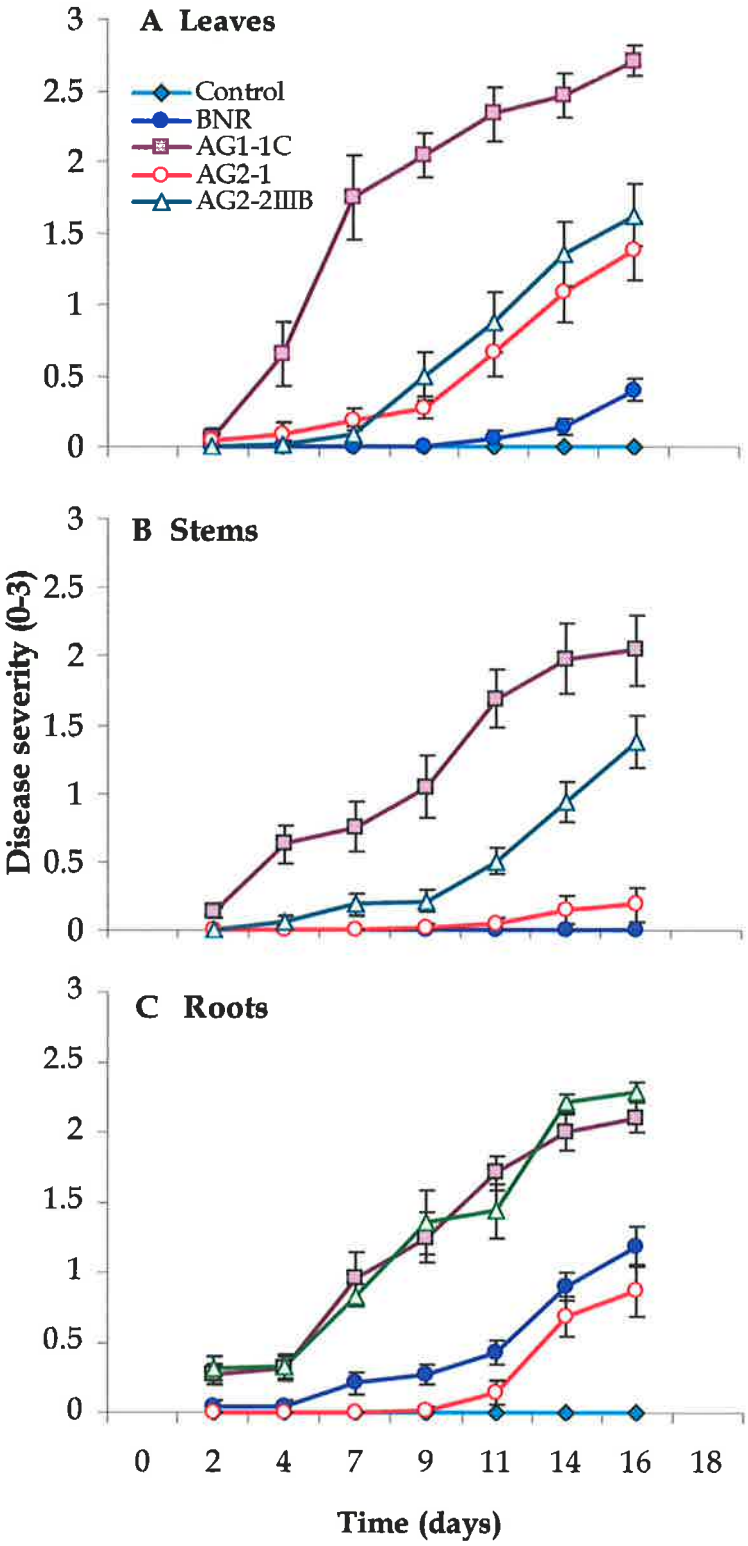


Figure 6.4. Disease severity due to BNR isolate CFM1, *R. solani* AG1-1C, AG2-1 and AG2-2IIIB on leaves (**A**), stems (**B**) and roots (**C**) of mung bean in an *in vitro* experiment at 2 to 16 days after inoculation. Bars represent standard errors of means (n=8). Figure **B.** Control similar to BNR and is not shown.

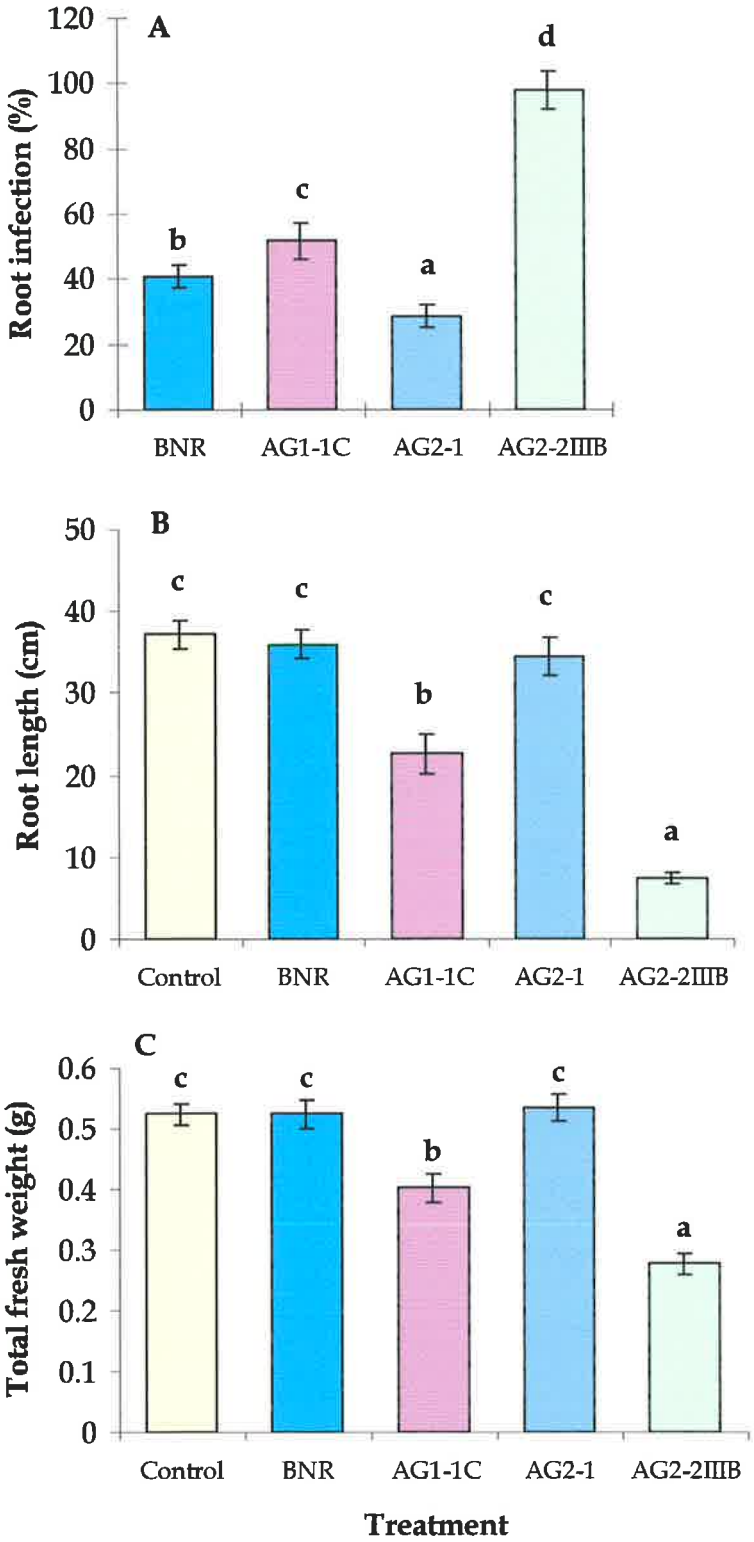


Figure 6.5. Effects of BNR isolate CFM1, *R. solani* AG1-1C, AG2-1 and AG2-2IIIB on root infection **(A)**, total root length **(B)** and total fresh weight **(C)** of mung bean in an *in vitro* experiment, 16 days after inoculation. Bars with the same letter are not significantly different ($P<0.05$). Vertical bars represent standard errors of means ($n=8$).

Furthermore, BNR isolate CFM1 caused only yellow discoloration on leaves, but lesions were not produced. Previous studies have shown that BNR isolates causes foliar diseases on a number of plant species (Burpee *et al.*, 1980b; Runion and Kelley, 1993; Olaya and Abawi, 1994a), but generally shows low virulence on foliage. The observations in this experiment support those findings. The isolate of *R. solani* AG1-1C caused the highest disease rating on the stem, followed by AG2-2IIIB, then AG2-1. As mentioned earlier, BNR isolate CFM1 did not cause any disease symptoms on stems. The disease severity caused by the isolates of *R. solani* AG1-1C and AG2-2IIIB on roots was similar and greater than that caused by the isolate of *R. solani* AG2-1 and BNR isolate CFM1.

Infection of mung bean roots by BNR isolate CFM1 was limited to the epidermal cells, as has been reported earlier (Chapter 5). The greatest root infection was observed on plants inoculated with the isolate of *R. solani* AG2-2IIIB, followed by AG1-1C and then BNR isolate CFM1 and the lowest infection was from AG2-1. The infection by the isolates of *R. solani* AG1-1C and AG2-2IIIB caused reduction in the root length and plant growth of mung bean. This suggests that the reduction in root length by *R. solani* was the result of either root rot or failure of roots to develop or both. The isolates of *R. solani* AG1-1C and AG2-2IIIB used in this study were not originally from legumes, but the results showed that both isolates were highly pathogenic on mung bean *in vitro*. These results are in agreement with previous studies showing that isolates of *R. solani* were able to infect host plants and cause disease in hosts other than the one from which they were originally obtained (Liu and Sinclair, 1991; Engelkes and Windels, 1996; Nelson *et al.*, 1996). There were no effects on the root length and growth of plants infected by BNR isolate CFM1 and the isolate of *R. solani* AG2-1. This contrasts with some previous work showing that isolates of AG2-1 are common pathogens on *Brassica* spp., highly pathogenic on cowpea and moderately virulent on snap bean and lima bean (Sumner, 1985).

The *in vitro* pathogenicity test was useful in a number of ways. A large number of plants could be tested quickly, inoculum could be applied uniformly to each plant, and disease symptoms could be recorded at intervals without destructively harvesting the plants. However, results of pathogenicity tests *in vitro* are not always be similar to those obtained in glasshouse experiments, as growth conditions influence the pathogenicity of the isolates (Burpee *et al.*, 1980b; Green *et al.*, 1993; Olaya and Abawi, 1994b). Therefore, the next experiment was conducted on plants grown in a soil-sand mixture in the glasshouse to assess the pathogenicity of BNR isolate CFM1, in comparison to the same of isolates of *R. solani* AG1-1C, AG2-1 and AG2-2IIIB on mung bean at 2, 4, 6 and 9 weeks.

6.3. EXPERIMENT 2. POT EXPERIMENT

6.3.1. MATERIALS AND METHODS

6.3.1.1. Preparation of seeds, inoculation and plant growth

Seeds of mung bean (*Vigna radiata* L. Wilczeck cv. Emerald) were surface sterilised, germinated, and one seed was planted into each pot containing 1.4 kg of soil/sand mix as described in Chapter 3 section 3.3. Inoculum of BNR isolate CFM1 or *R. solani* AG1-1C, AG2-1 and AG2-2IIIB was prepared (see Chapter 3 section 3.2.2.1), and six infected millet seeds were placed evenly at depths of 5 cm. Controls received six non-infected millet seeds (see Chapter 3 section 3.2.2.1). The plants were grown in the glasshouse and pots were given 10 ml of modified Long Ashton solution minus P weekly (see Chapter 3 section 3.3). Data for disease severity, plant growth, root infection, and root length were collected using the methods described in Chapter 3 section 3.5.

6.3.1.2. Experimental design

The experiment was conducted using a complete randomised design with three replications. Treatments were control, BNR isolate CFM1, *R. solani* AG1-1C, AG2-1 and AG2-2IIIB. The plants were harvested at 2, 4, 6 and 9 weeks after planting. ANOVA was used to determine statistical significance and data were analysed using the GENSTAT package (see Chapter 3 section 3.6).

6.3.2. RESULTS

6.3.2.1. Disease severity on roots

Disease severity was assessed based on lesions observed on roots at 2, 4, 6 and 9 weeks after planting. At 2 weeks symptoms were apparent on plants inoculated with BNR isolate CFM1, the isolates of *R. solani* AG1-1C and AG2-2IIIB, but not on plants inoculated with the isolate of *R. solani* AG2-1. Plants inoculated with the isolate of *R. solani* AG2-2IIIB had consistently (but not always significantly) higher disease ratings than other treatments throughout the experiment, followed by plants inoculated with the isolate of *R. solani* AG1-1C. Except at 2 weeks, there were no significant differences between disease ratings of plants inoculated with BNR isolate CFM1 or the isolate of *R. solani* AG2-1, and in both cases disease ratings were lower than for plants inoculated by the other *R. solani* isolates (Figure 6.6).

6.3.2.2. Root infection, root length and plant growth

In general, root infection of plants inoculated with BNR isolate CFM1 or the isolates of *R. solani* AG1-1C, AG2-1 and AG2-2IIIB increased between the 2-week and 9-week harvests. BNR isolate CFM1 showed significantly greater infection, compared with the isolates of *R. solani* AG1-1C, AG2-1 and

AG2-2IIIB at all harvest times except at 2 weeks when infection by all the fungi was very similar (6% or less) (see Figure 6.7).

Figure 6.8A shows that at 2 weeks none of the fungal isolates had any effects on root length. However, by 4 weeks all but the isolate of *R. solani* AG2-1 caused small reductions in root length. At 6 weeks, BNR isolate CFM1 reduced root length, but not significantly, whereas inoculation with *R. solani* significantly reduced the root lengths. At 9 weeks, plants inoculated with all fungi, including BNR isolate CFM1, had significantly shorter roots than the control plants.

Effects on root dry weight were generally similar to root length, although negative effects of inoculation with the isolate of *R. solani* AG2-1 were not apparent until 6 weeks and with BNR isolate CFM1 not until 9 weeks (Figure 6.8B).

There was a trend for all inoculation treatments to reduce shoot growth, but the differences were not significant until 6 weeks for the isolates of *R. solani* AG1-1C and AG2-2IIIB and 9 weeks for AG2-1. BNR isolate CFM1 caused no significant reduction in shoot dry weights at any harvest (Figure 6.8C).

6.3.3. DISCUSSION

The pathogenicity test in the glasshouse clearly showed that BNR isolate CFM1 caused disease symptoms on mung bean, but did not kill the plants up to 9 weeks after inoculation. The most severe disease on roots of mung bean was caused by the isolate of *R. solani* AG2-2IIIB, followed by AG1-1C, and then AG2-1, which was similar to BNR isolate CFM1. The results from this study were in agreement with other studies on different hosts, which showed that the pathogenicity of BNR isolates was generally lower than *R. solani* isolates (Sneh *et al.*, 1991; Nelson *et al.*, 1996).

