EFFECTS OF SELECTED FUNGICIDES ON VESICULAR-ARBUSCULAR MYCORRHIZAL SYMBIOSIS

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SUMMARY

Nampiah Sukarno. 1994. **Effects of selected fungicides on vesicular-arbuscular mycorrhizal symbiosis.** Ph D thesis, Department of Soil Science, The University of Adelaide, Australia.

The application of fungicides to increase crop yields may lead to detrimental effects on plants and their vesicular-arbuscular (VA) mycorrhizal fungi. The effects of the systemic fungicides Aliette, Benlate and Ridomil on the symbiosis between onion (*Allium cepa* L.) and the VA mycorrhizal fungus *Glomus* sp. "City Beach" (WUM 16) were studied in terms of plant and fungal growth and phosphorus (P) uptake. Growth and mycorrhizal infection were assessed by conventional methods, supplemented by vital staining and image analysis. The efficiency of symbiosis was assessed using P uptake per unit length of root (inflow), P uptake per unit length of living external hyphae in the soil, and transfer of P from the fungus to the host plant per unit area of interface.

In this thesis the following aspects are reported:

1. Development of a satisfactory method for measurement of living external hyphae in the soil. The vital stain fluorescein diacetate (FDA) produced a good contrast against the dark background and this was selected for future work.

2. Suitable non-mycorrhizal control plants were established in order to avoid confounding effects of P deficiency and fungicide application in non-mycorrhizal plants. Although this proved difficult to achieve, the most closely "matched" non-mycorrhizal plants were produced by addition of 15 μg P g^{-1} soil.

3. The effect of systemic fungicides on non-mycorrhizal plants was assessed. Benlate had no effect on onion plants whereas Aliette and Ridomil markedly

reduced growth, particularly of roots. Benlate had no effect on shoot P concentration. Ridomil had no effect on shoot P concentration at early harvests, but later shoot P concentration increased. In plants treated with Aliette the shoot P concentration and content were significantly increased despite the reduction in plant growth. This led to the Experiments reported in section 5 below.

4. Two experiments were conducted under growth room conditions to determine the effects of the three fungicides on onion plants associated with *Glomus* sp. " City Beach" (WUM 16). All three fungicides had negative effects on growth of the mycorrhizal fungus both in soil and in the root.

Benlate appeared to act directly on the fungus, reducing percentage infection markedly, together with intensity of infection (number of arbuscules, intercellular hyphae and vesicles), area of interface and development of external hyphae in the soil. P inflow and P transfer across the living interface were reduced. The rate of P uptake per cm living external hyphae was not affected but, as development of external hyphae was reduced, the contribution of the fungus to P uptake was limited.

Aliette and Ridomil were found to act both on the plant and the fungus. Aliette reduced mycorrhizal colonization and development of external hyphae in the soil. The intensity and characteristics of infection per unit length of root were not affected but, as this fungicide reduced root growth, the area of interface per plant was lower. The reduction in length of external hyphae and in area of interface on a per plant basis and negative effect of Aliette on plant growth led to stunted growth of mycorrhizal plants following Aliette application. Mycorrhizal plants recovered after 13 weeks but non-mycorrhizal plants remained stunted. Aliette increased P concentration and content as observed in non-mycorrhizal plants. This led to the experiments reported in section 5 .

Ridomil reduced mycorrhizal colonization, intensity and characteristics of infection, area of interface between plant and fungus and length of external hyphae in the soil. P inflow per cm root and P uptake per cm living external hyphae were reduced but not significantly. There was no effect on the rate of P transfer across the interface, but shoot P concentration and content were reduced.

5. The effects of Aliette on growth of both non-mycorrhizal and mycorrhizal plants was investigated in greater detail. The effects of aluminium (applied as aluminium chloride) and phosphonate (applied as dimethyl phosphonate), degradation products of Aliette, were compared with Aliette itself. Aluminium chloride alone had no effects on the growth of either non-mycorrhizal or mycorrhizal plants at the pH of the soil used. Dimethyl phosphonate and Aliette markedly reduced plant growth, especially root growth. The effects of Aliette were more severe than those of dimethyl phosphonate.

NMR spectroscopy was used to determine the relative concentration of phosphonate and phosphate. Both non-mycorrhizal and mycorrhizal plants took up phosphonate as well as phosphate following Aliette or dimethyl phosphonate application. The relative concentration of the two compounds in the plant tissue correlated with the reduction of plant growth. The results suggest that phosphonate is converted to phosphate by plant and fungus. The recovery from initial stunted growth of mycorrhizal plants appears to be due to the mycorrhizal fungus being more efficient in converting phosphonate to phosphate than non-mycorrhizal plants. Other mechanisms are also discussed.

DECLARATION

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. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference has been made in the text.

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

August, 1994 *Signed:*

Nampiah Sukarno

PUBLICATIONS FROM THE THESIS

The following papers have arisen from work done in this thesis.

Journal Articles:

1. Sukarno N, Smith SE, Scott ES. 1993. The effetcs of fungicides on vesicular-arbuscular mycorrhizal symbiosis. I. The effects on vesicular-arbuscular mycorrhizal fungi and plant growth. *New Phytologist* **125**: 139-147. **(Results of Chapter 3).**

2. Sukarno N, Smith SE, Scott ES. 1994. The effects of fungicides on transport of phosphorus from fungus to plant in vesicular-arbuscular mycorrhizal symbiosis. In: Pankhurst, ed. *Soil biota: management in sustainable farming systems (poster papers).* Adelaide: CSIRO Australia, 111-113. **(Results of parts of Chapters 3 and 4).**

3. Sukarno N, Smith SE, Scott ES. 1994. The effects of fungicides on vesicular-arbuscular mycorrhizal symbiosis. II. The effects on P uptake from the soil and transfer across plant-fungal (in preparation). **(Results of Chapter 4).**

Conference Articles:

1. Sukarno N, Dickson S. 1992. Vital staining of vesicular-arbuscular mycorrhizal fungi. *The International Symposium on Management of Mycorrhizas in Agriculture, Horticulture and Forestry.* Perth, Australia. (An invited poster).

2. Sukarno N, Smith SE, Scott ES. 1993. Effects of fungicides on symbiotic development and transfer of P from fungus to plant in VA mycorrhizal onion. *The 9th North American Conference on Mycorrhiza.* Guelph, Canada.

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To my late father

CHAPTER 1. INTRODUCTION AND REVIEW OF LITERATURE

1.1. Introduction

Nutrient availability in the soil and application of fungicides are two factors that can be optimised to increase crop production in the field. Plant growth can also be increased in the presence of a mutualistic symbiosis such as vesicular-arbuscular (VA) mycorrhiza, which is found in most of the major plant families. This symbiosis markedly improves plant growth in a wide range of plant species, primarily through enhancement of phosphate (P) uptake (Cooper & Tinker, 1978, 1981; Harley & Smith, 1983; Tinker, 1975b). For some crop species the benefits of the mycorrhizal condition are only apparent under conditions of low nutrient availability, but for others (e.g. white clover) growth can be improved even when plants receive P fertilizer at normal application rates (Hayman, 1987).

While most experiments on mycorrhizal effects on plant growth have been undertaken in a controlled environment, it has also been reported that VA mycorrhizal fungi play important roles in crops grown in the field. For example, cassava seems to be obligately dependent on mycorrhizal infection under field conditions (Hayman, 1987; Howeler et al., 1987; Sieverding, 1991). This species has a high demand for P but is frequently grown successfully in tropical soils which are P-deficient and unable to support growth of other crops (Howeler et al., 1979, 1982; Howeler & Sieverding, 1983). The explanation

appears to be that it becomes heavily infected by the native VA mycorrhizal fungi.

Positive contributions of VA mycorrhizas to other field-grown crops such as cotton (Rich & Bird, 1974), legume species, cereals (Jakobsen & Nielsen, 1983; Jensen, 1984) and potato (Black & Tinker, 1977) have been reported. In addition, a number of vegetable crops such as onions, leeks, celery, asparagus, carrots and cucurbits are mycorrhiza-dependent in the field (Plenchette et al., 1983; Stribley & Snellgrove, 1985; Stribley et al., 1980).

Regardless of nutrient status in the soil, high productivity of crops depends on the success of methods used to suppress pathogens. Application of fungicides is commonly used to control pathogenic fungi. However, the combined dependence on VA mycorrhizal fungi and application of fungicides to increase crop production may lead to unforeseen side-effects. Non-specific action of both non-systemic and systemic fungicides may inhibit the establishment of mycorrhizas (see Tables 1.1 and 1.2). Reports of the effects of some of the fungicides are quite consistent. Captan and Benlate, for example, appear to reduce percentage of the root length colonised by mycorrhizal fungi, regardless of the plant or fungal species used. However, Ridomil has been reported both to decrease and (surprisingly) to increase percentage colonisation, while Aliette application appears to be associated with either no effect or increases. These discrepancies may have resulted from the way in which the fungicidal effects were analysed. The parameter used to assess mycorrhizal infection was the percentage of root length colonised by the fungus (percent infection) and results were interpreted as being due to direct effects of the fungicide on fungal colonisation. In fact, several processes

contribute to percentage infection. These are formation of new infection units, growth of infection units within the root and, very importantly, root growth (Bruce et al., 1994; Sutton, 1973; Walker & Smith, 1984). Clearly, if fungicide application affects root growth as well as fungal colonization, values for percentage infection will not reflect direct effects on the fungus alone. Other approaches, such as measurement of total infected root length per plant and/or studies on non-mycorrhizal plants are required to analyse the complex interactions. The work described in this thesis was motivated by this requirement.

Systemic fungicides were used in all experiments reported in this thesis, as they are taken up by the plant and were, therefore, expected to have effects on host plants as well as on VA mycorrhizal fungi in the soil and roots. Onion *(Allium cepa* L.) associated with *Glomus* sp. "City Beach" (WUM 16) was used to analyse the interactions. Onion was chosen as it is mycorrhiza-dependent, and has frequently been used as an experimental plant in nutrient uptake studies. Furthermore, its roots are suitable for sectioning and image analysis, which would facilitate sudies of intensity of fungal colonisation within the roots.

The review of the literature which follows covers relevant aspects of the biology of VA mycorrhizal infection and physiological effects on plant growth, together with what is known of the effects of fungicides on this symbiosis. It was largely completed in 1992. Discussion of papers published since then is included in the discussion sections of relevant chapters and in the general discussion.

1.2. Review of literature

1.2.1. What is vesicular-arbuscular mycorrhizal symbiosis?

Vesicular-arbuscular (VA) mycorrhiza is a common type of mycorrhiza, which is a mutualistic symbiosis between plant roots and fungi which belong to the order Glomales (Schenk & Pérez, 1990). This symbiosis occurs in most plant families except Cruciferae, Chenopodiaceae, Cyperaceae, Orchidaceae, Ericaceae and the plants that form ectomycorrhiza such as Pinaceae and Cupuliferae (Gerdemann, 1968; Harley & Smith, 1983). The fungi are obligate symbionts and they cannot be grown on synthetic media without the presence of living plant root tissues. The symbiosis is characterized by the formation of vesicles and/or arbuscules in the host root cortex (Sanders & Sheikh, 1983; Smith, 1980).

A number of different structures can act as propagules for VA mycorrhizal fungi. Spores, pieces of dried infected root and external hyphae in the soil can all give rise to infective hyphae capable of initiating infection. The development of VA mycorrhizal infection inside the root begins with contact between an infective hypha and the root surface which is followed by formation of an appressorium on the root epidermis and penetration of the outer cells of the root (see Fig. 1.1). Spread of mycorrhizal fungus within the root tissue occurs via the formation of intercellular hyphae, arbuscules and vesicles in the root cortex. Arbuscules are formed intracellularly and are believed to be the site of nutrient exchange between the plant and the fungal symbionts. Arbuscules are produced by dichotomous branching of the hyphae and begin to develop 2 - 3

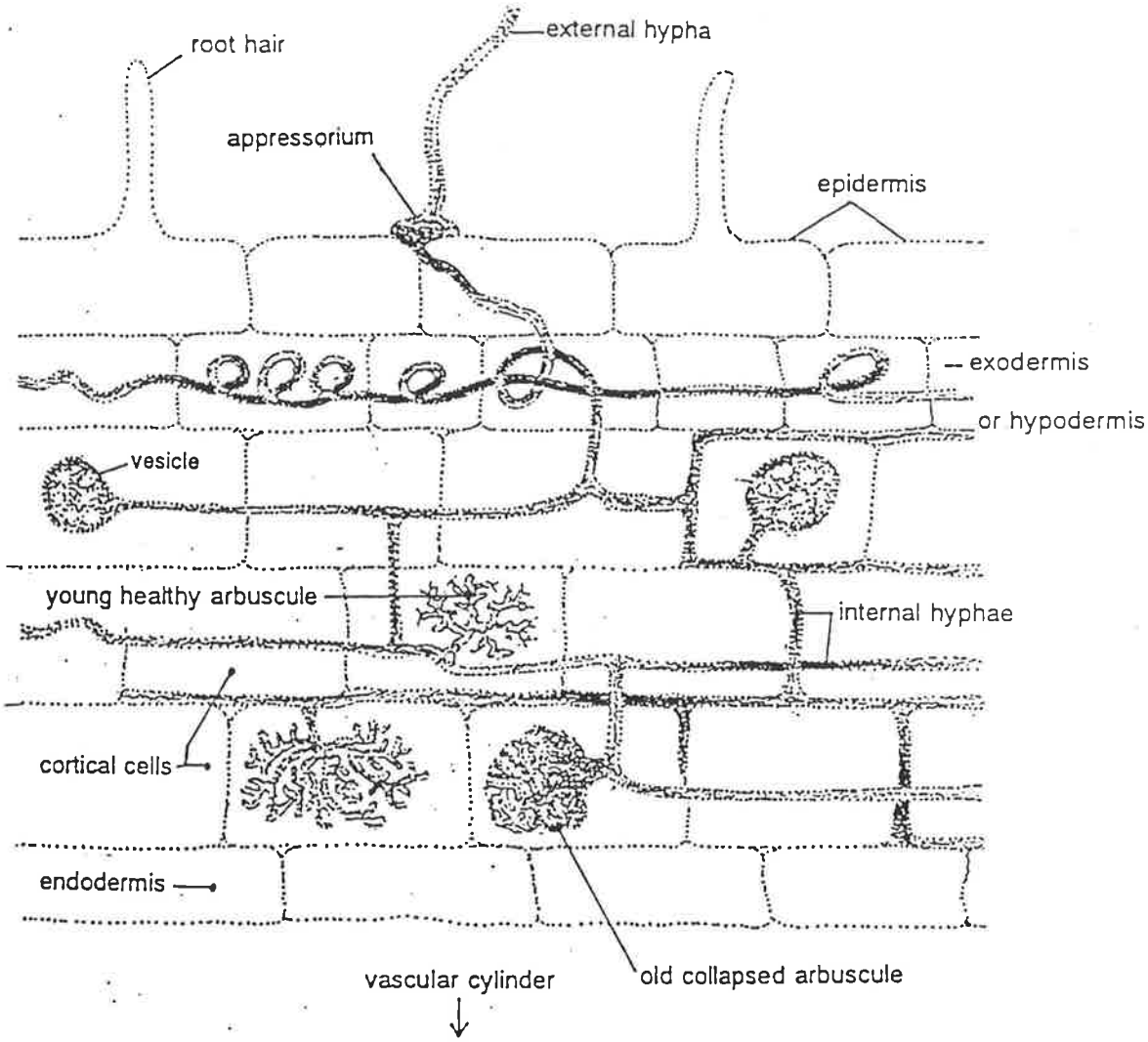


Figure 1.1. The stages of VA mycorrhizal infection (Melville, 1993).

days after root infection. These structures have a limited life, with a span of 2 - 15 days (Alexander et al., 1988, 1989; Cox & Tinker, 1976). Vesicles are formed intercellularly as well as intracellularly and usually occur at the tips of the hyphae. In addition, vesicles contain very large amounts of lipid and their function is as a storage organ (Harley & Smith, 1983).

VA mycorrhizal fungi, in association with roots, also produce an extensive network of fungal hyphae in the soil. This mycelium has a large surface area that acts as an extension of the root-absorbing surface, and is very important for nutrient uptake (P, Zn, Cu, N) by the host plant, especially when nutrient supply is limited. The marked effects on P nutrition arise from the fact that inorganic phosphate in soil is adsorbed easily by clay-soil complexes and diffuses slowly. As plant roots absorb P, a depletion zone develops around them (Nye & Tinker, 1977). The external hyphae absorb P beyond the depletion zone and translocate it into the roots (Tinker, 1975b). It has been demonstrated that external hyphae take up P from 7 - 11 cm away from the root (Jakobsen et al., 1992b; Li et al., 1991). External hyphae are well adapted morphologically for absorbing nutrients. They are aseptate and anastomose frequently to produce a well-interconnected mycelium. The diameter of hyphae ranges from 2 - 20 μm, which is the same or less than the diameter of root hairs (Abbott & Robson, 1985; Sylvia, 1992). The small diameter of the hyphae means that they can penetrate into small soil pores and probably compete effectively with other organisms in the absorption of nutrients from the soil.

VA mycorrhizal infection is different from a pathogenic infection. The VA mycorrhizal fungi invade only the epidermal and cortical cells of the root and

do not penetrate the endodermis or vascular tissue. In addition, no disease symptoms, such as lesions, distortion and discoloration of tissue, are observed on an infected plant (Harley & Smith, 1983).

1.2.2. Development of infection

Infection is initiated by hyphae that grow from the soil-borne propagules or from neighbouring infected roots. Normally, the spread of infection is measured as the fraction or percentage of the root length colonised. A graph of the fraction of the root colonised by the fungus versus time takes a sigmoid form. The curve is recognised as representing three phases of development of infection: a lag phase, a period of rapid fungal spread during which the rate of fungal growth is higher than the rate of root growth, and a plateau phase where the rates of fungal and root growth are the same (Bowen, 1987; Tinker, 1975a).

A VA mycorrhizal root forms a dynamic system, as both components (fungus and root) are growing. The root grows by extension of the main and lateral roots and by initiation of new roots, while the fungus inside the root also grows longitudinally and radially. Models of the spread of infection have been used to analyse the effects of environmental variables on the rate of infection (Buwalda et al., 1982; Sanders & Sheikh, 1983; Smith & Walker, 1981; Walker & Smith, 1984). The model of Smith and Walker (1981) treated infection as three processes that contribute to the percentage of the root length infected by VA mycorrhizal fungi: (1) the growth of hyphae in the soil, resulting in the

formation of entry points on the root epidermis; (2) the growth of hyphae from each entry point longitudinally in the root cortex; and (3) the growth of the plant root. In other words, the fraction of the root that is colonized by the fungus (percentage infection) depends on the number of entry points that are produced from external hyphae, the growth of infection units inside the root and the rate of growth of the host root (Smith & Walker, 1981; Tester et al., 1986; Walker & Smith, 1984). The first process includes several stages: germination of propagules, the growth of the propagules until they make contact with the root, and the formation of entry points. Thus this stage is dependent on propagule density, susceptibility of the host plant (Carling et al., 1979; Smith & Walker, 1981) and soil conditions such as temperature (Smith & Bowen, 1979) and phosphorus supply (Bruce et al., 1994). The second and third stages are influenced by the physiology of the host plant and environmental factors such as nutrient availability and light (Bruce et al., 1994; Jasper et al., 1979; Son & Smith, 1988; Tester et al., 1985, 1986).

The stages of infection may have different sensitivity to environmental and chemical factors and they can, therefore, be used as parameters to analyse damage caused by application of fungicides. In general, the models do not take into account variation in the intensity of infection in colonised regions of the root, so that alternative approaches are required to assess this (Amijee et al., 1986; Bruce et al., 1994; Smith & Dickson, 1991).

Following germination of propagules, infection networks of hyphae are produced. The growth of mycelium sometimes increases if the fungus detects a susceptible root. It appears that there is some recognition phenomenon by VA mycorrhizal hyphae that enables them to approach roots (Friese & Allen,

1991). Recent studies suggest that flavonoids, when applied with CO_2 at optimal concentration, act as molecular signals for hyphal growth (Becard et al., 1992; Chabot et al., 1992; Gianinazzi-Pearson et al., 1989).

Mosse and Hepper (1975) reported that mycelium grows directionally toward the root if the distance between them is a few millimetres. Powell (1976) found that hyphal growth changed direction toward the root at 1.6 mm, while Koske (1982) reported that this effect was apparent at 11 mm. These differences may be due to differences in fungal and plant species used in these investigations.

Infection may occur in all parts of the root (Smith & Walker, 1981; Smith et al., 1992a). An uninfected zone is commonly observed immediately behind the root tip, but is not apparently due to that region being resistant to infection but, rather, due to a delay between the root encountering a fungus and the growth of detectable infection (Smith et al.,1992a).

Environmental conditions influence the formation of entry points. The effect of temperature on the formation of entry points has been reported by Smith and Bowen (1979) on *Medicago truncatula* and *Trifolium subterraneum*. They found that reducing the temperature of the soil from 20°C to 12°C resulted in a 90% decrease in the number of entry points per cm of the root. Another factor is density of inoculum. The rate of production of new infection units was proportional to the propagule density for a short period of time until the primary infection matured (Carling et al., 1979; Smith & Walker, 1981). Subsequently, secondary entry-points develop from external hyphae growing from the primary infections.

After infection is established, the fungus produces extensive networks of external hyphae in the soil. The hyphal networks have been found to consist of several hyphal types, with each type appearing to be linked to a specialised function (Friese & Allen, 1991). The types of external hyphae observed in the soil are primarily classified as runner hyphae, hyphal bridges, absorptive networks, germ tubes and infection networks produced by spores and root fragments. Runner hyphae and infection networks are involved in acquisition of nutrients from the soil. Specialisation of external hyphae in the soil may relate to efficiency of the symbiosis.

While fungal colonisation proceeds, the roots continue to grow and branch, so that the percentage of root length colonised will depend on rates of all processes. Factors that influence the rates of root growth and branching will also affect percentage infection.

It is clear that the processes involved in infection are complex. Environmental factors may act differently on plant growth, root growth, initiation of infection by the fungus and fungal growth within the root cortex. Hence, the study of environmental effects on VA mycorrhizal symbiosis must take into account more than simply the percentage of the root length infected and should also consider the separate processes contributing to it. This approach can also be employed to assess fungicide effects on VA mycorrhizal symbiosis, as these chemicals may influence fungal and/or plant growth.

1.2.3. Measurement of the extent of fungal colonisation

Quantification of the amount of the fungus in the root is needed to determine the degree and intensity of the infection. Several methodological attempts have been made to quantify the fungus: (1) measurement of the total chitin content (Hepper, 1977), which is best adapted for experiments using a sterile medium because in an unsterile medium, chitin-containing non-VA mycorrhizal fungi or insects present in the roots may result in contamination of the assay; (2) measurement of the length of infected root based on the yellow pigment produced in the root (Becker & Gerdemann, 1977). This method is not universally applicable because not all mycorrhizal roots produce this pigment; (3) measurement of the length of infected root after staining by trypan blue (Phillips & Hayman, 1970), which is used in most investigations.

There are several methods for measuring the length of the root infected. Newman (1966) developed the hair-line intersection method in which the roots are arranged on a plastic dish and their lengths are measured by counting the intersections of the root and hair-line. Marsh (1971) modified the hair-line intersection method into the grid intersection method where root length can be estimated rapidly. Later, Giovannetti and Mosse (1980) modified the grid intersection method for use in mycorrhizal investigations, by cutting the root into 1 cm lengths and spreading them out randomly on a plastic dish which has a grid of vertical and horizontal lines; the sample is then observed under the microscope. The calculation of root length in this method is based on Newman`s formula (1966). The combination of trypan blue staining and grid intersection methods provides data for root length and the linear fraction of root

colonised by mycorrhizal fungi, but does not give any information on the quality or intensity of infection. Numbers and intensity of development of intercellular hyphae, arbuscules and vesicles have been measured with varying degrees of precision. Examples include semi-subjective ranking (Trouvelot et al., 1986), a high magnification modification of the line intersection method (McGonigle et al., 1990) and image analysis (Smith & Dickson, 1991). It is clear that qualitative changes in infection, in terms of hyphal or arbuscular development, can only be determined using methods such as these.

1.2.4. Measurement of the external hyphae

The external hyphae are important structures providing the interface between plant and soil which is important for nutrient uptake. There has been relatively little study on the external hyphae due to the difficulty of making quantitative measurements (Abbott et al., 1984; Bowen, 1987; Jakobsen et al., 1992a). However, several methods have now been used to determine the extent of development of external hyphae in soil. Sanders et al. (1977) measured the external hyphae by removing and weighing the hyphae attached to the roots. However, large errors are likely, due to the difficulty of recovering all hyphae and freeing them from soil debris and organic matter. Pacovsky and Bethenfalvay (1982) used chitin determination to quantify VA mycorrhizal external hyphae, but this method has been criticised because of the finding that the chitin content of the fungus depends on age and environmental conditions. In addition, controls must take into account the need to calibrate for

non-VA mycorrhizal fungi and soil microfauna. Consequently, quantification of external hyphae based on chitin content is likely to be impracticable. It seems likely that in physiological studies, determination of length of external hyphae is more appropriate than weight. The length of external hyphae has been measured following extraction from the soil, filtering through millipore filters, staining and measuring by counting intersections of the fungi and eye-piece grid line under high magnification (Abbott et al., 1984; Abbott & Robson, 1985; Jakobsen et al., 1992a,b). The disadvantages of this method are the difficulties in differentiating VA mycorrhizal and non-VA mycorrhizal fungi and distinguishing hyphae from the background colour of the soil material.

Most investigations of the occurrence of VA mycorrhizal fungi in soil have used non-vital stains, such as trypan blue or acid fuchsin. However, nutrient uptake from the soil by VA mycorrhizal fungi is carried out by living fungus. Consequently, measurement of living hyphae using vital staining is necessary when the efficiency of the symbiosis is being assessed, and can also be employed in analysing the effects of environmental and chemical factors such as fungicides acting upon the symbiosis. The requirements of vital stains are discussed in more detail below.

1.2.5. Vital staining

As outlined above, quantification of VA mycorrhizal fungus in both the soil and roots normally uses non-vital staining techniques such as trypan blue (Phillips & Hayman, 1970) and does not distinguish between the living and dead

fungus. The results often show no relationship between the length of the root infected and the effectiveness of infection with respect to plant growth (Smith & Dickson, 1991; Smith & Gianinazzi-Pearson, 1990). This may be because the role of the fungus in absorption of nutrients (especially P) from the soil and transport to the root depends not only on the quantity of external hyphae and internal infection, but also on their activity. Therefore, quantification of the activity of the fungus both in the root and in the soil is required.

The viability of VA mycorrhizal fungi both in the root and in the soil has been assessed by measuring enzyme activity of the hyphae (Hamel et al., 1990; MacDonald & Lewis, 1978). The activity of succinate dehydrogenase (SDH) can be used as an indicator for measuring metabolically active hyphae (Addy et al., 1993; Kough et al., 1987; Sylvia, 1988). In such an assay, tetrazolium salts, such as nitroblue tetrazolium (NBT), act as electron acceptors in enzyme-catalysed oxidation-reduction reactions, which reduce them to coloured formozans (MacDonald & Lewis, 1978). Activity of esterases can be assessed using fluorescein diacetate (FDA) (Schubert et al., 1987; Söderström, 1977), which is taken up by diffusion into the cell and hydrolysed. The hydrolysis produces fluorescein which accumulates in the cell and can be detected by fluorescence microscopy under illumination at 500 - 520 µm (Ingham & Klein, 1984; Rotman & Papermaster, 1961).

NBT staining is more successful than FDA for assessment of living fungal structures within the root (Sukarno & Dickson, 1992). However, formozan deposition in living cortical cells of the root can interfere with quantification of the fungus in longitudinal root squashes (Pearson et al., 1991; S. E. Smith & C. M. Long, unpublished result). This interference can be overcome either by

using transverse sections (which essentially cut and kill the cortical cells) or by using high magnification (Smith & Dickson, 1991; Smith & Gianinazzi-Pearson, 1990).

Living external hyphae have been measured following both FDA and NBT staining. The FDA assay has been used to assess the activity of fungi grown both in soil and in pure culture (Schubert et al., 1987; Soderstrom, 1977) and VA mycorrhizal fungi in soil (Hamel et al., 1990). NBT has also been used to stain VA mycorrhizal hyphae in soil (Addy et al., 1993). However, several cleaning steps are required which may reduce the recovery of hyphae and consequently underestimate their length.

In most investigations it has been assumed that there is no competition between hyphae of mycorrhizal fungi and soil saprophytic fungi. Consequently, lengths of mycorrhizal hyphae have been obtained by subtracting a "background value" for hyphae measured in uninoculated pots (Abbott & Robson, 1985; Jakobsen et al., 1992a). This approach seems reasonable when background values are very low compared with inoculated pots, but needs more investigation in situations where soil saprophytes are likely to contribute significantly to total lengths.

1.2.6. Mycorrhizal effects on plant growth

The effects of mycorrhizal inoculation on plant growth have been reviewed thoroughly (e.g. Smith, 1980; Smith & Gianninazzi-Pearson, 1988). The responses to VA mycorrhizal fungal infection are normally considered to be the

results of a mutualistic rather than a parasitic interaction and based on bidirectional nutrient transfer between the symbionts. Growth increases of mycorrhizal plants are frequently associated with increases in their nutrient content and concentration. Mycorrhizal plants often have a higher rate of growth, lower root:shoot ratio, higher fresh weight:dry weight ratio than non-mycorrhizal plants and different dry weight and nutrient distribution between root and shoot. Effects of VA mycorrhiza on the root:shoot ratio may be partly mediated by improvement of mineral nutrition (Bowen & Cartwright, 1977; Hunt et al., 1975). For example, non-mycorrhizal *Medicago sativa* and citrus grown in soil given additional P had the same dry weight and root:shoot ratio as mycorrhizal plants in the absence of added P (Eissenstat et al., 1993; Smith & Daft, 1977, 1978).

Despite these differences, the comparison commonly made is between large mycorrhizal and small non-mycorrhizal plants at a single harvest, where the two types of plant are different in "physiological age" (Smith, 1980). For example, mycorrhizal plants of cotton, petunia, maize and strawberry matured earlier than non-mycorrhizal plants (Daft & Okusanya, 1973). Therefore, the changes of root:shoot ratio may be mediated by differences in plant growth and development rather than direct effects of formation of mycorrhiza. The differences in size, "physiological age" and nutrient concentration between non-mycorrhizal and mycorrhizal plants grown in nutrient-poor soil cause problems in obtaining non-mycorrhizal control plants for use in physiological studies of the symbiosis.

A number of different methods have been used in attempts to make valid comparisons between non-mycorrhizal and mycorrhizal plants. These include

calculation of nutrient uptake per unit length or weight of root (Sanders & Tinker, 1973; Smith, 1982) rather than per plant, and supplementing the nutrition of non-mycorrhizal plants so that they have the same dry matter, tissue nutrient concentration or growth rate as mycorrhizal plants. The use of "matched" or "equivalent" plants is an ideal situation. In practice it is very difficult to grow plants that are equivalent with respect to all aspects of root and shoot growth and nutrient concentration (Eissenstat et al., 1993). Nevertheless, any investigation of non-nutritional aspects of mycorrhizal development must attempt to eliminate confounding factors resulting from differences in plant nutrition and size. In this investigation, the effects of fungicides on growth of non-mycorrhizal P-sufficient plants were investigated in parallel with effects on mycorrhizal plants. The influence of fungicides can also be investigated in terms of the growth of the symbiotic plant.

1.2.7. Mycorrhizal effects on phosphorus nutrition

Sanders (1975) emphasised that the most important contribution of VA mycorrhizal infection to plant nutrition is the increase in the efficiency of P uptake from the soil by the plant. The role of VA mycorrhizal fungi in P nutrition has received more attention than other nutrients. This is because P is required by the plant in relatively large amounts, while its availability in the soil solution is very limited.

Three processes are essential for mycorrhizal nutrient uptake:

1) nutrient uptake from the soil by external hyphae;

2) the translocation of nutrients over considerable distances through the external hyphae; and

3) transfer of nutrients from the fungus to the root of the host plant across fungal-plant interfaces (Jakobsen et al., 1992a,b; Smith & Gianninazi-Pearson, 1988; Smith & Smith, 1990). Therefore, the overall rate of uptake will be determined by the rates of these processes and all will contribute to increased uptake efficiency of the root system, measured as inflow (P uptake per unit length of the root per unit time). The efficiency of external hyphae in absorbing P depends partly on their size, extent and distribution in the soil. Fine hyphae penetrate more easily into soil pores and also provide a large surface area of contact with soil, per unit weight of mycelium (Gerdemann, 1968). However, their absorptive and translocating abilities and hence their activity is also important (Gianinazzi-Pearson & Gianinazzi, 1986; Kough & Gianinazzi-Pearson, 1986). Experiments investigating fluxes of ^{32}P orthophosphate have revealed two P uptake systems (active high affinity and passive low affinity) which operate simultaneously (analogous to the situation in cells of higher plants) in hyphae of VA mycorrhizal fungi (Thomson et al., 1990). Other attempts to measure the capacity of the external hyphae to absorb P have involved indirect determination of uptake by the hyphae from inflow measurements in mycorrhizal and non-mycorrhizal roots (Jakobsen et al., 1992a; Sanders et al., 1977; Sanders & Tinker, 1973). Without concurrent measurement of the length of active external hyphae, the uptake per unit length of root cannot be determined and there are no reports of hyphal P uptake per unit length of external hyphae prior to 1992.

The ability of VA mycorrhizal fungi to translocate P along the external hyphae has been demonstrated by applying ^{32}P to hyphae associated with mycorrhizal clover (Pearson & Tinker, 1975) and onion (Cooper & Tinker, 1978) plants in split plates. The efficiency of translocation in external hyphae was expressed as a translocation flux (amount of P transferred per unit cross-sectional area of hyphae per unit time). Values were 3.0×10^{-6} and 2.0×10^{-6} mol m^{-2} s^{-1} for clover and onion respectively. The hyphal diameters were measured at some distance from the roots, where hyphae crossed a physical diffusion barrier in the Petri dishes. These values are two orders of magnitude less than those obtained for the P flux through entry-point hyphae determined by Sanders & Tinker (1973) from onion plants grown in soil. The difference in magnitude of the fluxes probably results from the fact that large numbers of external hyphae scavenge P and transport it to the plant through relatively few large entry-point hyphae. In addition, the soil-grown plants were probably growing and transpiring more rapidly than those in Petri dishes, which may have resulted in higher rates of P translocation in hyphae.