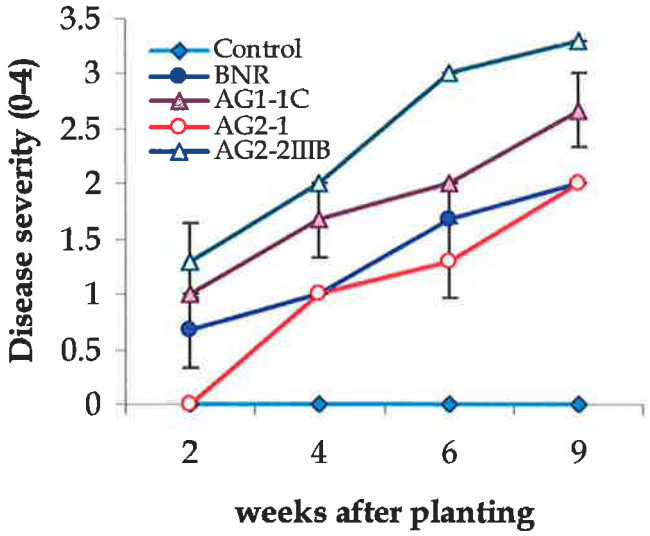


Figure 6.6. Roots disease severity of mung bean uninoculated and inoculated with BNR isolate CFM1, *R. solani* AG1-1C, AG2-1 and AG2-2IIIB at 2, 4, 6 and 9 weeks after planting. Bars represent standard errors of means (n=3). If there is no bar, standard error is 0. Disease severity rating (0-4) indicates 0 = no symptoms to 4 = lesions over 50% of the root necrotic.

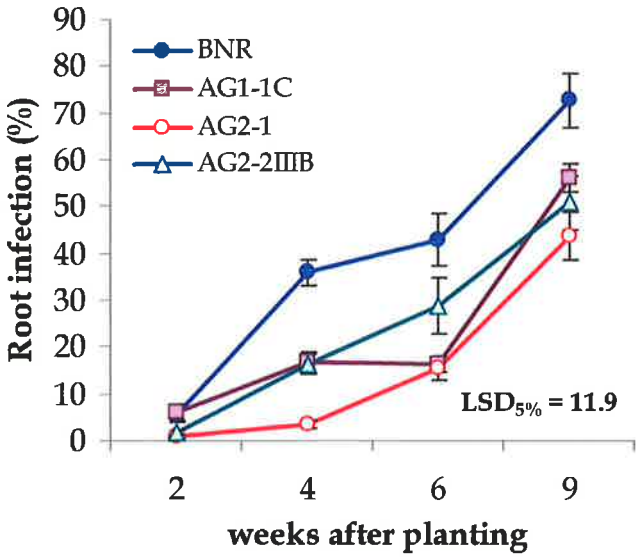


Figure 6.7. Percentage root infection of mung bean inoculated with BNR isolate CFM1, *R. solani* AG1-1C, AG2-1 and AG2-2IIIB at 2, 4, 6 and 9 weeks after planting. Bars represent standard errors of means (n=3).

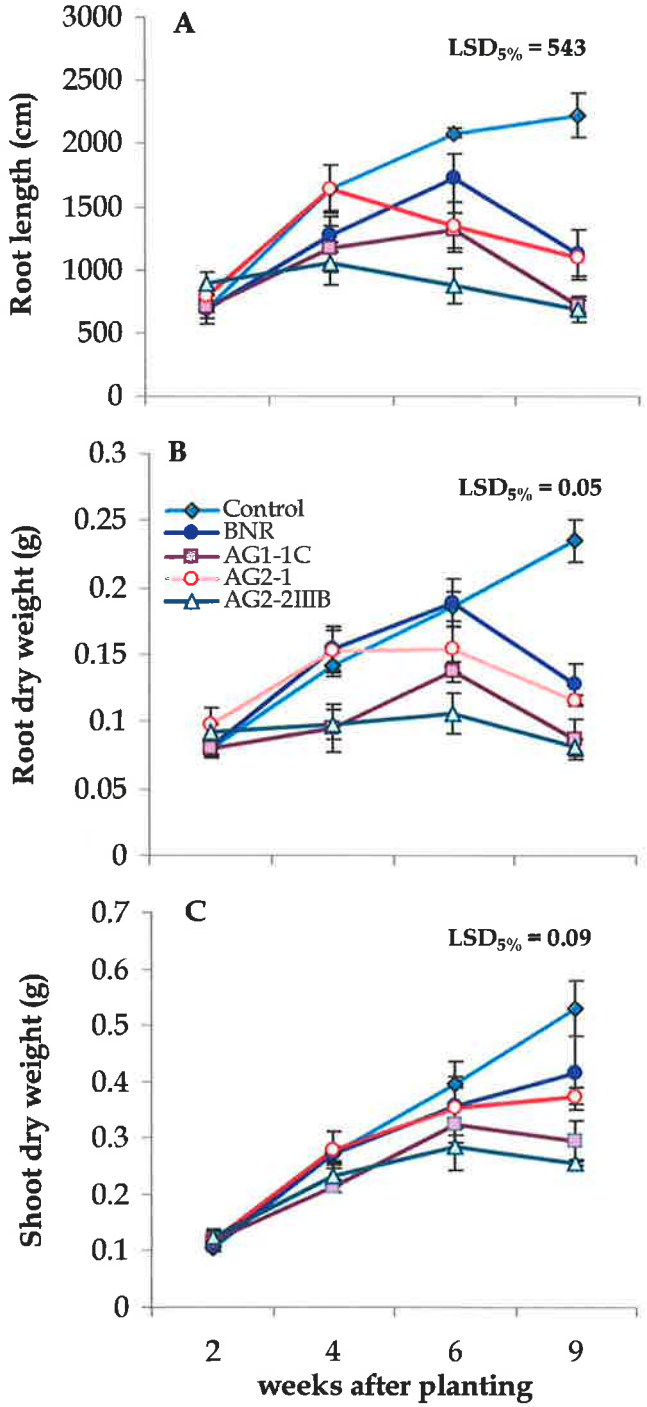


Figure 6.8. Total root length (A), shoot dry weight (B), and root dry weight (C) of mung bean uninoculated and inoculated with BNR isolate CFM1, *R. solani* AG1-1C, AG2-1 and AG2-2IIIB at 2, 4, 6 and 9 weeks after planting. Bars represent standard errors of means (n=3).

Root infection by BNR isolate CFM1 was rapid and more extensive than infection by the isolates of *R. solani* at most harvest times. Previous studies have indicated that dense infection of the epidermal cells by BNR reflects occupation sites (Sneh *et al.*, 1989a), and infection has been associated with a deposition of an electron-dense cell wall material rich in lignin, suberin and phenolic compounds (Xue *et al.*, 1998). The deposition of those compounds may provide a physical and chemical barrier to other fungi (Xue *et al.*, 1998), and rapid infection by BNR has been shown to protect the host tissues from infection by other fungi (Cardoso and Echandi, 1987; Harris *et al.*, 1994; Herr, 1995; Cartwright and Spurr, 1998).

Although BNR isolate CFM1 did not reduce the shoot dry weight of mung bean, there was an indication that the isolate was capable of reducing the root dry weight and the root length. The growth of plants was only slightly reduced by BNR isolate CFM1 and the isolate of *R. solani* AG2-1, whereas significant reduction of plant growth was observed in plants inoculated with the isolates of *R. solani* AG1-1C and AG2-2IIIB. Little research has been done on the effect of BNR on plant growth, and most reports provide only one time of harvest. For example, Sumner and Bell (1982) found that BNR (AG not determined) did not reduce the growth of corn (foliage, root dry weight or height) after 5 weeks. Mazzola (1997) stated that the effect of BNR on plant growth was influenced by the individual isolate within an AG. He found that isolates of AG-Q reduced shoot and root dry weights of apple, some isolates of AG-G reduced shoot and root dry weights, but isolates of AG-A did not reduce shoot and root dry weight 3 weeks after inoculation. The present study showed that mung bean plants inoculated with BNR AG-Bo isolate CFM1 showed less root growth, but did not differ significantly from the control plants up to 6 weeks after inoculation. However, at 9 weeks, extensive infection by BNR AG-Bo isolate CFM1 was associated with reduced root dry weight and root length. This finding suggests that inoculation with

BNR AG-Bo from a mycorrhizal pot culture, as used in this study, might have negative long-term effects on plant growth.

The glasshouse experiment showed that the isolates of *R. solani* AG1-1C, AG2-1 and AG2-2IIIB reduced plant growth. These AG had previously been reported to be pathogenic to a variety of bean plants (Sumner, 1985; Ogoshi, 1987; Engelkes and Windels, 1996). The present study showed that the isolate of *R. solani* AG2-2IIIB was more pathogenic than the other *R. solani* isolates tested and, therefore, it was chosen for comparison with BNR AG-Bo isolate CFM1 in the next experiment (see Chapter 7 and 8).

6.4. CONCLUSIONS

The results from the *in vitro* pathogenicity test were similar to the test conducted in pots in the glasshouse. The pathogenicity of BNR isolate CFM1 and the isolates of *R. solani* AG1-1C, AG2-1 and AG2-2IIIB on mung bean was characterised by disease severity and growth in both experiments. The relative damage to mung bean caused by individual isolates of BNR AG-Bo (CFM1) or *R. solani* AG1-1C, AG2-1 and AG2-2IIIB was similar for the two experiments. Isolates that caused greater disease severity and reduced plant growth (*R. solani* AG1-1C or 2-2IIIB) in the *in vitro* experiment did so in the pot experiment, and isolates that caused less disease severity and had little effect on plant growth (BNR isolate CFM1 and *R. solani* AG2-1) in the *in vitro* experiment, had similar effects in the pot experiment. However, disease development and growth of mung bean were slower and disease on mung bean was less severe in the *in vitro* experiment compared to the pot experiment. The only major difference appeared to be the greater infection of roots by BNR isolate CFM1 than the isolates of *R. solani* AG1-1C, AG2-1 and AG2-2IIIB when grown in soil.

The experiment conducted *in vitro* was useful because a large number of plants could be tested, inoculum could be applied uniformly to each plant, and the method facilitated visualisation of the roots. In addition, other organisms as well as abiotic and biotic soil factors could be excluded in the system. However, long-term experiments would not be possible with this system. On the other hand, the pot experiment provided natural conditions for fungal growth, the experiment could be conducted with other microorganisms (e.g. AM fungi) and plants could be grown for a longer time than in the *in vitro* experiment.

The results of the *in vitro* and pot experiments showed that BNR isolate CFM1 occurring as a fungal contaminant on mycorrhizal pot cultures was capable of rapidly infecting the roots and causing disease symptoms, and had negative effects on root growth of mung bean in a long term-inoculation experiment in soil. In view of these findings, careful choices of the host plants to be used for pot culture purposes should be made, avoiding hosts which, like mung bean, are rapidly and extensively infected by BNR isolate CFM1. A concurrent study of BNR isolate CFM1 and AM fungi would be useful, because they may influence each other within plant root systems and affect physiological processes of the plant. However, the interactions between BNR isolate CFM1 and AM fungi have not previously been considered and it is necessary to investigate both the interactions between AM fungi and BNR isolate CFM1 or *R. solani* in the roots and their effects on plant growth (see Chapter 7). The isolate of *R. solani* AG2-2IIIB, which was more pathogenic than the isolates of *R. solani* AG1-1C and AG2-1 on mung bean, was used in the next experiments (Chapter 7 and 8).

CHAPTER 7. EFFECTS OF PHOSPHORUS ON THE INTERACTIONS BETWEEN *GLOMUS CORONATUM* AND BNR OR *R. SOLANI*

7.1. INTRODUCTION

Studies of interactions between AM fungi and root-infecting fungal pathogens have shown that the effect of AM fungi on pathogen and disease development can sometimes be attributed to enhanced phosphorus (P) nutrition (see Chapter 2 section 2.5.5.1). However, reports on the role of enhanced P nutrition and AM fungi in reduction of root diseases are conflicting, probably due to the heterogeneity of AM fungi, pathogens, and host plants used in these studies, and differences in experimental design and methods for assessing disease severity. Prior to this work, there was no information available on the effects of P on the interactions between AM fungi and BNR or *R. solani.*

AM fungi, which form symbiotic associations with a wide range of plant species, can reduce root disease caused by a number of soil-borne pathogens (Linderman, 1994). A well-established mycorrhizal infection has been reported to reduce disease severity caused by a number of pathogenic fungi, such as *Fusarium, Verticillium, Phytophthora,* and *Pythium* species (see Chapter 2 Table 2.1). Few studies have been done on the interactions between AM fungi and *R. solani* (Zambolin and Schenck, 1983; Wyss *et al.,* 1991; Vierheilig *et al.,* 1993; Guenoune *et al.,* 2001). BNR isolates (teleomorph *Ceratobasidium*) have been studied in relation to orchid mycorrhiza (Warcup and Talbot, 1971, 1980). BNR isolate CFM1 occurred in mycorrhizal roots in pot cultures (Chapter 5), caused disease and reduced root growth of mung bean (Chapter 6), and may also influence the development of AM fungi and affect physiological processes of the plant. However, the interaction between BNR obtained from pot cultures and AM fungi has not been reported.

The objectives of this study were to investigate the role of *G. coronatum* in the reduction of disease caused by BNR isolate CFM1 (AG-Bo) or *R. solani* AG2-2IIIB in mung bean and the influence of P on disease development. Simultaneous inoculation and prior inoculation with *G. coronatum* were compared, to identify the effect of *G. coronatum* on the host-pathogen interactions and disease development by BNR isolate CFM1 (AG-Bo) or *R. solani* AG2-2IIIB.

7.2. MATERIALS AND METHODS

7.2.1. Fungal material

Spores of *G. coronatum* (WUM16) were derived from dry pot cultures of *Trifolium subterraneum* L. cv. Mt Barker following procedures described in Chapter 3 section 3.2.1.2. To inoculate each plant, 250 spores of *G. coronatum* were placed directly under each seed. The experiments used BNR isolate CFM1 previously investigated in Chapters 4-6, and the isolate of *R. solani* AG2-2IIIB (referred to only as *R. solani* in this chapter) was chosen for comparison because it was the most pathogenic isolate in mung bean of the *R. solani* isolates tested (results of Chapter 6). The inoculum of BNR isolate CFM1 or *R. solani* was prepared according to a method modified from McDonald and Rovira (1985) (see Chapter 3 section 3.2.2.1). Inoculation was carried out by placing inoculated six millet seeds evenly at depths of 5 cm, 2 cm from the stem. Control received six non-inoculated millet seeds.

7.2.2. Plant material and soil

Seeds of mung bean (*Vigna radiata* L. Wilczeck cv. Emerald) were surface sterilised, germinated, and one seed was planted into a pot containing 1.4 kg of soil/sand mix as described in Chapter 3 section 3.3. The plants were

grown in the glasshouse and pots were given weekly 10 ml of modified Long Ashton solution minus P (see Chapter 3 section 3.3).

7.2.3. Phosphorus supply

30.4 mg P was added as $NaH_2PO_4.2H_2O$ to each pot (1.4 kg soil/sand) one week before planting. This amount of P was chosen on the basis of preliminary experiments.

7.2.4. Assessment of disease severity, root infection and root length

At harvest, shoots and roots were separated, roots were thoroughly washed, blotted and fresh weights recorded. The disease severity was assessed according to the rating schemes of Liu and Sinclair (1991) and the disease index (DI) was calculated using the equation of Liu and Sinclair (1991) (see Chapter 3 section 3.5). Sub-samples of roots were taken for determination of dry weight, fungal infection and total root length. Roots were stained by the method of Phillips and Hayman, (1970), and infection assessed by the method of Giovannetti and Mosse (1980), and the root length was measured and calculated using the equation of Tennant (1975) (see Chapter 3 section 3.5).

7.2.5. Determination of P concentration in plant tissues

Dried shoots and sub-samples of roots were ground with a pestle and mortar and analysed for total P by the phospho-vanado-molybdate method (Hanson, 1950). Approximately 50 mg of ground plant material was placed into 50 ml digestion tubes and 3 ml of a nitric-perchloric digestion mixture was added. Samples were allowed to stand overnight and the tubes were then digested on a programmed Tecator R digestion block. After digestion, the digests were made up to 25 ml using RO water. A 5 ml aliquot was made

up to 25 ml with 3 ml of mixed reagent (containing 1 part of nitric acid, 1 part 0.25% ammonium vanadate and 1 part 5.0% ammonium molybdate) and RO water. After 30 minutes, the absorbance was read on a LKB Biochrom-Ultraspect 4050 sprectophotometer at 390 nm using a blue filter. A standard curve was obtained from 1000 µg P/ml stock solution using a concentration range 0-5 µg P/ml to calculate values in samples.