The effect of VA mycorrhiza on plant growth is based not only on the contribution of external hyphae to P acquisition, but also on the extent of infection in the roots and, hence, area of interface where nutrient transfer between the two symbionts occurs (Smith & Gianinazzi-Pearson, 1988; Smith & Smith, 1990). P transfer from the fungus to the plant involves membrane transport processes in the interface between the two symbionts. P is assumed to be exported from the fungus to the apoplastic compartment (efflux) and subsequently absorbed by the plant membrane (uptake). The fungus may

reabsorb P from the apoplastic compartment, hence reducing the amount of P available for the plant cell to take up (Smith & Gianinazzi-Pearson, 1988; Smith & Smith, 1990, 1993). Thus the amount of P that reaches the plant is determined by the capacity of P unloading by the fungus, the extent of reabsorption by the fungus and uptake by the plant membrane. P flux across the interface has been estimated as 13 x 10^{-9} mol m^{-2} s^{-1} (Cox & Tinker, 1976; Smith et al., 1994). This value is the same order as those for P influx into plant cells.

Which of the steps outlined actually limits P uptake and transfer is, however, not fully understood. This may explain why percentage infection of root or development of arbuscules is not always related to an increase in P uptake or plant growth (Kough & Gianninazzi-Pearson, 1986). Again, the efficiency of P uptake and transfer to the plant may differ in different species of the fungus and depend on the amount of available P in the soil (Harley & Smith, 1983; Sanders, 1975). For a single species combination (fungus and plant), however, measurement of P uptake and transfer to the plant are useful parameters to determine physiological function of the symbiosis and they can also be used to assess physiological damage caused to the fungus or plant by fungicides or other environmental factors.

In summary, the effects of environmental variables, including fungicide application, on VA mycorrhizal symbiosis need to be assessed in terms of physiological effects as well as fungal development. The physiological activity can be determined using vital staining as well as by the capacity of the VA mycorrhizal fungi to increase the efficiency of nutrient acquisition.

1.2.8. Use of fungicides

Protection of crops from diseases is an important aspect of achieving high productivity and fungicides play an essential role in this.

A fungicide is an agent that kills or inhibits the development of either spores or mycelium of a fungus. Although fungicides are used to kill pathogenic fungi, they all have potential side-effects other than inhibition of target pathogens. The side-effects include inhibition of growth of host plants or of non-target microorganisms, including some which are beneficial to plant growth (Bollen, 1979; Vyas, 1988). Previous work on fungicidal side-effects on mycorrhizal symbiosis will be reviewed in the next section, with a focus on systemic fungicides.

1.2.8.1. The effects of systemic fungicides on plants

Systemic fungicides are usually selective against particular fungal pathogens and may be effective as seed treatments, soil drenches or foliar sprays depending partly on whether translocation is upward (as for most systemic fungicides), downward or both (Rowe, 1982). Since the metabolic processes of fungi are basically similar to those of plants, there is a considerable potential for interference in plant metabolism.

Negative effects of fungicides on plant growth have been reported. Carboxin and oxycarboxin were toxic to soybean seedlings when applied to the

soil at 100 ppm. Benlate had effects similar to carboxin on groundnut, soybean, green gram and black gram. These fungicides delayed seed germination and inhibited radial elongation of roots when they were applied as seed treatments (Vyas, 1988). Additionally, Benlate and carboxin have been reported to cause chromosomal aberration in onion root tips, similar to those caused by radiation and other mutagenic treatments (Vyas, 1988). Another systemic fungicide acylalanins and ethazol has been reported to have toxic effects on rhododendron when applied as a soil drench at high dosages (Benson, 1979).

Triforine is an effective fungicide against mildew. However, soil and foliar application of this fungicide (125 to 250 ppm) reduced the amino acid content of root exudates of wheat and the exudation of aromatic amino acids (phenylalanine and tyrosine) was also suppressed (Jalali & Domsch, 1975). The extent of the phytotoxic effect may vary with plant species. Therefore, in a study of the effects of fungicides on a symbiosis such as VA mycorrhiza, the potential damage to plant growth as well as to the fungus needs to be considered.

1.2.8.2. The effects of fungicides on VA mycorrhizal symbiosis

A number of investigations have been carried out on the effects of fungicides on the development of VA mycorrhizas, but the results do not give a consistent picture (Menge, 1982, Trappe et al., 1984). This may arise from the way in which the effects of fungicides on mycorrhizal infection have been assessed. In

most investigations, measurement of the percentage (or fraction) of the root length infected by mycorrhizal fungi (which is the outcome of several different processes, see section 1.2.2) following staining with trypan blue, has been used to determine the effects of fungicide. Results are then interpreted in terms of direct effects of the chemicals on the fungi. This approach does not, however, take into account complexities of the infection processes, nor the fact that root growth as well as fungal growth contributes to the measured percentage of root length colonised (see section 1.2.2 - 1.2.3 above).

Setting aside the problems of assessment until later, it has been found that effects of fungicide on VA mycorrhizal symbiosis vary depending on the host, the species of the fungus, type of fungicide, fungicide concentration and method of fungicide application (see Tables 1.1 and 1.2). It appears that systemic fungicides are more harmful than non-systemic fungicides and soil drenches are more damaging than foliar applications (Jalali & Domsch, 1975; Nemec, 1980). Sometimes the results are contradictory. For example, foliar application of Ridomil, at the recommended rate, to leek associated with *Glomus intraradices* reduced percentage infection, whereas infection was increased in citrus plants associated with *Glomus etunicatum* (Table 1.2) (Jabaji-Hare & Kendrick, 1987; Nemec, 1980, see Menge, 1982). The effect of a fungicide on percentage infection depends on the magnitude of its effects on both root and fungal growth. Three possible outcomes can be envisaged. First, if a fungicide reduces root growth more than fungal growth, the result will be an increase in the percentage of the root length colonised (percentage infection). Second, if a fungicide reduces fungal growth proportionally more than root growth, the result will be a decrease in the percentage infection. Third, if the

fungicide affects both root and fungal growth similarly, the percentage infection may not change significantly compared to the untreated situation even though both plant and fungal growth may have been affected. It is not possible to determine which of these mechanisms apply to the investigations listed in the tables (Tables 1.1 and 1.2) and it seems, intuitively, unlikely that any fungicide would actually increase the growth of the fungus. It must be suspected that reduced root growth is implicated in these cases. However, only a few fungicides have been tested for their effects on plant growth in mycorrhizal and non-mycorrhizal plants. The results were different depending on the host plant used (Paul et al., 1989; West et al., 1993a,b). For example, in mycorrhizal plants Benlate (benomyl) application apparently resulted in an increase in root length of pea (Fitter & Nichols, 1988) but stunting in sour orange (Nemec, 1980).

Some indications of direct effects of fungicides on activity of VA mycorrhizal fungi have been obtained from work using vital staining (Kough et al., 1987) and measurement of P inflow (Hale & Sanders, 1982). Kough et al. (1987) showed that there were direct physiological effects of Benlate and captan on the ability of *Glomus intraradices* to reduce NBT, which were apparent within 3 days of fungicide application, while Hale & Sanders (1982) demonstrated a reduction in percentage infection and a reduced contribution of mycorrhizal hyphae to P inflow, following the application of Benlate. However, results are not always so straightforward. Fitter (1986) showed an apparent increase in P uptake following application of Benlate, despite the fact that the percentage infection (trypan blue staining) was reduced. It seems clear that investigations need to combine the approaches used in these studies and use vital as well as

Table 1.1. Summary of the effects of non-systemic fungicides on development of VA mycorrhizal fungi and mycorrhizal plant growth

Fungicides	Plants	Fungi	Concentrations or rates	Effects on VA mycorrhiza	References
Arrasan	maize	*Glomus. intraradices*	25 & 50 ppm	(-)%, (-) plant growth	El-Giahmi et al., 1976
Botran	maize	*G. mosseae*	25 & 50 ppm	(-)%, (-) plant growth	El-Giahmi et al., 1976
Chloroneb	sour orange	*G. etunicatum*	recommended rate	(0) sporulation, (+) root growth	Nemec, 1980
Plantavax	onion	*G. fasciculatum*	2.5 & 25 μg g^{-1} soil	(-) %	Manjunath & Bagyaraj, 1984
Chlorothalonil	sour orange	*G. mosseae*	recommended rate	(-) sporulation, (-) plant growth	Nemec, 1980
Dichlofluanid	wheat	*Endogone*	0.5 kg a.i. ha^{-1}	(-) %, (-) spore production	Jalali & Domsch, 1975
Maneb	sour orange	*Endogone*	0.8 kg a.i ha^{-1}	(-) sporulation	Jalali & Domsch, 1975
Maneb	wheat	*Endogone*	0.8 kg a.i ha^{-1}	(0) %, (0) spore production	Gnekow & Marschner, 1989
Pentachloronitrobenzene	oat	*G. mosseae*	20 mg kg^{-1} soil	(+) root length	Gnekow & Marschner, 1989
Pentachloronitrobenzene	oat	*G. mosseae*	50 mg kg^{-1} soil	(-) root length	Gnekow & Marschner, 1989

(-), Reduced; (0), no effect; (+), increased; %, percentage infection.

Table 1.2. Summary of the effects of systemic fungicides on VA mycorrhizal fungus and mycorrhizal plant growth

Fungicides	Plants	Fungi	Concentrations or rates	Effects on VA mycorrhiza	References
Aliette	leek	*Glomus intraradices*	3.0 kg a.i. ha^{-1}	(+) %; (+) shoot dry weight	Jabaji-Hare & Kendrick, 1987
			0.3, 1.0, 3.0 mg a.i. $plant^{-1}$	(+) %; (+) root exudation	Jabaji-Hare & Kendrick, 1985
	leek	*Glomus* sp. (Herb. DAOM 181602)	6000 ppm	(+) %; (-) shoot dry weight	J. H. Graham, pers. com
	Cleopatra mandarin	*G. intraradices*	3200 µg ml^{-1}	(+) %	Plenchette & Perrin, 1992
	wheat	*G. intraradices*	3200 µg ml^{-1}	(+) %	Plenchette & Perrin, 1992
Benlate	onion	*G. intraradices*	recommended rate	(-) %; (-) fungal activity	Kough et al., 1987
		G. fasciculatum	2.5 µg g^{-1} soil	(0) %	Manjunath & Bagyaraj, 1984
			25 µg g^{-1} soil	(-) %	Manjunath & Bagyaraj, 1984
	sour orange	*G. mosseae*	recommended rate	(-) plant growth	Nemec, 1980
	pea	indigenous	0.9 g l^{-1}	(-) %; (-) P inflow	Fitter & Nichols, 1988
	red clover	indigenous	3.75 g l^{-1}	(-) %; (-) P shoot	Fitter & Nichols, 1988
	wheat	*Endogone*	0.1mg a.i. g^{-1}seed	(-) %	Jalali & Domsch, 1975
	leek and wheat	*G. intraradices*	400 µg ml^{-1}	(-) %	Plenchette & Perrin, 1992
			1.5 mg a.i. g^{-1}seed	(-) %	Jalali & Domsch, 1975
	red clover	*Glomus clarus*	33 mg kg^{-1}	(-) %; (-) dry weight (-) root length; (-) P inflow	Hale & Sanders, 1982
	Vulpia ciliata	indigenous	2.2 g l^{-1}	(-) %	Carey et al., 1992
Captan	wheat	*Endogone*	0.6 kg a.i ha^{-1}	(-) %	Nemec, 1980
	onion	*G. intraradices*	recommended rate	(-) %; (-) fungal activity	Kough et al., 1987
	wheat	*G. fasciculatum*	recommended rate	(0)%	Manjunath & Bagyaraj, 1980
	sour orange	*G. etunicatum*	recommended rate	(-) %; (-) plant growth	Nemec, 1980
Ridomil	leek	*G. intraradices*	recommended rate	(-) %; (-) shoot dry weight	Jabaji-Hare & Kendrick, 1987
	citrus	*G. etunicatum*	recommended rate	(+)%	Nemec, 1980
Thiabendazole	citrus	*G. etunicatum*	recommended rate	(-) %	Nemec, 1980
Topsin	wheat	*Endogone*	0.38 kg a.i ha^{-1}	(0)%; (-) spore development	Boatman et al., 1978
Tridemifon	wheat	*Endogone*	0.06 kg a.i. ha^{-1}	(-)%; (-) spore production	Jalali & Domsch, 1975
Calaxin	wheat	*Endogone*	0.55 kg a.i. ha^{-1}	(-)%; (-) spore production	Jalali & Domsch, 1975

(-), Reduced; (0), no effect; (+), increased; %, percentage infection.

non-vital staining and measurements of P uptake in order to obtain results that can be reasonably easily interpreted. The vital staining approach of Kough et al. (1987) certainly seems useful as a rapid method of assessing direct fungicidal effects and could be extended to external hyphae, on which no investigations of fungicide effects have been made.

A further problem, contributing to contradictory results such as those of Fitter (1986), may be the possible side-effects of fungicides on the plants, which were not taken into account. There is a significant problem of comparing non-mycorrhizal and mycorrhizal plants which have marked differences in P acquisition, metabolism and allocation and consequently have different relative growth rates. Plants grown in P-deficient soil show poor growth unless they are mycorrhizal and phytotoxic effects of fungicides (if any) are difficult to separate out from effects of nutrient deficiency. One way of overcoming this problem would be to use P-sufficient non-mycorrhizal plants as controls, so that they have similar growth rates to mycorrhizal plants, as discussed above. This approach has been used successfully to clarify effects of VA mycorrhizal infection itself on photosynthesis and carbon allocation in citrus (Eissenstat et al., 1993; Peng et al., 1993) and could be more widely applied.

It is clear that interactions between fungicide application and VA mycorrhizal development and function are complex. Analysis requires effects on both fungus and plant to be taken into account using a range of different techniques, including use of "matched" plants, measurements of P uptake via the fungus and vital staining. Systemic fungicides, which are taken up by the plant and act on the fungus after infection has become established, may be

expected to have more complex effects than non-systemic fungicides and, furthermore, they appear to be more generally harmful to VA mycorrhizal fungi. Consequently, three systemic fungicides were chosen for this investigation and their mechanisms of action are discussed in the next section.

1.2.9. The mechanisms of action of Benlate, Aliette and Ridomil

1.2.9.1. Benlate (benomyl)

Benlate is one of the benzimidazole group of fungicides, with benomyl as its active ingredient (a.i.). Benzimidazoles are active against most Ascomycetes, Deuteromycetes and Basidiomycetes, but are generally inactive towards Oomycetes (Ishii, 1992).

Benlate is converted in the soil, plant and aqueous solution to the methyl-2yl-carbamate (MBC or carbendazim). Together these chemicals have been used against a wide spectrum of plant diseases in numerous crops (Buchenauer, 1990).

It has been demonstrated in *Aspergillus nidulans* that benomyl acts by interfering with mitosis in the fungal cells, inducing nuclear instability and blocking nuclear division (Davidse, 1986). Studies with ^{14}C-labelled carbendazim in sensitive and resistant strains of *A. nidulans* revealed high binding activity of the compound to the tubulin prepared from sensitive strains but no, or extremely low, binding affinity to tubulin from resistant strains (Davidse, 1986; Davidse & Flach, 1977). In *Fusarium acuminatum* almost all

cytoplasmic microtubules, except those near spindle pore bodies, disappeared after exposure to carbendazim for 10 min (Howard & Aist, 1977; 1980). These authors found that disappearance of microtubules causes a variety of effects including displacement of mitochondria from hyphal apices, disappearance of Spitzenkorper (involved in linear elongation of hyphae), reduction in rate of linear growth and arrest of mitosis. These phenomena strongly support the idea that in fungi benzimidazoles cause abnormal function of microtubules as a result of these compounds binding to tubulin.

Microtubules are a major part of the cytoskeleton of cells, a unique feature of eukaryotes. The interference in functioning or structural integrity of the cytoskeleton will lead to disturbance in the growth and development of the cell. There is a lack of information on interactions of Benlate with microtubules of higher plants.

1.2.9.2. Aliette (fosetyl-Al)

Aliette, the aluminium salt of tris-o-ethyl phosphonate (fosetyl-Al), is the only commercial compound of the alkyl phosphonate group of fungicides. This fungicide was first released in 1977 (Cohen & Coffey, 1986). Generally, Aliette is highly active in controlling downy mildews as well as *Phytophthora* diseases in various crops (Buchenauer, 1990).

Aliette breaks down rapidly in soil and plant tissue to phosphorous acid (H_3PO_3) or phosphonic acid {H-PO$(OH)_2$}, CO_2 and aluminium (Cohen & Coffey, 1986; Coffey & Ouimette, 1989; Despatie, et al., 1989; Saindrenan et al.,

1985; Fenn & Coffey, 1984, 1985, 1989). Phosphorous or phosphonic acid may be regarded as the active component of fosetyl-Al (Cohen & Coffey, 1986; Guest & Grant, 1991). Phosphorous and phosphonic acids are in tautomeric equilibrium, with the equilibrium favouring phosphonic acid (Guthrie, 1979; see Coffey & Ouimette, 1989). Alkyl esters of phosphorous and phosphonic acids are termed phosphite and phosphonate, respectively. In this thesis, the terms phosphonate and phosphate will be used to refer to HPO_3^{2-} and PO_4^{3-} detected in plant tissues by NMR spectroscopy (see Chapter 5).

In vitro, fosetyl-Al generally shows low activity against various *Pythium* and *Phytophthora* spp.. This leads to the hypothesis that fosetyl-Al may act indirectly by triggering resistance mechanisms in the host (Bompeix et al., 1980). However, comparative studies using resistant and sensitive strains of *Phytophthora* indicated that this fungicide and its degradation products have direct action on the target pathogens by interference with phospholipid synthesis (Bouchenauer, 1990; Dolan & Coffey, 1988).

Toxic effects of Aliette (or its degradation products) on plants have been reported. Phosphonate was accumulated in avocado, orange and tangerine following application of Aliette (Coffey & Ouimette, 1989; Pelegri et al., 1993). Reduction in trunk cross-sectional area was also observed in citrus treated by Aliette (Sandler et al., 1988). The excessive accumulation of phosphonate in Aliette-treated plants may be toxic to the plant as well as to the fungus. Aluminium is also released following Aliette application and may accumulate in the plant. In soil of low pH excessive aluminium in the soil may cause poor root growth, via inhibition of root cell mitosis and root elongation (Fiskesjo, 1990; Rengel, 1992).

As Aliette degrades to phosphorous acid in the soil (see above), an excessive amount of degradation products of Aliette may itself lead to reduction in soil pH. Unfortunately this possibility does not appear to have been addressed in the literature.

1.2.9.3. Ridomil (metalaxyl)

Metalaxyl has the widest anti-fungal spectrum and highest activity *in vitro* among the phenylamide group of fungicides (Griffith et al., 1992). Metalaxyl is primarily translocated acropetally (upwards), but may move basipetally in a number of cultivated plants (Rowe, 1982). This fungicide is reported to have a high degree of mobility in the soil when applied as a soil drench (Zaviezo et al., 1993). Metalaxyl, as seed treatments, foliar and soil drenches, has contributed to an important improvement in the control of economically important fungal plant pathogens belonging to the order Peronosporales (Buchenauer, 1990).

Studies on the mode of action of phenylamides indicate that this group of fungicides specifically inhibits RNA-polymerase I and blocks r-RNA synthesis of fungi belonging to the Peronosporales with consequent inhibition of infection Buchenauer, 1990; Fisher & Hayes, 1992).

1.3. The aims of the project

The overall aim of this project was to gain an understanding of the complex effects of fungicides on the VA mycorrhizal symbiosis. This required determination of effects of fungicides on:

a) the growth of the plant;

b) the development of the fungus, both in soil and within the root;

c) the physiological activity of the fungus and consequent effects on symbiotic function.

To achieve these aims it was necessary to:

a) establish mycorrhizal and non-mycorrhizal (control) plants which were similar, as far as possible, in growth and P content and concentration, so that effects of fungicide on both host and fungus could be assessed;

b) study effects of three systemic fungicides, Benlate, Aliette and Ridomil on growth of non-mycorrhizal plants and the development of infection (including external hyphae);

c) clarify effects of Aliette and its degradation products on plant growth and mycorrhizal development.

The work is presented in six chapters. Following the Introduction and Literature Review (Chapter 1), Chapter 2 describes Materials and Methods together with some preliminary investigations on vital staining of fungus within the root and in the soil.

In Chapter 3, the effects of the three fungicides on plant growth, mycorrhizal development and P nutrition are compared. The work reported in this chapter has been published in New Phytologist (Sukarno et al., 1993).

The effects of the fungicides on rates of P uptake and transfer to the plant are presented in Chapter 4. The data allow comparison of the effects of fungicides on the physiological efficiency of the symbiosis.

Chapter 5 presents a group of experiments designed to clarify the toxicity of Aliette towards mycorrhizal and non-mycorrhizal plants observed in the experiment reported in Chapter 3, and the observation that mycorrhizal plants were less sensitive to these toxic effects than non-mycorrhizal plants.

Chapter 6 is a General Discussion in which the significance of the results is considered with respect to application of systemic fungicides on the VA mycorrhizal symbiosis.

CHAPTER 2. GENERAL MATERIALS AND METHODS

2.1. Introduction

This chapter summarises the methods used in the work discussed in this thesis. Details of the individual experiments are given in the appropriate chapters.

2.2. Host and fungus

The plant species used throughout the experiments was onion (*Allium cepa* L. var White Globe). Onion was chosen as it is responsive to mycorrhiza, has large cells and simple root morphology; thus, fungal structures can be distinguished easily within the root. This species has been the subject of a considerable amount of previous work on mycorrhizal infection and the effects of the symbiosis on plant nutrition. Furthermore, transverse sections of roots have proved to be suitable for vital staining and image analysis (Smith & Dickson, 1991; Smith et al., 1994).

Glomus sp. "City Beach" (WUM 16) was obtained from the Department of Soil Science and Plant Nutrition, The University of Western Australia. This fungus was chosen as it has a very extensive hyphal network in soil and was therefore appropriate for studying development and function of this component of mycorrhizal roots.

2.3. Source and maintenance of inoculum

Mycorrhizal inoculum (kindly supplied by Dr. S.E. Smith) was derived from pot cultures which were infected by *Glomus* sp. "City Beach" (WUM 16). The pot cultures were prepared by growing clover (*Trifolium subterraneum* cv Mt Baker) plants in a soil/sand mix (see section 2.4) amended with 10% (by weight) of inoculum of *Glomus* sp. "City Beach" (WUM 16) (from previous pot cultures) grown in a glasshouse at 15 (night) - 25 (day) °C. Non-mycorrhizal pot cultures of clover were prepared without added inoculum. The pots were watered to 12% w/w with reverse osmosis (RO) water three times per week and given 10 ml of nutrient solution minus P weekly (based on Long Ashton Nutrient Solution, see Smith & Smith, 1981; see section 2.8 for details). After several months, root infection of the clover plants was measured. If infection levels were adequate (over 70%), watering was stopped and plants and soil were allowed to dry out. Shoots were then discarded and the roots and soil used as inoculum, which comprised spores, external hyphae, root fragments and soil.

2.4. Plant growth medium

The plant growth medium used in all investigations including pot cultures was a soil and sand mixture (1:9, w/w). Soil was collected from Mallala, South Australia, and contained 15.6 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 for 45 min before mixing. One kg capacity black plastic pots, 6 cm in diameter and 20 cm deep, were used.

2.5. Inoculation procedure

Mycorrhizal plants (M) were inoculated with *Glomus* sp. "City Beach" (WUM 16) derived from pot cultures as described above. Ten percent (by weight) of inoculum was mixed throughout the plant growth medium. Non-mycorrhizal plants (NM) received non-inoculated pot culture material to ensure that the microflora and nutrient status were similar.

2.6. Phosphorus supply

Additional phosphorus (P) was required in particular experiments. P was applied as a 0.1 M solution of NaH_2PO_4 and mixed thoroughly through the soil/sand mixture before planting. The amount applied per pot is given in the appropriate sections.

2.7. Fungicides and fungicide application

The three systemic fungicides used were Benlate, Aliette and Ridomil. The commercial formulations of the fungicides are Benlate DF (E.I. Du Pont de Nemours & Co.Inc.), Aliette (Rhone-Poulenc), and Ridomil 250 WP (Ciba Geigy). Active ingredients were benomyl (500 g kg^{-1}), fosetyl-Al (740 g kg^{-1}) and metalaxyl (250 g kg^{-1}), respectively.

Solutions of the three fungicides were freshly prepared (0.5, 1.0 and 0.4 g l^{-1} in double distilled water, respectively) and 125 ml was applied separately, as a soil drench to pots. The final concentrations in the soil of active ingredient (a.i.) were: Benlate 31.25 mg kg^{-1}, Aliette 82.33 mg kg^{-1} and Ridomil 12.5 mg kg^{-1}. These concentrations were calculated from recommended rates. Fungicides were applied to the soil with the aim of studying the effects of the fungicide on the development of fungus in soil and its ability to infect root as well as spread of infection within the root.

2.8. Plant growth

Seeds of *Allium cepa* were surface sterilised by soaking in a solution containing 9% ethanol, 0.1% chlorine as sodium hypochlorite (Liquid pool chlorine) and 90.9% sterile distilled water for 20 min and then rinsed three times with sterile distilled water (L.M. Haugen, personal communication). Four seeds were planted per pot. After germination, seedlings were thinned to one plant per pot. The surface of the soil in the pots was covered with black plastic

beads to control the growth of algae, which caused technical problems when quantifying the length of external hyphae in preliminary experiments.

The plants were placed in a growth room with photon flux density of 400 µmol $m^{-2}s^{-1}$ during a 14 h photoperiod. Temperatures were 21°C day and 19°C at night. The plants were watered to 12% (w/w) with RO water, randomly rearranged three times per week and given 10 ml of nutrient solution minus P and plus N weekly (Smith & Smith, 1981). The nutrient solution consisted of macronutrients 2 mM K_2SO_4, 1.5 mM $MgSO_4$, 4 mM $CaCl_2$, 8 mM $NaNO_3$ and micronutrients (all in mg l^{-1}) 2.86 H_3BO_3, 1.81 $MnCl_2.4H_2O$, 0.22 $ZnSO_4.7H_2O$, 0.08 $CuSO_4.5H_2O$, 0.025 $Na_2MoO_4.2H_2O$ and Fe-EDTA to give 5 mg Fe l^{-1}.

At harvest, two groups of observations were made:

(a) determination of the growth of plants and extent of colonization by the fungus in the roots (see sections 2.9, 2.10 and 2.12);

(b) measurement of the development of external hyphae in the soil (see section 2.11).

2.9. Harvesting and determination of the plant growth response

Roots were washed free of soil and shoots were detached. The root systems were weighed and divided into three subsamples. Details of sample used and parameters measured are presented in Table 2.1.

Table 2.1. Sample used and parameters measured at harvest

Sample	Parameters measured
Shoots	
Whole sample	FW, DW, P concentration and P content
Roots	
Subsample 1 (Sample 1)	FW, DW, P concentration and P content
Subsample 2 (Sample 2)	FW, Root length, % infection
Subsample 3 (Sample 3)	Sectioning, staining and image analysis

FW = fresh weight, DW = dry weight.

Dry weight was determined after oven-drying at 80°C for 24 h. Oven-dried root and shoot materials were ground before measurement of phosphorus concentration (see section 2.15).

2.10. Assessment of percentage infection and root length

Sample 2 of root was cleared in 10% KOH (w/v) for 3 - 15 days (depending on the age of the plants) at room temperature and rinsed briefly with RO water and 1 N HCl. After the roots had been cleared, they were stained by soaking in trypan blue stain (Phillips & Hayman, 1970). Root length and total mycorrhizal infection were determined by the line intercept method described by Tennant

(1975). Stained roots were cut into approximately 1 cm lengths and evenly spread over a Petri dish (9 cm in diameter) containing a shallow layer of glycerol and lined with a plastic grid (0.5 cm grid dimension). Intersections between stained root (infected and uninfected) and grid (vertical and horizontal) were counted under a binocular microscope using a hand tally counter. The numbers of intersections were converted to total root length and percentage infection (Giovanetti & Mosse, 1980).

2.11. Measurement of development of external hyphae in the soil

External hyphae were extracted from the soil and measured following the method of Abbott & Robson (1985) with some modification. At each harvest, five cores (13 mm in diameter) of moist soil were taken from each pot and mixed together. A 2 g subsample was added to 200 ml of double distilled water and stirred vigorously for 5 min using a magnetic stirrer. A 6 ml aliquot was passed through a 8 µm Millipore filter. The retained material was stained with 1 ml of a freshly prepared solution of fluorescein diacetate (FDA) or nitroblue tetrazolium (NBT) to distinguish living hyphae. The non-vital stain trypan blue was used to determine total (living and dead) hyphae.

FDA is taken up by active transport and is hydrolysed by esterases within cells and the fluorescence produced accumulates in the cells. FDA stain stock solution was freshly prepared from 5 mg of FDA in 1 ml acetone. This was diluted to 10 µg ml^{-1} in phosphate buffer (60 mM K_2HPO_4, pH 7.4) (Ingham & Klein, 1980; Söderström, 1977). SDH activity was determined histochemically

by deposition of purple formozan following reduction of NBT in the presence of succinate (Kough et al., 1987; MacDonald & Lewis, 1978; Smith & Gianinazzi-Pearson, 1990). The stain was freshly prepared from 2.5 ml of NBT 4 mg ml^{-1}; 2.5 ml of 0.2 M Tris/HCl buffer (pH 7.4); 1.0 ml of 5 mM $MgCl_2$ and 3 ml of distilled water.

The length of living hyphae was determined by counting the intersections between fluorescent hyphae and a grid eyepiece micrometer at 160 x magnification, on a Zeiss Standard Lab 16 microscope equipped for epifluorescence microscopy, with excitation filter BP 450 - 490 and barrier filter LP 520. All procedures (from extraction of hyphae to counting intersects) were completed within 30 min, to overcome problems of loss of enzyme activity and fading of fluorescence. Lengths were determined as for root length (Tennant, 1975 and see above). The results were expressed as the length of hyphae per m root and per g soil.

A preliminary experiment was carried out to determine the most appropriate method of staining hyphae. In brief, experimental design was as follows:

Non-mycorrhizal and mycorrhizal plants were grown for 6 weeks. Methodology for sowing, watering regimes, fertilising techiques, growth conditions and inoculation techniques were as described above. There were two levels of P; 0 (P0) and 15 µg P (P1) added g^{-1} soil, applied to both uninoculated and inoculated pots.

At 6 weeks, soil was sampled, hyphae separated from soil by filtration and filters were stained as described above. Two vital stains, FDA and NBT, and the non-vital stain trypan blue were compared. Lengths of living and dead

hyphae were measured and the proportion of living to total (dead plus living) hyphae was estimated.

The results were as follows:

Plate 2.1 shows that NBT stain produced poor contrast with the soil sediment and furthermore did not show the activity of the melanised hyphae clearly. The poor contrast was due to the fact that the product of reduction of NBT is a purple formozan, thus it was difficult to discriminate stained hyphae from the dark background of the soil sediment. Consequently, the NBT stain was deemed unsuitable for determination of external hyphae.

FDA gave an excellent contrast (see Plate 2.2) and was used as a vital stain for external hyphae in all subsequent experiments. The autofluorescence of unstained hyphae in the absence of FDA was very low for external hyphae on the filters and it is not likely to have affected the values of length of living hyphae.

Comparison was made of lengths of external hyphae measured using FDA and trypan blue in mycorrhizal and non-mycorrhizal pots. Fig. 2.1 shows that in the absence of P supplement, the length of living hyphae was four times greater in mycorrhizal pots (MP0) than in non-mycorrhizal pots (NMP0). In contrast, the length of external hyphae stained with trypan blue in the two types of pots was not significantly different between treatments. When the ratios of living to total hyphae were calculated, approximately 30% of the total hyphae in mycorrhizal pots (MP0) were metabolically active compared with only 9% in non-mycorrhizal pots (NMP0). A similar trend was observed in the presence of P application. The length of living hyphae stained with FDA in mycorrhizal pots (MP1) was twice that of non-mycorrhizal pots (NMP1) but

when hyphal length was measured using trypan blue no differences were observed between mycorrhizal and non-mycorrhizal pots (Fig. 2.1).

Comparison between length of external hyphae determined using the vital stain FDA and the non-vital stain trypan blue in non-mycorrhizal and mycorrhizal pots showed that there were no differences in length of total hyphae (dead plus living). It is assumed that saprophytic fungi in soil in the uninoculated pots gave rise to the lengths measured. The data indicate that competition between saprophytic fungi and mycorrhizal fungi occurred in mycorrhizal pots, leading to equal total lengths of hyphae (stained with trypan blue). Interestingly, the lengths of living external hyphae were greater in mycorrhizal pots whether or not P was added. The differences in the ratio of living to total hyphae suggest differences in rate of hyphal turnover between the two treatments. Further experiments would be required to confirm this suggestion.

In the systems used here it is clearly not possible to determine the length of living mycorrhizal hyphae using trypan blue staining, as has been done previously by Abbott & Robson (1985), Abbott, Robson & De Boer (1984) and Jakobsen et al., (1992a,b). Staining with FDA did show differences between mycorrhizal and non-mycorrhizal pots and was used to determine length of mycorrhizal hyphae (see Jones et al., 1990). In any event, length of living hyphae is clearly more relevant to physiological studies than is total length.

For all subsequent investigations the length of hyphae in soil was measured following staining with FDA, and in determination of length of VA mycorrhizal hyphae no attempt will be made to subtract values in non-mycorrhizal pots from those in mycorrhizal pots.

Plate 2.1. The external hyphae stained with NBT. Precipitation of purple formozan produced poor contrast between the hypha and the background. Magnification was 100 times.