7.2.6. Statistical analysis

ANOVA was used to determine statistical significance and data were analysed using the GENSTAT package (see Chapter 3 section 3.6). Means were separated using the LSD test at 5% level of probability. Percentage data for fungal infection were arcsin transformed prior to analysis.

7.3. EXPERIMENT 1. *G. CORONATUM*, BNR OR *R. SOLANI* INOCULATED AT THE TIME OF PLANTING

The aim of this experiment was to identify whether addition of P and/or simultaneous inoculation with *G. coronatum* influenced the development of disease caused by BNR isolate CFM1 or *R. solani* in mung bean. The experiment was conducted in a 2 x 6 factorial design with two P treatments (no additional P (P0) and 30.4 mg P ($NaH_2PO_4.2H_2O$) (P1) added to the soil in each pot one week before planting) and six inoculation treatments: no inoculum (control), *G. coronatum*, BNR isolate CFM1, *G. coronatum* + BNR isolate CFM1, *R. solani*, *G. coronatum* + *R. solani*. In this experiment, all fungi were inoculated at the time of planting. One seed was planted in each pot, with three replicate pots for each treatment, and the plants were harvested 6 weeks after planting.

7.3.1. RESULTS

7.3.1.1. Plant growth responses

At harvest, shoot dry weight of mycorrhizal plants grown in P0 was greater than for non-mycorrhizal (NM) plants (Table 7.1A). Inoculation of NM plants in P0 with BNR isolate CFM1 had no significant effect on shoot dry weight, whereas there was a reduction in shoot dry weights when NM plants were inoculated with *R. solani*. Adding P to the soil (P1) increased shoot dry weights; however, there was no significant effect due to inoculation with BNR isolate CFM1, *R. solani* or *G. coronatum*.

Plants in P0 infected by *G. coronatum* had greater root dry weight than corresponding NM controls (Table 7.1B). Inoculation of NM plants with BNR isolate CFM1 had no effect on root dry weight, but inoculation with *R. solani* reduced root dry weight. Root dry weights of mycorrhizal plants in P0 inoculated with BNR isolate CFM1 or *R. solani* were similar to those of NM plants. Addition of P to the soil (P1) increased the root dry weights of both NM and mycorrhizal plants.

Total root length of mycorrhizal plants did not differ from that of NM plants in either P0 or P1. However, root length in P1 was greater than in P0 (Table 7.1C). In P0, NM plants infected by *R. solani* had shorter roots than NM controls, whereas in P1, NM plants infected by BNR isolate CFM1 had reduced root length compared to NM control plants.

7.3.1.2. Root infection

After 6 weeks, the percentage root length infected by *G. coronatum* was not affected by the presence of either BNR isolate CFM1 or *R. solani* at either P level (Figure 7.1).

Table 7.1. Shoot dry weight, root dry weight and total root length of mung bean uninoculated and inoculated with BNR isolate CFM1 or *R. solani* AG2-2IIIB in non-mycorrhizal (NM) and mycorrhizal (*G. coronatum*) plants in two P treatments (P0 and P1).

Inoculation treatment	P0		P1	
	NM	*G. coronatum*	NM	*G. coronatum*
A. Shoot dry weight (g)				
Control	0.22±0.02^{a}	0.37±0.04^{c}	0.79±0.08^{d}	0.91±0.10^{d}
BNR	0.18±0.01^{a}	0.36±0.06^{c}	0.75±0.10^{d}	0.70±0.06^{d}
R. solani	0.09±0.01^{b}	0.26±0.02^{c}	0.83±0.08^{d}	0.84±0.05^{d}
B. Root dry weight (g)				
Control	0.07±0.01^{a}	0.10±0.01^{c}	0.26±0.02^{d}	0.26±0.03^{d}
BNR	0.06±0.01^{a}	0.09±0.01^{c}	0.19±0.02^{d}	0.25±0.02^{d}
R. solani	0.02±0.01^{b}	0.08±0.02^{c}	0.25±0.03^{d}	0.25±0.01^{d}
C. Total root length (cm)				
Control	377.64±64.20^{a}	475.10±63.93^{a}	1291.70±107.20^{c}	1385.40±470.00^{c}
BNR	210.53±45.56^{a,b}	314.12±63.31^{a}	796.74±132.10^{d}	1431.80±347.50^{c}
R. solani	80.18±32.25^{b}	342.50±99.45^{a}	1360.70±117.50^{c}	1340.00±151.00^{c}

Values are the means ± SE, n=3. Values for A) Shoot dry weight, B) Root dry weight and C) Total root length that are not significantly different (P<0.05) have the same superscript letters.

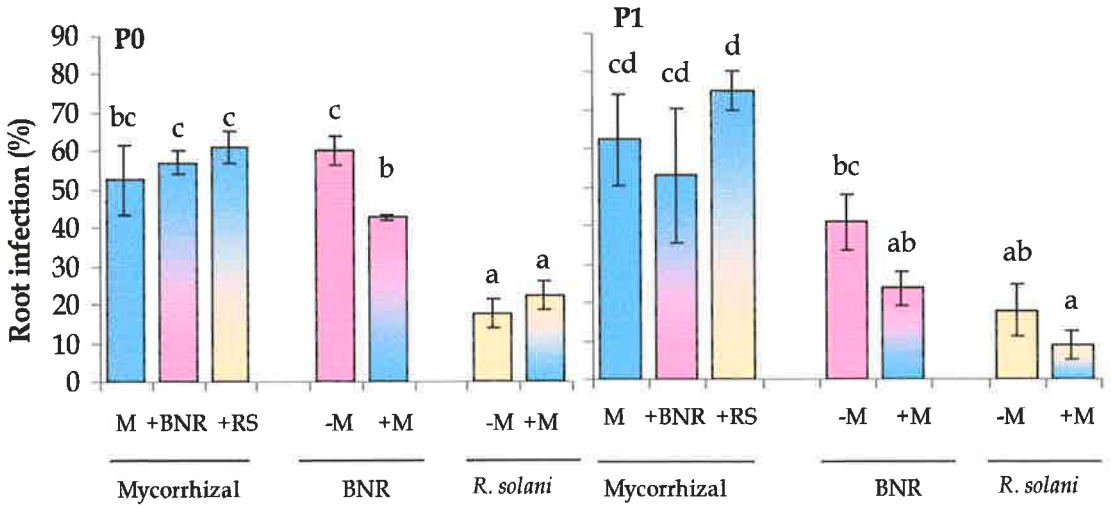


Figure 7.1. Percentage of root length of mung bean infected by *G. coronatum* (M), BNR isolate CFM1 (BNR) or *R. solani* AG2-2IIIB (RS) at 6 weeks after planting in P0 and P1 treatments. Bars with the same letter are not significantly different ($P<0.05$). Vertical bars indicate standard errors of the mean (n=3).

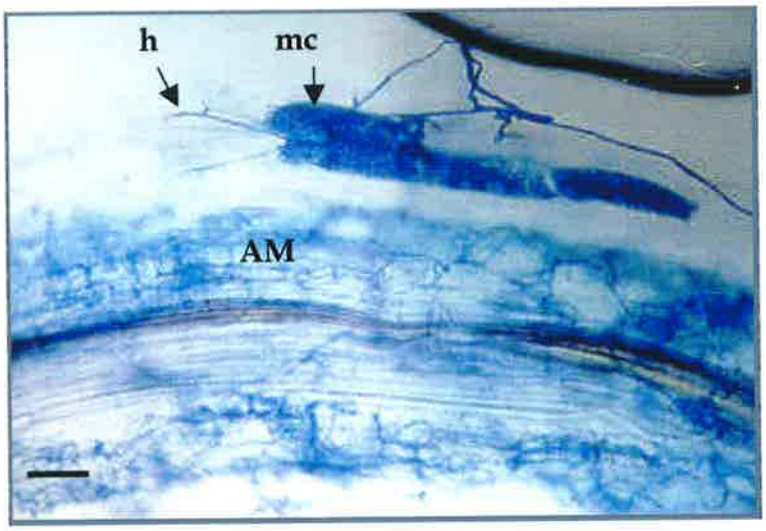


Figure 7.2. Root infection of mung bean by BNR isolate CFM1 in the epidermal cells showing hyphae (h) and monilioid cells (mc), and by the AM fungus, *G. coronatum* (AM) in the cortical cells. *Bar* = 50 μm.

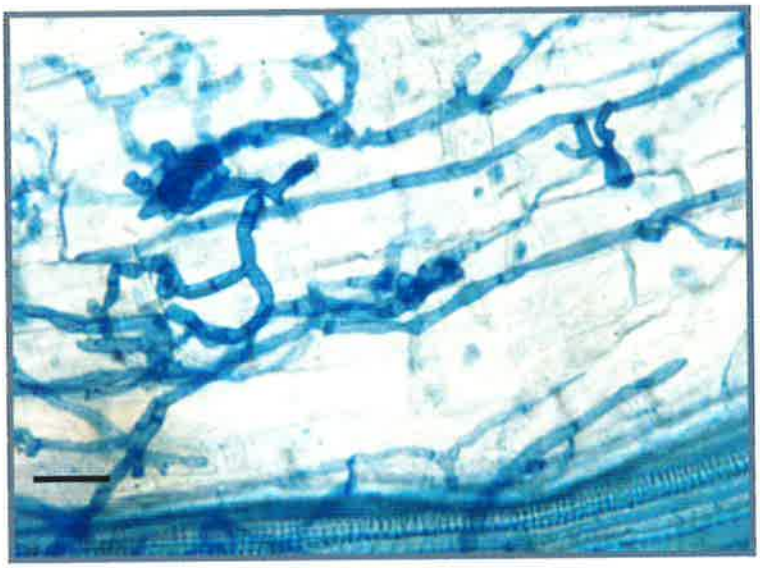


Figure 7.3. Root infection of mung bean by *R. solani* AG2-2IIIB, characterised by the septation of hyphae. *Bar* = 25 μm.

Furthermore, P addition had no significant effect on mycorrhizal infection. In P0, the presence of *G. coronatum* reduced infection by BNR isolate CFM1, but not by *R. solani*. However, in P1, root infection by BNR isolate CFM1 and *R. solani* were not significantly reduced by *G. coronatum*. BNR isolate CFM1 infected the epidermal cells of mung bean roots, characterised by the production of monilioid cells and thin hyphae (Figure 7.2), whereas the infection by *R. solani* was characterised by septation of hyphae in the root cortex (Figure 7.3).

7.3.1.3. Shoot and root P concentrations

As shown in Table 7.2A, there was a tendency for mycorrhizal plants to have higher shoot P concentrations than NM plants in P0 and P1, although differences were not always significant. Plants infected by *G. coronatum* in P1 had higher shoot P concentrations than those in P0. Shoot P concentrations of plants infected by *G. coronatum* + *R. solani* in P0 and P1 were higher than those of plants infected by *R. solani* only.

Mycorrhizal plants had significantly greater root P concentrations than the corresponding NM plants in both P0 and P1, with the exception of plants infected by *G. coronatum* alone in P0 (Table 2B). Infection of mycorrhizal plants in P0 by *R. solani* increased P concentrations in roots. Adding P to the soil increased root P concentration of plants infected by *G. coronatum* alone.

7.3.1.4. Disease severity

The mean disease severity and the disease index in plants inoculated with BNR isolate CFM1 were the same in both P0 and P1 (Table 7.3). Plants inoculated with *R. solani* had a mean disease rating and disease index in P0 slightly lower than in P1.

Table 7.2. Shoot and root P concentrations in mung bean uninoculated and inoculated with BNR isolate CFM1 or *R. solani* AG2-2IIIB in non-mycorrhizal (NM) and mycorrhizal (*G. coronatum*) plants in two P treatments (P0 and P1).

Inoculation treatment	P0		P1	
	NM	*G. coronatum*	NM	*G. coronatum*
A. Shoot P concentration (mg g^{-1})				
Control	0.73±0.05^{a}	1.14±0.22^{a,b}	0.72±0.14^{a,b}	2.00±0.34^{c}
BNR	0.64±0.19^{a}	1.05±0.06^{a,b}	1.44±0.47^{a,b,c}	2.10±0.56^{c}
R. solani	0.67±0.26^{a}	1.48±0.06^{b}	0.95±0.11^{a,b}	2.83±0.32^{c}
B. Root P concentration (mg g^{-1})				
Control	0.43±0.10^{a}	0.98±0.37^{a}	0.81±0.10^{a}	2.63±0.56^{c}
BNR	0.37±0.15^{a}	2.37±0.57^{b,c}	0.80±0.32^{a}	3.09±0.71^{c}
R. solani	0.66±0.06^{a}	2.69±0.50^{c}	1.20±0.15^{a}	3.30±0.32^{c}

Values are the means ± SE, n=3. Values for A) Shoot P concentration and B) Root P concentration that are not significantly different (P<0.05) have the same superscript letters.

Table 7.3. Effects of *G. coronatum* and P treatments (P0 and P1) on disease severity in mung bean inoculated with BNR isolate CFM1 or *R. solani* AG2-2IIIB.

Treatment	Disease rating (0-4)[a]		Disease Index (%)[b]	
	P0	**P1**	**P0**	**P1**
Control	0	0	0	0
G. coronatum	0	0	0	0
BNR	1.7	1.7	41.7	41.7
BNR (with *G. coronatum*)	1	1.3	25 (40)	33.3 (20)
R. solani	3	2.7	75	66.7
R. solani (with *G. coronatum*)	2.3	2.3	58.3 (22)	58.3 (12)

Values are the means of n=3, values in parenthesis indicate the percentage reduction of disease index when *G. coronatum* was present.

[a]Disease rating was based on scale of 0 to 4 with 0 = no lesions and 4 = lesions over 50% of the root area and necrosis.

[b]Disease Index was calculated from Σ (number of plants of a given disease rating x disease rating) x 100/ 4 (total number of plants rated) x 100.

Lower disease rating and disease index were observed in plants inoculated with BNR isolate CFM1 or *R. solani* when *G. coronatum* was present in the same roots. In P0, the disease index due to BNR isolate CFM1 was reduced by 40% in the presence of *G. coronatum* and in P1 soil by 20%. The disease index due to *R. solani* was reduced by 22% in the presence of *G. coronatum* in P0 soil and by 12% in P1 soil.

7.3.2. DISCUSSION

Infection by *G. coronatum* improved growth of mung bean, and this growth enhancement was not influenced by the presence of BNR isolate CFM1 or *R. solani*. Despite extensive infection of root tissues by BNR isolate CFM1, this fungus did not significantly reduce the dry weights and total root length of mung bean plants in P0 soil, although inoculation did result in the development of root lesions. These results were similar to those of the previous experiments (see Chapter 6.), and supported the evidence that BNR isolate CFM1 was not strongly pathogenic to mung bean. In contrast, inoculation with *R. solani* reduced the shoot and root dry weights, and total root length of mung bean, and roots developed lesions and necrosis. These results were also consistent with previous results (Chapter 6.). *R. solani* has been known to damage or kill host cells prior to, or immediately following, penetration and infection (Weinhold and Sinclair, 1996).

Although it had little effect on plant growth, BNR isolate CFM1 infected the roots of mung bean extensively, and was limited to the epidermal cells, as has been observed by other researchers (Cardoso and Echandi, 1987; Herr, 1995) and in previous experiments (see Chapter 4, 5 and 6). BNR isolates have been shown to grow in the presence of root exudates and to infect healthy, undamaged plant tissues (Cardoso and Echandi, 1987). In P0 and P1, when BNR isolate CFM1 infected the roots in the presence of *G. coronatum*, the percentage infection by BNR isolate CFM1 was reduced, but

that of *G. coronatum* remained unaffected. This indicates that direct interactions between the two fungi occurred, possibly involving competition for space and nutrients, as suggested by other authors for other root-infecting fungi (Afek *et al.* 1990; Hooker *et al.* 1994; Azcón-Aguilar and Barea, 1996, Graham, 2001). However, it is worth noting that BNR isolate CFM1 infected only the root epidermal cells of mung bean and other hosts, whereas AM fungi infect the cortex, so direct competition for nutrients seems unlikely. Induction of defence responses in mycorrhizal plants (Cordier *et al.* 1996) or production of antimicrobial compounds by the plant (Morandi *et al.* 1984) may also help to explain the reduced infection by BNR isolate CFM1 in mycorrhizal plants.

Inoculation by *G. coronatum* did not reduce the infection of roots by *R. solani*. The septate hyphae of *R. solani* were found in the root cortex of mung bean. Competition between these two fungi for space and nutrients might be limited in this case, but failure of *G. coronatum* to influence infection by *R. solani* was perhaps caused by low infection by *R. solani* in the roots (Table 7.3) or limited opportunity for the fungi to interact directly or to compete, since only 4.8-8.7% (P0) and 0.5-2% (P1) of the root length were occupied together by *G. coronatum* and *R. solani* (results not shown).

P concentrations in mung bean were strongly influenced by mycorrhizal infection, while BNR isolate CFM1 or *R. solani* had little effect. P concentration was high when disease severity was low, as observed in mycorrhizal plants. Infection by *G. coronatum*, even in P1 soil, resulted in the development of lower disease ratings and indices. The present results were in agreement with previous reports in which a higher concentration of P in the plant was not entirely responsible for lower disease development (e.g. Kaye *et al.*, 1984; Trotta *et al.*, 1996).

In this experiment, inoculation by *G. coronatum* reduced disease caused by BNR isolate CFM1 or *R. solani*. This effect was unexpected, as mycorrhizal

infection was not established before BNR isolate CFM1 or *R. solani* were introduced. Accordingly, any effect of presence of mycorrhizal fungi is unlikely to have been via host nutrition. Possibly, direct interaction between *G. coronatum* and BNR isolate CFM1 or *R. solani* occurred. Similar suggestions have been made for other root pathogens (Caron *et al.*, 1985).

7.4. EXPERIMENT 2. BNR OR *R. SOLANI* INOCULATED 3 WEEKS AFTER *G. CORONATUM*

The aim of the second experiment was to determine whether, at the two P levels, prior establishment of *G. coronatum* in the roots of mung bean could reduce the effect of BNR isolate CFM1 or *R. solani* on plant growth and disease severity. The experiment had a 2 x 6 factorial design with the same two P treatments, and spores of *G. coronatum* were applied at the time of planting as described for experiment 1. After 3 weeks, in each pot, 6 millet seeds infected with either BNR isolate CFM1 or *R. solani* were placed evenly at the depth of 5 cm in the soil approximately 2 cm from the stem. The plants were harvested 6 weeks after inoculation with BNR isolate CFM1 or *R. solani* (9 week-old plants).

7.4.1. RESULTS

7.4.1.1. Plant growth responses

In P0, shoot dry weights of mycorrhizal plants were higher than NM plants (Table 7.4A). Shoot dry weights in P1 were significantly higher than in P0 for all treatments. Shoot dry weights of mycorrhizal plants were higher than NM plants in P0, but did not differ in P1. Inoculation with BNR isolate CFM1 or *R. solani* did not reduce shoot dry weights in either P0 or P1.

Similar trends were observed in root dry weight and total root length of plants (Table 7.4B,C). Root dry weight and total root length of plants in P1 were higher than in P0. There were no differences in root dry weight and total root length between mycorrhizal plants and NM plants in either P0 or P1.

7.4.1.2. Root infection

Plants were extensively infected (66-81%) by *G. coronatum* after 9 weeks, and generally *G. coronatum* had infected more of the root than did BNR isolate CFM1 or *R. solani* in both P0 and P1 (Figure 7.4). Again, P addition had no effect on mycorrhizal infection. Addition of P and the presence of *G. coronatum* in roots reduced the infection by BNR isolate CFM1 in both P0 and P1, whereas infection by *R. solani* was not reduced. In both P0 and P1, the presence of either BNR isolate CFM1 or *R. solani* did not influence infection of roots by *G. coronatum*.

7.4.1.3. Shoot and root P concentrations

Shoot P concentrations of mycorrhizal plants were greater than corresponding NM plants, regardless of inoculation with either BNR isolate CFM1 or *R. solani*. The same trend was apparent for root P concentration (Table 7.5). Adding P to the soil did not influence shoot P concentrations significantly, except in plants infected by *R. solani*. In both P treatments, plants inoculated by *G. coronatum* + BNR isolate CFM1 or *G. coronatum* + *R. solani* had higher shoot P concentrations than those infected by BNR isolate CFM1 or *R. solani* alone (Table 7.5A).

Table 7.4. Shoot dry weight, root dry weight and total root length of mung bean uninoculated and inoculated with BNR isolate CFM1 or *R. solani* AG2-2IIIB in non-mycorrhizal (NM) and mycorrhizal (*G. coronatum*) plants in two P treatments (P0 and P1).

	P0		**P1**	
Inoculation treatment	NM	*G. coronatum*	NM	*G. coronatum*
A. Shoot dry weight (g)				
Control	0.26±0.05^{a}	0.50±0.07^{b}	2.32±0.26^{c}	1.53±0.27^{c}
BNR	0.23±0.04^{a}	0.51±0.03^{b}	1.78±0.25^{c}	2.25±0.08^{c}
R. solani	0.19±0.05^{a}	0.56±0.05^{b}	2.36±0.22^{c}	2.40±0.39^{c}
B. Root dry weight (g)				
Control	0.06±0.01^{a}	0.08±0.02^{a}	0.43±0.07^{b}	0.34±0.03^{b}
BNR	0.07±0.01^{a}	0.08±0.01^{a}	0.38±0.15^{b}	0.48±0.03^{b}
R. solani	0.07±0.01^{a}	0.08±0.02^{a}	0.26±0.09^{b}	0.40±0.06^{b}
C. Total root length (cm)				
Control	243.59±84.72^{a}	248.29±51.84^{a}	1502.10±253.05^{b}	1019.48±107.50^{b}
BNR	298.13±108.35^{a}	238.96±33.13^{a}	953.12±224.05^{b}	1254.50±58.65^{b}
R. solani	258.41±120.35^{a}	305.89±44.85^{a}	1114.12±335.90^{b}	1093.60±231.54^{b}

Values are the means ± SE, n=3. Values for A) Shoot dry weight, B) Root dry weight and C) Total root length that are not significantly different (P<0.05) have the same superscript letters.

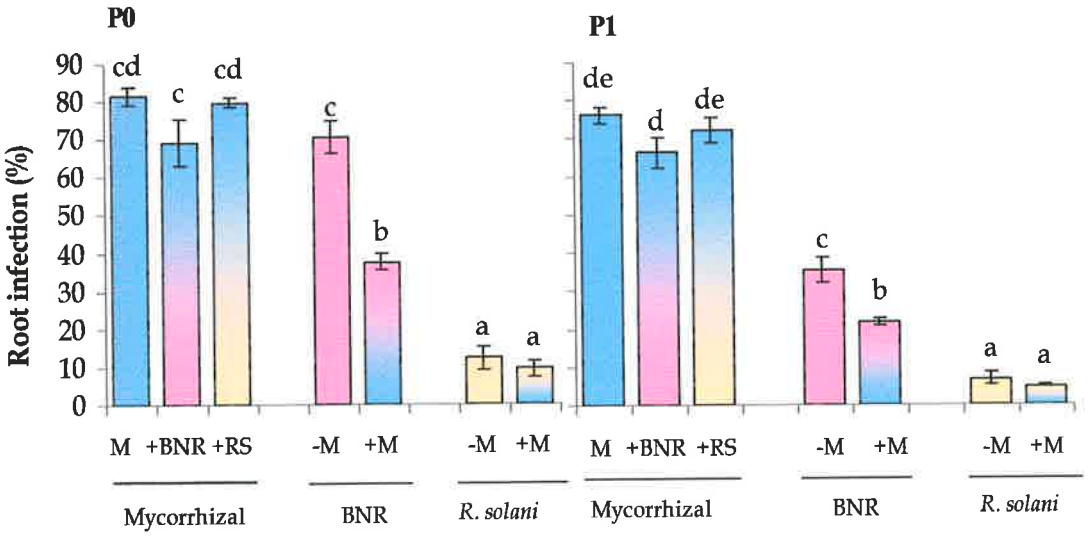


Figure 7.4. Percentage of root length of mung bean infected by *G. coronatum* (M), BNR isolate CFM1 (BNR) or *R. solani* AG2-2IIIB (RS) at 9 weeks after planting in P0 and P1 treatments. Bars with the same letter are not significantly different ($P<0.05$). Vertical bars indicate standard errors of the mean ($n=3$).

Table 7.5. Shoot and root P concentrations of mung bean uninoculated and inoculated with BNR isolate CFM1 or *R. solani* AG2-2IIIB in non-mycorrhizal (NM) and mycorrhizal (*G. coronatum*) plants in two P treatments (P0 and P1).

	P0		P1	
Inoculation treatment	NM	*G. coronatum*	NM	*G. coronatum*
A. Shoot P concentration (mg g^{-1})				
Control	0.60±0.03^{a}	1.71±0.42^{c}	1.19±0.21^{a,b}	1.87±0.19^{c}
BNR	0.52±0.05^{a}	1.49±0.17^{c}	0.90±0.18^{a,b}	1.90±0.08^{c}
R. solani	0.54±0.02^{a}	1.48±0.06^{b,c}	1.12±0.27^{b}	1.76±0.15^{c}
B. Root P concentration (mg g^{-1})				
Control	0.73±0.29^{a}	1.86±0.26^{b}	1.15±0.29^{a,b}	1.64±0.27^{b}
BNR	0.44±0.25^{a}	1.77±0.28^{b}	0.82±0.20^{a}	1.78±0.15^{b}
R. solani	0.72±0.06^{a}	2.19±0.19^{b}	1.07±0.14^{a}	2.02±0.04^{b}

Values are the means ± SE, n=3. Values for A) Shoot P concentration and B) Root P concentration that are not significantly different (P<0.05) have the same superscript letters.

Table 7.6. Effects of *G. coronatum* and two P treatments (P0 and P1) on disease severity in mung bean inoculated with BNR isolate CFM1 or *R. solani* AG2-2IIIB.

	Disease rating (0-4)[a]		Disease Index (%)[b]	
Treatment	**P0**	**P1**	**P0**	**P1**
Control	0	0	0	0
G. coronatum	0	0	0	0
BNR	1.7	1.3	41.7	33.3
BNR (with *G. coronatum*)	1.3	1.3	33.3 (20)	33.3 (0)
R. solani	2.3	2	58.3	50
R. solani (with *G. coronatum*)	2	1.7	50 (14)	41.7 (17)

Values are the means of n=3, values in parenthesis indicate the percentage reduction of disease index when *G. coronatum* was present.

[a]Disease rating was based on scale of 0 to 4 with 0 = no lesions and 4 = lesions over 50% of the root area and necrosis.

[b]Disease Index was calculated from Σ (number of plants of a given disease rating x disease rating) x 100/ 4 (total number of plants rated) x 100.

Root P concentration of mycorrhizal plants was significantly higher than NM plants in P0. In P1, plants infected by *G. coronatum* + BNR isolate CFM1 or *G. coronatum* + *R. solani* had higher root P concentration than NM plants infected by BNR isolate CFM1 or *R. solani*. Adding P to the soil (P1) did not have an effect on root P concentrations of NM plants or mycorrhizal plants (Table 7.5B).

7.4.1.4. Disease severity

In P0, plants inoculated with *R. solani* showed higher mean disease ratings than those inoculated with BNR isolate CFM1 and uninoculated controls (Table 7.6). Prior infection of roots by *G. coronatum* reduced the mean disease rating due to BNR isolate CFM1 in P0 and that due to *R. solani* in P0 and P1. The disease index due to BNR isolate CFM1 was reduced by 20% by prior inoculation with *G. coronatum* in P0, but no reduction was observed in P1. The disease index due to *R. solani* was reduced by 14% and 17% by prior inoculation with *G. coronatum* in P0 and P1, respectively.

7.4.2. DISCUSSION

The second experiment suggested that when *G. coronatum* was first established in the roots before inoculation of other fungi, mung bean plants were less affected by BNR isolate CFM1 or *R. solani*. We did not find a reduction in dry weights or total root length in either BNR isolate CFM1 or *R. solani* treatments in P1, irrespective of mycorrhizal treatment. This suggests that the plants had become resistant or more tolerant as they grew older. Mycorrhizal infection may be involved in increasing resistance of the plants at low P (Dehne, 1982). The effect of addition of P in the experiment was to stimulate plant growth and it did not influence development of disease.

Experiment 2 showed that a well-established mycorrhizal infection did not prevent BNR isolate CFM1 from infecting the roots. However, application of P reduced the infection by BNR isolate CFM1, without affecting mycorrhizal infection. In contrast, *R. solani* did not infect the roots very well. Failure of *R. solani* to infect some host plants has been reported (Keijer *et al.* 1997). A previous study by Zambolin and Schenck (1983) showed that infection by *G. mosseae* in soybean was reduced by the presence of *R. solani*. Infection by *G. coronatum* was not affected by the presence of *R. solani*, even when P was applied to the soil. The differences in results may be due to the use of different AM fungi and/or the pathogen isolates and host plants as well as differences in experimental designs or methods for recording the disease. As observed in Experiment 1, inoculation with *G. coronatum* did not reduce the infection of roots by *R. solani*. Low infection was still observed in plants inoculated with *R. solani*, and only 1.4-5.4% of the root length was occupied together by *G. coronatum* and *R. solani* in P0 and 1.8-3.4% in P1 (results not shown).

As was observed in Experiment 1, P concentrations in mung bean were strongly influenced by mycorrhizal infection and, again, BNR isolate CFM1 or *R. solani* had little effect.

The results obtained for the mean disease rating and the disease index showed that prior infection by *G. coronatum* resulted in reduced disease development due to BNR isolate CFM1 or *R. solani*. There was a slightly lower mean disease rating and index for BNR isolate CFM1 and *R. solani* in the presence of *G. coronatum* in this experiment and, in this case, increased P nutrition provided by *G. coronatum* might make the plant more resistant or reduce the effects of the disease.

7.5. CONCLUSIONS

The simultaneous inoculation of mung bean by *G. coronatum* and BNR isolate CFM1 or *R. solani* reduced the effects of BNR isolate CFM1 or *R. solani* in terms of enhancement of plant growth, reduced infection by BNR isolate CFM1 or *R. solani* and lowered disease severity in P0, but not in P1. Apparently, BNR isolate CFM1 did not reduce the growth of the plants, whereas *R. solani* reduced growth of NM mung bean plants during the 6 weeks after inoculation. In Experiment 2, when *G. coronatum* was established in roots and the plants were older, there was no significant effect of infection by BNR isolate CFM1 or *R. solani* on growth of mung bean. Early infection by *G. coronatum* may be responsible for increased resistance towards BNR isolate CFM1 or *R. solani*. Effects of improved P nutrition with or without mycorrhizal infection appeared only in the form of stimulated plant growth and had little effect in reducing the disease rating. In conclusion, P nutrition did not directly influence the resistance of mung bean to BNR isolate CFM1 or *R. solani* infection, and other disease suppression mechanisms may be involved. Therefore, further investigation on the mechanism of the interactions other than P, such as the induction of defence-related compounds produced during the interactions, is necessary (see Chapter 8).