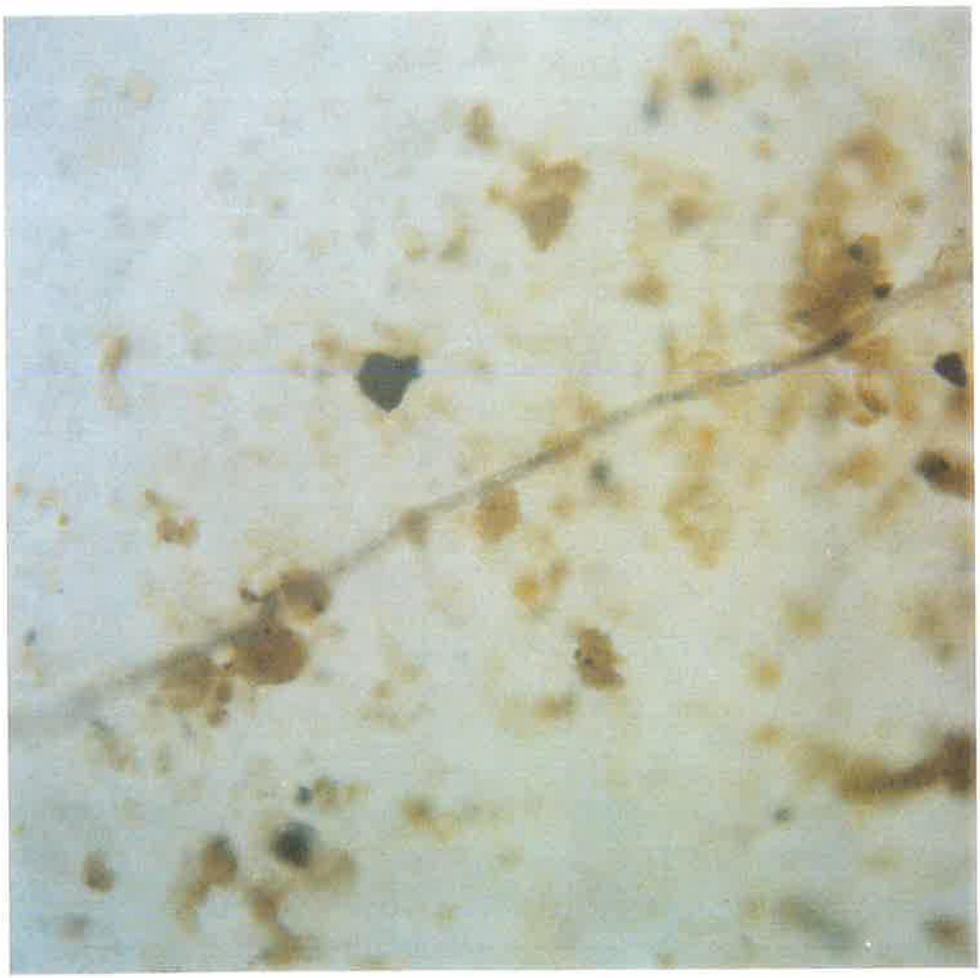


Plate 2.2. The external hyphae stained with FDA. The bright fluorescence of the hyphae contrasted well with the background. Magnification was 160 times.

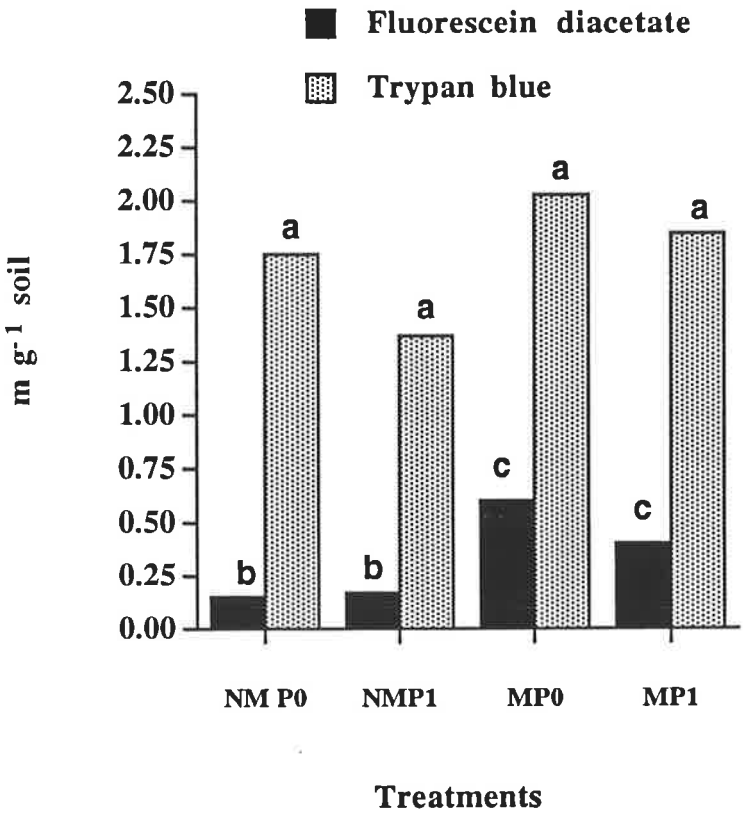


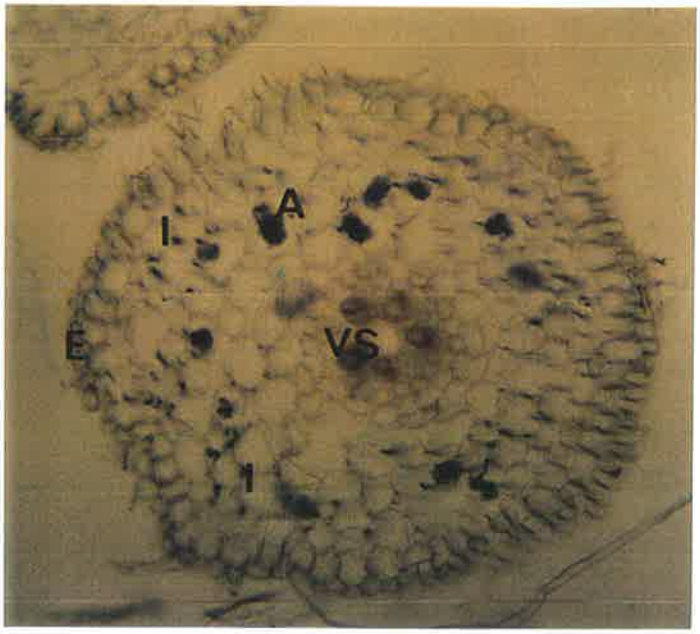
Figure 2.1. Length of external hyphae determined by vital stain fluorescein diacetate and trypan blue in non-mycorrhizal (NM) and mycorrhizal (M) pots in the absence (P0) and presence (P1) of P supplement at the 6-week harvest. Values are means from 2 replicate (for NM) and 3 replicate (for M) pots. Values with the same letter are not significantly different, based on analysis of variance at the 10% level.

2.12. Preparation and staining of sections of roots

Root sample 3 (see Table 2.1) was used for preparation of root sections according to the method of Smith & Dickson (1991) to determine number and perimeter of fungal structures (intercellular hyphae, arbuscules and vesicles). Roots were cut into 1 cm lengths and stored on ice for no more than 1 h. The root segments were inserted in a block of gelatin (containing 0.8% dimethyl sulphoximine, 2.0% glycerol and 20% gelatin), frozen on a freezing stage (Zeiss) and cut into 120 µm thick sections using a Leitz freezing microtome (Smith & Dickson, 1991). Two staining methods were tested. Fresh sections were floated on a solution of fluorecein diacetate (10 µg ml^{-1} phosphate buffer, pH 7.4, see section 2.11) for approximately 15 min and observed at 100 times magnification with a Zeiss Standard Lab 16 microscope with epifluorescence attachment (excitation filter BP 450 - 490 and barrier filter LP 520). Alternatively, sections were stained with nitroblue tetrazolium overnight to disclose living fungus (Smith & Gianinazzi-Pearson, 1990), washed in tap water at 40°C to remove the gelatin and arranged on glass microscope slides. The fungal structures were stained purple due to formozan deposition. Vascular systems were also stained and dark colouration of epidermis was observed (see Plate 2.3).

FDA was taken up into the fungal structures but background flare and autofluorescence of plant and fungal walls caused serious interference. NBT, therefore, was selected for all further investigations of sectioned roots.

Plate 2.3. Root section stained by NBT. Vascular system (VS), epidermis (E) of root as well as intercellular hypha (I) and arbuscule (A) showed succinate dehydrogenase activity.


A
I
E
VS
1

2.13. Image analysis

The Chromatic Colour Image Analysis Package (L.R. Jarvis, Leading Edge) was used for interactive measurement of the number and perimeter of stained fungal structures in sections. The automatic discrimination of the program was set at the colour and intensity of arbuscules in the root. Stained cells in the plant vascular system (phloem and xylem parenchyma) and dark coloured cells in the epidermis were identified by this process and were eliminated by a manual deletion function, leaving only vesicles, arbuscules and hyphae in the discriminated image.

The cross-sectional area and perimeter of the root and numbers and perimeters of intercellular hyphae, arbuscules and vesicles were measured. Vesicles were counted and deleted using another interactive step in the program before automatic measurement of intercellular hyphae and arbuscules using the sizer function (see Plate 2.4) (Smith & Dickson, 1991; Smith et al., 1994; Sukarno et al., 1993).

Results for perimeters of fungal structures are expressed as the mean per section, including all sections (both infected and uninfected) in the population.

2.14. Plant-fungal interface

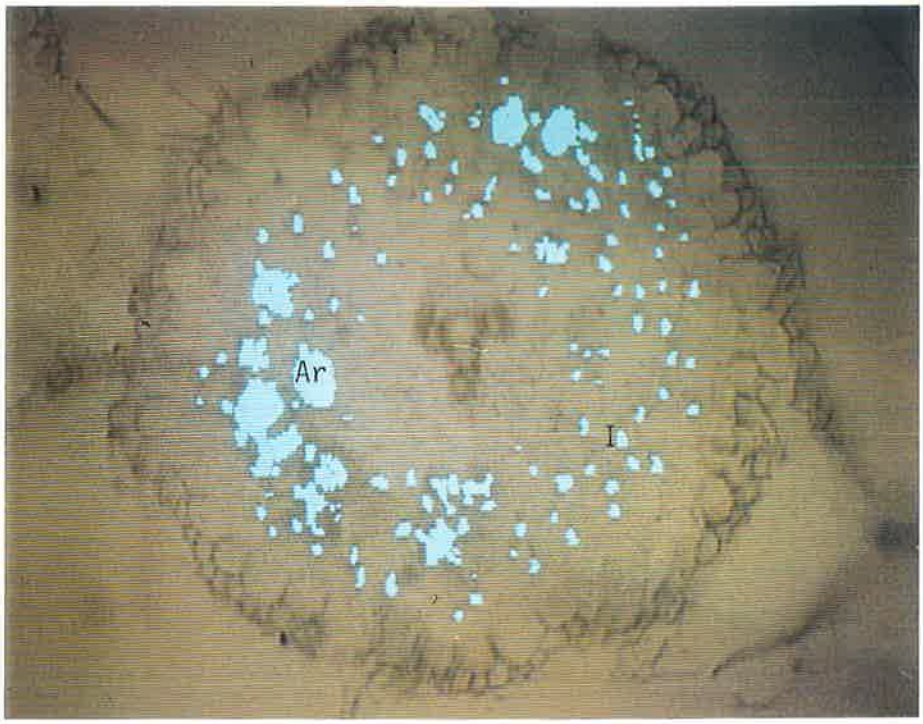
The measured perimeters of arbuscules (see section 2.13) did not take into account the extensive invagination of the plant plasma membrane by the arbuscule. The area of plant-fungal interface was calculated from the measured perimeter of each fungal structure (Smith & Dickson, 1991). Hyphae were assumed to be cylindrical and the perimeters of uninvaginated arbuscules were converted to an estimated value for invaginated arbuscule perimeter using the factor of 2.07 determined by Cox & Tinker (1976) for *Glomus mosseae* in onion roots. Results are expressed as area of interface per m root and per plant.

2.15. Determination of phosphorus concentration and uptake

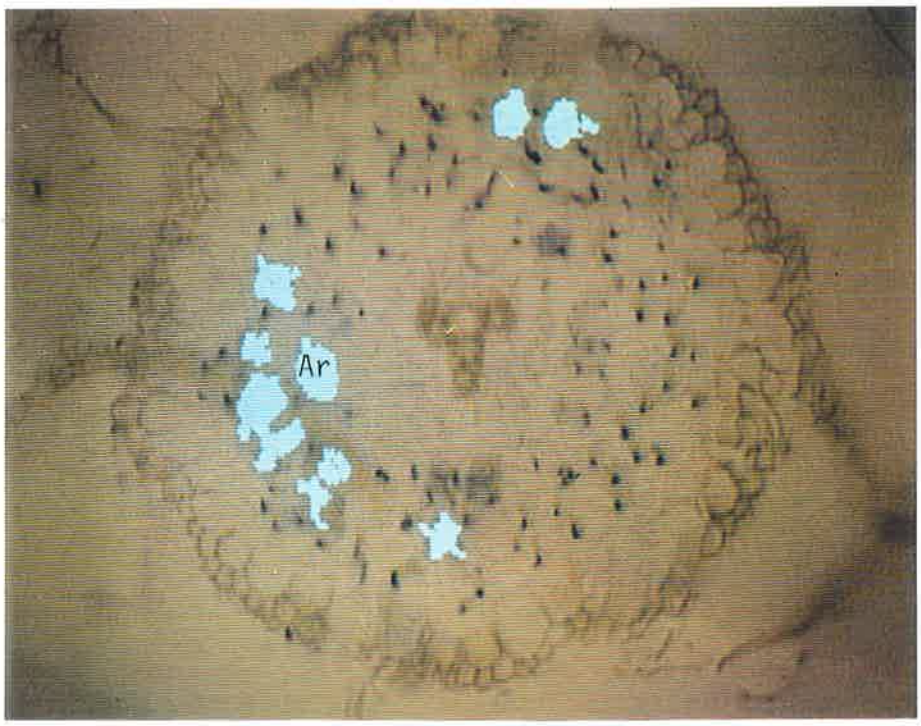
Dried material of shoot and sample 1 of roots (see Table 2.1) was ground in a pestle and mortar and used to determine P concentration using a molybdenum blue method (Bartlett, 1959). P content in plant tissue was calculated from the P concentration and total dry weight.

Plate 2.4. Root section stained with nitroblue tetrazolium (NBT) revealing succinate dehydrogenase activity in intercellular hyphae (I) and arbuscules (Ar). A white mask (Plate A = intercellular hypha and arbuscule; B = arbuscule only) indicates regions discriminated by the image analysis program. These photographs were kindly supplied by Dr. S.E. Smith and Ms. S. Dickson.

A



B



2.16. Calculation of P inflow, hyphal inflow, hyphal uptake, hyphal flux and P flux across the plant-fungal interface

P inflow to roots of both non-mycorrhizal and mycorrhizal plants (rate of P uptake per unit length per unit time) was calculated following the method of Brewster & Tinker (1972). The initial P content per plant was 21.8 µg, based on the average P content in the seed, and initial root length was taken as 0.1 cm. The contribution of the hyphae to the root inflow per m root length (hyphal inflow or *HI*, *sensu* Sanders & Tinker, 1973) was calculated by subtracting inflow in non-mycorrhizal plants from that in mycorrhizal plants. Hyphal P uptake (*HU*, strictly inflow) by the hyphae themselves (mol P m^{-1} external hyphae s^{-1}) was calculated using the length of living external hyphae per m root length and the value for hyphal inflow (*HI*). *HU* is strictly an inflow based on length, not a flux based on area. The *HU* calculated is a minimum value as it takes into account only that P which is detected by analysis of roots and shoots and does not include the P retained in the hyphae in the soil. P uptake by the external hyphae as a flux (mol P m^{-2} area of external hyphae s^{-1}) was also calculated and is referred to as *HF*. The transfer of P from fungus to plant across the symbiotic interface was calculated as a flux (mol P m^{-2} interface area s^{-1}), from hyphal inflow (*HI*) and the area of interface determined as described above (section 2.14).

Details of calculations of P inflow, uptake and flux will be described further in Chapter 4.

2.17. Determination of phosphate and phosphonate

Phosphate and phosphonate levels in plant tissue were measured using ^{31}P nuclear magnetic resonance (NMR) spectroscopy. ^{31}P NMR spectra were obtained on a JEOL FX90Q multinuclear havier transform spectrometer operating at 36.23 MHz using a spectral width of 5 KHz employing 4,000 to 25,000 transients dependent upon tissue sample size. A 35° pulse width and a recycle delay of 2.5 s were used to ensure complete relaxation of the phosphorus signals and ensure accurate quantitation (Jones, Misso & Paleg, 1992). Samples were prepared as follows: 80 to 4,000 mg of dried root or shoot material were homogenised separately in 10 ml of a solvent system consisting of methanol:chloroform:water (12:5:3) using an Ultraturrax homogeniser. The sample was cooled in an ice bath during extraction to prevent excessive heat build-up. The homogenate was centrifuged at 3,000 *g* at 20°C for 10 min and the upper aqueous layer collected. The extraction/centrifugation procedure was repeated three times and the pooled aqueous layer was adjusted to pH 6 - 7, freeze-dried and stored at -70°C.

Samples were dissolved in 2.5 ml of heavy water (D_2O) just prior to NMR measurement. To the solution was added 50 mg solid Na_2EDTA and sufficient solid NaOH to bring the pH of the sample to 13. The solution was then filtered through a glass wool filter into a 10 mm NMR tube and the measurement taken. The pH adjustment and EDTA addition were necessary to remove the extreme line broadening effects of paramagnetic species present in the plant

tissue. Time course experiments carried out on the pH adjusted samples showed that phosphate and phosphonate were very stable at pH 13. Alkyl phosphonate is not as stable at this pH, however. When the presence of alkyl phosphonate was to be confirmed the measurements were carried out quickly (< 30 min). Phosphate and phosphonate peak positions and intensities were referenced against an external capillary reference containing 25% Na_3PO_4 in D_2O. Quantitation of phosphate and phosphonate in the samples was achieved by constructing calibration curves by measuring the NMR intensities of various known concentrations of phosphate and phosphonate with the same capillary reference.

The phosphate, alkyl phosphonate and phosphonate ^{31}P NMR positions were identified both by spiking the samples with authentic compounds and from proton coupled spectra. The alkyl phosphonate was also confirmed from its ^{13}C and ^{1}H NMR spectra. Referenced against Na_3PO_4, alkyl phosphonate, inorganic phosphate and phosphonate had chemical shifts of 1.11 ppm, 0.84 ppm and -1.70 ppm respectively at pH 13 (see Fig. 2.2).

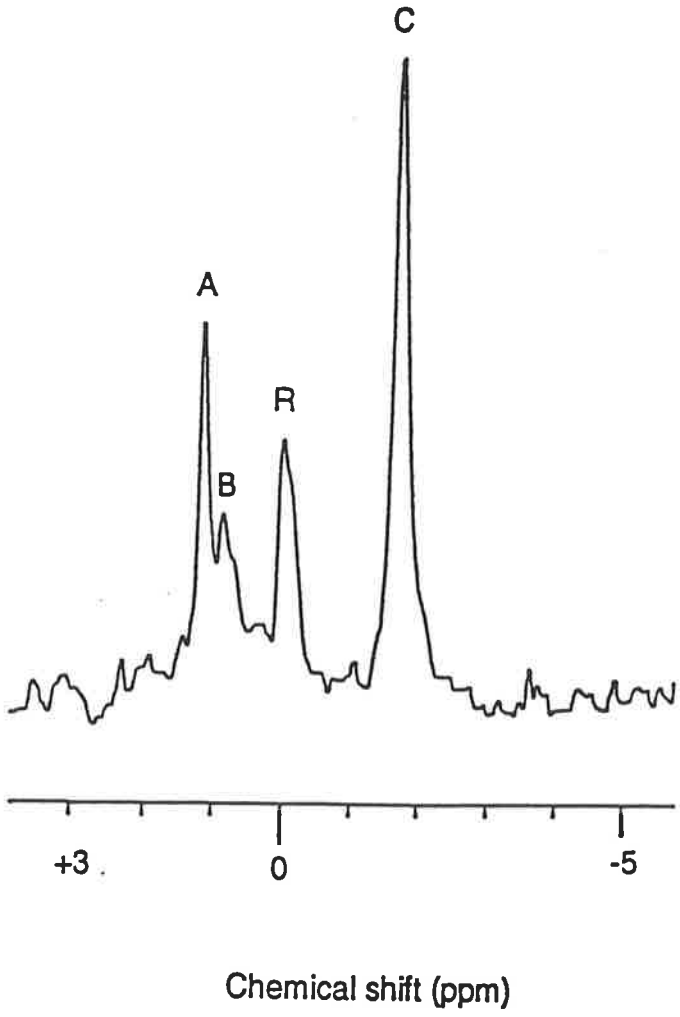


Figure 2.2. ^{31}P spectrum showing the signals of alkyl phosphonate (A), inorganic phosphate (B) and phosphonate (C) externally referenced against a capillary containing Na_3PO_4 (R). The chemical shifts at pH 13 were 1.11, 0.84 and -1.70 ppm, respectively.

2.18. Statistical analysis

Results were analyzed with the Genstat 5 computer package (Lawes Agricultural Trust; Rothamsted Experimental Station) using an analysis of variance on a single or double factor RCBD. Linear contrasts were performed to determine the source of any significant differences between treatments.

CHAPTER 3. THE EFFECTS OF PHOSPHORUS AND FUNGICIDES ON PLANT GROWTH, PHOSPHORUS UPTAKE AND MYCORRHIZAL DEVELOPMENT

3.1. Introduction

The effect of fungicides on plant growth (both non-mycorrhizal and mycorrhizal plants) and mycorrhizal infection in the presence and absence of supplementary P will be discussed in this chapter.

Previous studies of the effects of systemic fungicides on the extent of mycorrhizal colonization have been discussed in Chapter 1 section 2. In summary, most previous research has assessed the effects of fungicides by measuring the percentage infection by the fungus using the non-vital stain trypan blue. Results were interpreted as being direct effects of the fungicide on the fungus. In these studies the effect of fungicides on plant growth in the absence of the fungus was not taken into account. The importance of considering plant growth in the absence of the fungus in determining side-effects of fungicides on VA mycorrhizal symbiosis has been discussed in Chapter 1 sections 1.2.2. and 1.2.8.1.

As systemic fungicides are taken up by the plant they can influence not only the fungus but also the physiology and metabolism of the plant (Vyas, 1988). The effects on the fungus can be direct and/or indirect through the host plant. Hence, when assessing the effects of fungicides on the VA mycorrhizal

symbiosis it is important to separate out the effects on the fungus from those on the plant. The separation of the direct effects of fungicides on the fungus can be achieved easily with other types of mycorrhizas (e.g. ectomycorrhiza and orchid mycorrhiza) because these fungi can be cultured in the absence of host plants. However, in the VA mycorrhizas the fungus is an obligate symbiont, therefore direct effects of fungicides on the fungus are more difficult to determine.

Direct effects of fungicides on plant growth in the VA mycorrhizal symbiosis must be investigated using non-mycorrhizal plants. However, the experimental design must take into account the following:

a) that positive effects of VA mycorrhizal fungi on plant growth are displayed in P- deficient soil;

b) in P-deficient soil non-mycorrhizal plants will have a lower P nutrition, lower dry weight and a higher root/shoot ratio compared with mycorrhizal plants (Harley & Smith, 1983), possibly confounding effects of experimental variables such as fungicide application. Therefore, in choosing appropriate conditions for non-mycorrhizal control plants, it is important to attempt to select plants which have "matching" growth (root length and shoot dry weight) and P nutrition (P concentration and P content) with mycorrhizal plants, to prevent fungicide effects being confounded with P deficiency or toxicity.

One of the approaches in establishing suitable control plants is to grow a non-mycorrhizal plant which is "matched" in growth, root/shoot ratio and P

concentration to the mycorrhizal plant. This is achieved by choosing the appropriate level of P in the soil. Several groups have previously used this approach with varying success (e.g. Eissenstat et al., 1993; Graham et al., 1991; Rousseau & Reid, 1991; Syvertsen & Graham, 1990). It is usually possible to grow plants that are "matched" for one or two parameters, but it has proved very difficult to achieve equivalence for growth rate and hence root and shoot dry weight, as well as P concentration and root length. In general, mycorrhizal plants of the same total dry weight as non-mycorrhizal P-supplemented plants have lower root/shoot ratios, higher shoot fresh weight/dry weight ratios and higher P concentrations (Oliver et al., 1983). Therefore, in this study, plant growth characteristics will be considered rather than nutrient status in selecting P levels for the control "matched" plants.

Previous research on the side-effects of fungicides on plants has been discussed in Chapter 1 section 1.2.8.1. To date there is only one report, using the fungicide Benlate, which took this problem into account when assessing mycorrhizal responses (Carey et al., 1992). This paper was published after the experiments reported in this chapter had been completed. No information has been reported in the literature on the effects of the fungicides Aliette or Ridomil on plant growth.

The objective of this chapter is to analyse the effects of fungicides on VA mycorrhizal symbiosis, by considering individual effects on plant and fungal

growth. To accomplish these objectives, an experiment was conducted with the following aims:

1. to study the effect of systemic fungicides on plant growth and P uptake in non-mycorrhizal plants, including non-mycorrhizal "matched" plants;
2. to assess the development of external hyphae in mycorrhizal plants;
3. to analyse mycorrhizal development in the root and,

consequently, the effect of fungicides on growth of mycorrhizal plants.

3.2. Materials and Methods

Methodology for sowing, application of fungicides, watering regimes, fertilising techniques, growth conditions and inoculation techniques were as described in Chapter 2. There were four fungicide treatments: Benlate, Aliette, Ridomil and the control. Fungicide concentrations (a.i.) in the soil were: Benlate 31.25 mg kg^{-1}, Aliette 82.33 mg kg^{-1} and Ridomil 12.5 mg kg^{-1}. Two levels of P: 0 (P0) and 15 mg P (P1) added g^{-1} soil, were applied to both uninoculated and inoculated pots. These levels of P were chosen on the basis of preliminary experiments.

Two destructive harvests were conducted, at 3 and 6 weeks after planting and there were three replicate pots per treatment at each harvest (except for characteristics of infection where n=2). Data for root length, shoot dry weight, shoot P concentration and uptake, percentage infection (obtained using the stain trypan blue), number and perimeter of living intercellular hyphae, uninvaginated arbuscules and vesicles (obtained from root sections stained

for perimeters of living intercellular hyphae and uninvaginated arbuscules were used to calculate the interface between plant and fungus (see Chapter 4). Using this area of interface and data for hyphal inflow, the P transfer from the fungus to the plant could be calculated.

Mycorrhizal plants grown in a soil/sand mix with no added P (MP0) were compared with non-mycorrhizal plants grown with 0 and 15 μg g^{-1} (NMP0 and NMP1). Non-mycorrhizal "matched" plants (NMP1) were used to study the effects of fungicides on plant growth in the absence of the fungus. Mycorrhizal plants with added P (MP1) were included for completeness of the experimental design.

3.3. Results and Discussion

Complete data for all treatments at both harvests are provided in Tables 3.1a,b and 3.2a,b. For clarity, the results are described and briefly discussed in three sections below. The same data (e.g. for the control plants) are included in more than one section for comparative purposes and to maintain continuity of the theme. The overall results and conclusions from this experiment are considered in section 3.4.

3.3.1. Production of "matched" plants

3.3.1.1. Results

At the 3-week harvest, inoculation with the mycorrhizal fungus had no significant effects on root length, shoot dry weight, P concentration and content, whereas P supply increased all parameters except shoot dry weight (Table 3.1a). At the 6-week harvest (Fig. 3.1, A,B and Table 3.1b), both inoculation with the mycorrhizal fungus (M) and application of P increased plant growth in terms of root length, shoot dry weight, P concentration and P content compared with uninoculated plants grown in the absence of P (NMP0).

Comparison of data for MP0 with NMP0 and NMP1 plants indicates that NMP1 plants were most closely "matched" to MP0 plants at the 6-week harvest (Fig. 3.1). However, NMP1 plants had greater root lengths and shoot dry weights (SDW) (Fig 3.1, A,B). Despite the smaller root length and SDW, by the 6-week harvest mycorrhizal plants (MP0) had both greater shoot P concentration and total shoot P compared with NMP1 plants (Fig 3.1, C,D).

Table 3.1a. Effects of inoculation with *Glomus* sp. "City Beach" (WUM 16), phosphorus (P) and fungicide application on root length, shoot dry weight, shoot P concentration and shoot P content of *Allium cepa* L. at the 3-week harvest

Treatments	Root length (cm)	Shoot dry weight (mg)	Shoot P concentration (µg P mg^{-1})	Shoot P content (µg P $plant^{-1}$)
NMP0				
Control	14.02 ± 3.16	11.00 ± 2.00	1.33 ± 0.06	14.43 ± 2.15
Benlate	8.28 ± 0.09	6.67 ± 0.67	2.16 ± 0.31	13.96 ± 0.85
Aliette	8.60 ± 2.21	7.67 ± 0.88	5.61 ± 0.35 ***	42.45 ± 2.62***
Ridomil	11.00 ± 3.40	5.67 ± 0.67	2.51 ± 0.22*	16.76 ± 2.33
NMP1				
Control	40.07 ± 2.72	16.67 ± 0.88	2.95 ± 0.38	48.69 ± 5.61
Benlate	18.19 ± 1.60**	12.67 ± 0.67*	2.93 ± 0.10	37.12 ± 2.06*
Aliette	9.33 ± 0.55**	8.00 ± 0.58***	7.98 ± 1.05***	62.41 ± 3.47*
Ridomil	24.01 ± 6.77*	7.00 ± 1.00***	3.91 ± 0.12	27.32 ± 3.65***
MP0				
Control	22.00 ± 5.19	9.33 ± 1.67	1.85 ± 0.16	16.76 ± 2.26
Benlate	13.36 ± 5.13	7.00 ± 1.00	2.07 ± 0.12	14.28 ± 1.62
Aliette	7.73 ± 1.67	5.67 ± 1.20	5.77 ± 0.70***	31.56 ± 4.44*
Ridomil	7.60 ± 3.02	6.00 ± 1.00	3.43 ± 0.12*	28.82 ± 4.03
MP1				
Control	54.33 ± 13.72	13.33 ± 1.67	2.78 ± 0.19	37.39 ± 6.19
Benlate	16.99 ± 2.07**	14.33 ± 3.28	2.38 ± 0.24	29.00 ± 3.40
Aliette	18.04 ± 2.82**	9.33 ± 0.33	5.46 ± 0.61***	50.64 ± 4.28*
Ridomil	15.91 ± 2.87**	6.33 ± 2.88	3.99 ± 0.41	24.62 ± 1.59

Values are means and standard errors of three replicate pots, one plant per pot; NM, non-mycorrhizal; M, mycorrhizal; P0, no added P; P1, 15 µg P g^{-1} soil; ***, **, * Significant at 0.001, 0.01, 0.05 levels of probability, respectively.

Table 3.1b. Effects of inoculation with *Glomus* sp. "City Beach" (WUM 16), P and fungicide application on root length, shoot dry weight, shoot P concentration and shoot P content of *Allium cepa* L. at the 6-week harvest

Treatments	Root length (cm)	Shoot dry weight (mg)	Shoot P concentration (μg P mg^{-1})	Shoot P content (μg P $plant^{-1}$)
NMP0				
Control	64.18 ± 12.14	14.00 ± 1.00	1.19 ± 0.34	16.45 ± 3.51
Benlate	26.09 ± 2.08**	12.67 ± 0.88	1.39 ± 0.42	18.31 ± 6.52
Aliette	6.24 ± 0.64**	9.00 ± 1.00*	6.74 ± 0.48***	61.21 ± 6.32*
Ridomil	42.81 ± 12.03*	11.00 ± 1.00@	1.14 ± 0.11	14.88 ± 2.68
NMP1				
Control	269.91 ± 50.68	88.00 ± 3.51	1.86 ± 0.26	164.27 ± 25.30
Benlate	250.39 ± 16.95	90.00 ± 23.54	2.34 ± 0.15	217.66 ± 72.01
Aliette	19.43 ± 2.26***	18.67 ± 0.88**	8.12 ± 0.35***	150.93 ± 11.17
Ridomil	55.18 ± 17.15***	15.67 ± 5.78**	3.53 ± 0.11***	54.68 ± 21.80@
MP0				
Control	163.49 ± 25.55	64.67 ± 6.12	3.12 ± 0.13	201.73 ± 23.56
Benlate	34.92 ± 10.43***	20.33 ± 3.48***	2.29 ± 0.43	48.94 ± 16.97***
Aliette	17.95 ± 1.87***	21.67 ± 0.88***	4.94 ± 0.52**	108.76 ± 11.75*
Ridomil	31.68 ± 7.56***	16.00 ± 3.06***	3.88 ± 0.33	61.95 ± 14.03*
MP1				
Control	451.87 ± 52.44	139.00 ± 14.93	3.75 ± 0.15	518.13 ± 40.18
Benlate	183.73 ± 24.79***	72.67 ± 15.32**	2.99 ± 0.27	225.23 ± 67.62***
Aliette	58.57 ± 5.22***	40.67 ± 3.48 ***	4.77 ± 0.35*	193.85 ± 23.98***
Ridomil	29.28 ± 5.15***	12.67 ± 3.48***	4.31 ± 0.35	58.53 ± 20.15***

Values are means and standard errors of three replicate pots, one plant per pot; NM, non-mycorrhizal; M, mycorrhizal; P0, no added P; P1, 15 μg P g^{-1} soil; ***, **, *, @ Significant at 0.001, 0.01, 0.05, 0.1 levels of probability, respectively.

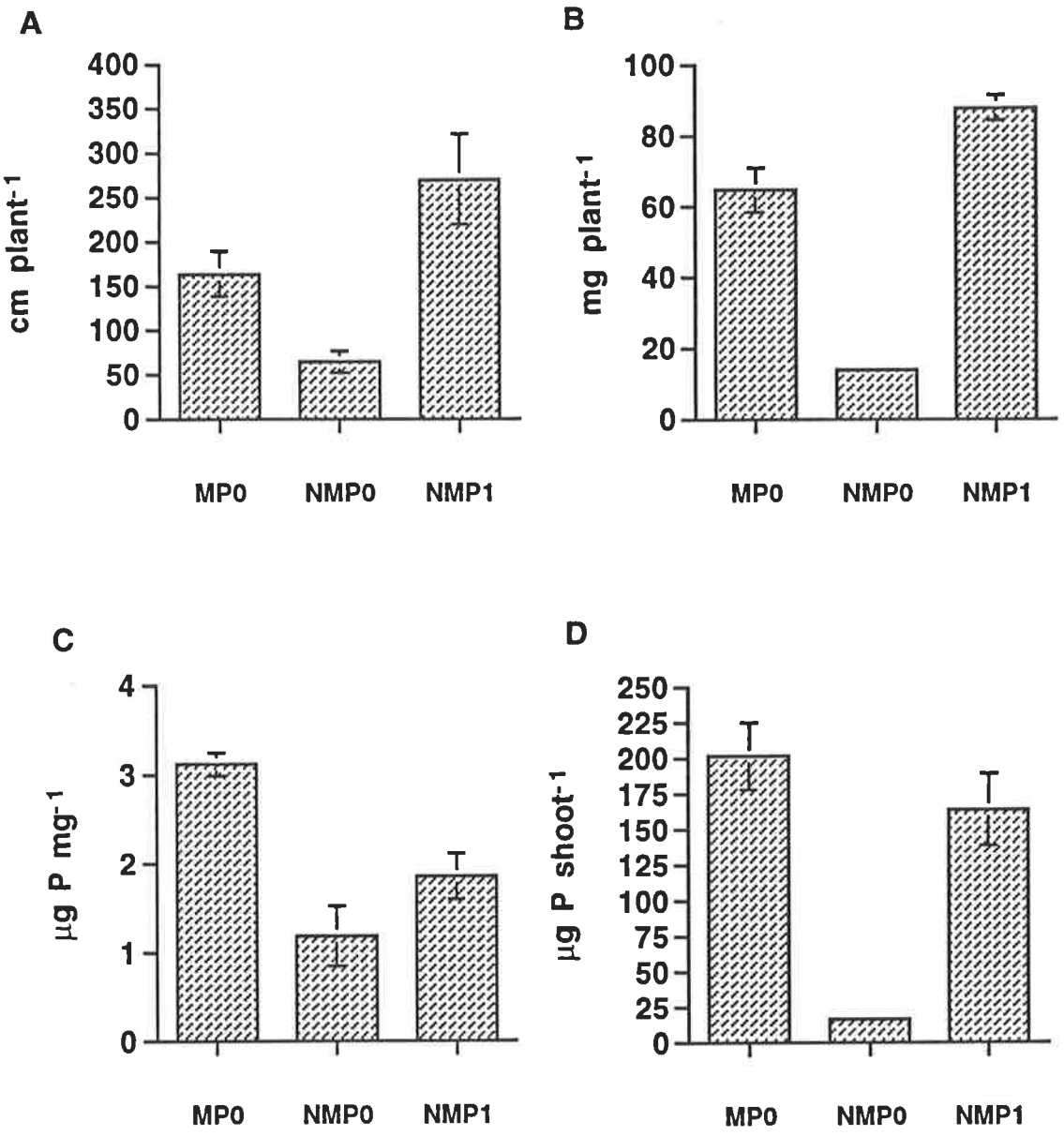


Figure 3.1. Effects of inoculation with *Glomus* sp. "City Beach" (WUM 16) and phosphorus (P) application on root length (A), shoot dry weight (B), shoot P concentration (C) and shoot P content (D) of *Allium cepa* L. at the 6-week harvest. Vertical bars represent standard errors of the means, n=3; if no bar is shown, standard error is smaller than the line.

3.3.1.2. Discussion

The comparison between mycorrhizal plants and non-mycorrhizal plants to which two levels of P had been added confirmed that it is difficult to establish plants equivalent in both growth and P nutrition. The differences in their physiology make it difficult to achieve an exact equivalence. Environmental factors such as light can influence the growth of plants. Preliminary experiments conducted in a glasshouse showed that adding 20 µg P mg^{-1} soil to NM pots resulted in the growth of NM plants similar to MP0 plants (data not shown). However, in the experiment undertaken in a growth room, where the light conditions were different, plants grown in 15 µg P mg^{-1} soil (NMP1) had greater root length and shoot dry weight and lower P concentration and content than MPO plants.

In conclusion, approximately "matched" non-mycorrhizal plants were produced by addition of 15 µg P mg^{-1} soil. In general, these plants were most similar to MP0 plants, particularly with respect to shoot dry weight and root length. No further experiments were carried out to optimise conditions for producing "matched" plants. As root length influences percentage of infection, similarity in root length is the most important characteristic for control plants in this experiment. The effects of fungicides on plant growth (both P-deficient and P-sufficient plants) in the absence of the fungus will be discussed in the following section.

3.3.2. The effects of fungicides on plant growth in the absence of infection

3.3.2.1. Results

3.3.2.1.1. The effects of fungicides on root length and shoot dry weight

In P-deficient plants (NMP0) none of the three fungicides had a significant effect on root length or shoot dry weight at the 3-week harvest (Table 3.1a). However, at the 6-week harvest there was significant reduction in root length following applications of all three fungicides and a significant reduction in shoot dry weight in plants treated with Aliette and Ridomil (Table 3.1b)

In P-sufficient plants (NMP1), root length and shoot dry weight were significantly reduced by all the three fungicides compared with the control without fungicide at the 3-week harvest (Table 3.1a). At the 6-week harvest the three fungicides had different effects on the root length and shoot dry weight of these plants (Fig. 3.2, A,B). Benlate had no effect on either root length or shoot dry weight in comparison to the control. Aliette and Ridomil, on the other hand, significantly reduced both the root length and shoot dry weight. Aliette had a greater effect on root length than Ridomil (Fig. 3.2, A).

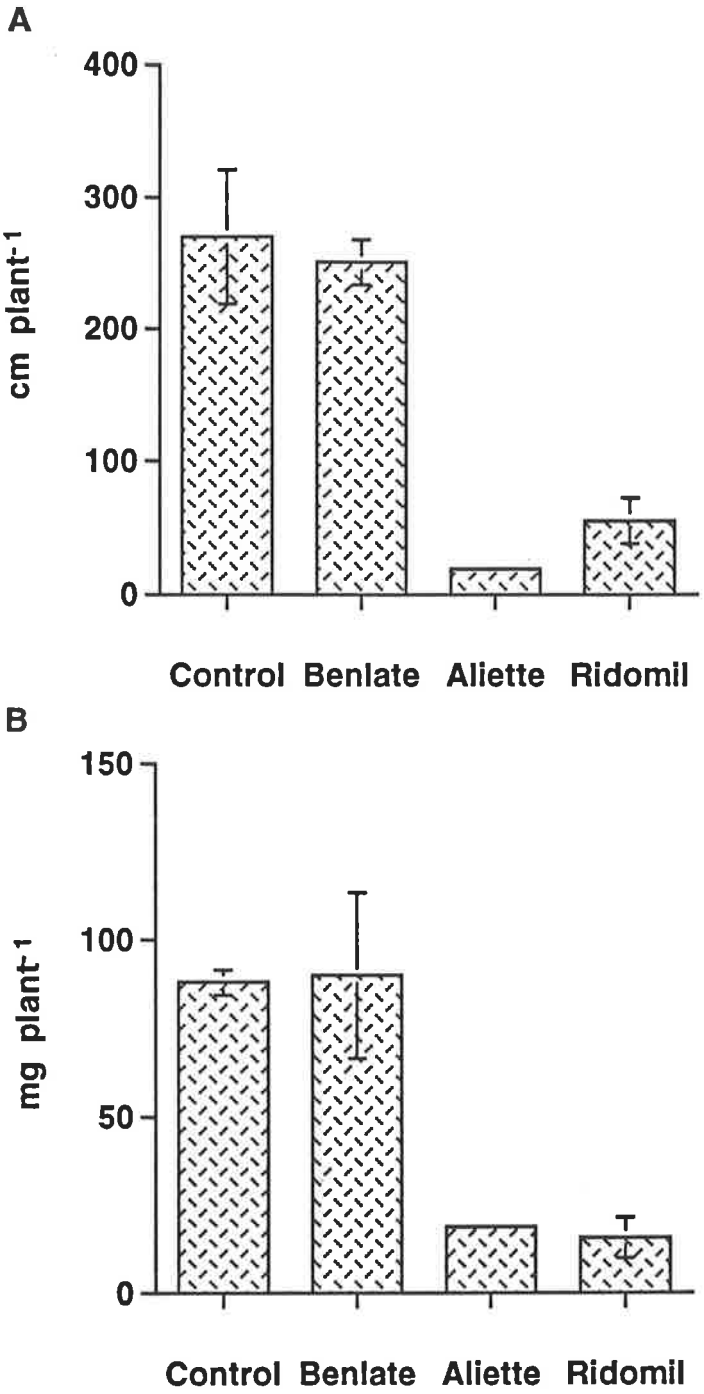


Figure 3.2. Effects of Benlate, Aliette and Ridomil on root length (A) and shoot dry weight (B) of non-mycorrhizal "matched" control (NMP1) plants at the 6-week harvest. Vertical bars represent standard errors of means, n=3; if no bar is shown, standard error is smaller than the line.

3.3.2.1.2. The effects of fungicides on P concentration and uptake

The P concentration of P-insufficient plants (NMPO) at the 3-week harvest was not affected by Benlate, whereas it was significantly increased by Aliette and Ridomil (Fig. 3.3, A). Aliette increased the concentration 3-fold compared with the control value. Shoot P content was also increased by Aliette, while Benlate or Ridomil application had no effects (Fig. 3.3, B). At the 6-week harvest, only Aliette increased the P concentration and shoot P content whereas Benlate and Ridomil had no effect (Fig. 3.3, C,D).

The three fungicides had different effects on the P concentration and content of P-sufficient plants (NMP1) (Fig. 3.4). Benlate had no effect on the P concentration and content at either harvest (Fig. 3.4). Ridomil decreased P content significantly at both harvests (Fig. 3.4, B,D) but had variable effects on the P concentration which was unaffected at the 3-week harvest (Fig. 3.4, A), but increased at the 6-week harvest (Fig. 3.4, C). Aliette increased both the P concentration and content at the 3-week harvest (Figs 3.4, A,B). P concentration was also increased by Aliette at the 6-week harvest (Fig. 3.4, C) but P content was not significantly affected (Fig. 3.4, D).

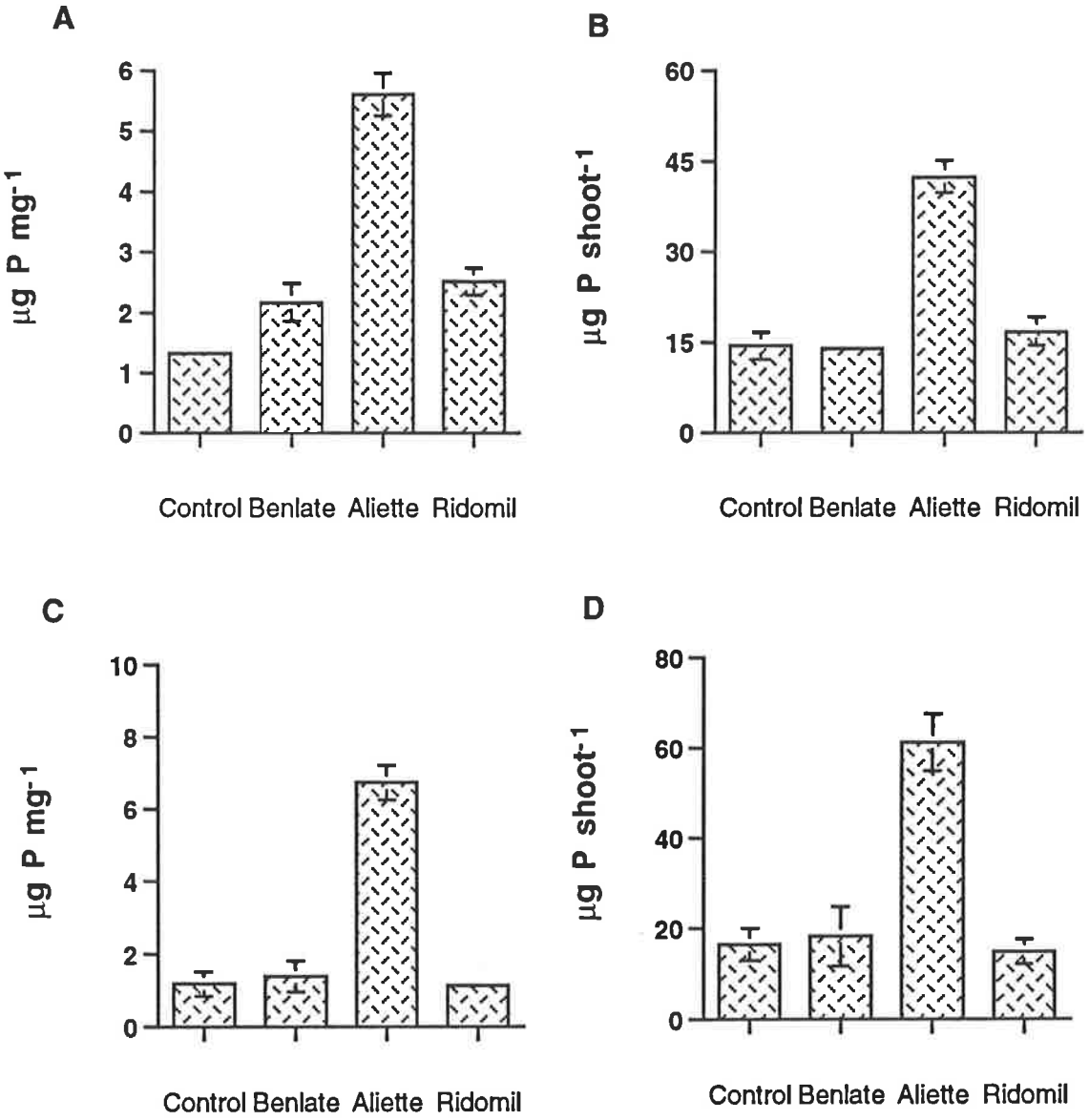


Figure 3.3. Effects of Benlate, Aliette and Ridomil on shoot P concentration and shoot P content at the 3-week harvest (A and B, respectively) and at the 6-week harvest (C and D, respectively) of non-mycorrhizal P-insufficient control (NMP0) plants. Vertical bars represent standard errors of means, n=3; if no bar is shown, standard error is smaller than the line.

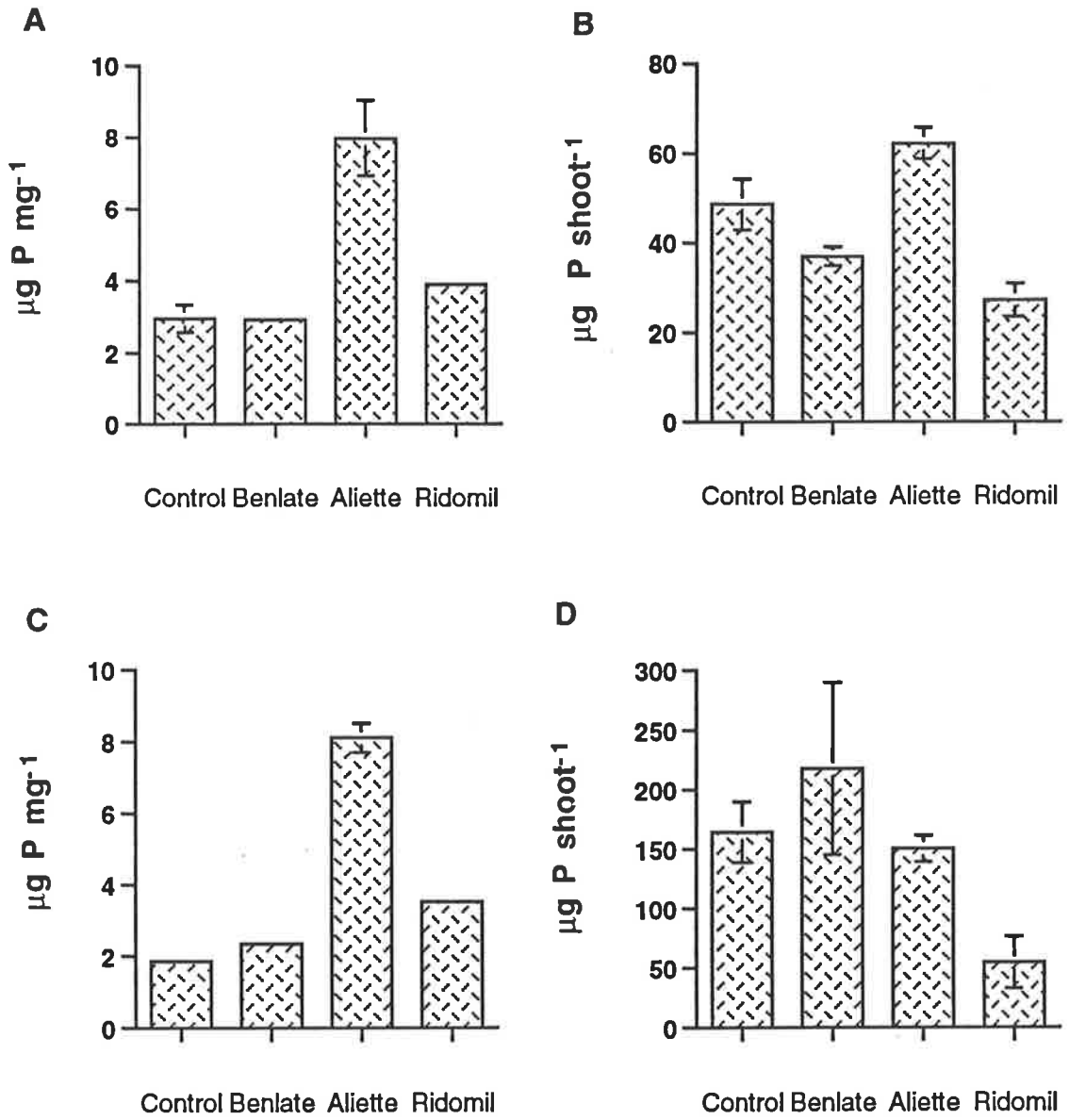


Figure 3.4. Effects of Benlate, Aliette and Ridomil on shoot P concentration and shoot P content at the 3-week harvest (A and B, respectively) and at the 6-week harvest (C and D, respectively) of non-mycorrhizal "matched" control (NMP1) plants. NM, non-mycorrhizal; P1, added 15 µg P g^{-1} soil. Vertical bars represent standard errors of means, n=3; if no bar is shown, standard error is smaller than the line.

3.3.2.2. Discussion

Non-mycorrhizal P-insufficient plants (NMP0) and P-sufficient plants (NMP1) had different responses to applications of fungicide. This illustrates the importance of considering "matched" plants as a control to assess fungicide effects on plant growth in the absence of the fungus. In general, Benlate had small effects on growth and P nutrition of P-insufficient plants. The reduction in root length, following Benlate application, of P-insufficient plants at the 6-week harvest may be due to confounding effects of P-deficiency and fungicide action. Benlate affected the shoot dry weight of the 3-week old plants (NMP1), but this effect was not apparent at 6-weeks. The reduction of growth at 3 weeks may have resulted from a delay in seed germination caused by Benlate application, since this fungicide was applied as a soil drench at the time of planting. A preliminary experiment showed that seed germination in Benlate-treated pots was delayed by 3 days. However, by the 6-week harvest the plants were able to compensate for slow initial growth caused by Benlate. The lack of observable effects at the 6-week harvest in Benlate-treated plants was in agreement with the work of Paul et al. (1989), who reported that Benlate application had no effects on the growth of a wide range of plants in the absence of fungi. P-sufficient plants were unaffected by Benlate application, both with respect to growth and P nutrition. Consequently, effects of this fungicide on mycorrhizal plants (see below) can be safely attributed to direct effects on the fungus.

Aliette and Ridomil, on the other hand, had consistent effects on both groups of plants in respect to plant growth whereas effect on P nutrition was

variable. The striking feature in plants treated with Aliette was that the shoot P concentration at both harvests and content at the 3-week harvest, were significantly increased. This is thought to be due to accumulation of phosphonate, an active ingredient of this fungicide, in the plant (Chapter 5). The method used to measure the amount of P in the plants in this experiment did not distinguish phosphonate (HPO_3^{2-}) from phosphate (PO_4^{3-}). Hence, values for total P include both forms.

Although the P concentration of Aliette-treated plants was four times higher than for the untreated controls, shoot P uptake was not always significantly different, because shoot growth was markedly stunted. Such stunted growth may be the result of high concentrations of phosphonate relative to phosphate in the plant. Phosphonate can be toxic to plants (Seymour et al., 1994; Wellings et al., 1990) and it is possible that its accumulation was responsible for the inhibition of growth of Aliette-treated plants. An alternative explanation is that aluminium, another degradation product of Aliette, accumulated in the soil. This element can also interfere with plant growth if it is taken up by the plant in excessive amounts and may cause aluminium toxicity in soils with low pH (Rengel, 1992). It is possible that the combined effects of phosphonate and aluminium accumulation may be greater than their individual effects on plant growth. Experiments were carried out to clarify this, using ^{31}P NMR spectra to differentiate the individual P compounds as discussed in Chapter 5.

Aliette seemed to have more severe effects on root growth than on shoot growth. In the situation where root growth is reduced by application of fungicide, the extensive hyphal network of VA mycorrhizal fungi may play an

important role in replacing the root function in nutrient uptake (see below and Chapter 5).

Apart from a slight increase in P concentration at the 6-week harvest, Ridomil clearly had negative effects on all aspects of plant growth at both 3 and 6-week harvests.

The reduction in growth of plants following Aliette or Ridomil application will lead to difficulties in interpreting the effects of these fungicides on VA mycorrhizal symbiosis (see section 3.3.3. below).

In summary, Benlate had no effects on plant growth, whereas application of Aliette and Ridomil resulted in reduction in growth of host plants. This information was taken into account when interpreting fungicidal effects on VA mycorrhizal symbiosis in the subsequent discussion.

3.3.3. Effects of fungicides on mycorrhizal plants

3.3.3.1. Summary of statistical analysis

There were significant (5% level) interactions between fungicide and P treatment and between fungicide and mycorrhiza for root length and shoot dry weight at the 6-week harvest. The interaction showed that P addition increased root length and shoot dry weight. The magnitude of the effects on both parameters was small in Aliette and Ridomil treatments and larger in Benlate and control treatments. In the presence of mycorrhiza, a small reduction was observed in root length following Benlate application, whereas root length

increased in control, Aliette and Ridomil treatments. Shoot dry weight in the presence of mycorrhiza increased in control and Aliette treatments whereas small reductions were observed following applications of Benlate and Ridomil.

3.3.3.2. The effects of fungicides on mycorrhizal plant growth

At the 3-week harvest, in the absence of P supplement, there was a tendency for all three fungicides to reduce the root length (Table 3.1a). Benlate reduced it to about 50%, whereas Aliette and Ridomil reduced it to 30% of the control values. There were no fungicide effects on shoot dry weight (Table 3.1a). At the 6-week harvest, the reductions in root length (Table 3.1b and Fig. 3.5, A) and shoot dry weight (Table 3.1b and Fig. 3.5, B) were significant in all fungicide treatments. Aliette had the most pronounced effect on root length followed by Benlate and Ridomil (Fig. 3.5, A). However, for shoot dry weight, the most severe effect was observed following application of Ridomil, followed by Benlate and Aliette (Fig. 3.5, B). Plants treated with Aliette but not harvested were observed to recover from stunting by approximately 12 weeks (see Chapter 5 section 5.2.2.1).

In the presence of the P supplement, at the 3-week harvest all three fungicides significantly reduced root length, whereas shoot dry weight was not affected significantly (Table 3.1a). However, at the 6-week harvest both root length (Fig. 3.6, A) and shoot dry weight (Fig. 3.6, B) were severely decreased following the application of all three fungicides.

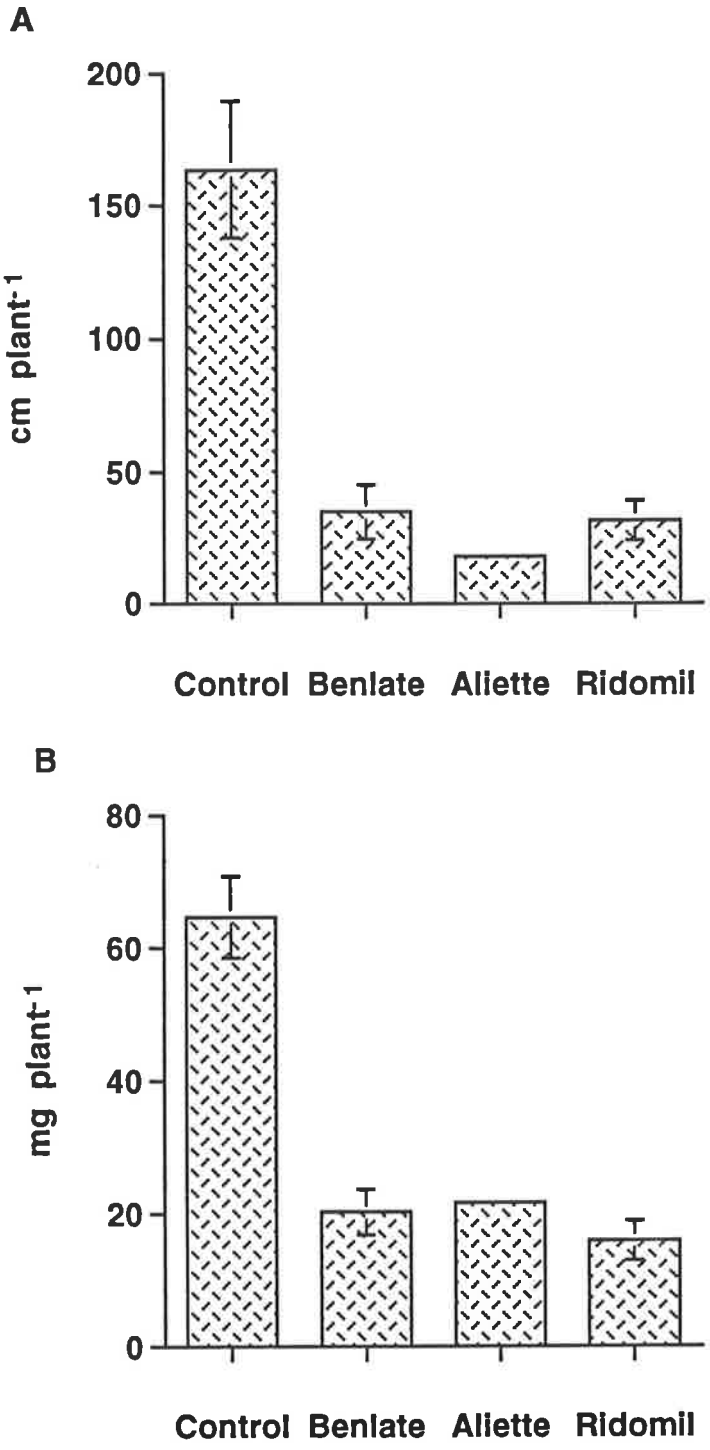


Figure 3.5. Effects of Benlate, Aliette and Ridomil on root length (A) and shoot dry weight (B) of mycorrhizal plants in the absence of P supplement (MP0) at the 6-week harvest. Vertical bars represent standard errors of means, n=3; if no bar is shown, standard error is smaller than the line.

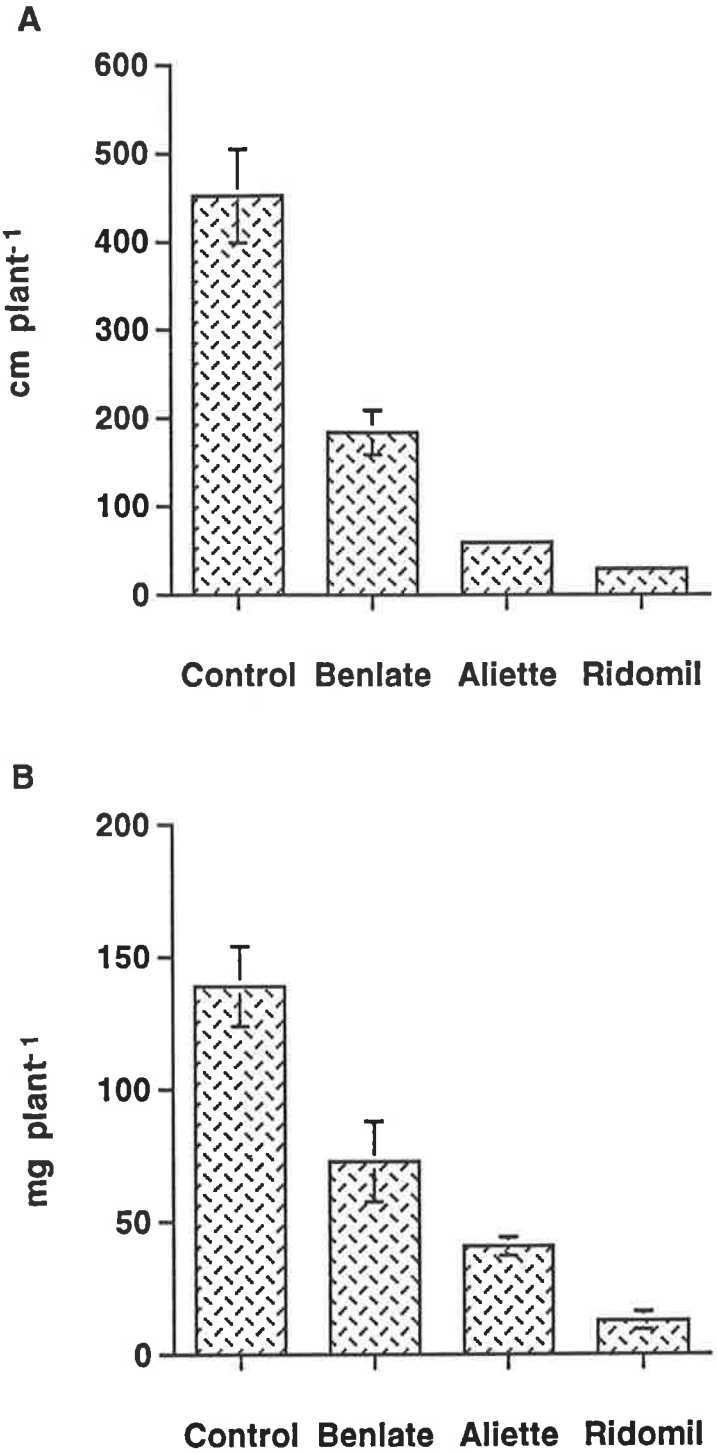


Figure 3.6. Effects of Benlate, Aliette and Ridomil on root length (A) and shoot dry weight (B) of mycorrhizal plants in the presence of P supplement (MP1) at the 6-week harvest. Vertical bars represent standard errors of means, n=3; if no bar is shown, standard error is smaller than the line.

3.3.3.3. The effects of fungicides on P nutrition

In the absence of the P supplement (MP0), the three fungicides had different effects on the P concentration in mycorrhizal onion plants. At the 3-week harvest, Benlate had no effect, whereas Ridomil increased the concentration of P two times, and Aliette three times, that of the control value (Fig. 3.7, A). The effects on P concentration following application of Aliette were also observed at the 6-week harvest, whereas Benlate or Ridomil had no significant effects at that time (Fig. 3.7, C). Fig. 3.7, (B) shows that at 3 weeks, P content was not affected by Benlate, whereas Aliette and Ridomil significantly increased P content. At the 6-week harvest the P content was significantly decreased with all fungicide treatments (Fig. 3.7, D). The high concentration of P at both harvests and high P content at the 3-week harvest following application of Aliette was due to accumulation of phosphonate in the plant (see Chapter 5), an effect also observed in the non-mycorrhizal plants (see above).

In the presence of the P supplement, Benlate and Ridomil had no effect on P concentration in the plants whereas Aliette increased the concentration at both harvests (Table 3.1a,b). Shoot P content was not affected by Benlate and Ridomil application at 3 weeks (Table 3.1a) but at 6 weeks the shoot P content was significantly reduced (Table 3.1b). Aliette significantly increased the shoot P content at the 3-week harvest (Table 3.1a). However, at the 6-week harvest the P content was significantly reduced (Table 3.1b) following Aliette application, and the explanation provided for non-mycorrhizal plants also applies here.

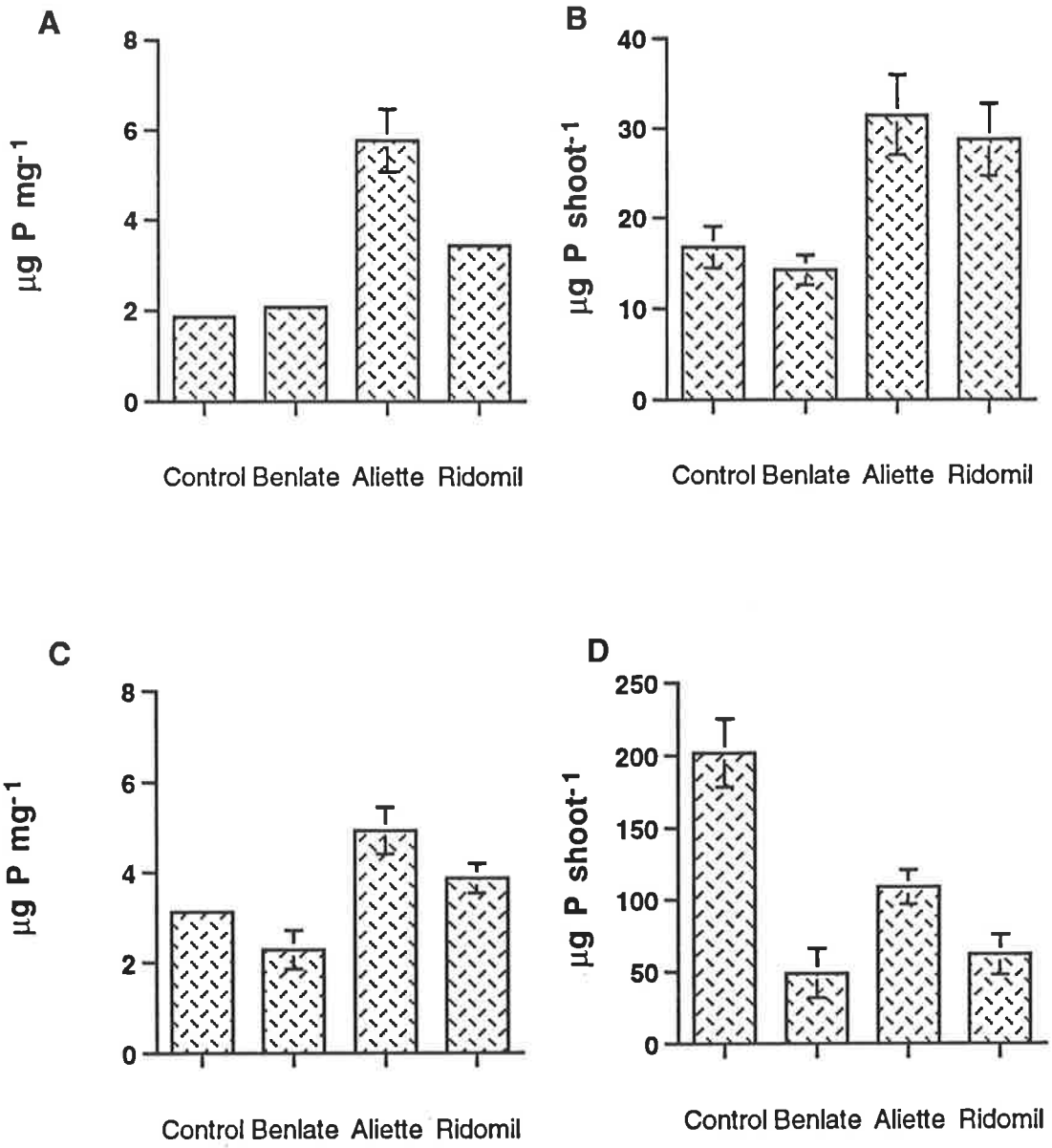


Figure 3.7. Effects of Benlate, Aliette and Ridomil on shoot P concentration and shoot P content at the 3-week harvest (A and B, respectively) and at the 6-week harvest (C and D, respectively) of MP0 plants. MP0, mycorrhizal and no added P. Vertical bars represent standard errors of means, n=3; if no bar is shown, standard error is smaller than the line.