CHAPTER 8. CYTOLOGICAL RESPONSES OF NON-MYCORRHIZAL AND MYCORRHIZAL ROOTS OF MUNG BEAN INFECTED BY BNR OR *R. SOLANI*

8.1. INTRODUCTION

Root infection by arbuscular mycorrhizal (AM) fungi induces important physiological and biochemical changes in the host plant (Azcón-Aguilar and Barea, 1996). Among these changes, the induction of enzymes such as β-1,3-glucanases, chitinases and peroxidases, and phenolic compounds in AM fungi is receiving increased attention due to their being implicated in the regulation of the symbiosis as well as in the protection of the plant against pathogen attacks (see Chapter 2 section 2.2.5).

Few studies have compared defence responses of pathogens and mycorrhizal symbionts present together in the root systems (e.g. Cordier *et al.*, 1996, 1998; Mohr *et al.*, 1998; Guenoune *et al.*, 2001). During infection by mycorrhizal and pathogenic fungi, the defence-related compounds may be produced. For example, studies by Cordier *et al.* (1996, 1998) showed that infection of *Phytophthora parasitica* in mycorrhizal tissues of tomato roots produced bright yellow autofluorescence under blue light, indicating an accumulation of phenolic compounds, which was not observed in mycorrhizal tissues not infected by *P. parasitica*. Similarly, Guenoune *et al.* (2001) found a strong autofluorescence reaction in the dual inoculation treatment of *Glomus intraradices* and *Rhizoctonia solani* in alfalfa. Defence-related compounds can be detected qualitatively by histochemical staining. However, the use of histochemical staining to detect defence-related compounds in roots infected by AM fungi and pathogens has been limited. Other than phenolic compounds, there have been no histochemical studies of defence-related compounds such as pectic substances, lignin, suberin and callose, or enzymatic activity in roots infected by AM fungi and pathogens.

Interactions between the AM fungus, *G. coronatum*, and BNR isolate CFM1 or *R. solani* AG2-2IIIB showed that infection by BNR isolate CFM1 or *R. solani* AG2-2IIIB was reduced in mycorrhizal roots systems of mung bean (Chapter 7). Since P nutrition did not directly influence the resistance of mung bean to BNR isolate CFM1 or *R. solani* AG2-2IIIB, it was considered that induction of defence-related compounds in mycorrhizal plants or production of antimicrobial compounds by the plant might be involved in the protective effects.

The present investigations were aimed at elucidating the cytochemical reactions underlying protection against BNR isolate CFM1 or *R. solani* AG2-2IIIB in *G. coronatum*-infected root systems of mung bean. Because the probability of BNR isolate CFM1 or *R. solani* AG2-2IIIB infecting the same sites in the root tissues as *G. coronatum* was very low (less than 5%) (Chapter 7), in the following study, BNR isolate CFM1 or *R. solani* AG2-2IIIB was applied directly to mycorrhizal roots to provide direct contact between the fungi and ensure root invasion at the same sites. The accumulation of phenolic compounds, pectic substances, suberin, lignin and callose in mycorrhizal or non-mycorrhizal roots of mung bean, with and without inoculation with BNR isolate CFM1 or *R. solani* AG2-2IIIB, was investigated by using histochemical staining techniques in combination with bright-field and fluorescence microscopy.

8.2. MATERIALS AND METHODS

8.2.1. Fungal isolates and inoculation

Spores of *G. coronatum* (WUM16) were recovered from pot cultures of clover through wet sieving techniques and counted to provide standardised inoculum as described in Chapter 3 section 3.2.1.2. Two hundred and fifty spores per pot of *G. coronatum* were inoculated at the time of planting. The experiments used BNR isolate CFM1 and the isolate of *R. solani* AG2-2IIIB

(referred to only as *R. solani* in this chapter) previously investigated in Chapters 6 and 7. To initiate the growth of BNR isolate CFM1 or *R. solani*, a disk of inoculum 3-mm in diameter was placed onto PDA and incubated at 25°C. Inoculum preparation of BNR isolate CFM1 or *R. solani* was based on the modified method of Poromarto *et al.* (1998). Tissue paper (three 10 x 90 mm sheets per plate) was autoclaved and placed in 10 x 90 mm glass petri plates, enriched with 8 ml per plate of 10% PDA. Agar strips (5 x 45 mm, 1 mm thick) with mycelium of BNR or *R. solani* from 3 to 4 day old cultures on PDA were placed on the nutrient enriched tissue paper and incubated in the dark for 3-4 days at 23°C. To inoculate the roots, paper strips (5 x 15 mm) with mycelium were wrapped tightly around roots (see Figure 8.1). This inoculation technique provided immediate contact between roots and mycelium of BNR isolate CFM1 or *R. solani* and allowed infected segments of root to be selected for observation.

8.2.2. Plant material and growth conditions

Seeds of mung bean (*Vigna radiata* L. Wilczeck cv. Emerald) were surface sterilised and germinated as described in Chapter 3 section 3.3. A single plant was grown in each pot containing 400 g autoclaved soil and sand mix (1:9) in a glasshouse. Details of soil and sand used in this experiment were given in Chapter 3 section 3.3. There were a total of 18 pots; 9 pots were not inoculated (non-mycorrhizal plants) and 9 pots were inoculated with 250 spores of *G. coronatum* (mycorrhizal plants). After 3 weeks growth, the pots were carefully cut open vertically to expose the roots. Non-mycorrhizal roots were not inoculated (control; 3 pots), inoculated with BNR isolate CFM1 (3 pots) or inoculated with *R. solani* (3 pots). Similar treatments were applied for mycorrhizal roots. Different parts of the roots in each pot were wrapped tightly with 20 nutrient enriched paper strips (5 x 15 mm) enclosing mycelium of BNR isolate CFM1 or *R. solani* (Figure 8.1).

Figure 8.1. Inoculation method on roots of mung bean using nutrient enriched paper strips enclosing mycelium of BNR or *R. solani* provides direct contact between the root and the mycelium.

Controls received paper strips and agar only, without inoculum. After inoculation the pots were returned to the original conditions.

8.2.3. Preparation of root material for staining

A total of five pieces (each 15 mm-long) of non-inoculated or inoculated roots wrapped with paper strips in each pot was harvested at 1, 2, 4 and 7 days after inoculation. The paper strips were removed carefully from the roots and the root lesions were recorded. The fresh root segments were then embedded into a gelatin block (10% gelatine, 2% glycerol, a drop of Tween 20) using a hot needle. The block was frozen on a Microm K-400 freezing stage and, using a Leitz 1320 freezing stage microtome, transverse sections were cut at 78 µm thickness. A preliminary experiment showed that this thickness gave good root sections for observation of mycorrhizal infection and cells. Root sections were then submerged in staining solution, mounted on slides and observed using a compound microscope equipped for fluorescence microscopy.

8.2.4. Histochemical staining

Sections of the roots were subjected to different staining techniques to detect various compounds as shown below.

8.2.4.1. Intensity of staining

Intensity was measured by the brightness of the colour or fluorescence after staining. The intensity increased when the colours were darker or brighter.

8.2.4.2. Phenolic compounds (no staining)

Root sections without staining were observed by fluorescence microscopy under blue light (excitation filter: 450-500 nm; barrier filter: 515 to 560 nm) or UV excitation (excitation filter: 340-380 nm; barrier filter: 420-460 nm) to reveal phenolic compounds (Fernandez and Heath, 1986).

8.2.4.3. Ruthenium red for pectic substances

Root sections were placed in aqueous ruthenium red (1:5000) until the walls were red and then mounted in water or glycerol. Pectic substances appeared pink to red (Jensen, 1962).

8.2.4.4. Sudan black B for suberin

Sections were stained for 5-30 min. in a saturated solution of sudan black B in 80% ethanol, rinsed briefly in 80% ethanol, and mounted in glycerol. Black, blue or brownish-black stains indicate the presence of suberin (Pearse, 1960).

8.2.4.5. Phloroglucinol-HCl for lignin

Sections were put on slides and a large drop of 0.1 g phloroglucinol (1,3,5-trihydroxybenzene) in 10 ml of 95% ethanol was applied. Then the slides were covered with coverslip and part of the solution allowed to evaporate. After evaporation, a little 25% HCl was allowed to diffuse under the edge of the coverslip. Sections were examined 15 min later by which time a purple-red colour indicated the presence of lignin (Clark, 1981).

8.2.4.6. Aniline blue for callose or β-1,3 glucans

Sections were treated with 0.05% aniline blue in 0.068 M K_2HPO_4 adjusted to pH 9.0 by the addition of KOH. Solutions of aniline blue were stored for several days at 0-4°C to induce greater levels of fluorescence. The

presence of callose or β-1,3 glucans was indicated by bright yellow colour by fluorescence microscopy under blue light (excitation filter: 450-500 nm; barrier filter: 515 to 560 nm) (Eschrich and Currier, 1964).

8.3. RESULTS

8.3.1. Fungal infection and cell necrosis

Infection by the AM fungus, *G. coronatum*, reached 13-20% when plants were 3 weeks old (results not shown). BNR isolate CFM1 (Figure 8.2) or *R. solani* hyphae started to infect the roots one day after inoculation of both non-mycorrhizal and mycorrhizal roots of mung bean. Non-mycorrhizal and mycorrhizal roots not inoculated with BNR isolate CFM1 or *R. solani* did not show any lesions. Following inoculation with BNR isolate CFM1 or *R. solani*, brown lesions and cell necrosis were observed in some roots. The brown lesions and necrosis in the inoculated roots increased with time following inoculation with *R. solani*, but did not change following BNR isolate CFM1 inoculation. By the end of the experiment, all root samples inoculated with *R. solani* showed dark brown lesions covering the infected roots. Mycorrhizal roots inoculated with BNR isolate CFM1 also showed lesions and cell necrosis (Table 8.1).

8.3.2. Cell reaction to histochemical tests

8.3.2.1. Intensity of staining

Generally, the intensity of staining of non-mycorrhizal root sections subjected to all types of histochemical staining did not change with time after inoculation. Those inoculated with BNR isolate CFM1 alone increased the intensity of staining from day 1 to 7, revealing deposition of phenolics, pectic substances and suberin, but did not change with respect to lignin and callose.

Table 8.1. Schematic colour chart of changes in visual necrosis and intensity of staining of non-mycorrhizal (NM) or mycorrhizal (M) roots of mung bean uninoculated or inoculated with BNR isolate CFM1 or *R. solani* AG2-2IIIB at 1, 2, 4 and 7 days after inoculation.

Treatment	Visual necrosis				Phenolic compounds				Pectic substances				Suberin				Lignin				Callose			
	1	2	4	7	1	2	4	7	1	2	4	7	1	2	4	7	1	2	4	7	1	2	4	7
NM control	1	1	1	1	9	9	9	9	14	14	14	14	18	18	18	18	23	23	23	23	24	24	24	24
NM + BNR	4	4	5	5	10	12	13	13	16	16	16	17	19	20	21	22	23	23	23	23	25	25	25	25
NM + *R. solani*	6	6	7	8	10	10	13	13	14	15	16	17	18	18	18	18	23	23	23	23	25	25	25	25
M control	1	1	1	1	9	9	9	9	17	17	17	17	19	19	19	19	23	23	23	23	25	25	25	25
M + BNR	2	3	3	4	9	9	11	11	17	17	17	17	20	21	21	22	23	23	23	23	26	26	26	26
M + *R. solani*	4	4	4	5	9	9	11	11	17	17	17	17	19	19	19	19	23	23	23	23	26	26	26	26

Similar numbers indicate similarity in the intensity of staining.

Those inoculated with *R. solani* alone increased the intensity of staining from day 1 to 7, revealing deposition of phenolics and pectic substances, but did not change with respect to suberin, lignin and callose (Table 8.1).

Mycorrhizal roots subjected to histochemical staining showed no changes on the intensity of staining over time. When mycorrhizal roots were inoculated with BNR isolate CFM1, the intensity of black or blue stain indicative of suberin increased from day 1 to 7, but this was not the case for other compounds. There were no changes of staining intensity from day 1 to 7 for any compounds observed on mycorrhizal roots inoculated with *R. solani*, except slight changes in the accumulation of phenolic compounds (Table 8.1).

8.3.2.2. Phenolic compounds

Light microscope observations of reactions of root cells to infection by BNR isolate CFM1 showed that the presence of BNR isolate CFM1 resulted in cell death and necrosis, not only of invaded cells, but also of adjacent, uninfected cortical cells in non-mycorrhizal roots (double arrows in Figure 8.2). This was clearly associated with strong autofluorescence under blue light of the root cell walls and contents, indicating an accumulation of phenolic components as shown in Figure 8.3. When observed under UV excitation, bright white-blue colours were observed in the infected area (Figure 8.4). The cortical cells of uninoculated non-mycorrhizal roots did not autofluoresce (Figures 8.5 and 8.6, Table 8.2). Non-mycorrhizal roots inoculated with *R. solani* showed strong autofluorescence in the epidermal cells and adjacent cells but not in the cell walls of the cortical cells (Figures 8.7 and 8.8).

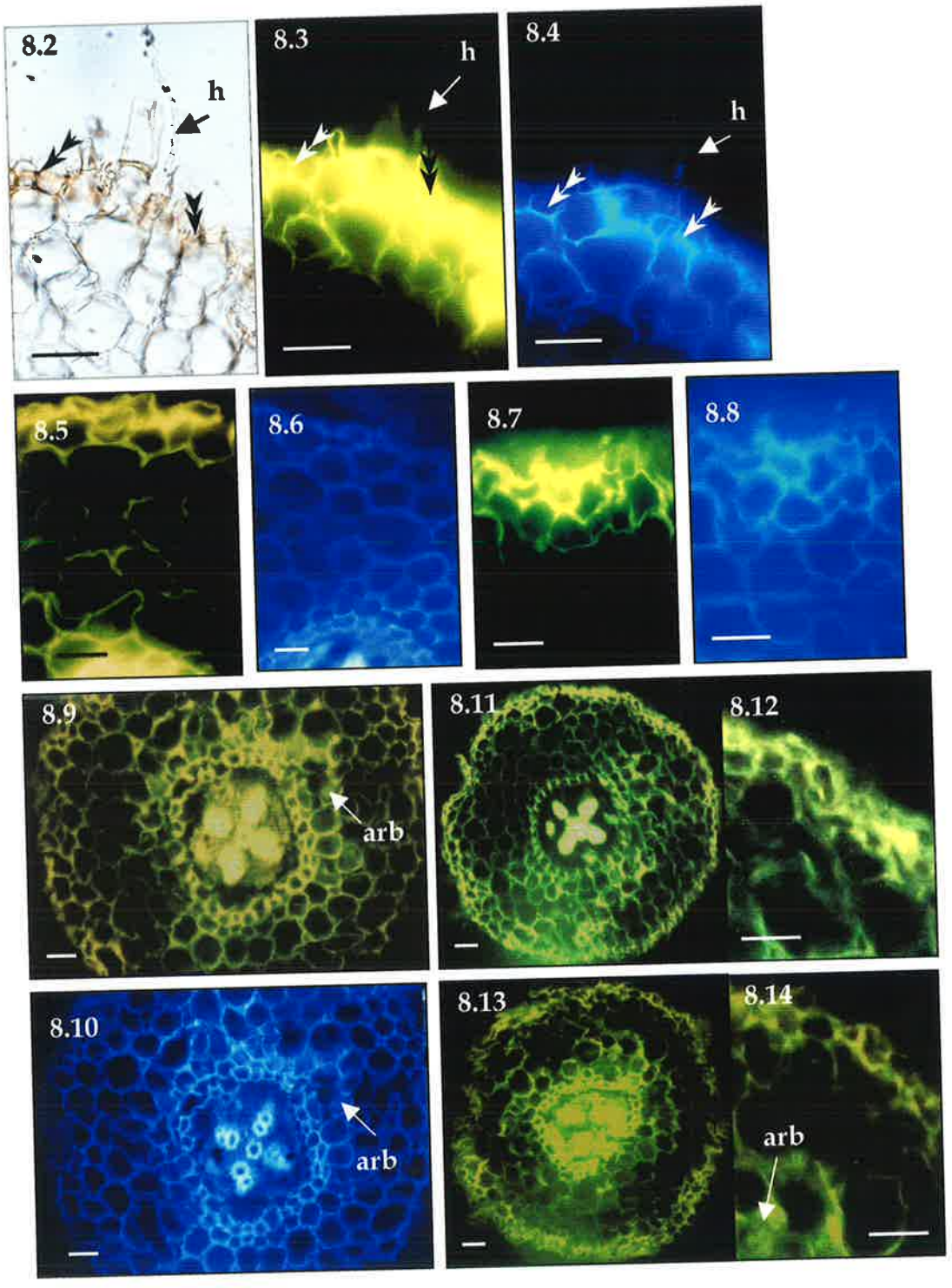
All mycorrhizal root sections, uninoculated and inoculated with BNR isolate CFM1 or *R. solani*, observed under blue light produced yellow autofluorescence in all tissues except the cell lumina of the cortical cells that did not contain arbuscules (Table 8.2).