3.3.3.4. The effects of fungicides on root length infected and percentage infection

Regardless of the presence or absence of the P supplement, roots were colonised by mycorrhizal fungi in all treatments and the length of root infected and percentage root infected increased between the 3-week and 6-week harvests (Figs 3.8 - 3.9). However, comparison between untreated control and fungicide treatments indicated that all three fungicides reduced the length of root infected and percentage infection at both harvests (Figs 3.8 - 3.9).

In the absence of the P supplement, the effects of Benlate and Aliette on percentage infection showed a similar trend to that for the length of root infected. At both the 3-week (Fig. 3.9, A) and 6-week (Fig. 3.9, C) harvests the percentage infection was significantly reduced by these fungicides, with the most severe effects obtained after Benlate application. There was a tendency for Ridomil to reduce the percentage infection at both harvest times but the results were less clear cut than for the other fungicides.

In the presence of the P supplement, the percentage infection was reduced markedly at both harvests (Figs 3.9, B,D). Benlate application had the most severe effects followed by Aliette and Ridomil.

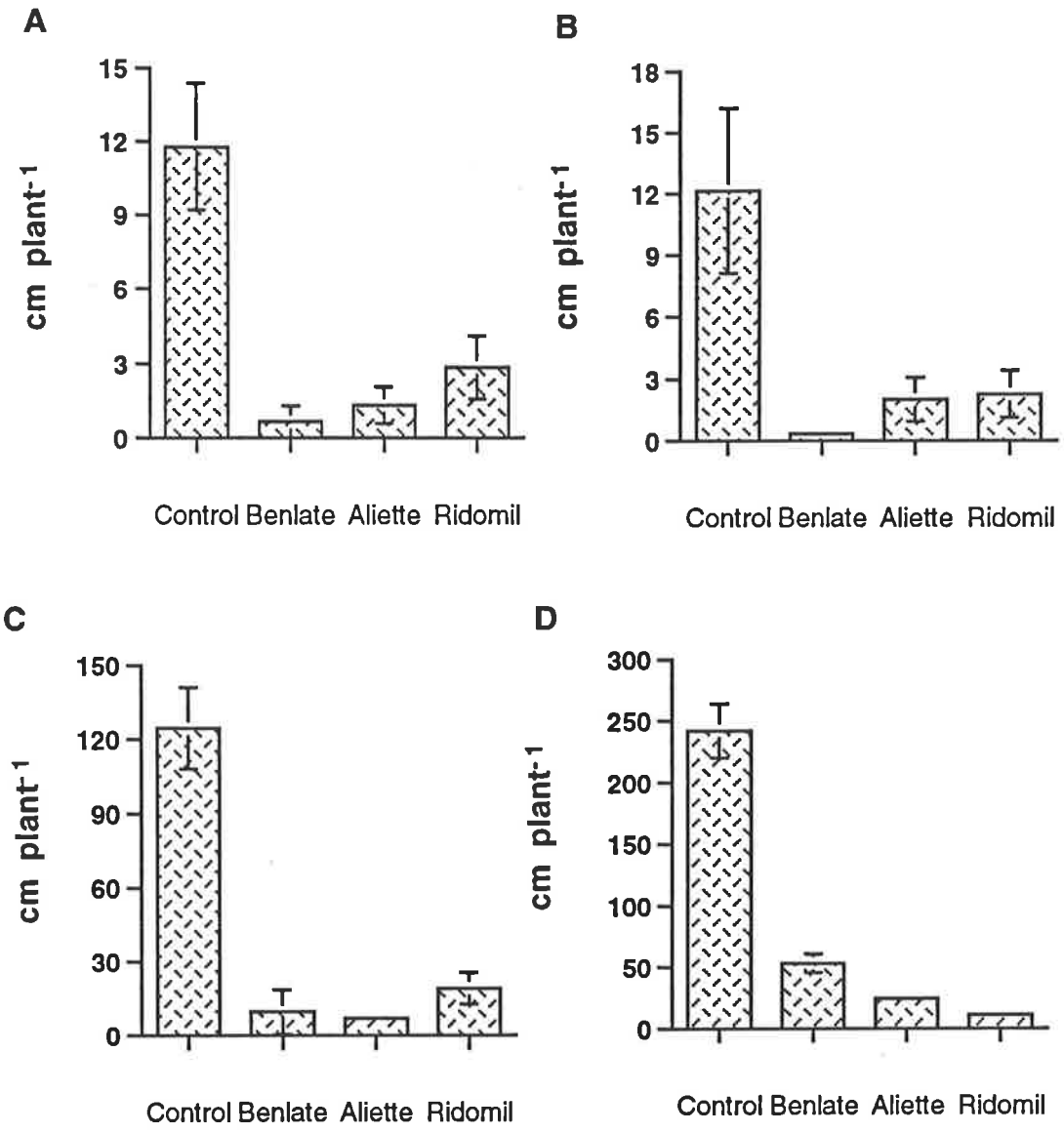


Figure 3.8. Effects of Benlate, Aliette and Ridomil on length of root infected per plant by *Glomus* sp. "City Beach" (WUM 16) at the 3-week and the 6-week harvests for MP0 (A and C, respectively) and MP1 (B and D, respectively). MP0, mycorrhizal and no added P; MP1, mycorrhizal and 15 μg P g^{-1} soil. Vertical bars represent standard errors of means, n=3; if no bar is shown, standard error is smaller than the line.

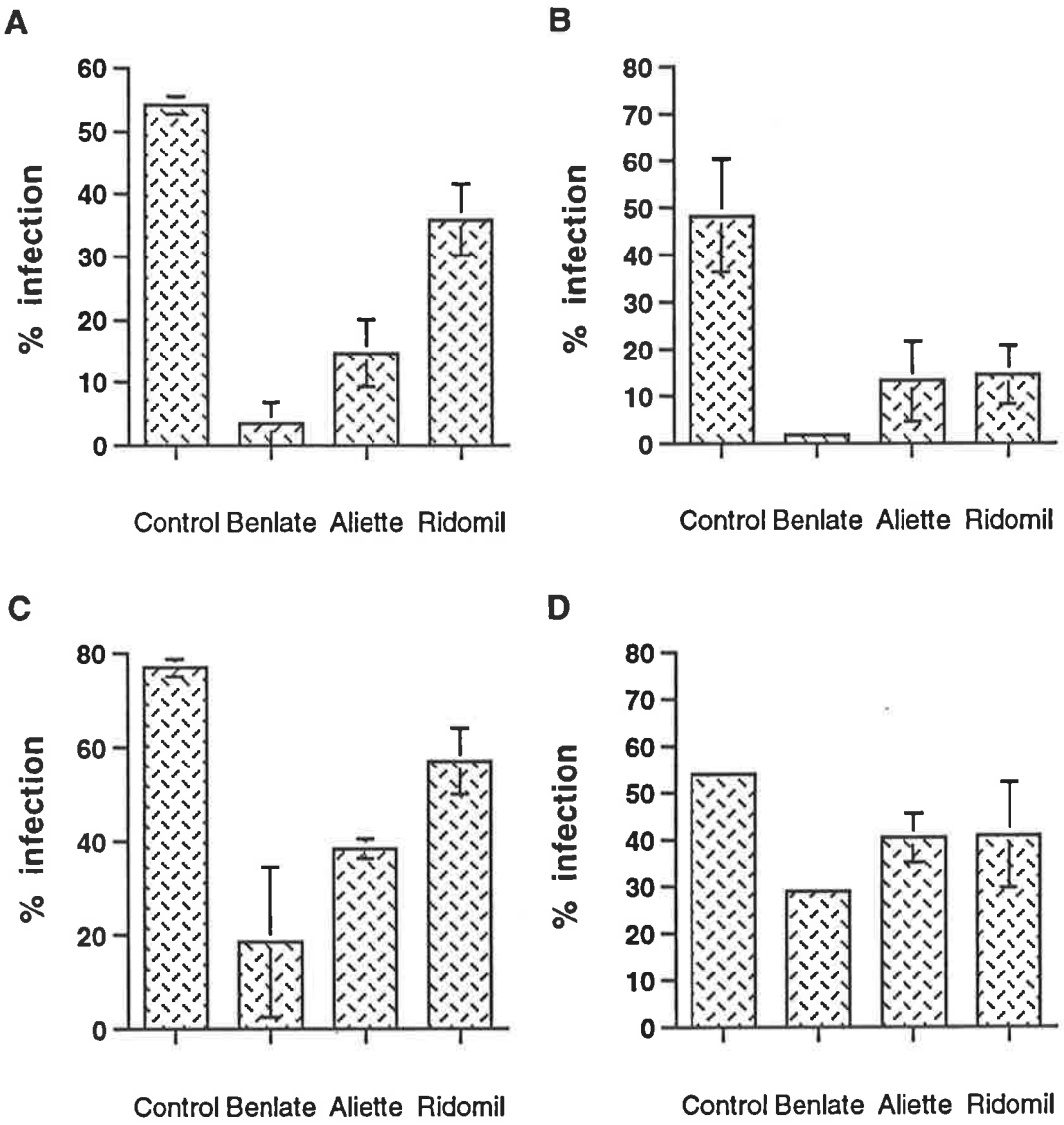


Figure 3.9. Effects of Benlate, Aliette and Ridomil on percentage infection per plant by *Glomus* sp. "City Beach" (WUM 16) at the 3-week and the 6-week harvests for MP0 (A and C, respectively) and MP1 (B and D, respectively). MP0, mycorrhizal and no added P; MP1, mycorrhizal and 15 μg P g^{-1} soil. Vertical bars represent standard errors of means, n=3; if no bar is shown, standard error is smaller than the line.

3.3.3.5. The effects of fungicides on living external hyphae

Study of the effects of fungicides on development of external hyphae of VA mycorrhizal fungi was complicated by the problem of differentiating between hyphae of non-VA mycorrhizal fungi (saprophytic Zygomycetes) and those of VA mycorrhizal fungi when hyphae fragmented into small pieces during processing. However, results of preliminary experiments in which hyphae were observed as long fragments indicated that most fungi in inoculated pots, with and without fungicide, were mycorrhizal.

In the absence of P supplement, at the 3-week harvest (Fig. 3.10) the length of hyphae per g soil was clearly greater in the untreated control inoculated (MP0) (Fig. 3.10, B) than in the uninoculated (NMP0) treatment (Fig. 3.10, A). Addition of P to the uninoculated control treatment (NMP1) had little effect on length of hyphae (Fig. 3.11, A). Therefore, at this time the effects of fungicides on hyphae in MP0 can be taken to include effects on mycorrhizal hyphae as well as on hyphae of saprophytes (Fig. 3.10, B).

No attempt was made to determine length of mycorrhizal hyphae by subtracting values in uninoculated pots from those in inoculated pots. Preliminary experiments (see Chapter 2 section 2.11.) had shown possible differences in turnover of saprophytic and mycorrhizal hyphae in the soil which made this approach impracticable.

At the 6-week harvest the results were more complex because there were no clear differences between hyphal length in the soil in controls for inoculated (MP0, Fig. 3.10, D and MP1, Fig. 3.11, D) and uninoculated pots (NMP0, Fig.

3.10, C and NMP1, Fig. 3.11, C). Although the living external hyphae in inoculated pots could be distinguished as mycorrhizal fungi, on the basis of morphological characteristics, and showed the same relative response to fungicides as at the 3-week harvest, the growth of saprophytic fungi (NMP0 and NMP1) made data for this harvest difficult to interpret. There was a clear effect of Benlate in reducing the length of hyphae in soil. The reductions were most marked in inoculated pots (Figs 3.10, D and 3.11, D), but were still clear in uninoculated pots (Figs 3.10, C and 3.11, C). Both Aliette and Ridomil application also produced significant reductions in hyphal length per g of soil in inoculated pots (Fig. 3.10, D). In uninoculated pots, the effects of Ridomil or Aliette were not significant at either level of P.

In the presence of the P supplement, all three fungicides initially reduced the length of external hyphae (Fig. 3.11, B) but by the 6-week harvest these effects were not apparent (Fig. 3.11, D).

Interestingly, when the length of external hyphae was expressed per cm of infected root (Fig. 3.12), it appeared that all three fungicides stimulated the growth of hyphae from the roots whether or not the P supplement was present.

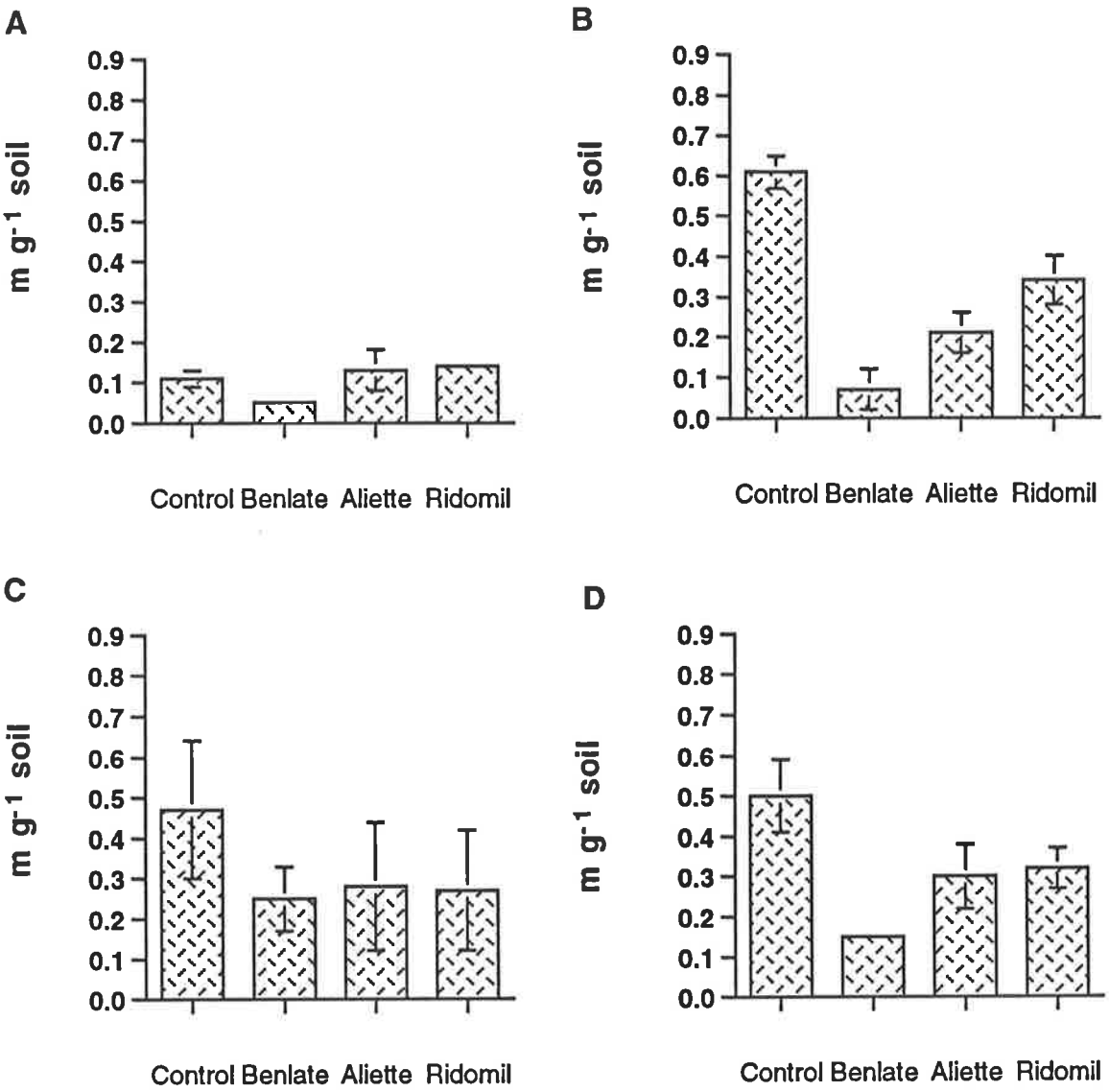


Figure 3.10. Effects of Benlate, Aliette and Ridomil on length of living external hyphae in the soil at the 3-week and the 6-week harvests of NMP0 (A and C, respectively) and MP0 pots (B and D, respectively). NM, non-mycorrhizal; M, mycorrhizal; P0, no added P. Vertical bars represent standard errors of means, n=3; if no bar is shown, standard error is smaller than the line.

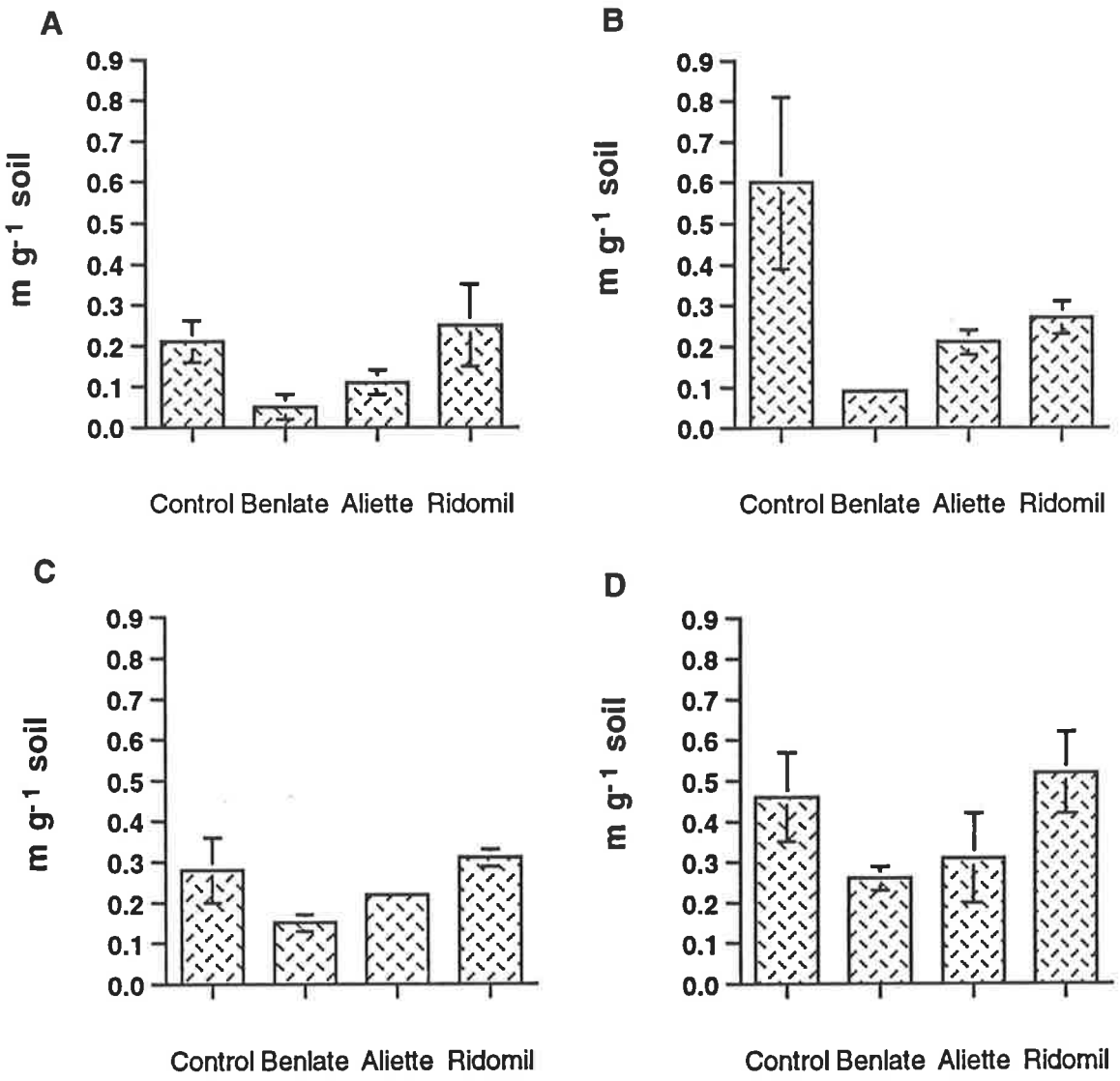


Figure 3.11. Effects of Benlate, Aliette and Ridomil on length of living external hyphae in the soil at the 3-week and the 6-week harvests of NMP1 (A and C, respectively) and MP1 pots (B and D, respectively). NM, non-mycorrhizal; M, mycorrhizal; P1 added 15 µg P g-1 soil. Vertical bars represent standard errors of means, n=3; if no bar is shown, standard error is smaller than the line.

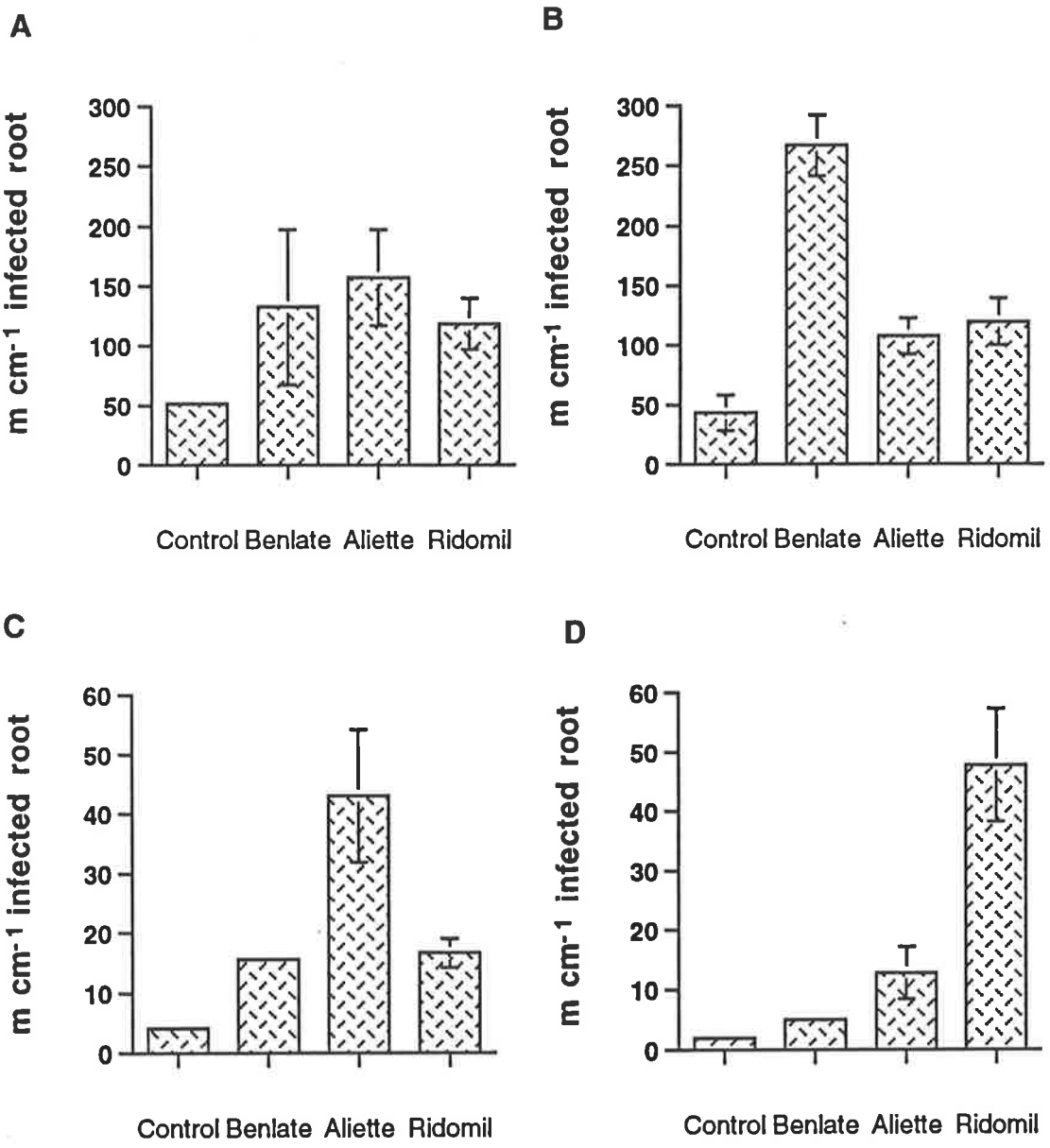


Figure 3.12. Effects of Benlate, Aliette and Ridomil on length of external hyphae per cm infected root at the 3-week and the 6-week harvests of MP0 (A and C, respectively) and MP1 pots (B and D, respectively). M, mycorrhizal; P0, no added P; P1, added 15 μg P g^{-1} soil. Vertical bars represent standard errors of means, n=3; if no bar is shown, standard error is smaller than the line.

3.3.3.6. The effects of fungicides on the intensity and characteristics of infection

The intensity of infection was assessed as mean numbers of intercellular hyphae, arbuscules and vesicles per section. In the absence of the P supplement, all three fungicides had effects on the intensity of infection even though the differences were not always significant (Figs 3.13 and 3.14). Benlate reduced numbers of hyphae, arbuscules and vesicles at both 3 and 6-week harvests (Tables 3.2a,b), this reduction being significant at the 6-week harvest . Aliette had no effect on the intensity of development of intercellular hyphae and arbuscules at either harvest nor on the number of vesicles at 3 weeks. However, at 6 weeks the number of vesicles was reduced. Ridomil reduced all parameters measured at both harvests but this was not significant.

In the presence of P supplement fungicide effects on development of intercellular hyphae, arbuscules and vesicles were small (Table 3.2a,b). The only significant effects were reductions in the number of intercellular hyphae at 3 weeks, following application of Benlate and Ridomil.

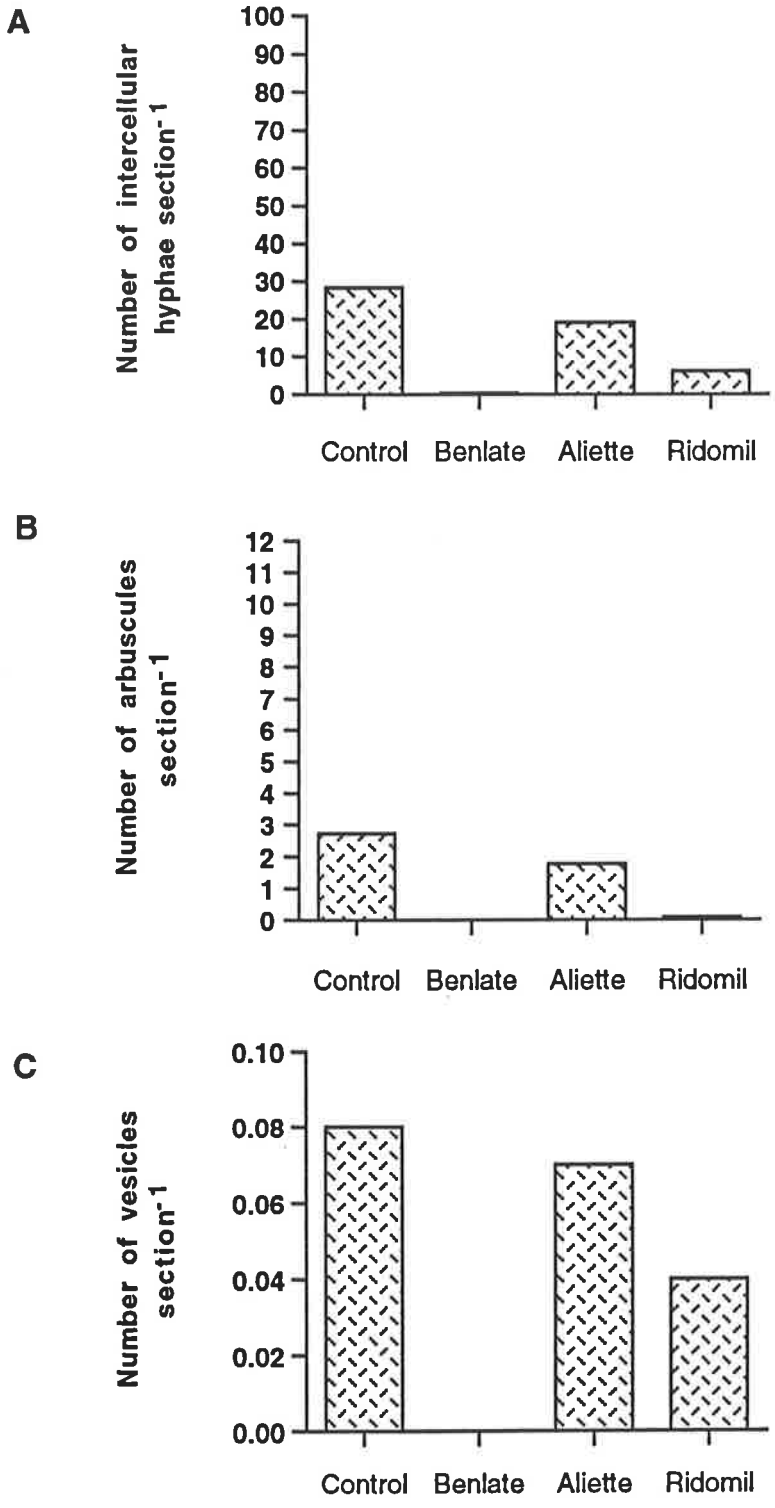


Figure 3.13. Effects of Benlate, Aliette and Ridomil on the number of intercellular hyphae (A), arbuscules (B) and vesicles (C) at the 3-week harvest of MP0 plants. M, mycorrhizal; P0, no added P. Values are means from 2 replicate pots. Significant differences were not seen at the 0.1 level of probability.

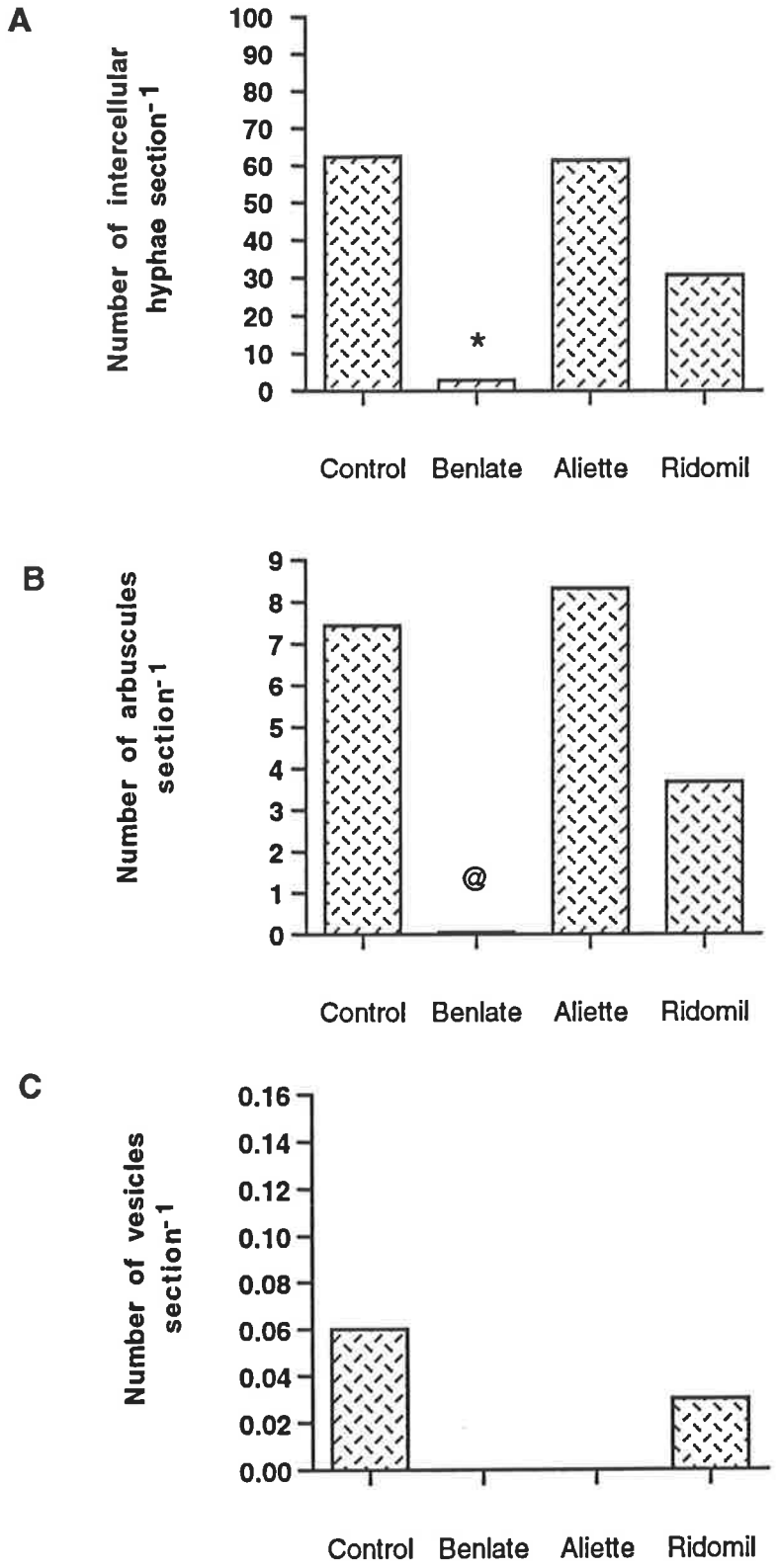


Figure 3.14. Effects of Benlate, Aliette and Ridomil on the number of intercellular hyphae (A), arbuscules (B) and vesicles (C) at the 6-week harvest of MP0 plants. M, mycorrhizal; P0, no added P. Values are means from 2 replicate pots. *, @ Significant at 0.05, 0.1 levels of probability, respectively.

Table 3.2a. Effects of inoculation with *Glomus* sp. "City Beach" (WUM 16), P and fungicide application on root length infected, percentage infection, length of living external hyphae and intensity and characteristics (number of living intercellular hyphae, arbuscules and vesicles per root section) of infection of *Allium cepa* L. at the 3-week harvest

Treatments	Root length infected (cm)	Percentage infection (%)	External hyphae (m g^{-1} soil)	External hyphae (m cm^{-1} infected root)	No. of intercellular hyphae $section^{-1}$	No. of arbuscules $section^{-1}$	No. of vesicles $section^{-1}$
NMPO							
Control	NA	NA	0.11 ± 0.02	NA	NA	NA	NA
Benlate	NA	NA	0.05 ± 0.00	NA	NA	NA	NA
Aliette	NA	NA	0.13 ± 0.05	NA	NA	NA	NA
Ridomil	NA	NA	0.14 ± 0.00	NA	NA	NA	NA
NMP1							
Control	NA	NA	0.21 ± 0.05	NA	NA	NA	NA
Benlate	NA	NA	0.05 ± 0.03	NA	NA	NA	NA
Aliette	NA	NA	0.11 ± 0.03	NA	NA	NA	NA
Ridomil	NA	NA	0.25 ± 0.10	NA	NA	NA	NA
MPO							
Control	11.79 ± 2.58	54.18 ± 1.34	0.61 ± 0.04	51.32 ± 3.45	28.23	2.72	0.08
Benlate	0.65 ± 0.65**	3.40 ± 3.40***	0.06 ± 0.05***	132.49 ± 64.68	0.36	0.001	0.00
Aliette	1.31 ± 0.73**	14.63 ± 5.35***	0.21 ± 0.05***	157.01 ± 40.29@	19.20	1.77	0.07
Ridomil	2.85 ± 1.27**	35.86 ± 5.60*	0.34 ± 0.06**	118.33 ± 21.41	6.08	0.09	0.04
MP1							
Control	12.12 ± 4.02	48.22 ± 11.86	0.59 ± 0.21	43.24 ± 14.54	13.64	1.26	0.00
Benlate	0.34 ± 0.33**	1.71 ± 1.65**	0.09 ± 0.01@	267.32 ± 25.36***	1.33@	0.32	0.00
Aliette	2.00 ± 1.07*	13.17 ± 8.60 *	0.22 ± 0.03*	107.63 ± 14.94*	8.75	1.69	0.02
Ridomil	2.26 ± 1.14*	14.48 ± 6.33*	0.27 ± 0.04*	119.58 ± 19.60**	0.33@	0.001	0.00

Values are means and standard errors of three replicate pots (except for intensity and characteristics of infection where n=2), one plant per pot; NM, non-mycorrhizal; M, mycorrhizal; P0, no added P; P1, 15 µg P g^{-1} soil; NA, not applicable; ***, **, *, @ Significant at 0.001, 0.01, 0.05, 0.1 levels of probability, respectively.

Table 3.2b. Effects of inoculation with *Glomus* sp. "City Beach" (WUM 16), P and fungicide application on root length infected, percentage infection, length of living external hyphae and intensity and characteristics (number of living intercellular hyphae, arbuscules and vesicles per root section) of infection of *Allium cepa* L. at the 6-week harvest

Treatments	Root length infected (cm)	Percentage infection (%)	External hyphae (m g^{-1} soil)	External hyphae (m cm^{-1} infected root)	External hyphae at harvest period 3 - 6 weeks (m cm^{-1} root)	No. of intercellular hyphae $section^{-1}$	No. of arbuscules $section^{-1}$	No. of vesicles $section^{-1}$
NMP0								
Control	NA	NA	0.47 ± 0.17	NA	NA	NA	NA	NA
Benlate	NA	NA	0.25 ± 0.08	NA	NA	NA	NA	NA
Aliette	NA	NA	0.28 ± 0.16	NA	NA	NA	NA	NA
Ridomil	NA	NA	0.27 ± 0.15	NA	NA	NA	NA	NA
NMP1								
Control	NA	NA	0.28 ± 0.08	NA	NA	NA	NA	NA
Benlate	NA	NA	0.15 ± 0.02@	NA	NA	NA	NA	NA
Aliette	NA	NA	0.22 ± 0.01	NA	NA	NA	NA	NA
Ridomil	NA	NA	0.31 ± 0.02	NA	NA	NA	NA	NA
MP0								
Control	124.67 ± 16.56	76.85 ± 1.93	0.50 ± 0.09	4.03 ± 0.74	15.29 ± 1.16	62.17	7.44	0.06
Benlate	9.69 ± 9.10***	18.52 ± 15.97**	0.15 ± 0.00**	15.55 ± 0.43	5.38 ± 1.51*	2.77 *	0.05 @	0.00
Aliette	6.94 ± 0.94***	38.44 ± 2.12*	0.30 ± 0.08	43.08 ± 11.20**	21.48 ± 1.83**	61.33	8.34	0.00
Ridomil	19.12 ± 6.35***	57.04 ± 7.03	0.32 ± 0.05*	16.64 ± 2.40	27.21 ± 3.91**	30.71	3.67	0.03
MP1								
Control	242.26 ± 21.81	242.26 ± 21.81	0.46 ± 0.11	1.91 ± 0.44	5.23 ± 1.56	34.85	5.56	0.03
Benlate	53.16 ± 7.76***	53.16 ± 7.76***	0.26 ± 0.03	4.95 ± 0.65	3.40 ± 0.17	23.79	2.99	0.00
Aliette	24.23 ± 4.95***	24.23 ± 4.95***	0.31 ± 0.11	12.83 ± 4.36	8.62 ± 1.38*	32.65	5.57	0.00
Ridomil	10.87 ± 1.25***	10.87 ± 1.25***	0.52 ± 0.10	47.75 ± 9.95***	17.36 ± 2.01***	11.46	1.12	0.00

Values are means and standard errors of three replicate pots (except for intensity and characteristics of infection where n=2), one plant per pot.; NM, non-mycorrhizal; M, mycorrhizal; P0, no added P; P1, 15 µg P g^{-1} soil; NA, not applicable; ***, **, *, @ Significant at 0.001, 0.01, 0.05, 0.1 levels of probability, respectively.

3.3.4. Discussion

Growth and P nutrition. The effects of fungicides on the growth and P nutrition of mycorrhizal plants can only be satisfactorily interpreted by comparison with the effects on non-mycorrhizal plants (see section 3.3.2) and will be postponed until the general discussion at the end of this chapter. This section will be restricted to discussion of mycorrhizal development both in soil and in the plant, because this information is essential to the interpretation of data for growth and P uptake.

Mycorrhizal infection. In all treatments mycorrhizal fungi colonised the roots and there were increases in both percentage infection and infected root length between the two harvests, indicating that the processes of colonisation were not completely inhibited by fungicide application. In the control treatments values were typical for previous experiments of this type (Hale & Sanders, 1982). The length of living external hyphae in the untreated control pots at 3 and 6 weeks was 0.5 and 0.6 m g^{-1} soil, respectively. This is close to the range (0.01 - 0.12 m g^{-1}) measured by Schubert et al. (1987) using the same vital staining method and much lower than values obtained using trypan blue staining (6.67 - 54 m g^{-1} soil, Abbott and Robson, 1985; Allen and Allen, 1986; Sylvia, 1992). Hamel et al. (1990) estimated that about 65% of the mycelium of *Glomus intraradices* associated with alfalfa was metabolically active at 6 weeks, while O'Connor (unpublished) found between 25 and 50% of the length of *Glomus etunicatum* in association with *Allium porrum* to be metabolically active after the same time. Applying maximum and minimum correction factors

of 25% and 65%, total hyphae per g of soil might be roughly estimated as between 0.8 and 2.4 m g^{-1}, which is still low compared with published estimates. The length of living hyphae expressed per cm infected root in untreated control (MP0) pots were 51.32 and 4.03 m cm^{-1} root at the 3 and 6-week harvest, respectively. The value was much higher at 3 weeks than at 6 weeks. The difference may be due to large amounts of hyphae produced by germinating propagules or may reflect real differences in the timing of internal and external colonisation. Estimates of the length of external hyphae per cm of infected root measured using the stain trypan blue have ranged from 0.8 to 14.22 m cm^{-1} infected root at harvests between 28 and 54 days (Sanders & Tinker, 1973; Abbott & Robson, 1985; Jakobsen et al., 1992a). Again a correction could be applied. The mean of the 3-week and 6-week values was used to calculate hyphal P uptake (see Chapter 4).

All three fungicides studied had negative effects on the fungus in soil and in the root. In summary, Benlate had the most consistently severe effects on fungal development and growth within the root, regardless of P supplement. Infection was delayed and arbuscules were almost totally eliminated. Development of living external hyphae was also consistently reduced when estimated per g of soil. However, the ratio of external to internal infection (length of hyphae per cm infected root) actually increased (as it was with both other fungicides). Aliette reduced the infected root length per plant at both harvests and the percentage infection at 3 weeks. However, values for percentage infection were confounded by the significant reductions in root growth produced by this fungicide (see above). There were no significant

effects of this fungicide on intensity and characteristics of infection within the root, but neither was there any evidence to support previous observations (see discussion below) of increased infection of roots. As with Benlate, length of hyphae per g of soil was reduced, but the ratio of external to internal infection was higher than for controls. Ridomil not only reduced the extent of infection (m per plant and percentage infection) but also tended to reduce the numbers of intercellular hyphae and arbuscules per section.

3.4. General discussion

The three fungicides studied had different effects on the partners of the VA mycorrhizal symbiosis and each will be discussed separately.

Benlate appears to act directly on the fungus and the results obtained here confirm previous work (Kough et al., 1987; West et al., 1993a,b). Benlate affected all aspects of fungal development. Not only were the fraction of the root length infected and total root length markedly reduced, as described previously (Carey et al., 1992; Jalali & Domcsh, 1975; Fitter & Nichols), but also reductions in intensity of infection and length of living external hyphae were demonstrated. Within the root, the number of intercellular hyphae and arbuscules were markedly reduced. This led to a reduction in the area of living interface between the symbionts through which nutrient transfer occurs (see Chapter 4). In addition, the length of external hyphae was also clearly reduced, an aspect which has not been studied previously. The reduction in development of external hyphae limited the contribution of the fungus in taking up P from the soil. Again, the negative effects on area of interface would have

hampered the efficiency of P transfer from the fungus to the root. These combined effects led to a reduction in total P uptake on a per plant basis, to the same level as NMP0 plants, and hence plant growth was reduced to a similar extent. The relatively small effect of Benlate on P uptake and growth of MP1 plants confirms the idea that elimination of fungal-mediated P uptake is the mechanism underlying growth reduction following application to MP0 plants. Despite the marked reduction in the intensity and the living fungal-plant interface (see Chapter 4) by Benlate application, the fungus was still able to colonize the root, and the small amount of fungal tissue in the root was metabolically active.

Aliette and Ridomil were found to act both on the plant and the fungus. In non-mycorrhizal plants, application of either of these fungicides resulted in reduction in shoot and root growth, complicating the analysis of the effects on the mycorrhizal fungus.

Both total root length infected and percentage infection were reduced following the application of Aliette and Ridomil, with the effects of Aliette being more marked than those of Ridomil. No evidence was found to support the previous work showing increases in mycorrhizal infection (percentage infection) following application of Aliette (Jabaji-Hare & Kendrick, 1987). These workers applied the fungicide after the infection had been established, which may help to explain the discrepancy. A situation can be envisaged in which the reduction in root growth was greater than the reduction in fungal growth within the root following fungicide application, leading to apparent increases in percentage infection. The results from these experiments emphasize the

importance of considering all of the processes contributing to percentage infection before drawing conclusions.

It is true that Aliette had a relatively minor effect on the intensity of infection with respect to numbers of living arbuscules and intercellular hyphae. However, marked reduction in root growth and fungal growth inside the root will reduce the amount of the fungus on a per plant basis. The effects of Aliette on external hyphae are very interesting. The length of hyphae per pot was clearly reduced but on the basis of the length per cm of root it appeared that development of external hyphae was actually stimulated by this fungicide. Hyphal development, however, was unable to compensate for very poor root growth, hence no differences in plant growth were observed in comparison with the control, at least up to 6 weeks. The very high concentrations of P are thought to be due to accumulation of the degradation product of Aliette, phosphonate in plants (Coffey & Joseph, 1985; Fenn & Coffey, 1985; Cohen & Coffey, 1986 and see Chapter 5). It is, therefore, not possible to correlate development of infection with P uptake and P accumulation in Aliette-treated plants from data presented here. There is a slight indication that excessive accumulation of P is less severe in mycorrhizal plants at the 6-week harvest (compare MP0 and NMP1). In addition, plants stunted due to Aliette application were observed to recover by 12 weeks when they were mycorrhizal whereas non-mycorrhizal plants did not overcome the phytotoxic effects of Aliette application (see Chapter 5 section 5.2.2.1 and Plate 5.1). This was further investigated using Nuclear Magnetic Resonance techniques to distinguish the different P compounds and will be discussed in a subsequent chapter (Chapter 5).

Ridomil reduced the length of infected root, the percentage infection and development of intercellular hyphae and arbuscules. This led to the reduction of the fungal interface within the root. Thus, the reduction of P concentration and uptake by mycorrhizal plants treated with Ridomil are the results of the multiple effects of this fungicide on the fungus-plant interaction. The results support those of Jabaji-Hare & Kendrick (1987), but not of Nemec (1980) which indicated an increase in percentage infection of citrus following Ridomil application. Again, the complications caused by inhibition of both the growth of the fungus and the root make the situation difficult to analyse.

Ridomil had a smaller effect on the development of arbuscules, intercellular hyphae and external hyphae per pot than did Benlate. The length of external hyphae per cm root, however, was increased significantly compared to the control. This situation was similar with Aliette-treated plants where the stimulation of external hyphae per unit length of root was negatively correlated with the reduction in root length. It appears that when the root growth was limited, the plant stimulated the development of hyphae as a compensatory reaction mechanism in order to obtain more nutrition to support plant growth.

Fungicides may affect not only the fungal development but also the efficiency of the fungus to take up P from the soil and transfer it to the plant. The possibility of fungicides affecting the efficiency of the fungus to take up P from the soil and transfer it to the plant will be discussed in Chapter 4.

CHAPTER 4. THE EFFECTS OF FUNGICIDES ON AREA OF INTERFACE AND EFFICIENCY IN P UPTAKE AND TRANSFER TO PLANT IN VESICULAR-ARBUSCULAR MYCORRHIZAL SYMBIOSIS

4.1. Introduction

The effects of systemic fungicides on the extent of mycorrhizal colonisation and plant growth have been discussed in Chapter 3. The function of the symbiosis with respect to P nutrition, however, is determined not only by the percentage of infection and development of external hyphae in the soil, but is influenced by factors such as:

1) the efficiency of the external hyphae in taking up P from the soil;

2) the area of interface between plant and fungus; and

3) the rate of P uptake and transfer to the plant.

All of these contribute to the efficiency of the symbiosis in terms of P nutrition and hence plant growth (Jakobsen et al., 1992a & b; Smith et al., 1994; Smith & Gianinazzi-Pearson, 1988). P inflow (rate of P uptake per unit length of root per unit time) and flux (rate of P transfer per unit area of interface per unit time) are parameters that can be used to measure the role of the VA mycorrhizal symbiosis with respect to P uptake by an infected plant. Environmental and chemical factors acting upon symbiotic function can also be assessed using these parameters.

Apart from one report on arbuscule activity following Benlate application (Kough et al., 1987) and on P inflow into mycorrhizal roots (Hale & Sanders,1982), no attempt has been made to determine the effects of fungicides on the parameters outlined above. In the experiments reported in this chapter, P inflow and flux were used to assess the effects of fungicides on the function of the VA mycorrhizal symbiosis in terms of nutrient uptake. The aim was to determine the effects of Benlate, Aliette and Ridomil on the development of the plant-fungal interface (site of nutrient transfer) and on the function of the VA mycorrhizal symbiosis (the rate of P uptake through external hyphae and its transfer across the living plant-fungal interface), and which stages of the processes of P uptake by VA mycorhizal fungi are more affected by fungicide application. This information would be valuable for manipulating the conditions of VA mycorrhizal infection in the field, if necessary, following fungicide application, to minimise the negative effects on the symbiosis and hence plant growth.

Three types of inflow will be measured:

1) Rate of P uptake per metre of root per second of non-mycorrhizal (NM inflow) and mycorrhizal (M inflow) plants;

2) Rate of P uptake to the roots via the mycorrhizal fungi (hyphal inflow *sensu* Sanders & Tinker, 1973). This will be referred to as "*HI* " and expressed as uptake of P per metre root per second;

3) Rate of P uptake by living external hyphae (uptake of P per m living external hyphae per second). This will be referred as "hyphal uptake" (*HU*) to avoid confusion with hyphal inflow. There are two reasons why hyphal uptake is of interest. First, the findings in the experiment reported in Chapter 3 showed that

Aliette and Ridomil increased the length of external hyphae expressed both per m root and per m infected root. This led to the question of whether the fungicides also stimulate the efficiency of the fungus to take up P to support the overall high uptake, particularly when the growth of roots was stunted. Second, it is inspired by the report in the literature that the reduction in mycorrhizal colonization following Benlate application was not always related to a reduction in P uptake and hence plant growth (West et al., 1993b).

Calculation of P flux across the plant-fungal interface requires data for both the area of symbiotic interface and hyphal inflow (Cox & Tinker, 1976; Smith et al., 1994; Tinker,1975a).The area of symbiotic interface involved in P transfer in this study was considered to be both intercellular hyphal and arbuscular interfaces. The reason was that until now differentiation of function between the two types of interfaces has not been clearly established. Therefore, the possible contribution of the intercellular hyphal interface in P transfer should not be ignored (Tinker, 1975, Smith & Dickson, 1991; Smith et al., 1994).

Data from two experiments were used. Experiment 1 (see Chapter 3 and Tables 3.1a,b and 3.2a,b) was used to calculate values based on uptake into the shoot, because root material was not available. A new experiment (Experiment 2) was carried out with Benlate to provide complete data, including both roots and shoots. This is described here for the first time.

4.2. Materials and Methods

Measurements and calculations used were as follows:

1) P inflow into non-mycorrhizal (NM inflow) and mycorrhizal (M inflow) roots (mol P m^{-1} root s^{-1}). This was calculated from the quantity of P in the plant and root length between two harvest times. In Experiment 1 data for shoot P only were available, whereas in Experiment 2 both shoot P and root P were included in the calculations.

2) Hyphal inflow (*HI*) *sensu* Sanders & Tinker (1973) (mol P^{-1} mroot s^{-1}). This was calculated from values of NM inflow subtracted from those of M inflow.

3) Hyphal uptake (*HU*) (mol P m^{-1} living external hyphae s^{-1}). This was obtained by dividing hyphal inflow values by the mean of length of external hyphae per m root between the two harvests.

4) The area of interface was calculated per m of root and per plant. The interface per m of root was used to calculate P fluxes (see point 5) whereas the interface per plant was used to analyse the effect of fungicide on the quantity of the fungus in the roots.

5) P fluxes across the interface were obtained by dividing *HI* by the area of interface per m root (Smith et al., 1994).

4.2.1. Experiment 1

Methodology for sowing, application of fungicides, watering regimes, fertilising techniques, growth conditions and inoculation technique were as described in Chapters 2 and 3. Two levels of P application (P0 and P1) were used as described in Chapter 3. Two inoculation treatments were used: uninoculated (NM) and inoculated (M) with *Glomus* sp. "City Beach" WUM 16.

There were 10 pots per treatment with one plant per pot. The pots were randomly rearranged three times per week and fertilised weekly. Plants were harvested destructively 3 and 6 weeks after planting.

At each harvest three pots from each treatment were used to measure length of external hyphae, plant growth response and mycorrhizal infection. Two pots were used for measurement of the intensity and characteristics of infection.

P content and concentration in the plant, the intensity of infection, the surface area of plant-fungal interface, the rate of P uptake and transfer across the plant-fungal interface were measured as described in Chapter 2. Data for P inflow in Aliette-treated plants were analysed, but because total P in these plants included both phosphate (absorbed from soil) and phosphonate (from Aliette) as outlined in Chapter 3, the results cannot be compared directly with those for other fungicides.

4.2.2. Experiment 2

Experimental conditions and methods were the same as those in Experiment 1 except that Benlate only was applied. Plants were harvested destructively 2, 3, 4, 5 and 6 weeks after sowing.

At each harvest there were four replicate pots with five plants per pot in order to supply sufficient root material for all measurements. Two root systems were used for examining root length and mycorrhizal infection, one root system for preparing root sections and the remaining two root systems for measuring dry weight and P concentration and content.

4.3. Results

4.3.1. Experiment 1

4.3.1.1. The effects of P application and fungicides on P inflow

The values for P inflow determined in this experiment were minimum estimates, because P content in the root was not included in the calculation of inflow. Table 4.1 shows the effects of P application on inflows in NM and M plants. The application of P increased the P inflow into non-mycorrhizal but not into mycorrhizal roots. This meant that hyphal inflow (obtained by subtracting NM from M values) was apparently reduced by P application. Mean length of hyphae (m m^{-1} root) was also reduced and consequently *HU*, a parameter

used to determine efficiency of P uptake by the fungus from the soil, was significantly increased.

Table 4.2a shows the effects of fungicides on plants without added P (NMP0 and MP0). Benlate and Ridomil application resulted in a relatively small increase and decrease in inflow of P into non-mycorrhizal roots, respectively. However, Aliette apparently increased P inflow markedly. The same effects were observed in P-supplemented plants (see Table 4.2b). Here, the inflows were all higher, corresponding with data for plants not treated with fungicides.

Inflow into mycorrhizal roots in the absence of P supplement (Table 4.2a) was relatively unaffected by Ridomil, whereas it was markedly reduced by Benlate, and Aliette significantly increased the P inflow as before. This increase in both NM and M plants is likely to be due to accumulation of phosphonate, the active ingredient of Aliette (see Chapter 5). In the presence of P supplement (Table 4.2b) the inflow was unaffected by Benlate, whereas it was reduced by application of Ridomil and again increased by Aliette.

Application of fungicides had varied effects on hyphal inflow (Table 4.2a,b). Regardless of P supplement, Benlate and Ridomil both reduced hyphal inflow. Aliette application apparently increased the inflow in the absence of added P (as might be expected given the complications already discussed), but could not be calculated in P-supplemented plants because the MP1 plants had lower root inflows than the NMP1 plants.

4.3.1.2. Length of external hyphae and uptake by external hyphae

Average length of external hyphae per m of root (m m^{-1}) between the two harvests was calculated (Tables 4.1 and 4.2a,b) to estimate hyphal uptake and hence hyphal flux. The length of external hyphae per m of root was reduced significantly by P application (Table 4.1). The fungicides had varied effects on external hyphal development (Table 4.2a,b). In both P treatments, Benlate reduced the length of external hyphae whereas it was increased by Aliette and Ridomil application (Table 4.2a,b).

Using hyphal length (Tables 4.1 and 4.2a,b) and an average diameter of living external hyphae of 3.7 μm, measured using an Image Analysis package (Chapter 2), the surface area of external hyphae was calculated and hence hyphal flux determined (see section 4.2 above). Table 4.1 shows that application of P significantly increased both hyphal uptake per unit length of hyphae (*HU*) and, consequently, hyphal flux. The values obtained for the flux were 0.24×10^{-9} and 0.41×10^{-9} mol P m^{-2} s^{-1} in the absence and presence of P supplement, respectively. The application of Benlate, Aliette and Ridomil had no effect on hyphal uptake or flux in the absence of P supplement (Table 4.2a). In the presence of P supplement (Table 4.2b), Benlate again had no significant effect on hyphal uptake or flux whereas they were reduced approximately to 16% following Ridomil application. Hyphal uptake and flux following Aliette application were not calculated, because hyphal inflow in NMP1 was higher than that in MP1.

Table 4.1. (Experiment 1). Effects of P application on P inflow into non-mycorrhizal (NM) and mycorrhizal (M) roots, hyphal inflow (*HI*), hyphal length, hyphal uptake (*HU*), hyphal flux (HF), area of symbiotic interface and P flux across the symbiotic interface of onion plants associated with *Glomus* sp. "City Beach" (WUM 16) at harvest period 3 - 6 weeks

P level	NM inflow (mol P m^{-1} s^{-1}) x 10^{-13}	M inflow (mol P m^{-1} s^{-1}) x 10^{-13}	*HI* Hyphal inflow (mol P m^{-1} root s^{-1}) x 10^{-13}	Hyphal length (m m^{-1} root)	*HU* Hyphal uptake (mol P m^{-1} hyphae s^{-1}) x 10^{-15}	*HF* Hyphal flux (mol P m^{-2} s^{-1}) x 10^{-9}	Area of interface (m^2 m^{-1} root) x 10^{-6}	Flux through interface (mol P m^{-2} s^{-1}) x 10^{-9}
P0	1.70 ± 0.79	52.66 ± 6.92	51.68 ± 6.81	1830.99 ± 5.18	2.82 ± 0.37	0.24 ± 0.03	1998.00	2.59 ± 0.34
P1	18.85 ± 2.66	50.40 ± 0.79	31.64 ± 0.79@	658.20 ± 313.68@	4.81 ± 0.12*	0.41 ± 0.01*	1161.50	2.72 ± 0.07

Values are means and standard errors of 3 replicates, except area of interface where n = 2; P0 = no added P, P1= added 15 µg P mg^{-1} soil; P inflow calculated from shoot P only; Hyphal length was average value between the 3-week and 6-week harvests; Hyphal flux was determined by dividing values of hyphal uptake by area of external hyphae (11.62 x 10^{-6} m^2), diameter of external hyphae was 3.7 µm; Area of interface was average value between the 3-week and 6-week harvests; *, @ Significant at 0.05, 0.1 levels of probability, respectively.

Table 4.2a. (Experiment 1). Effects of fungicides on P inflow into non-mycorrhizal (NM) and mycorrhizal (M) roots, hyphal inflow (*HI*), hyphal length, hyphal uptake (*HU*), hyphal flux (*HF*), area of symbiotic interface and P flux across the symbiotic interface of onion plants associated with *Glomus* sp. "City Beach" (WUM 16) in the absence of P-supplement (MP0) at harvest period 3 - 6 weeks

Treatments	NM inflow (mol P m^{-1} s^{-1}) x 10^{-13}	M inflow (mol P m^{-1} s^{-1}) x 10^{-13}	*HI* Hyphal inflow (mol P m^{-1} root s^{-1}) x 10^{-13}	Hyphal length (m m^{-1} root)	*HU* Hyphal uptake (mol P m^{-1} hyphae s^{-1}) x 10^{-15}	*HF* Hyphal flux (mol P m^{-2} s^{-1}) x 10^{-9}	Area of interface (m^2 m^{-1} root) x 10^{-6}	Flux through interface (mol P m^{-2} s^{-1}) x 10^{-9}
P0 Control	1.70 ± 0.79	52.66 ± 6.92	51.68 ± 6.81	1831.0 ± 115.18	2.82 ± 0.37	0.24 ± 0.03	1998.00	2.59 ± 0.34
P0 Benlate	5.65 ± 7.88	26.04 ± 9.64	20.40 ± 9.64*	538.3 ± 151.3*	3.79 ± 3.17	0.33 ± 0.27	33.00@	68.00 ± 29.20*
P0 Aliette	46.81 ± 16.96	116.13 ± 19.06*	69.32 ± 7.50	2148.3 ± 183.1**	3.23 ± 0.63	0.28 ± 0.06	2258.15	3.15 ± 0.33
P0 Ridomil	0.20 ± 1.94	39.51 ± 10.51*	39.31 ± 10.51	2720.7 ± 390.9**	1.40 ± 0.58	0.12 ± 0.05	934.10	4.21 ± 1.13

Values are means and standard errors of 3 replicates except area of interface where n = 2; P0 = no added P; Data for MP0 control treatment are taken from Table 4.1; P inflow calculated from shoot P only; Hyphal length was average value between the 3-week and 6-week harvests; Hyphal flux was determined by dividing values of hyphal uptake by area of external hyphae (11.62 x 10^{-6} m^2), diameter of external hyphae was 3.7 µm; Area of interface was average value between the 3-week and 6-week harvests; **, * Significant at 0.01, 0.05 levels of probability, respectively.

Table 4.2b. (Experiment 1). Effects of fungicides and P application on P inflow into non-mycorrhizal (NM) and mycorrhizal (M) roots, hyphal inflow (*HI*), hyphal length, hyphal uptake (*HU*), hyphal flux (*HF*), area of symbiotic interface and P flux across the symbiotic interface of onion plants associated with *Glomus* sp. "City Beach" (WUM 16) in the presence of P-supplement (MP1) at harvest period 3 - 6 weeks

Treatments	NM inflow (mol P m^{-1} s^{-1}) x 10^{-13}	M inflow (mol P m^{-1} s^{-1}) x 10^{-13}	*HI* Hyphal inflow (mol P m^{-1} root s^{-1}) x 10^{-13}	Hyphal length (m m^{-1} root)	*HU* Hyphal uptake (mol P m^{-1} hyphae s^{-1}) x 10^{-15}	*HF* Hyphal flux (mol P m^{-2} s^{-1}) x 10^{-9}	Area of interface (m^2 m^{-1} root) x 10^{-2}	Flux through interface (mol P m^{-2} s^{-1}) x 10^{-9}
P1 Control	18.85 ± 2.66	50.40 ± 0.79	31.64 ± 0.79	658.20 ± 313.68	4.81 ± 0.12	0.41 ± 0.01	1161.50	2.72 ± 0.07
P1 Benlate	41.36 ± 17.58	52.76 ± 13.87	11.40 ± 13.87	346.17 ± 21.26*	3.30 ± 4.01	0.28 ± 0.34	790.50	1.47 ± 1.73
P1 Aliette	125.97 ± 9.23**	82.55 ± 14.52@	NA	898.17 ± 57.91	NA	NA	1183.50	NA
P1 Ridomil	11.08 ± 10.35	27.69 ± 14.80	16.61 ± 14.80	836.87 ± 337.74*	0.90 ± 0.81	0.08 ± 0.07	316.50	6.53 ± 3.77

Values are means and standard errors of 3 replicates except area of interface where n = 2; Data for MP1 control treatment was taken from Table 4.1; P inflow calculated from shoot P only; NA = not applicable because P inflow in NM was higher than that in M plants; Hyphal length was average value between the 3-week and 6-week harvests; Hyphal flux was determined by dividing values of hyphal uptake by area of external hyphae (11.62 x 10^{-6} m^2), diameter of external hyphae was 3.7 µm; Area of interface was average value between the 3-week and 6-week harvests; **, *, @ Significant at 0.01, 0.05, 0.1 levels of probability, respectively.

4.3.1.3. The effects of fungicides and P application on area of interface

Table 4.3a and b show that the area of total interface (intercellular and arbuscular interfaces) per m of root increased between the 3-week and 6-week harvests in all treatments. P application alone tended to reduce the area of interface at both harvests.

The fungicides had varied effects on the plant-fungal interface per m of root. In the absence of P supplement Aliette had no effects, while Ridomil caused slight, and Benlate marked, reduction in the area of interface at both harvests. In the presence of P supplement similar effects were observed at the 3-week harvest but by the 6-week harvest no significant differences in area of interface between treatment were apparent.

Average values of total interface per m root between the two harvests were also calculated (Table 4.2a,b) to estimate P flux across the symbiotic interface (see section 4.3.1.4). When the area of total interface was calculated on a per plant basis (Table 4.3a and b) the effects were different from those calculated per m root. The P supplement had no effect on the total area of interface at the 3-week harvest but it was increased at the 6-week harvest. The application of all fungicides, on the other hand, significantly reduced the area of total interface at both harvests. This severe reduction in the area of interface per plant was observed both at the 3- and 6-week harvests, both in the absence and presence of P supplement.

The reduction of root length following application of Aliette or Ridomil at both levels of P supplement led to a significant reduction in the total area of

interface on a per plant basis even though the intensity of infection (see Chapter 3) and area of interface expressed per m of root were not affected by application of Aliette and only slightly reduced by Ridomil. In Benlate-treated plants at the 3-week harvest, the reduction in the area of interface on a per plant basis was a result of combined effects of a delay in root growth and a reduction in the area of interface per m root. In contrast, at the 6-week harvest, the reduction in the area of interface was solely due to inhibition of fungal development as this fungicide had no effect on root length at this harvest.

Table 4.3a. (Experiment 1). Effects of fungicides and P application on symbiotic interface per m of root and per plant of onion associated with *Glomus* sp. "City Beach" (WUM 16) at the 3-week harvest

Treatments	Intercellular hyphal interface per m of root (m^2 m^{-1} root) x 10^{-6}	Arbuscular interface per m of root (m^2 m^{-1} root) x 10^{-6}	Intercellular hyphal + arbuscular interface per m of root (m^2 m^{-1} root) x 10^{-6}	Intercellular hyphal interface per plant (m^2 $plant^{-1}$) x 10^{-6}	Arbuscular interface per plant (m^2 $plant^{-1}$) x 10^{-6}	Intercellular hyphal + arbuscular interface per plant (m^2 $plant^{-1}$) x 10^{-6}
MP0						
Control	318.00 ± 162.00	774.00 ± 464.00	1093.00 ± 625.00	70.03 ± 35.56	170.39 ± 101.97	240.42 ± 137.52
Benlate	2.00 ± 1.00	0.00 ± 0.00	2.00 ± 1.00	0.07 ± 0.07	0.00 ± 0.00	0.26 ± 0.26
Aliette	424 .00 ± 199.00	929.00 ± 676.00	1353.00 ± 875.00	32.81 ± 15.40	71.77 ± 52.22	104.58 ± 67.61
Ridomil	78.00 ± 24.00	24.00 ± 24.00	102.00 ± 99.00	5.94 ± 5.69	1.83 ± 1.83	7.77 ± 7.52
MP1						
Control	244 .00 ± 59.00	291.00 ± 70.00	535.00 ± 129.00	132.57 ± 32.05	158.32 ± 37.98	290.88 ± 70.03
Benlate	15.00 ± 13.00*	58.00 ± 20.00*	73.00 ± 33.00*	2.55 ± 2.21**	9.80 ± 3.45**	12.35 ± 5.66**
Aliette	104.00 ± 28.00*	291.00 ± 91.00	395.00 ± 119.00	12.55 ± 11.17*	52.51 ± 16.48**	71.10 ± 27.65**
Ridomil	19.00 ± 19.00*	0.10 ± 0.00*	19.00 ± 19.00*	3.06 ± 3.06**	0.00 ± 0.00**	3.06 ± 3.06**

Values are means and standard errors of 3 replicates; M = mycorrhizal; P0 , no added P; P1, 15 µg P g^{-1} soil; **, * Significant at 0.01, 0.05 levels of probability, respectively.