Table 8.2. Presence (+) or absence (–) of cell response to BNR isolate CFM1 (B) or *R. solani* AG2-2IIIB (R) in non-mycorrhizal and mycorrhizal roots of mung bean.

Stain	No staining			Ruthenium Red			Sudan Black			Phloroglucinol-HCl			Aniline Blue		
Compounds	Phenolic compounds			Pectic substances			Suberin			Lignin			Callose		
	-B/-R	+B	+R	-B/-R	+B	+R	-B/-R	+B	+R	-B/-R	+B	+R	-B/-R	+B	+R
Non-mycorrhizal roots															
Epidermis	+	+	+	–	+	+	+	+	+	–	–	–	–	+	+
Cortex															
Cell walls	+	+	+	+	+	+	–	+	+	–	–	–	–	–	–
Cell lumen	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Endodermis	+	+	+	–	+	+	+	+	+	+	+	+	+	+	+
Vascular tissues	+	+	+	–	+	+	+	+	+	+	+	+	+	+	+
Mycorrhizal roots															
Epidermis	+	+	+	+	+	+	+	+	+	–	–	–	+	+	+
Cortex															
Uninfected															
Cell walls	+	+	+	+	+	+	+	+	+	–	–	–	+	+	+
Cell lumen	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Arbuscule-containing tissues															
Cell walls	+	+	+	+	+	+	+	+	+	–	–	–	+	+	+
Cell lumen	+	+	+	+	+	+	+	+	+	–	–	–	+	+	+
Endodermis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Vascular tissues	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Symbols in the same colour indicate differences in staining reactions between treatments or cell types.

Figures 8.2-8.14. Bright-field and fluorescence microscopy of 78 μm thick transverse sections of non-mycorrhizal and mycorrhizal mung bean roots. **Figure 8.2.** Root infection by BNR isolate CFM1 on non-mycorrhizal root of mung bean caused necrosis (double arrows) at 1 day after inoculation (dai). Autofluorescence under blue light of BNR-infected non--mycorrhizal root shows bright yellow colour of infected cells **(Figure. 8.3)**, and by UV excitation, the infected area has a bright white-blue colour **(Figure 8.4).** Uninfected non-mycorrhizal roots autofluorescce weakly under blue light **(Figure 8.5)** or UV excitation **(Figure 8.6).** Strong autofluorescence of non-mycorrhizal roots infected by *R. solani* AG2-2IIIB at 1 dai under blue light **(Figure 8.7)** or UV excitation **(Figure 8.8).** Weak fluorescence under blue light **(Figure 8.9)** and UV excitation **(Figure 8.10)** of the epidermal cells and arbuscule-containing cells of mycorrhizal roots. **Figure 8.11.** Mycorrhizal roots infected by BNR isolate CFM1 autofluoresce only weakly under blue light, and the magnification **(Figure 8.12)** shows less autofluorescence in the epidermal cells and adjacent cells than BNR-infected non-mycorrhizal root **(Figure 8.3).** Mycorrhizal root infected by *R. solani* AG 2-2IIIB under blue light shows weak fluorescence in the whole area of roots **(Figure 8.13)** and the magnification shows *R. solani*-infected cells fluoresced weakly **(Figure 8.14).** h = hyphae, arb = arbuscules. *Bar* = 50 μm.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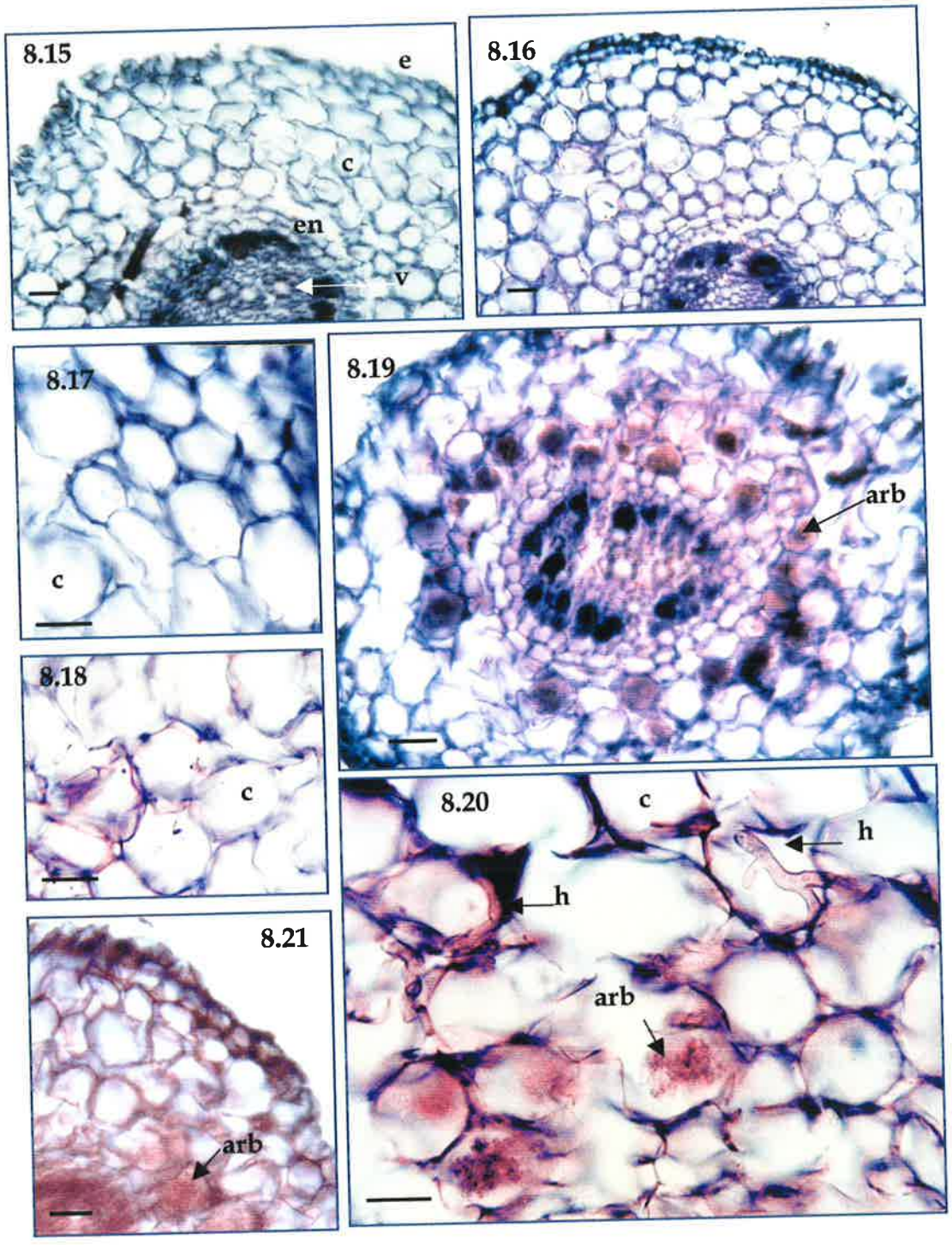
However, autofluorescence remained weak in mycorrhizal tissues not actually invaded by BNR isolate CFM1 or *R. solani* (Figures 8.9 and 8.10). The epidermal cells of root sections of BNR isolate CFM1-or *R. solani*-infected mycorrhizal root tissues fluoresced homogeneously yellow under blue light (Figures 8.11-8.14), and this was never observed in the mycorrhizal roots not infected by BNR isolate CFM1 or *R. solani*.

8.3.2.3. Pectic substances

Uninoculated non-mycorrhizal roots stained with ruthenium red to detect pectic substances showed a positive reaction in part of the cortex only (Figure 8.15). Those inoculated with BNR isolate CFM1 gave positive reactions in all tissues except the cell lumina of the cortical cells, with most intense staining in the epidermal cells, cortical and vascular tissues (Table 8.2; Figure 8.16). The cortical cells of non-mycorrhizal roots inoculated with *R. solani* gave more intense staining at day 7 than at day 1 (Figures 8.17 and 8.18).

Mycorrhizal roots uninoculated or inoculated with BNR isolate CFM1 or *R. solani* all gave positive red staining with ruthenium red (including arbuscules) with the exception of the cell lumina of cortical cells not containing arbuscules (Table 8.2, Figures 8.19 and 8.21). The intensity of staining of mycorrhizal root sections treated with ruthenium red depended upon the extent of AM fungal infection. Highly infected mycorrhizal roots not inoculated with BNR isolate CFM1 or *R. solani* showed strong intense staining particularly in the cell walls of the cortical cells, the arbuscules and the AM hyphae.

Figures 8.15-8.21. Semi-thick (78 μm) transverse sections of non-mycorhizal (**Figures 8.15-8.18**) and mycorrhizal (**Figures 8.19-8.21**) mung bean roots stained with ruthenium red to reveal pectic substances. **Figure 8.15.** Non-inoculated root shows weak staining of ruthenium red in some part of the inner cortex. **Figure 8.16.** Non-mycorrhizal root at 1 dai with BNR isolate CFM1. Intense staining is observed in the inner part of the cortical area compared to the outer part. **Figure 8.17.** Non-mycorrhizal roots inoculated with *R. solani* AG2-2IIIB at 1 dai shows weak staining in the cortical area, and a more intense staining at 4 dai (**Figure 8.18.**). **Figure 8.19.** Greater infection of roots by *G. coronatum* produces more intense staining with ruthenium red mainly in the arbuscule-containing tissues and vascular tissues. **Figure 8.20.** Higher magnification of the cortical tissue of mycorrhizal roots shows intense staining in the arbuscules, hyphae and cell wall of cortex. **Figure 8.21.** Mycorrhizal root at 4 dai with BNR isolate CFM1 shows intense staining in the whole area of roots except the content of cortical cells. ep = epidermis, c = cortex, en = endodermis, v = vascular tissue, h = hyphae, arb = arbuscules. *Bar* = 50 μm.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8.3.2.4. Suberin

Suberin was present in the epidermal cells, endodermis and vascular tissues of non-mycorrhizal roots not inoculated with BNR isolate CFM1 or *R. solani*, but was absent from the cell walls and the lumina of the cortical cells (Table 8.2; Figure 8.22). When the roots were inoculated with BNR isolate CFM1, intense blue staining in the epidermal cells, wall of cortical cells, and cell wall and cell lumina of the endodermis was produced (Figure 8.23). Non-mycorrhizal roots inoculated with *R. solani* did not stain as intensly as those with BNR isolate CFM1, and gave positive reactions in the epidermal cells and the endodermis only (Figure 8.24).

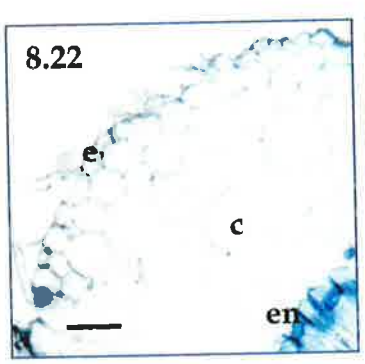
Mycorrhizal root sections stained with sudan black B showed a moderate dark blue staining in all parts of the root tissues except the lumen of uninfected cortical cells (Table 8.2). Epidermal cells of mycorrhizal roots not inoculated with BNR isolate CFM1 or *R. solani* showed dark blue staining. The cell walls of uninfected cells of the cortical tissues of mycorrhizal roots stained dark blue, but the cell lumen did not. Arbuscule-containing tissues produced strong dark blue staining, as did the cell walls of the endodermis, some cell lumina of the endodermis and the vascular tissues (Figure 8.25). Mycorrhizal roots inoculated with BNR isolate CFM1 produced intense staining, especially in the epidermal cells and the cell walls of the cortex (Figure 8.26), whereas those inoculated with *R. solani* did not stain intensely (Figure 8.27).

8.3.2.5. Lignin

All sections of non-mycorrhizal roots uninoculated and inoculated with BNR isolate CFM1 or *R. solani*, resulted in a near absence of staining with phloroglucinol-HCl in all parts of roots except in the cell walls of the endodermis and xylem, indicating the presence of lignin in the vascular tissues as expected.

Figures 8.22-8.27. Accumulation of suberin in non-mycorrhizal root **(Figures 8.22-8.24)** and mycorrhizal root sections **(Figures 8.25-8.27)** of mung bean determined by sudan black B staining. Accumulation of suberin in the epidermal cells of uninoculated non-mycorrhizal root **(Figure 8.22).** Non-mycorrhizal root inoculated with BNR isolate CFM1 shows intense dark-blue staining in the epidermal cells, cell wall of cortex and endodermis **(Figure 8.23). Figure 8.24.** Non-mycorrhizal roots inoculated with *R. solani* AG2-2IIIB shows positive staining in the epidermal cells. **Figure 8.25.** Mycorrhizal root section shows dark blue staining in the epidermal cells, arbuscule-containing cells and endodermis. Mycorrhizal roots inoculated with BNR isolate CFM1 produce intense dark-blue colour in the epidermal cells **(Figure 8.26),** but less so when inoculated with *R. solani* AG2-2IIIB **(Figure 8.27).** ep = epidermis, c = cortex, en = endodermis, h = hyphae, arb = arbuscules. *Bar* = 50 μm.