Table 4.3b. (Experiment 1). Effects of fungicides and P application on symbiotic interface per m of root and per plant of onion associated with *Glomus* sp. "City Beach" (WUM 16) at the 6-week harvest

Treatments	Intercellular hyphal interface per m of root (m^2 m^{-1} root) x 10^{-6}	Arbuscular interface per m of root (m^2 m^{-1} root) x 10^{-6}	Intercellular hyphal + arbuscular interface per m of root (m^2 m^{-1} root) x 10^{-6}	Intercellular hyphal interface per plant (m^2 $plant^{-1}$) x 10^{-6}	Arbuscular interface per plant (m^2 $plant^{-1}$) x 10^{-6}	Intercellular hyphal + arbuscular interface per plant (m^2 $plant^{-1}$) x 10^{-6}
MP0						
Control	610.00 ± 185.00	2293.00 ± 698.00	2903.00 ± 883.00	997.29 ± 302.46	3748.83 ± 1141.16	4746.12 ± 1443.62
Benlate	57.00 ± 20.00@	8.00 ± 8.00	64.00 ± 12.00@	19.73 ± 6.81*	2.62 ± 2.62*	22.35 ± 4.19*
Aliette	636.00 ± 165.00	2526.00 ± 886.00	3162.00 ± 1051.00	114.17 ± 29.62*	453.33 ± 158.95*	567.50 ± 188.57*
Ridomil	452.00 ± 94.00	1315.00 ± 595.00	1766.00 ± 688.00	143.04 ± 29.63*	416.44 ± 188.34*	559.47 ± 217.96*
MP1						
Control	371.00 ± 45.00	1417.00 ± 78.00	1788.00 ± 123.00	1674.18 ± 201.08	6403.00 ± 352.46	8077.18 ± 553.54
Benlate	397.00 ± 164.00	1111.00 ± 594.00	1508.00 ± 758.00	729.41 ± 301.32*	2040.97 ± 1091.64**	2770.38 ± 1393.96*
Aliette	335.00 ± 82.00	1638.00 ± 2.00	1973.00 ± 84.00	196.21 ± 48.03*	959.09 ± 0.88**	1155.30 ± 48.91**
Ridomil	188.00 ± 146.00	405.00 ± 405.00	593.00 ± 550.58	55.05 ± 42.75**	118.44 ± 118.44**	173.49 ± 161.19**

Values are means and standard errors of 3 replicates; M, mycorrhizal; P0, no added P; P1, 15 µg g^{-1} soil; **, *, @ Significant at 0.01, 0.05, 0.1 levels of probability, respectively.

4.3.1.4. The effects of fungicides and P application on flux across living plant-fungal interface

P flux across the living plant-fungal interface was not affected by P application (Table 4.1). In the absence of P supplement, the rate of P transfer from the fungus to the plant for the harvest period 3 to 6 weeks was 2.59×10^{-9} mol P m^{-2} interface s^{-1} . The flux was not affected by the application of Aliette or Ridomil but was markedly increased by Benlate (Table 4.2a). This increase of P flux was unexpected. The number of arbuscules per section was very low and, therefore, the area of interface markedly reduced, leading to very high calculated values for P flux. However, P flux following Benlate application to P-supplemented (MP1) plants (Table 4.2b) was of the same magnitude as that in the control (MP1). This suggests that experimental error, resulting from the very small number of sections available for image analysis, may have been important here. The high P flux observed in MP0 following application of Benlate will not be considered further here. Experiment 2 (section 4.3.2) was carried out to follow up this observation.

In the presence of P supplement, the value of P flux was 2.72×10^{-9} mol P m^{-2} interface s^{-1} and was unaffected by Benlate or Ridomil (Tables 4.1 and 4.2b). The flux in the presence of Aliette was apparently negative (values for NM plants greater than for M plants).

4.3.2. Experiment 2

Mycorrhizal infection in plants treated with Benlate was not observed until the harvest at 4 weeks (Table 4.4a) when one of the four plants was infected. Hence, the data could not be analysed statistically, except for the 5- and 6-week harvests when all four replicate inoculated plants were infected.

Benlate had no significant effects on shoot dry weight at the first two harvests (2 and 3 weeks), while there were small effects on root length. By 4 weeks both shoot dry weight and root length were lower than untreated controls, corresponding with the delay in mycorrhizal colonisation just noted (Table 4.4a).

Tables 4.4a and 4.5a show that up to 4 weeks, Benlate reduced all the parameters measured, *viz.* percentage infection, P concentration, P content, inflow into mycorrhizal roots, hyphal inflow, intensity and characteristics of infection, area of interface expressed both as per m root and per plant. These findings are in agreement with those in Experiment 1 and Chapter 3. Additionally, Benlate reduced the rate of P transfer to the root across the living interface. The reduction of P flux across the symbiotic interface following application of Benlate contradicted the findings from Experiment 1.

At the 5- and 6-week harvests, Benlate had similar effects to those between 2 and 4 weeks, except that the area of interface per m root (Table 4.5b) was no different from the control at this time. Benlate reduced markedly the percentage infection, P concentration, P content, inflow into mycorrhizal roots, hyphal inflow, intensity and characteristics of infection, area of interface expressed per plant and P flux across the interface (Tables 4.4b and 4.5b).

The area of interface expressed per m of root was not affected by application of Benlate whereas the area of interface per plant was significantly reduced as in Experiment 1. The rate of P transfer across the interface was reduced to 37-40% of the control value (Table 4.5b).

Table 4.4a. (Experiment 2). Effects of Benlate application on shoot dry weight, root length, length of infected root, percentage infection, shoot P concentration and shoot P content of onion plants associated with *Glomus* sp. "City Beach" (WUM 16) harvested at 2, 3 and 4 weeks

Harvest	Treatments	Shoot dry weight (mg)	Root length (cm)	Length of root infected (cm)	Percentage infection (%)	Shoot P concentration ($\mu g\ P\ mg^{-1}$)	Shoot P content ($\mu g\ plant^{-1}$)
2-week	Control	2.74 ± 0.29	6.91 ± 0.36	2.79 ± 0.52	40.40 ± 7.71	5.58 ± 0.38	14.95 ± 0.49
	Benlate	2.13 ± 0.35	4.23 ± 0.67	NA	NA	NA	NA
3-week	Control	5.69 ± 0.61	17.39 ± 1.27	5.48 ± 1.59	21.26 ± 8.24	2.96 ± 0.39	16.14 ± 0.18
	Benlate	4.77 ± 0.43	10.42 ± 2.61	NA	NA	NA	NA
4-week	Control	11.83 ± 1.36	32.74 ± 6.25	20.37 ± 5.00	62.2 ± 9.78	3.16 ± 0.20	36.57 ± 2.48
	Benlate[1]	5.83	18.05	1.14	5.20	2.01	15.49

Values are means and standard errors of 4 replicates, except for Benlate treatment at 4-week harvest as only 1 of 4 replicate plants was infected; 1 = one plant infected; NA = not applicable because the plants were not infected.

Table 4.4b. (Experiment 2). Effects of Benlate application on shoot dry weight, root length, length of infected root, percentage infection, shoot P concentration and shoot P content of onion plants associated with *Glomus* sp. "City Beach" (WUM 16) harvested at 5 and 6 weeks

Harvest	Treatments	Shoot dry weight (mg)	Root length (cm)	Length of root infected (cm)	Percentage infection (%)	Shoot P concentration ($\mu g\ P\ mg^{-1}$)	Shoot P content ($\mu g\ plant^{-1}$)
5-week	Control	30.13 ± 1.54	61.79 ± 4.45	48.16 ± 4.28	77.75 ± 3.50	3.16 ± 0.08	94.74 ± 3.07
	Benlate	12.37 ± 0.60**	34.52 ± 6.76**	4.22 ± 3.27**	8.98 ± 5.66**	1.50 ± 0.30*	18.87 ± 4.47**
6-week	Control	72.22 ± 9.04	157.01 ± 34.14	124.24 ± 27.08	79.23 ± 1.26	2.54 ± 0.20	180.13 ± 16.33
	Benlate	18.19 ± 2.78**	34.35 ± 6.07**	2.97 ± 2.73*	6.08 ± 5.18**	1.61 ± 0.34	31.30 ± 11.18**

Values are means and standard errors of 4 replicates; **, * Significant at 0.01, 0.05 levels of probability, respectively.

Table 4.5a. (Experiment 2). Effects of Benlate application on P inflow into non-mycorrhizal (NM) and mycorrhizal (M) roots, hyphal inflow, area of symbiotic interface and P flux across the symbiotic interface of onion plants associated with *Glomus* sp. "City Beach" (WUM 16) at harvest periods 0 - 2, 2 - 3, and 3 - 4 weeks

Harvest Periods	Treatments	NM inflow (mol P m^{-1} s^{-1}) x 10^{-15}	M inflow (mol P m^{-1} s^{-1}) x 10^{-15}	Hyphal inflow (mol P m^{-1} root s^{-1}) x 10^{-15}	Area of interface per m of root (m^2 m^{-1} root) x 10^{-6}	Flux through interface (mol P m^{-2} s^{-1}) x 10^{-9}
0 - 2 weeks	Control	-11.19 ± 35.32	24.94 ± 22.35	36.13 ± 22.35	864.50 ± 289.71	4.18 ± 2.58
	Benlate	2.46 ± 15.71	NA	NA	NA	NA
2 - 3 weeks	Control	-3.07 ± 8.39	10.97 ± 5.09	14.04 ± 5.92	858.00 ± 120.00	1.64 ± 0.59
	Benlate	1.52 ± 6.83	NA	NA	NA	NA
3 - 4 weeks	Control	-0.35 ± 1.69	72.53 ± 4.91	72.88 ± 4.98	1550.50 ± 89.54	4.70 ± 0.18
	Benlate[1]	-1.81	4.60	6.41	200	3.21

Values are means and standard errors of 4 replicates, except for Benlate treatment at harvest period 3 - 4 weeks as only 1 of 4 replicate plants was infected; 1 = one plant infected; NA = not applicable because the plants were not infected; P inflow calculated from P content in the shoots and roots; Area of interface was the average value between the two harvest periods.

Table 4.5b. (Experiment 2). Effects of Benlate application on P inflow into non-mycorrhizal (NM) and mycorrhizal (M) roots, hyphal inflow, area of symbiotic interface and P flux across the symbiotic interface of onion plants associated with *Glomus* sp. "City Beach" (WUM 16) at harvest periods 4 - 5 and 5 - 6 weeks

Harvest periods	Treatments	NM inflow (mol P m^{-1} s^{-1}) x 10^{-15}	M inflow (mol P m^{-1} s^{-1}) x 10^{-15}	Hyphal inflow (mol P m^{-1} root s^{-1}) x 10^{-15}	Area of interface per m of root (m^2 m^{-1} root) x 10^{-6}	Area of interface per plant (m^2 $plant^{-1}$) x 10^{-6}	Flux through interface (mol P m^{-2} s^{-1}) x 10^{-9}
4 - 5 weeks	Control	1.34 ± 1.24	99.97 ± 4.28	98.62 ± 0.01	870.00 ± 238.00	528.15 ± 139.22	13.19 ± 2.34
	Benlate	-1.83 ± 1.86	10.45 ± 12.94@	12.23 ± 12.94***	520.00 ± 327.00	201.48 ± 116.43	1.78 ± 1.77*
5 - 6 weeks	Control	-0.83 ± 0.20	75.87 ± 9.96	76.70 ± 9.96	464.30 ± 118.16	658.15 ± 123.45	20.11 ± 6.23
	Benlate	4.26 ± 1.89@	26.58 ± 21.10	22.33 ± 21.10@	534.40 ± 93.74	173.15 ± 22.07*	8.49 ± 6.77

Values are means and standard errors of 4 replicates; P inflow calculated from P content in the shoots and roots; Area of interface was the average value between the two harvest periods; ***, **, *, @ Significant at 0.001, 0.01, 0.05, 0.1 levels of probability, respectively.

6.4. Discussion

The values for all measurements of P uptake and transfer to the plants in Experiment 1 are minimum estimates for several reasons:

a) P in roots was not included in the calculation of inflow;

b) It is assumed that there have been no changes in the uptake by the plants as a consequence of infection (see Pearson & Jakobson, 1993);

c) The hyphal lengths are maximum estimates because no attempt was made to determine length of mycorrhizal hyphae by subtracting the values of hyphal length in uninoculated pots from those in inoculated pots (see Chapter 3). The data are useful for assessing the relative effects of fungicides but not for comparison of rate of uptake with values obtained in other investigations.

The estimates of hyphal inflow in the absence and presence of P supplement were 51.68×10^{-13} and 31.64×10^{-13} mol P m^{-1} s^{-1}, respectively. These values are close to estimates by Sanders and Tinker (1973) for onion plants infected by an unnamed mycorrhizal fungus and about one quarter of those of Smith et al. (1994) who used leeks and *Glomus* sp. " City Beach" (WUM 16). The data presented here are based on shoot P only. If it is assumed that roots contributed one third of the total P (see Experiment 2) in the plants, then the total hyphal flux would be very close to the published values.

The values for hyphal inflow were minimum estimates because values were obtained by subtracting the value of P inflow into non-mycorrhizal control plants from those in mycorrhizal plants, with assumption that mycorrhizal infection does not change the response of the root in taking up P. However, it

has been demonstrated that *Scutellospora calospora* infection in cucumber plants "switches off" the host root P uptake (Pearson & Jakobsen, 1993) and hence subtraction was not valid in that case and the values for hyphal inflow were underestimated by the approach used there. If this is also the case in onion plants inoculated with *Glomus* sp. "City Beach" then the value of P flux must be greater than 11.48×10^{-9} mol P m^{-2} $s.^{-1}$

Values for P flux across the interface of the control treatment in the absence and presence of P supplement in Experiment 1 were 2.59×10^{-9} mol P m^{-2} s^{-1} and 2.72×10^{-9} mol P m^{-2} s^{-1}, respectively. The P flux, based on both P in the shoots and roots of control plants, was 20.11×10^{-9} mol P m^{-2} s^{-1} at 5 - 6 weeks (Experiment 2). These values are again of the same magnitude as those measured by Smith et al. (1994) ($3.7\text{-}11.3 \times 10^{-9}$ mol m^{-2} s^{-1}) for onion plants associated with *Glomus* sp. "City Beach" (WUM 16) for the harvest period 3 - 6 weeks.

Comparing P flux across the symbiotic interface with P flux through entry points, the gateway of P entry to the plant from networks of hyphae in the soil, measured by Sanders & Tinker (1973) (3.8×10^{-4} mol m^{-2} s^{-1}), indicated that the capacity of entry points to transfer P was 10,000 times faster than the plant-fungal interface. These results may suggest that P flux through the interface and/or the area of interface is a limiting factor in P transfer from the fungus to the plant (Smith & Smith, 1993; Smith et al., 1994). P transfer across the interface in Experiment 1 was not altered by P supplement.

Comparison of the hyphal flux (*HF*, mol P m^{-2} m^{-1}) from the soil into external hyphae and P flux to the plant across the interface in the inoculated control, indicates that P flux across the interface was 10 times higher than hyphal flux. However, the relative magnitudes of hyphal uptake from soil (*HF*), flux through entry points (Sanders and Tinker, 1973) and transfer to the plant indicate that the membrane transport process is several orders of magnitudes lower than translocation through the entry points. A large hyphal surface area in soil is clearly required to supply P through the entry points to the interfacial regions, where transfer occurs at a rate about 10 times higher than hyphal uptake (*HF*) from soil.

This study demonstrated that the three fungicides had different effects on the symbiosis. Ridomil had a negative effect on the development of the fungus in the soil and in the plant. The efficiency of P uptake from the soil was also reduced. All of this led to an overall reduction in the contribution of the fungus to P nutrition of the plant.

Aliette had no effect on the intensity and characteristics of infection or area of plant-fungal interface per unit length of root. However, the reduction in root growth following Aliette application led to the reduction in the area of interface on a per plant basis. Furthermore, the presence of phosphonate in the plant tissue, as a degradation product of this fungicide, complicated the analysis of the effects. Phosphonate caused an apparently significant increase in P inflow both in non-mycorrhizal and mycorrhizal plants and also led to a slightly increased P flux across the symbiotic interface. The interference of

phosphonate in P metabolism in both non-mycorrhizal and mycorrhizal plants will be discussed in detail in Chapter 5.

Benlate had a greater effect on the fungus than either Ridomil or Aliette. The effects of Ridomil on reducing fungal development were discussed in Chapter 3 and this chapter. Hyphal uptake and hyphal flux were also reduced slightly, but not so much as to reduce the transfer of P to the plants (which was actually slightly increased). Ridomil, therefore, acts via reduction in the amount of fungal infection per plant, rather than on the function of the internal infection.

Benlate also reduced fungal colonisation in every respect including lengths of external hyphae. In addition, Experiment 2 indicated clear effects on the fungal activity in terms of P uptake from soil (*HI*) and transfer to the plant.

CHAPTER 5. ELUCIDATION OF THE INFLUENCE OF VA MYCORRHIZA ON PHYTOTOXIC EFFECTS FOLLOWING ALIETTE APPLICATION

5.1. Introduction

Aliette, or fosetyl-Al, degrades rapidly in soil and plant tissues to phosphorous acid (H_3PO_3) or phosphonic acid [H-PO(OH) $_2$], CO_2 and aluminium (Cohen & Coffey, 1986; Coffey & Ouimette, 1989; Despatie et al., 1989; Fenn & Coffey, 1985; Guest & Grant, 1991; Saindrenan et al., 1985). Phosphorous acid and phosphonic acid are in tautomeric equilibrium, but the equilibrium favours phosphonic acid (Guthrie, 1979; see Coffey & Ouimette, 1989). Alkyl esters of phosphorous and phosphonic acids are termed phosphite and phosphonate, respectively. In this thesis phosphonate and phosphate will be used to refer to HPO_3^{2-} and PO_4^{3-}, respectively. Phosphonate, an active component of the fungicide Aliette, is detected both in the plant and in the soil soon after the fungicide is applied (see Cohen & Coffey, 1986; Coffey & Ouimette, 1989). The application of this fungicide, to control plant diseases caused by pathogenic fungi belonging to Oomycetes, leads to accumulation of phosphonate in plant tissues. Such accumulation is beneficial in controlling the pathogen, but it may also cause unforeseen effects on plant growth. The findings described in Chapter 3 showed that Aliette had negative effects on growth of both non-mycorrhizal and mycorrhizal plants. Interestingly, despite the fact that plants treated with Aliette had stunted growth, they had very high P concentrations

and P contents. This was found in both non-mycorrhizal and mycorrhizal plants. In addition, mycorrhizal plants were able to overcome the initial stunted growth due to application of Aliette by approximately 12 weeks, whereas the non-mycorrhizal plants remained stunted regardless of P supplement. As the method used previously to measure P concentration in the plants (Bartlett, 1959) cannot discriminate between phosphonate and phosphate, the high P concentration and content measured in Aliette-treated plants is likely to be due to accumulation of both components. The relative concentrations and/or contents of phosphonate and phosphate in the plant tissues may be responsible for stunted growth of plants following application of Aliette. Recovery to normal growth in the case of mycorrhizal plants by approximately 12 weeks may be due to the fungus being able to convert phosphonate to phosphate and transfer it to the plants. This may explain why mycorrhizal plants grew better than non-mycorrhizal plants by approximately 12 weeks. Alternatively, mycorrhizal fungi associated with the root may be able to compensate for poor root growth with respect to uptake of the whole range of nutrients from the soil, including phosphate.

The aim of the experiments reported in this chapter was to investigate individual effects of Aliette and its degradation products, phosphonate and aluminium, on growth of both non-mycorrhizal and mycorrhizal plants in greater detail, following up the findings described in Chapter 3. It has been reported that application of Aliette at a lower concentration than that applied in the experiment reported in Chapter 3 (82.33 mg a.i. kg^{-1}) had no effects on the growth of mycorrhizal plants (Menge, 1982; Guillemin & Gianinazzi-Pearson,

1991). Therefore, an attempt was also made to clarify whether application of Aliette at a lower concentration would result in reduction of the growth of onion. Hence, the main points that were investigated were:

1. Confirmation that application of Aliette caused stunted growth of onion plants whether or not the plants were mycorrhizal and to determine effect of different rates of application;

2. Confirmation that Aliette-treated plants accumulated phosphonate as well as phosphate;

3. The observation that mycorrhizal plants recovered by approximately 12 weeks. This could have been due to mycorrhizal hyphae providing a mechanism for the plants to take up phosphate (and possibly other nutrients) even though the root systems were stunted. Furthermore, the VA mycorrhizal fungus may be able to convert phosphonate to phosphate and hence improve P nutrition;

4. The individual effects of aluminium (applied as aluminium chloride), phosphonate (applied as dimethyl phosphonate) and Aliette on plant growth and phosphorus uptake with the aim of separating out confounding effects of aluminium and phosphonate in the Aliette treatments.

Three experiments (Experiments 1 - 3) were conducted to accomplish the objectives outlined above.

The aim of Experiment 1 was to confirm that both non-mycorrhizal and mycorrhizal plants had stunted growth following Aliette application at dosages ranging from 20.00 to 82.33 mg kg^{-1} soil of the active ingredient (a.i) of Aliette (Objective 1).

Experiment 2 was expected to provide information on whether mycorrhizal plants and both types of non-mycorrhizal (NMP0 and NMP1) plants treated with Aliette take up phosphate as well as phosphonate (Objective 2).

Experiment 3 was designed to gain information to determine mechanism of recovery from initial stunted growth in mycorrhizal plants (Objective 3) and to study effects of aluminium, phosphonate and Aliette individually on plant growth (Objective 4). To obtain continuity of the theme, materials and methods, results and discussion for each experiment will be presented individually.

5.2. Experiment 1: Effects of different concentrations of Aliette on the growth of non-mycorrhizal and mycorrhizal plants

The aim of this experiment was to confirm that stunted growth following Aliette application observed in the experiment reported in Chapter 3 was not due to the high concentration of Aliette that was applied. To accomplish this objective a range of Aliette concentrations as well as the concentration used in Chapter 3, was used to study the effect of different concentrations of the fungicide on growth of both non-mycorrhizal (NMP1) and mycorrhizal (MP0) plants.

5.2.1. Materials and methods

Methodology for sowing, watering regimes, fertilising techniques, growth conditions, inoculation techniques and application of fungicide were as described in Chapter 2. Non-mycorrhizal "matched" control plants (NMP1) were used as uninoculated control plants and mycorrhizal plants without added P (MP0) were used as inoculated control plants (see Chapter 3). There were four concentrations of Aliette; 0.00, 20.00, 40.00, 82.33 mg a.i. kg^{-1}soil.

There were four replicates per treatment and the plants were harvested at 4 and 8.5 weeks after sowing. Data for shoot dry weight, root dry weight, root length and percentage infection were collected.

5.2.2. Results

5.2.2.1. The effects of Aliette concentration on shoot dry weight

Fig 5.1 (A and B) shows that shoot dry weights of non-mycorrhizal "matched" plants (NMP1) were reduced markedly at both harvests following application of the three concentrations of Aliette. The trend of the effects on shoot growth in the two harvests was consistent, growth decreased as the concentration of Aliette increased.

In mycorrhizal plants at the 4-week harvest, shoot dry weight was decreased, but not always significantly, by application of the three

concentrations of Aliette, with severe effects observed at the highest Aliette concentration (Fig. 5.2, A). At the 8.5-week harvest, the reduction in shoot growth was observed only at the highest concentration of Aliette (Fig. 5.2, B). The effect of Aliette was less on mycorrhizal than on non-mycorrhizal plants. In addition, MP0 plants not harvested were observed to have recovered from initial stunting, at all Aliette concentrations, by approximately 12 weeks (see Plate 5.1, A) whereas NMP1 remained stunted (Plate 5.1, B). This observation was in agreement with the experiment reported in Chapter 3.

5.2.2.2. The effects of Aliette concentration on root dry weight

Root dry weight was reduced in all Aliette treatments at both harvests. Again, the root growth decreased as the concentration of fungicide increased (Fig 5.3, A and B). The effects were more severe in non-mycorrhizal control plants (NMP1) than in mycorrhizal plants (MP0) and this observation was consistent for both harvests (compare Figs 5.3 and 5.4).

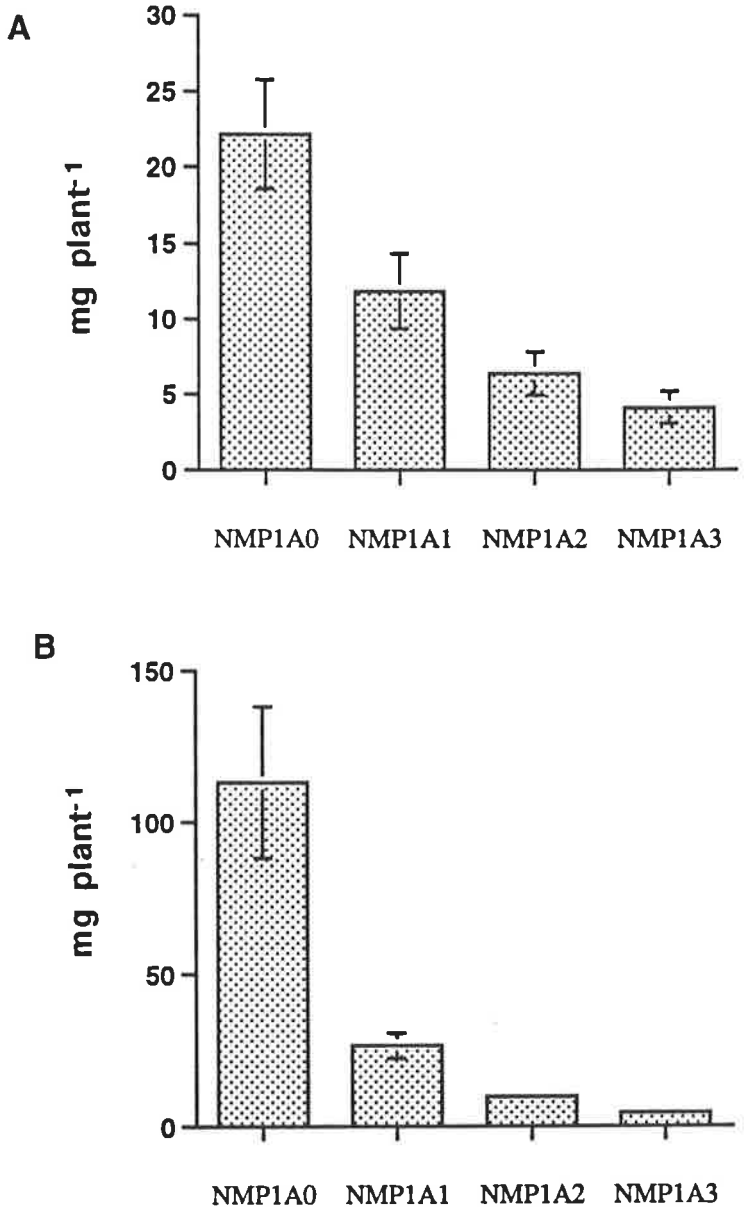


Figure 5.1. (Experiment 1). Effects of different concentrations of Aliette; 0.00 (A0), 20.00 (A1), 40.00 (A2) and 82.33 (A3) mg a.i. kg^{-1} soil on dry weight of shoot of non-mycorrhizal *Allium cepa* L. (NMP1) at the 4-week (A) and the 8.5-week (B) harvests. Vertical bars represent standard errors of means, n=4; if no bar is shown, standard error is smaller than the line.

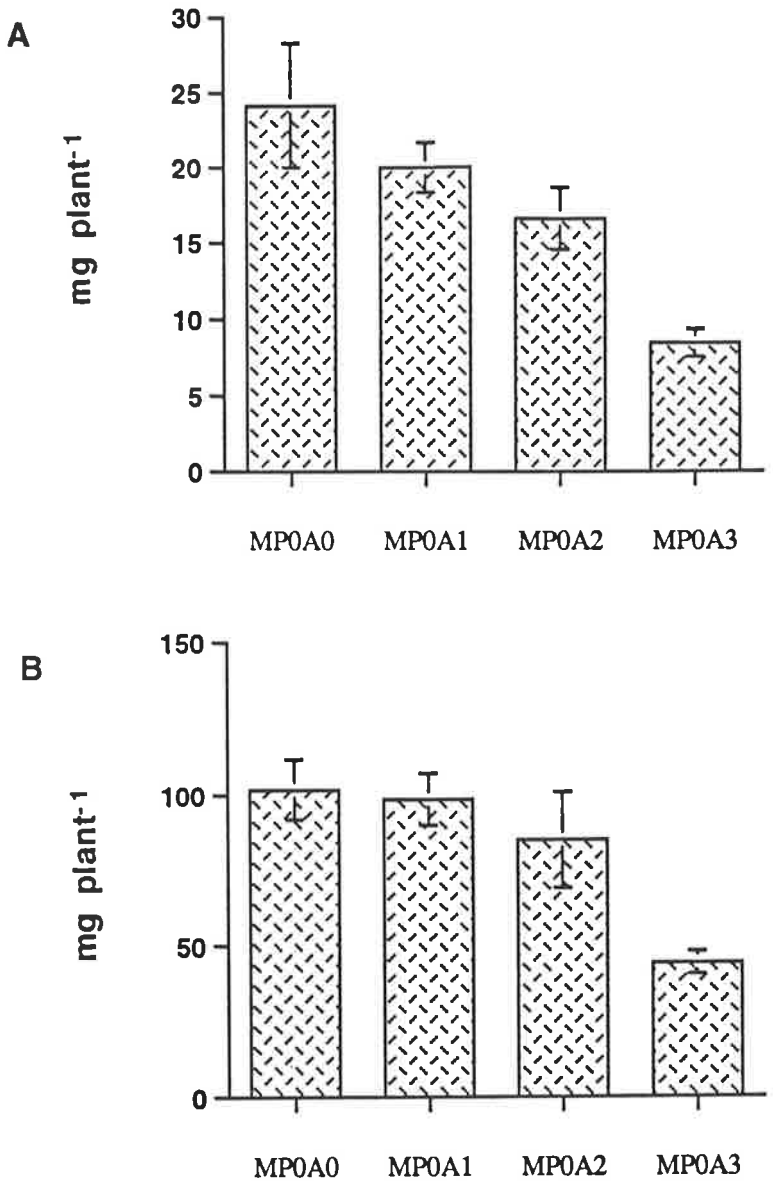
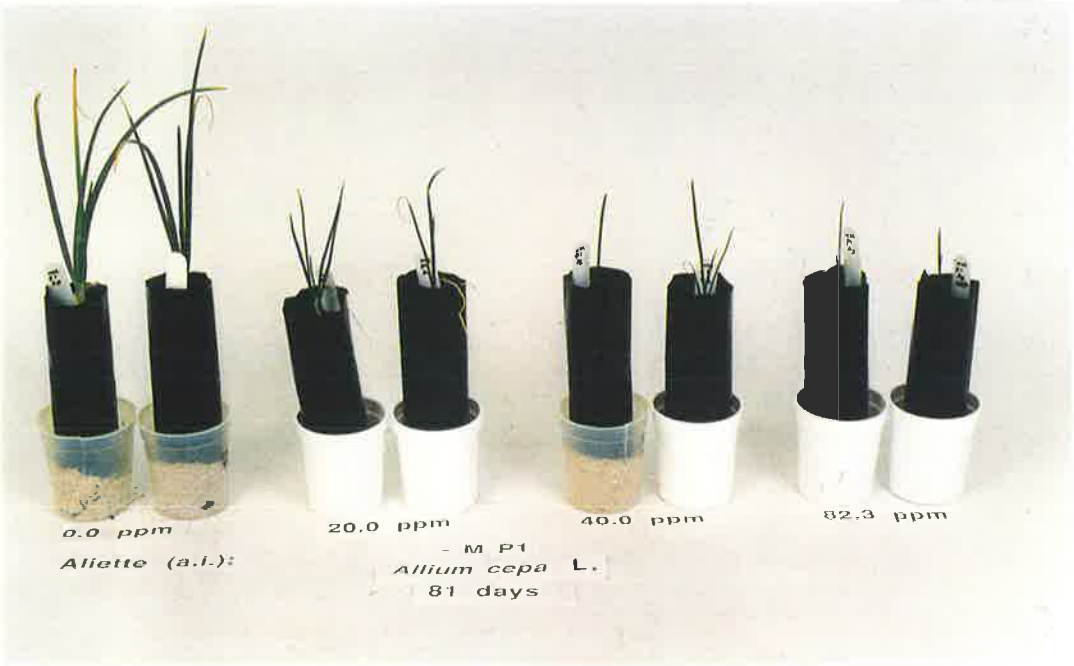


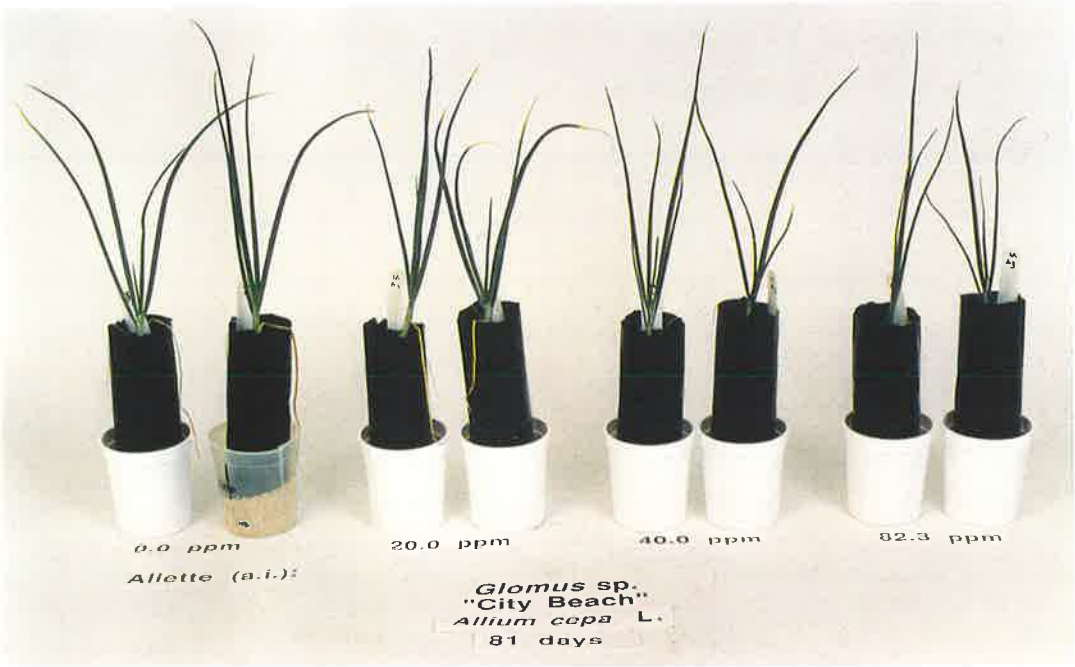
Figure 5.2. (Experiment 1). Effects of different concentrations of Aliette; 0.00 (A0), 20.00 (A1), 40.00 (A2) and 82.33 (A3) mg a.i. kg^{-1} soil on dry weight of shoot of mycorrhizal *Allium cepa* L. (MP0) associated with *Glomus* sp. "City Beach" (WUM 16) at the 4-week (A) and the 8.5-week (B) harvests. Vertical bars represent standard errors of means, n=4; if no bar is shown, standard error is smaller than the line.