Figures 8.28-8.30. Root sections stained with phloroglucinol-HCl to detect deposition of lignin produces a near absence of staining except the endodermis and the vascular tissues. **Figure 8.28.** Uninfected non-mycorrhizal root. **Figure 8.29.** Non-mycorrhizal root infected by BNR isolate CFM1. **Figure 8.30.** Mycorrhizal root infected by BNR isolate CFM1. *Bar* = 50 μm.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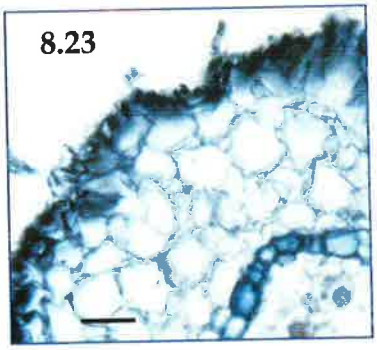
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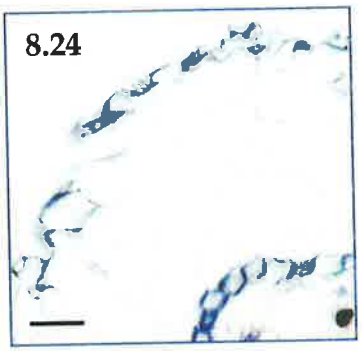
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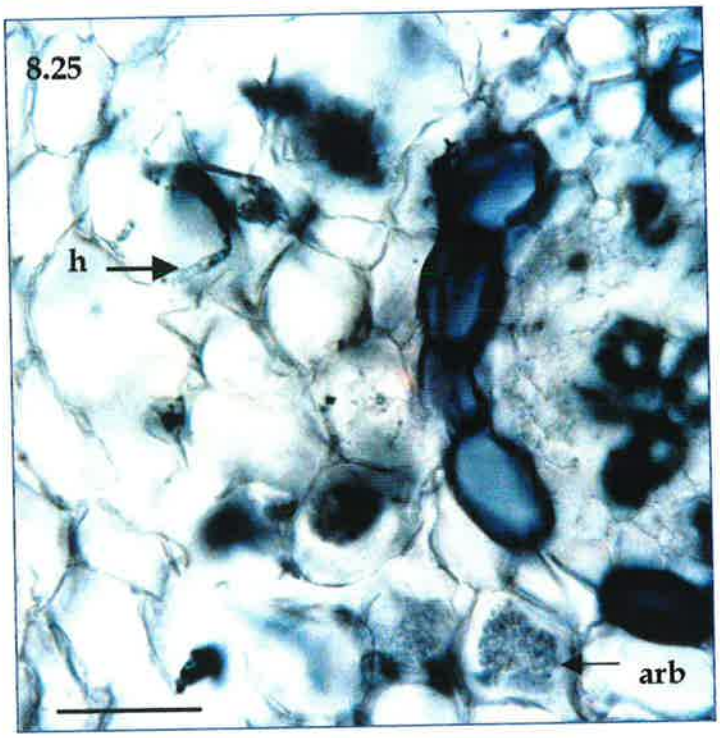
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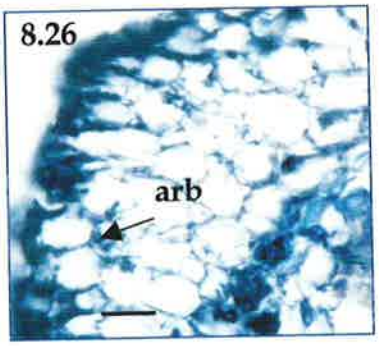
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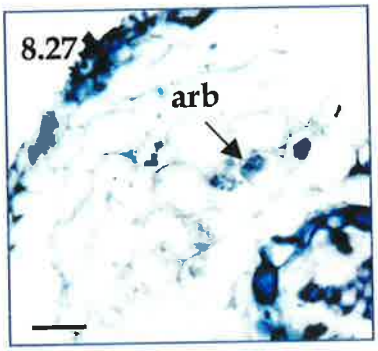
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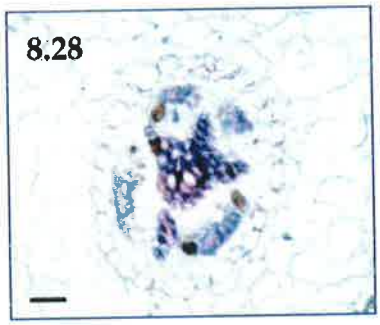
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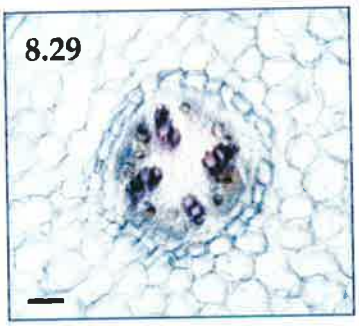
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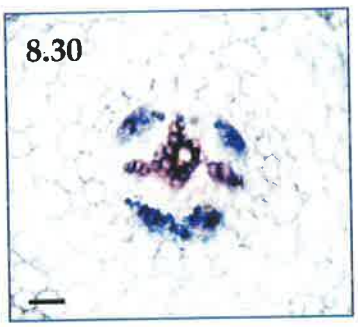

8.30

Similar results were obtained in mycorrhizal roots uninoculated and inoculated with BNR isolate CFM1 or *R. solani* (Table 8.2; Figures 8.28-8.30). Thus there appeared to be no effects of fungal invasion on lignin deposition.

8.3.2.6. Callose

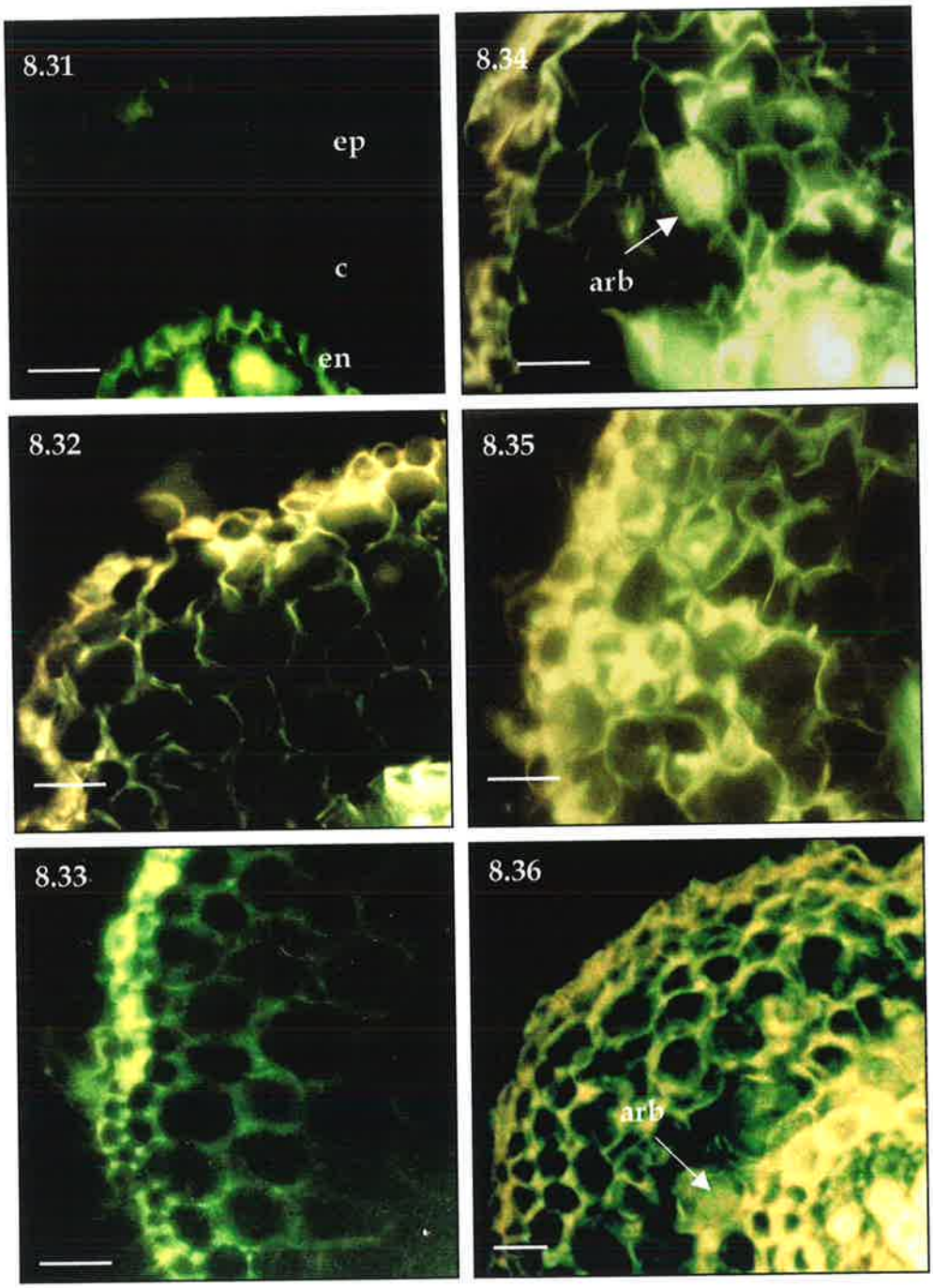
The accumulation of callose was observed with fluorescence microscopy under blue light, and aniline blue staining caused the callose to fluoresce yellow (Clark, 1981). Deposition of callose was not observed in the epidermal and the cortical cells of uninoculated non-mycorrhizal roots (Figure 8.31). When BNR isolate CFM1 or *R. solani* were inoculated onto non-mycorrhizal roots, strong yellow autofluorescence indicating the accumulation of callose was observed in the epidermal cells and the walls of the cortical cells (Table 8.2; Figures 8.32 and 8.33).

Callose, as indicated by yellow autofluorescence under blue light was present in all parts of the mycorrhizal roots except the lumina of the cortical cells (Table 8.2; Figures 8.34-8.36). When the mycorrhizal root was inoculated with BNR isolate CFM1 (Figure 8.34), the epidermal cells autofluoresced a strong yellow indicating that callose was present, but this was not observed in mycorrhizal roots not inoculated with BNR isolate CFM1 (Figure 8.35). Mycorrhizal roots inoculated with *R. solani* stained with aniline blue and observed under blue light also showed strong fluorescence in the epidermal cells, the cell walls of cortex and arbuscule-containing tissues (Figure 8.36).

8.4. DISCUSSION

The accumulation of plant-derived compounds in response to AM infection has been proposed as a mechanism to explain the contribution of AM fungi to control diseases caused by pathogens (Linderman, 1994; Azcón-Aguilar and Barea, 1996).

Figures 8.31-8.36. Non-mycorrhizal and mycorrhizal root sections of mung bean stained with aniline blue fluoresce yellow under blue light, indicating deposition of callose. **Figure 8.31.** Uninfected non-mycorrhizal root does not fluoresce yellow. **Figure 8.32.** Strong yellow autofluorescence in the epidermal cells and adjacent area of non-mycorrhizal roots infected by BNR isolate CFM1. **Figure 8.33.** Non-mycorrhizal root at 2 dai with *R. solani* AG2-2IIIB produces yellow fluorescence in the epidermal cells and the cell wall of cortex. **Figure 8.34.** Yellow autofluorescence is observed in the arbuscules of mycorrhizal root section. Mycorrhizal root infected by BNR isolate CFM1 produces intense yellow colour in the epidermal cells and outer cortex (**Figure 8.35**). More intense yellow autofluorescence in the whole area of mycorrhizal root at 7 dai with *R. solani* AG2-2IIIB (**Figure 8.36**). ep = epidermis, c = cortex, en = endodermis, arb = arbuscules. *Bar* = 50 μm.


8.31
ep
c
en
8.34
arb
8.32
8.35
8.33
8.36
arb

This mechanism might be involved in the interactions between the AM fungus, *G. coronatum* and BNR isolate CFM1 or *R. solani* in mung bean (see Chapter 7). Therefore, the present work studied the defence-related compounds produced during the interactions, using histochemical staining techniques. The results provide preliminary qualitative data on the cytochemical responses of mycorrhizal and non-mycorrhizal roots of mung bean infected by BNR isolate CFM1 or *R. solani*. Overall results showed a difference in host reaction between non-mycorrhizal and mycorrhizal tissues inoculated with BNR isolate CFM1 or *R. solani*.

The present study showed that inoculation of mung bean roots with BNR isolate CFM1 or *R. solani* resulted in the appearance of necrotic areas in the root system, as has been observed in previous studies (see Chapters 6 and 7). Infection by *G. coronatum* decreased the negative effect of BNR isolate CFM1 or *R. solani* infection, in relation to root necrosis, as was reported in Chapter 7.

Increased deposition of phenolic compounds in host cell walls usually implies an increase in resistance to enzymes produced by invading fungi as well as a physical barrier against fungal penetration (Codignola *et al.*, 1989; Grandmaison *et al.*, 1993). Previous studies showed that infected host cells showed little reaction or fluoresced weakly when infected by AM fungi, indicating that phenolic compounds are only weakly accumulated (Cordier *et al.*, 1996; Gianinazzi-Pearson *et al.*, 1996; Morandi, 1996; Guoneune *et al.*, 2001). The present study on mycorrhizal roots of mung bean showed similar results. Mycorrhizal roots inoculated with BNR isolate CFM1 or *R. solani* showed a weaker fluorescence in the area infected by BNR isolate CFM1 or *R. solani* compared to non-mycorrhizal roots. This indicated that phenolic compounds might be responsible for the resistance of mycorrhizal roots to infection by BNR isolate CFM1 or *R. solani*. This result is in agreement with Guenoune *et al.* (2001), who found that the infection by the AM fungus *G.*

intraradices suppressed defence responses induced by *R. solani* in alfalfa roots, but interactions with the BNR were not reported.

Pectic substances are important chemical constituents of the cell walls (Jensen, 1962). They have been detected in the walls of epidermal cells of bean hypocotyls infected by non-pathogenic BNR and in the walls of epidermal cells and the cortex of hypocotyls infected by *R. solani* (Jabaji-Hare *et al.*, 1999). The present study showed that pectic substances were detected in non-mycorrhizal mung bean roots infected by BNR isolate CFM1 or *R. solani*, in the walls of the epidermal cells, the cortical cells and vascular tissues. Pectic substances have also been shown on the epidermal surface of cucumber inoculated with a non-pathogenic BNR isolate (AG-A) (Villajuan-Abgona *et al.*, 1996a). Production of pectic substances in mycorrhizal roots has not been reported. However, this study showed that pectic substances were detected in the cell walls of the cortex as well as in the hyphae and the arbuscules.

Previous work has shown that BNR induces accumulation of an electron dense barrier rich in suberin in bean hypocotyls (Jabaji-Hare *et al.*, 1999; Xue *et al.*, 1998). This structural defence reaction was not seen in *R. solani*-infected bean hypocotyl (Jabaji-Hare *et al.*, 1999). The present study showed similar results, in which non-mycorrhizal roots of mung bean infected by BNR isolate CFM1 showed an intense accumulation of suberin, which was not seen in roots infected by *R. solani*. Suberin is accumulated in plants as a result of defence reaction to microorganisms (Rioux and Baayen, 1997), and this study demonstrated that suberin was also accumulated in cells containing hyphae and arbuscules of *G. coronatum*.

In relation to other histochemical staining, phloroglucinol-HCl to reveal the depositions of lignin was the only stain that did not give positive reactions in the infected areas. This indicated that there were no effects of fungal inoculation on the deposition of lignin. These results agree with previous reports (Jabaji-Hare *et al.*, 1999), that lignin was not detected in BNR-

or *R. solani*-infected hypocotyl of bean (other than vascular tissues) also using phloroglucinol-HCl staining.

The present study revealed callose in roots infected by BNR isolate CFM1 or *R. solani*. These findings were in contrast with those of Jabaji-Hare *et al.* (1999). They found that histochemical tests on the hypocotyls of bean infected by BNR or *R. solani* did not give a positive reaction for callose. Callose formation is a natural wound response mechanism in plants (Galway and McCully, 1987). It has been reported that aniline blue-induced fluorescence indicates the presence of 1,3-β-glucans and it has been used extensively as a specific marker for the 1,3-β-glucan in plant tissue (Eschrich and Currier, 1964; Morrow and Lucas, 1986). Previous studies have shown that AM fungi contain β-1,3-glucans (Gollote *et al.*, 1997, Gianinazzi-Pearson *et al.*, 1994), which are known to be important as elicitors of plant defence reactions (Azcón-Aguilar and Barea, 1996). The present study also showed that arbuscule-containing cells and cell walls of the cortical cells of *G. coronatum* accumulated callose or β,1-3-glucans. There has been no previous work on the deposition of callose or β,1-3-glucans in mycorrhizal roots infected by BNR or *R. solani*. However, presence of callose has been previously shown in mycorrhizal-transformed carrot roots challenged with *F. oxysporum* (Benhamou *et al.*, 1994).

8.5. CONCLUSIONS

This study shows observations on the host's reaction to BNR isolate CFM1 or *R. solani* AG2-2IIIB inoculation in mycorrhizal and non-mycorrhizal roots. Altogether, these observations suggest that the nature and activities of the fungi play an important role in determining the frequency and the extent of the host's response. This chapter presents qualitative data on histochemical reactions to fungal invasion of mung bean. The results should be regarded as preliminary, giving indications of which compounds could usefully be

targeted in future, quantitative investigations. The data reported here provide, for the first time, evidence that inoculation with BNR isolate CFM1 or *R. solani* in non-mycorrhizal and mycorrhizal roots induced physiological and biochemical changes at sites of attempted penetration by BNR isolate CFM1 or *R. solani.* In the absence of BNR isolate CFM1 or *R. solani* inoculation, non-mycorrhizal roots did not exhibit substantial reactions. Contact with BNR isolate CFM1 or *R. solani* provided an essential signal to the plant to express a defence response. Although host reactions to infection by the AM fungus *G. coronatum* were generally very weak or transient, the presence of *G. coronatum* not only reduced the spread of disease, but also specifically limited damage to host tissue caused by BNR isolate CFM1 or *R. solani*. The present cytological investigations provided evidence that the protective effects induced by *G. coronatum* involve the accumulation of plant defence-related compounds in host roots.

CHAPTER 9. GENERAL DISCUSSION

9.1. INTRODUCTION

The general aim of this project was to elucidate the biology and pathogenicity of a fungal contaminant (binucleate *Rhizoctonia* sp. [BNR]) in pot cultures of AM fungi and to devise specific detection methods for it. The interactions between *Glomus coronatum,* and BNR or the pathogenic fungus *Rhizoctonia solani* were investigated to understand mechanisms underlying the role of AM fungi in improving resistance of plants towards invasion by other root-infecting fungi.

9.2. SUMMARY OF FINDINGS

1. BNR are often present in roots used to culture AM fungi. The lack of information on the identity and characteristics of the BNR causes difficulty in developing an effective control for this fungus in pot cultures. This study identified and characterised for the first time an isolate of BNR (CFM1) belonging to AG-Bo, which occurs as a contaminant in mycorrhizal pot cultures (Chapter 4).

2. Routine testing for observation of fungal contaminants in roots and soil of pot cultures involves conventional techniques that are laborious and time consuming. This study developed a rapid and sensitive molecular technique for detection of BNR belonging to AG-Bo or AG-A in roots and soil of pot cultures. The detection of BNR in pot cultures was achieved using the BNR-specific primers designed from the ITS rDNA sequence of BNR isolate CFM1 (Chapter 5). The method could also be used for other applications.

3. There was a lack of information on the ability of BNR isolate CFM1 to induce disease and affect plant growth. This study revealed that isolate CFM1 achieved rapid infection of roots, was capable of causing disease symptoms on mung bean, was less pathogenic than the isolates of *R. solani* tested, had no effects on shoot growth, but reduced the root growth of mung bean in long-term inoculation experiments in soil (Chapter 6).

4. Interaction studies between AM fungi and root-infecting fungal pathogens have shown that the effect of AM fungi on pathogen and disease development could sometimes be attributed to enhanced P nutrition. There is no information available on the effects of P on the interactions between AM fungi and BNR or *R. solani*. This study found that improved P had no effect on the role of *G. coronatum*, in reducing disease severity and infection of BNR isolate CFM1 and *R. solani* (Chapter 7).

5. Increased activity of enzymes or defence-related compounds may be of importance in the resistance of mycorrhizal roots to pathogenic fungi. The use of histochemical staining to detect defence-related compounds and enzymatic activities in roots infected by AM fungi and pathogens has been limited. Using histochemical staining, this study revealed that the protective effects induced by *G. coronatum* against BNR isolate CFM1 and *R. solani* involved the accumulation of plant defence-related compounds in host roots (Chapter 8).

9.3. CHARACTERISATION AND PATHOGENICITY OF BNR

The characterisation and identification of BNR isolate CFM1 (AG-Bo) provides a firm basis to recognise BNR in pot cultures (Chapter 4). Although the similarity in morphology between BNR and *R. solani* in pure culture

causes difficulty in identification, DAPI staining was useful to confirm the number of nuclei inside the hyphal cells and confirmed the isolate to be binucleate *Rhizoctonia.* Hyphal anastomosis was necessary for the identification of isolate CFM as a member of AG-Bo, as PCR-RFLP could not differentiate between AG-Bo and AG-A due to similarities in restriction fragment banding patterns. The analysis of the ITS rDNA sequence of BNR isolate CFM1 confirmed its identity as AG-Bo, supporting the morphological characterisation, and provided a supplementary tool in identification. Only a small number of ITS sequences of BNR isolated from soil and plant tissues have been listed in GenBank (Kuninaga *et al.*, 1997; Gonzales *et al.*, unpublished; MacNish and O'Brien, unpublished). This study provided, for the first time, a complete sequence of the ITS rDNA of BNR AG-Bo isolate CFM1 obtained from a mycorrhizal pot culture. Further work is necessary to obtain more isolates and ITS sequences and other more variable regions of BNR involving other AGs from pot cultures, to understand the biodiversity and genetic relationships of this fungus. See Addendum 1, p197.

Pathogenicity tests conducted on BNR isolate CFM1 in comparison with pathogenic isolates of *R. solani* gave information on the ability of these fungi to induce disease and affect plant growth (Chapter 6). Two types of pathogenicity tests were applied, *in vitro* and in pot experiments. Tests conducted using mung bean as a host plant showed the ability of BNR isolate CFM1 to rapidly infect the roots and cause disease symptoms. This, of course, created problems particularly when a mycorrhiza-responsive host, like mung bean, was chosen as a host plant in pot cultures. Therefore, further pathogenicity tests on different host plants normally used in pot cultures are required to undertand the host range of BNR isolate CFM1. This information would be important as it could lead to establishing pot cultures avoiding using host plants that are extensively infected by BNR. On the other hand, as BNR isolate CFM1 extensively infects mung bean as a host, it might have potential as a biological control agent (Herr, 1988) in pot cultures, as well as

more generally. BNR isolate CFM1 infects less rapidly and extensively than *R. solani* (Chapter 6). Extensive infection by BNR isolate CFM1 did not reduce infection by *Glomus coronatum* (Chapter 7), and might, therefore, not be expected to reduce AM infectivity; however, further tests are required. Extensive infection by BNR isolate CFM1 in roots might inhibit infection of *R. solani* or other pathogenic fungi. There is no information on the potential of BNR AG-Bo as biological agents for the control of diseases caused by other pathogenic fungi, but previous studies showed that isolates of BNR belonging to AG-A, AG-F, AG-K, AG-R were highly effective in controlling disease caused by *R. solani* on a variety of beans (Bell *et al.*, 1984; Cardoso and Echandi, 1987; Escande and Echandi, 1991; Khan *et al.*, 1992). More detailed investigations on the potential of BNR AG-Bo as a biological control agent are required. See Addendum 2, p 197.

9.4. DETECTION OF BNR IN ROOTS AND SOIL OF POT CULTURES OF MYCORRHIZAL FUNGI

This study reported for the first time, the design of BNR-specific primers from the ITS region of the rDNA sequence of BNR isolate CFM1, which can be used for rapid and sensitive detection of BNR AG-Bo or AG-A in mycorrhizal pot cultures (Chapter 5). The aim was to achieve specific specific amplification of AG-Bo. The failure of the BNR-specific primers to differentiate AG-Bo from AG-A could be explained by the fact that the primers were designed from a sequence of the ITS region which is similar in both groups. The close similarity of the ITS regions of AG-Bo and AG-A makes the development of specific primers of closely related groups unlikely. Schilling *et al.* (1996) found similar problems when they tried to design primers specific for the very closely related *Fusarium culmorum* and *F. graminearum*. Though the importance of PCR-based fungal detection tools is widely recognised, this study shows that caution is required in their design,

use and interpretation of results. This is particularly important where the fungi under study are closely related.

The intention was to detect BNR in DNA extracted directly from pot cultures by amplification with the BNR-specific primers. However, problems in obtaining amplification from low quantities of mycelial DNA of BNR in dried roots and soil rendered this approach unsuitable. Therefore, the use of nested PCR was introduced for detection of BNR in pot cultures and allowed detection of small numbers of hyphae in soil samples and roots in pot cultures (Chapter 5). The use of nested PCR, rather than single step PCR as a diagnostic tool, has also been used to improve detection of fungal DNA in infected plants (Schesser *et al.*, 1991), and of fungal propagules in soil (Hamelin *et al.*, 1996; Errampalli *et al.*, 2001). Positive signals from pot cultures of different sources indicated that BNR AG-Bo or AG-A might be present in various parts of the world. It is likely that BNR found in the pot cultures tested is similar to other BNR in pot cultures of other laboratories currently using pot cultures obtained from the same sources. The BNR-specific primers could be used to test pot cultures from different laboratories to understand the distribution of this fungus in pot cultures. The BNR-specific primers present a considerable practical advantage for monitoring the presence of BNR in roots and soils of pot cultures and for evaluating attempts to eliminate fungal contaminants.

Detection of BNR in soil by conventional methods could not be achieved in this study due to the presence of fast growing fungi, such as *Fusarium, Aspergillus, Rhizopus* and *Trichoderma*. Errampalli *et al.* (2001) found similar problems when they tried to detect *Helminthosporium solani* in soil. The presence of the fast growing fungi indicated that not only BNR, but other fungal contaminants were present in pot cultures, as has been reported in other studies (e.g. Ross and Ruttencutter, 1977; Schenck and Nicolson, 1977; Menge, 1984; Daniels and Menge, 1980; Brundrett, 1991). Techniques

developed in this study for BNR, such as sequencing and designing specific primers, could be applied to other fungal contaminants in pot cultures. Primers with a high level of specificity might be designed, and they would be useful for the detection of specific groups of fungi.

The quantification of BNR using dot-blot hybridisation permitted the relative proportion of fungal infection in roots to be estimated (Chapter 5). This technique is potentially more accurate and reliable than traditional quantification methods because it is not necessary to culture the fungus or stain the roots. The DNA hybridisation assay could be used to determine the relationships between the inoculum level of BNR and disease severity, and to predict the expected disease severity caused by BNR, so that control practices could be applied if warranted. Using a similar approach *Gaeumannomyces graminis* has been quantified in infected roots of wheat plants (Herdina *et al.*, 1996). Quantification of fungal propagules in a range of soil samples is also possible with PCR assay (Heinz and Platt, 2000; Bürgmann *et al.*, 2001). Quantification of BNR in this study is considered preliminary and needs more work. A challenge in the future would be to apply this technique to relate numbers of propagules of BNR to levels of DNA recovered from soil, which might help in understanding the spread of BNR in soil. The availability of a rapid and reliable method to quantify this fungus in soil of pot cultures will allow studies on the population dynamics of this fungus and competition with other soil-borne pathogens.

Information obtained from this study could be used to develop methods to monitor pot cultures for unwanted contaminants. For example, roots from pot cultures could be plated on agar to test the possible pathogens and they could be stained and examined to make sure unwanted contaminants are not present in or on the roots of initial inoculum (Chapter 4). Detection of contaminants in roots and soil of pot cultures could be done quickly by molecular detection techniques (Chapter 5). Pot cultures could be produced

on selected hosts, in conditions which minimize microbial contamination to prevent build-up of pathogens (see Chapter 6). Pathogens and other microorganisms, frequently present in soil (Chapter 5), might be difficult to eliminate completely from pot cultures. If populations of these organisms are high, the best way is to discard contaminated cultures and save money in maintenance and storage. Frequent and careful examination of the inoculum will be the most effective way to control contamination of pot cultures.

9.5. INTERACTIONS BETWEEN THE AM FUNGUS *G. CORONATUM* AND BNR OR *R. SOLANI*

Improved P nutrition has been suggested as a possible mechanism of AM fungi to reduce disease caused by pathogens (Hooker *et al.*, 1994; Linderman, 1994). This study found that increased P supply improved growth of mung bean with and without *G. coronatum*, but had little effect on reducing the disease severity caused by BNR or *R. solani*. Instead, a good establishment of *G. coronatum* in roots was important in conferring protection against BNR or *R. solani*, particularly in soil without added P (P0). The presence of BNR was unlikely to confound experiments on mycorrhizal effects on plant growth and P uptake (Chapter 7). Infection of roots by *G. coronatum* did not prevent BNR or *R. solani* from infecting the roots, but reduced the growth of BNR or *R. solani* in the roots. This reduction could be caused by direct interaction between the two fungi or competition for space and nutrition (Chapter 7) and the accumulation of defence-related compounds (Chapter 8).

Information on the accumulation of defence-related compounds in mycorrhizal roots in the presence or absence of BNR or *R. solani* (Chapter 8) provided information on which compounds could be investigated quantitatively in the future. Studies have been done on the quantitative measurement of the defence-related compounds in the interactions between

AM fungi and pathogenic fungi (Grandmaison *et al.*, 1993; Mohr *et al.*, 1998), including *R. solani* (Wyss *et al.*, 1991; Vierheilig *et al.*, 1993; Guenoune *et al.*, 2001), but they have never been investigated with BNR.

There is already sufficient evidence from interaction studies to suggest that a range of different mechanisms exists, such as improvement of plant nutrition (Hooker *et al.*, 1994; Linderman, 1994), competition for infection sites (Dehne, 1982; Linderman, 1994; Cordier *et al.*, 1996), reduction of physical stresses (Linderman, 1994; Azcón-Aguilar and Barea, 1996), anatomical and morphological changes in the root systems (Atkinson *et al.*, 1994; Linderman, 1994), microbial population changes in the mycorrhizosphere (Citernesi *et al.*, 1996; Linderman, 1994), and stimulation of plant defence mechanisms (Linderman, 1994; Azcón-Aguilar and Barea, 1996). The mechanisms by which *G. coronatum* protects mung bean against infection and disease by BNR or *R. solani* have been identified in this study (Chapters 7 and 8). Other mechanisms are likely to be involved, but further research is needed to verify this suggestion. Different AM fungi might not be equally effective in providing protection against pathogens and different results might be obtained when different AM fungi are used. Therefore, future studies involving different AM fungi are necessary to identify variation in the ability of AM fungi to protect the plants against BNR, or *R. solani*, or other pathogens.

9.6. FUTURE RESEARCH

1. There is a need to obtain more isolates of BNR and isolates of pathogenic fungi from mycorrhizal pot cultures, sequence the mycelial DNA and develop specific primers for detection of specific groups of fungi for rapid and effective control of fungal contaminants in pot cultures.

2. It would be valuable if the BNR-specific primers designed in this study could be applied for routine detection of the presence of BNR in roots and soil of pot cultures, not only in Australia, but also in other laboratories in various part of the world. This would give information on the distribution of this fungus in pot cultures.

3. Further work is needed to test plant species currently used as hosts in pot cultures to provide information on the host range of BNR AG-Bo and would allow choice of hosts with low susceptibility. The potential of BNR isolate CFM1 as a biological control agent to protect plants from infection by other pathogenic fungi needs further investigation.

4. Quantification assays of fungal propagules of BNR in soil samples of pot cultures need to be examined to study the population dynamics of BNR and competitiveness with other soil-borne pathogens.

5. Further work is needed to quantify the defence-related compounds produced during the interactions between the AM fungi and BNR or *R. solani*.

6. Interaction studies could be extended by using different AM fungi and investigating mechanisms that are likely to be involved in the protection, by AM fungi, of host plants from other root-infecting fungi.

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Addendum 1 from p. 165

Approaches such as RAPD (random amplified polymorphic DNA), AFLP (amplified fragment length polymorphisms) or 'minisatellite' DNA probes have been used to examine variations in base sequence (DNA polymorphisms) in fungi, and might be used and applied to separate AG-A and AG-Bo. A more direct approach to separate AG-A and AG-Bo is to develop specific primers based on the differences in the sequences of the ribosomal genes and intervening spacer regions.

Addendum 2 from p. 166

The mechanisms of action have been identified with respect to biocontrol of other isolates of BNR which include competition for infection sites, direct interaction, induction of defence mechanisms in the host and involvement of systemic resistance or mycoparasitism (Burpee and Goulty, 1984; Cardoso and Echandi, 1987; Jabaji-Hare *et al.*, 1999; Siwek, 1996; Siwek *et al.*, 1995; Sneh *et al.*, 1989a, 1989b). Competition for infection sites, direct interaction and induction of defence mechanisms in the host were identified in the interactions between the AM fungus and BNR or *R. solani* (Chapter 7 and 8), but other mechanisms still need further investigations.