Plate 5.1 The effects of Aliette concentration on non-mycorrhizal "matched" (-MP1) (A) and mycorrhizal (MP0) (B) plants of *Allium cepa* L. associated with *Glomus* sp. "City Beach" (WUM 16) at approximately 12 weeks. -MP1 plants were stunted whereas MP0 had recovered from initial stunting.

A


0.0 ppm
Aliette (a.i.):
20.0 ppm
40.0 ppm
82.3 ppm
- M P1
Allium cepa L.
81 days

B


0.0 ppm
Aliette (a.i.):
20.0 ppm
40.0 ppm
82.3 ppm
Glomus sp.
"City Beach"
Allium cepa L.
81 days

5.2.2.3. The effects of Aliette concentration on length of infected root and percentage infection

Aliette at all concentrations tested reduced the length of infected root at the two harvests (Fig. 5.5). The reduction was more apparent in the 8.5-week harvest (Fig. 5.5, B). Again the highest concentration of Aliette had the most severe effect. This result was in agreement with the experiment reported in Chapter 3.

Percentage infection, however, was not affected at either harvest by application of Aliette (Fig. 5.6). This was unexpected since the fungicide reduced both length of infected root and percentage infection in the experiment reported in Chapter 3.

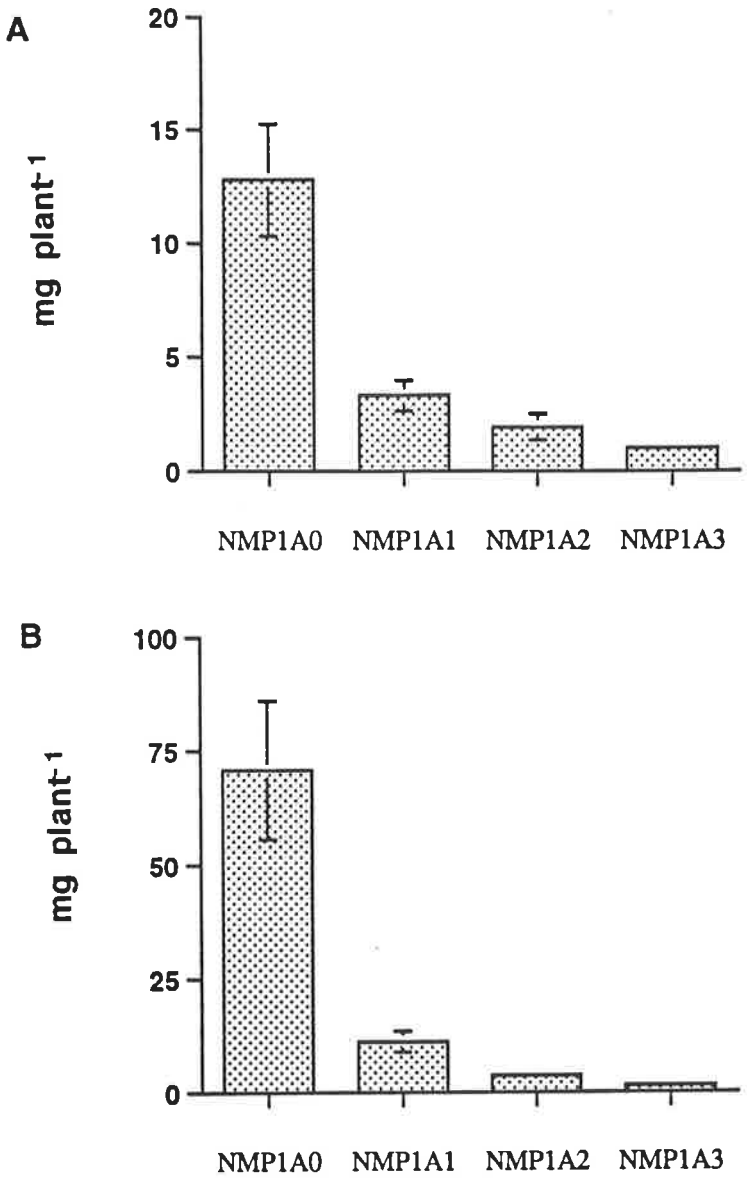


Figure 5.3. (Experiment 1). Effects of different concentrations of Aliette; 0.00 (A0), 20.00 (A1), 40.00 (A2) and 82.33 (A3) mg a.i. kg^{-1} soil on root dry weight of non-mycorrhizal *Allium cepa* L. (NMP1) at the 4-week (A) and the 8.5-week (B) harvests. Vertical bars represent standard errors of means, n=4; if no bar is shown, standard error is smaller than the line.

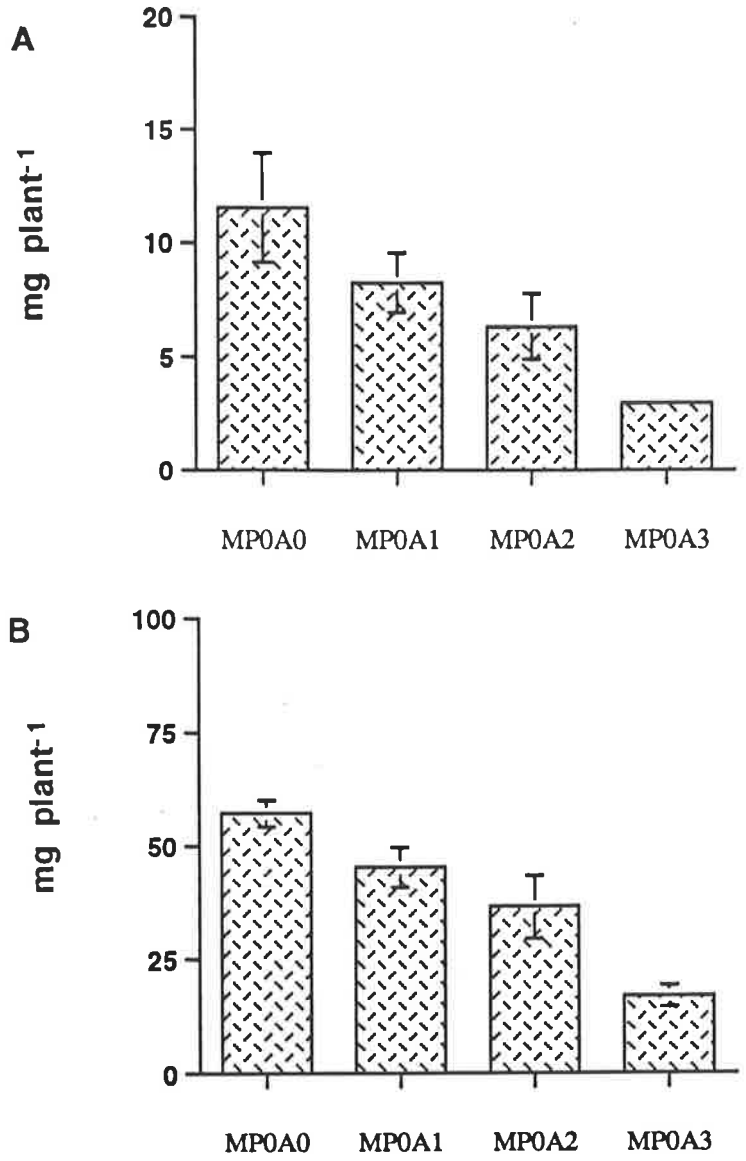


Figure 5.4. (Experiment 1). Effects of different concentrations of Aliette; 0.00 (A0), 20.00 (A1), 40.00 (A2) and 82.33 (A3) mg a.i. kg^{-1} soil on dry weight of root of mycorrhizal *Allium cepa* L. (MP0) associated with *Glomus* sp. "City Beach" (WUM 16) at the 4-week (A) and the 8.5-week (B) harvests. Vertical bars represent standard errors of means, n=4; if no bar is shown, standard error is smaller than the line.

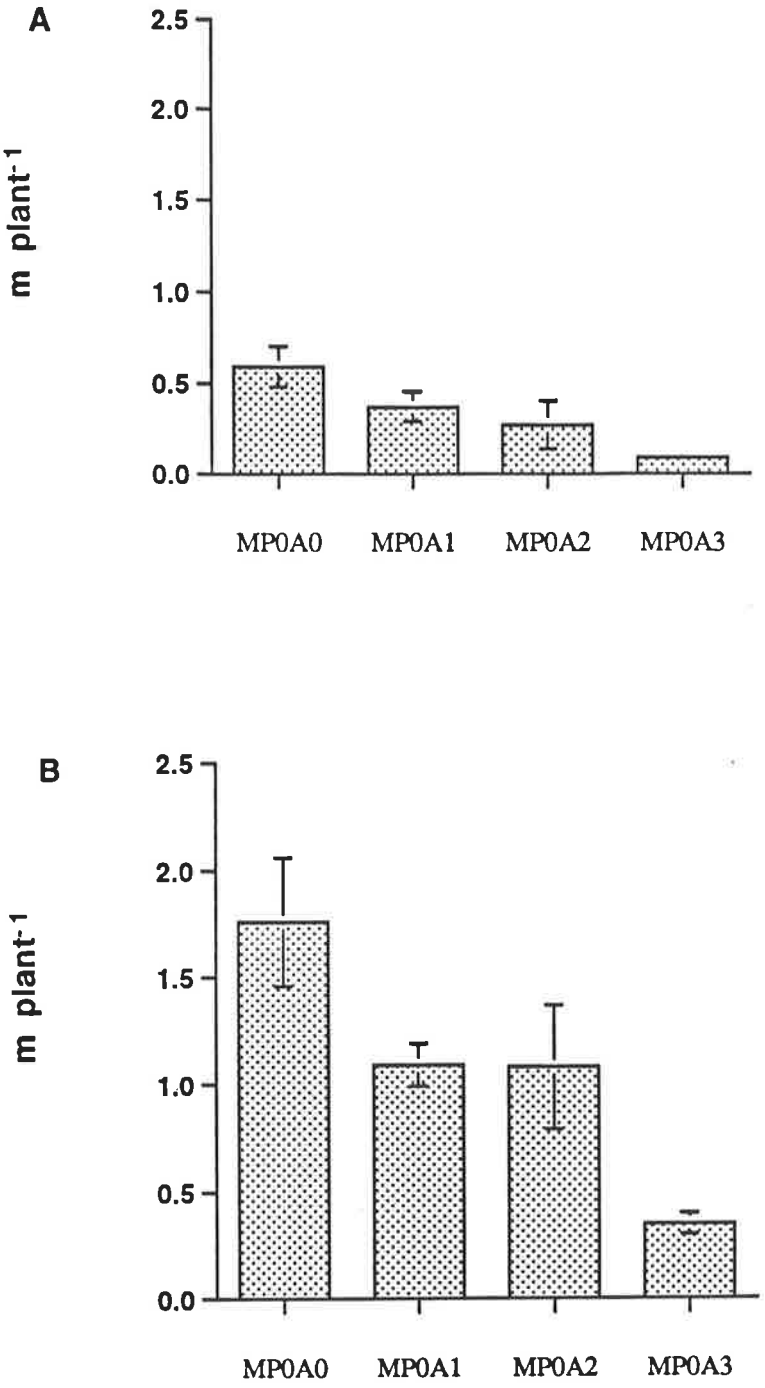


Figure 5.5. (Experiment 1). Effects of different concentrations of Aliette; 0.00 (A0), 20.00 (A1), 40.00 (A2) and 82.33 (A3) mg a.i. kg^{-1} soil on length of infected root of *Allium cepa* L. associated with *Glomus* sp. "City Beach" (WUM 16) at the 4-week (A) and the 8.5-week (B) harvests. Vertical bars represent standard errors of means, n=4; if no bar is shown, standard error is smaller than the line.

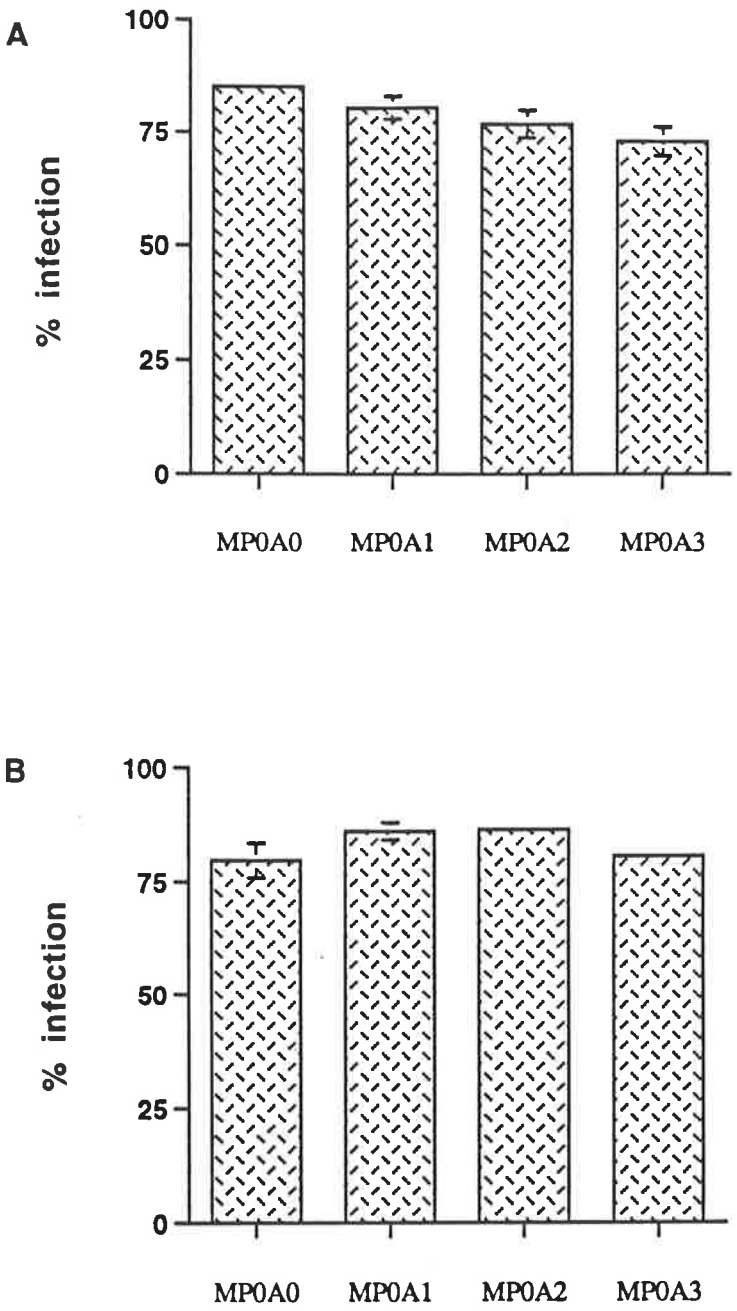


Figure 5.6. (Experiment 1). Effects of different concentrations of Aliette; 0.00 (A0), 20.00 (A1), 40.00 (A2) and 82.33 (A3) mg a.i. kg^{-1} soil on percentage infection of *Allium cepa* L. associated with *Glomus* sp. "City Beach" (WUM 16) at the 4-week (A) and the 8.5-week (B) harvests. Vertical bars represent standard errors of means, n=4; if no bar is shown, standard error is smaller than the line.

5.2.3. Discussion

In general, the three concentrations of Aliette were toxic to the onion plants whether or not the plants were mycorrhizal and this observation was in agreement with the effects reported in Chapter 3. Again it is clear that "matched" non-mycorrhizal plants (NMP1) were more sensitive than mycorrhizal plants (MP0).

It is true that percentage infection was not affected by Aliette application in this experiment, but length of infected root was markedly reduced at both harvests. This was associated with a reduction in growth of both roots and shoots in all Aliette treatments. The lack of reduction in percentage infection following application of Aliette was due to the reduction in fungal growth inside the root being proportional to root growth. This further emphasizes the importance of considering root growth as well as fungal growth in analysing fungicide effects on VA mycorrhizal symbiosis (see Chapter 3). This supports the observation described in Chapter 3, with respect to recovery of Aliette-treated plants after approximately 12 weeks. One explanation for the decreased sensitivity of mycorrhizal plants might be that mycorrhizal hyphae are able to convert phosphonate to phosphate and either transfer it to the plant across the mycorrhizal interface or make it available for absorption from soil, with the consequence that mycorrhizal plants suffered less than non-mycorrhizal plants from the toxic effects of the fungicide. Further experiments were required to determine whether both non-mycorrhizal and mycorrhizal plants were able to take up phosphonate as well as phosphate. If this is the case, it is necessary to quantify those compounds in the plants (Experiment 2).

The possible negative effects of aluminium (as a degradation product of Aliette) and of phosphonate on plant growth also need to be clarified (Experiment 3).

5.3. Experiment 2: Relative concentrations of phosphonate and phosphate in the plants following Aliette application

The aim of this experiment was to determine whether the high P concentrations and P contents in Aliette-treated plants (Chapter 3) were due to accumulation of phosphonate as well as phosphate. Nuclear Magnetic Resonance (NMR) spectroscopy was used to discriminate the two components, and the concentrations of phosphonate and phosphate present in the plants were quantified. The main points addressed in this experiment were:

1. To determine whether phosphonate as well as phosphate was taken up by both non-mycorrhizal and mycorrhizal plants following application of Aliette;
2. To quantify phosphonate and phosphate present in the plants; and
3. To clarify whether accumulation of phosphonate in the plants treated with Aliette was positively correlated with the amount of Aliette that was applied;
4. To assess the effect of the presence of the mycorrhizal fungus on relative concentrations of phosphonate and phosphate following Aliette application;
5. To study whether P (phosphate) application influences the relative concentrations of phosphonate and phosphate in non-mycorrhizal plants.

5.3.1. Materials and methods

Methodology for sowing, watering regimes, fertilising techniques, growth conditions, inoculation techniques and application of fungicide were as described in Chapter 2. Aliette fungicide only was used in this experiment at four concentrations: 0.0, 20.0, 40.0, 82.33 mg of a.i. kg^{-1} soil. Two groups of non-mycorrhizal plants, NMP0 and NMP1 and one type of mycorrhizal plant, MP0, were used.

There were four replicate pots per treatment with seven plants per pot to produce sufficient material for NMR spectroscopic analysis. The plants were harvested at 6, 8 and 11.5 weeks after sowing. At harvest, plants were washed free of soil, and shoots and roots were separated. Fresh weights of shoot and root were determined individually and then homogenised using an Ultraturrax in 10 ml of a solvent mixture composed of methanol:chloroform:water (12:5:3) and processed as described in section 2.17. The extracts were individually subjected to NMR spectroscopic analysis to determine the relative concentrations of phosphonate and phosphate (Fig 5.7). The presence or absence of phosphonate and phosphate in Aliette was also determined using NMR spectroscopy.

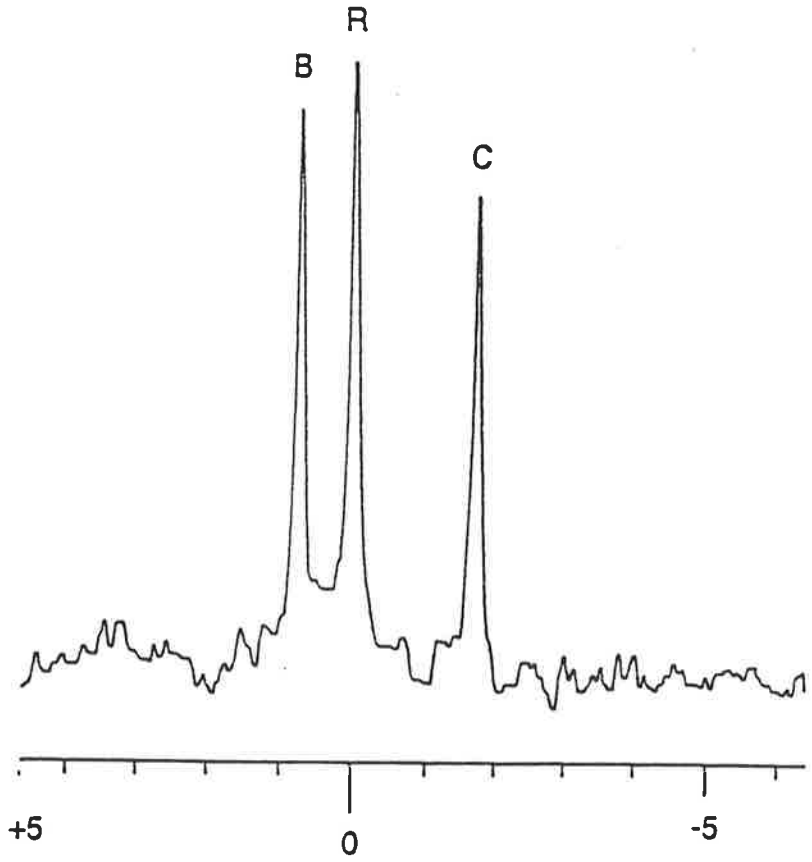


Figure 5.7. (Experiment 2). ^{31}P NMR spectrum of a representative shoot extract of an Aliette-treated plant showing the signals due to inorganic phosphate (B, 0.84 ppm) and phosphonate (C, -1.70 ppm) referenced against external capillary reference of Na_3PO_4 (R).

5.3.2. Results and Discussion

Based on ^{31}P-NMR analysis, the commercial Aliette used contains at least 90% alkyl phosphonate and less than 10% of free phosphonate (HPO_3^{2-}). No phosphate was detected in the sample (Fig. 5.8).

It proved difficult to coordinate harvesting and NMR spectroscopy of extracts of fresh materials, so a complete set of data was not obtained from this experiment. Comparison between treatments can be made, but it must be made clear that different times of harvest may have confounded the interpretation. However, there were interesting findings from this experiment (see Table 5.1). It was demonstrated that plants took up phosphate as well as phosphonate and that the relative concentrations in the plant were positively correlated with the concentrations of Aliette applied.

Table 5.1 shows that both non-mycorrhizal and mycorrhizal plants took up phosphonate as well as phosphate. In general, the relative concentrations of the two compounds in the tissue were different between treatments. Non-mycorrhizal plants had a higher ratio of phosphonate to phosphate than mycorrhizal plants.

In non-mycorrhizal plants where a P supplement was not present (NMP0), application of different concentrations of Aliette influenced the relative concentrations of phosphonate and phosphate. This relative concentration was positively correlated with the concentration of Aliette that was applied (Table 5.1). Even at low concentrations (20 mg a.i. kg^{-1} soil) a high proportion (60%) of

the total inorganic P in the tissue was phosphonate. This concentration may be sufficient to reduce plant growth (see Experiment 1). Comparison between NMP1 and MPO can be made only for the highest level of Aliette application between 6- and 8-week harvests. However, it is clear that the ratio of phosphonate : phosphate in NMP0 plants was similar to NMP1 (despite differences in availability of phosphate), while this ratio was much lower in MP0 plants. At the later times only two sets of data are available, but the results are consistent with the observation of the 8-week harvest.

At the 6-week harvest application of 82.33 mg active ingredient of Aliette kg^{-1} Aliette did not change the level of phosphonate in non-mycorrhizal plants whether or not P was applied. This suggested that up to 6 weeks the presence of P in the soil did not influence the uptake of phosphonate by the plants. By the 11.5-week harvest, the relative concentrations of phosphonate and phosphate in NMP1 plants had changed from 80:20 to 70:30. The change in the relative concentrations may be due to several factors:

1. Phosphonate had been converted to phosphate by the plants;

2. The plants were able to take up P which was applied to the soil as NaH_2PO_4, hence the concentration of phosphate was increased;

3. The plants exported phosphonate to the soil. This has been suggested by Despatie et al. (1989).

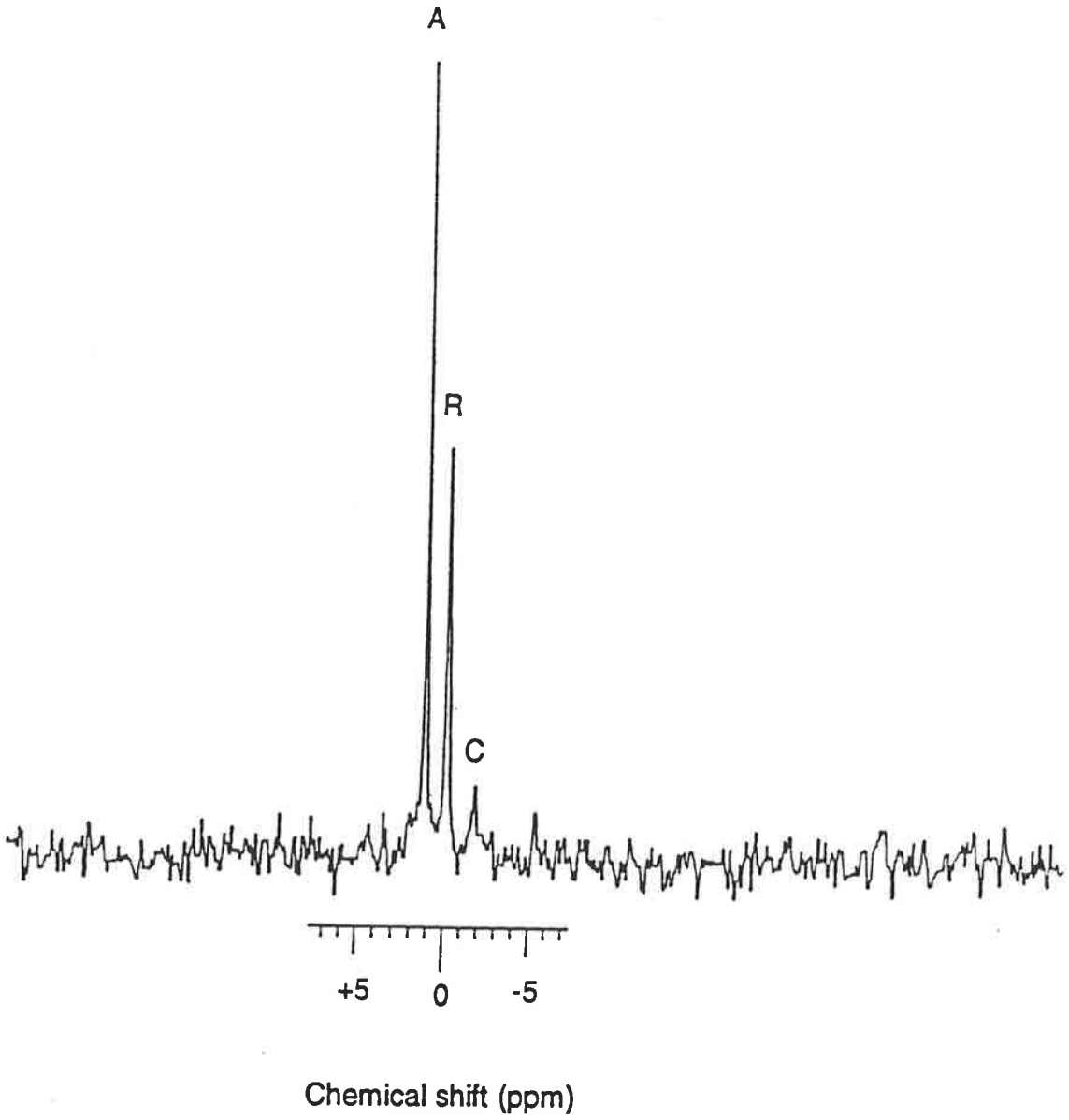


Figure 5.8. (Experiment 2). ^{31}P NMR spectrum of Aliette fungicide referenced against external Na_3PO_4 (R). Signals due to alkyl phosphonate (A) and inorganic phosphonate (C) were observed at 1.11 ppm and -1.70 ppm, respectively.

Table 5.1. (Experiment 2). Effects of Aliette concentrations on the relative concentration of phosphonate to phosphate of non-mycorrhizal and mycorrhizal *Allium cepa* L. associated with *Glomus* sp. "City Beach" (WUM 16) harvested at 6, 8 and 11.5 weeks

Treatments	Extracted sample (g)	Phosphonate: phosphate ratio
6 weeks		
NMP0		
0.00 mg kg^{-1}	3.11	00 : 100
20.00 mg kg^{-1}	3.00	60 : 40
40.00 mg kg^{-1}	3.46	75 : 25
82.33 mg kg^{-1}	2.92	80 : 20
NMP1		
0.00 mg kg^{-1}	4.02	00 : 100
20.00 mg kg^{-1}	NT	NT
40.00 mg kg^{-1}	NT	NT
82.33 mg kg^{-1}	2.08	80 : 20
8 weeks		
MP0		
0.00 mg kg^{-1}	5.11	00 :100
20.00 mg kg^{-1}	NT	NT
40.00 mg kg^{-1}	NT	NT
82.33 mg kg^{-1}	6.99	50 : 50
11.5 weeks		
NMP0		
0.00 mg kg^{-1}	NT	NT
20.00 mg kg^{-1}	NT	NT
40.00 mg kg^{-1}	NT	NT
82.33 mg kg^{-1}	NT	NT
NMP1		
0.00 mg kg^{-1}	NT	NT
20.0 mg kg^{-1}	NT	NT
40.0 mg kg^{-1}	NT	NT
82.33 mg kg^{-1}	3.00	70 : 30
MP0		
0.00 mg kg^{-1}	NT	NT
20.00 mg kg^{-1}	NT	NT
40.00 mg kg^{-1}	NT	NT
82.33 mg kg^{-1}	3.00	50 : 50

NM, non-mycorrhizal; M, mycorrhizal; P0, no added P; P1, 15 µg P kg^{-1} soil; NT, not tested.

In mycorrhizal plants, the relative concentration of phosphonate to phosphate did not change between the 8-week and the 11.5-week harvests. The incomplete data set cannot clearly distinguish between the alternative hypotheses. However, it can be concluded that:

1. Both non-mycorrhizal and mycorrhizal plants take up phosphonate as well as phosphate and that the relative concentrations in non-mycorrhizal plants were positively correlated with the concentration of Aliette applied;

2. Relative concentrations of phosphonate to phosphate were lower in mycorrhizal than in non-mycorrhizal plants. This together with the smaller effects of Aliette on the growth of mycorrhizal plants (Experiment 1) suggested that detoxification mechanisms may operate in these plants.

A third experiment was designed to provide information which had not been gained from this experiment and also to investigate recovery of mycorrhizal plants that was observed in Experiment 1 and Chapter 3. An attempt to separate out confounding effects of phosphonate and aluminium on Aliette-treated plants was also made. This was particularly important because aluminium-toxicity in soils of low pH results in damage to root meristems and stunting of the root system (Rengel, 1992).

5.4. Experiment 3: The effects of Aliette on the relative concentrations of phosphonate and phosphate in the plants: consequences for plant growth and influence of VA mycorrhizal fungi in overcoming phytotoxic effects

This experiment was undertaken to study:

1. The separate effects of aluminium, phosphonate and Aliette on plant growth and accumulation of phosphonate and phosphate in the plants;
2. Whether the relative concentrations of phosphonate and phosphate correlated with the observed reduction in growth of plants following application of Aliette;
3. The possibility of conversion of phosphonate to phosphate either by the plants or fungus.

5.4.1 Materials and methods

Methodology for sowing, watering regimes, fertilising techniques, growth conditions and inoculation techniques was as described in Chapter 2. The application of P was at one level (P0) in M pots and at two levels of P, 0 and 15 μg P g^{-1} soil, in NM pots.

There were four treatments: control, aluminium (applied as aluminium chloride), phosphonate (applied as dimethyl phosphonate) and Aliette. The concentrations were as follows: 31.01 mg $AlCl_3$ (equivalent to 6.28 mg aluminium) kg^{-1} soil; 82.33 mg dimethyl phosphonate (equivalent to 60.43 mg

phosphonate) kg^{-1} soil; 82.33 mg alkyl phosphonate (equivalent to 60.43 mg phosphonate) kg^{-1} soil for aluminium chloride, dimethyl phosphonate and Aliette treatments, respectively. The concentrations of aluminium and phosphonate were calculated from the concentrations of those compounds in Aliette. Dimethyl phosphonate was kindly supplied by Dr. Graham Jones, Department of Horticulture, Viticulture and Oenology, The University of Adelaide. Detailed information on Aliette application is provided in Chapters 2 and 3.

Aliette, dimethyl phosphonate and aluminium chloride were made up in 125 ml double distilled water and applied as soil drenches prior to sowing. The untreated control was watered with 125 ml double distilled water.

There were three replicate pots per treatment and 10 plants per pot to obtain sufficient material for NMR spectroscopic analysis. The pots were randomly rearranged three times per week and fertilised weekly (see Chapter 2). Three destructive harvests were conducted at 3, 6 and 9 weeks after sowing.

Methodology of harvesting and collecting data of plant growth and P analysis were as described in Chapter 2. Three measurements were made on the dried ground materials:

1. The three replicates were analysed separately to determine total P concentrations of shoots and roots by Bartlett's (1959) method. Values obtained were, therefore, phosphate plus phosphonate as in the experiment described in Chapter 3. Data obtained from this analysis will be referred to as " P from dried materials".

2. The replicates were pooled and shoots and roots were homogenised separately in 10 ml of a solvent system consisting of methanol:chloroform:water (12:5:3) using an Ultraturrax homogenizer to provide samples for NMR spectroscopic analysis, to provide data for relative and absolute amounts of phosphate and phosphonate in the tissues. Prior to NMR measurement, the supernatant of each sample was freeze-dried, dissolved in 2.5 ml D_2O and filtered. The method for preparation of samples and measurement of phosphate and phosphonate using NMR spectra was as described in Chapter 2 section 2.17.

3. After removal of a sub-sample for NMR analysis, another sub-sample (250 - 500 µl) was used for P analysis using Bartlett's (1959) method to provide data of the total P which was extracted. Data obtained from this analysis will be referred as "P from extract material".

5.4.2. Results

For simplicity, data for shoot dry weight, root dry weight and P concentration determined from dried material using Bartlet's (1959) method, for each treatment, will be presented individually under the headings aluminium, phosphonate and Aliette. Data for NMR spectroscopy and P analysis determined from extract material will be presented based on parameters measured.

5.4.2.1. The effect of aluminium on shoot dry weight, root dry weight and P nutrition determined from dried material, and on mycorrhizal infection

Aluminium chloride had no effect on shoot (Figs 5.9 - 5.11) and root dry weights (Figs 5.12 - 5.14) of either non-mycorrhizal or mycorrhizal plants at any harvest, with one exception. MP0 plants at 6 weeks were stunted. NMR analysis of these plants indicated the presence of phosphonate, suggesting contamination with either dimethyl phosphonate or Aliette (see Table 5.3 in section 5.4.2.4). These plants are not considered further.

Aluminium chloride also had no effect on P concentration in shoots or roots determined from dried material (Table 5.2a). Similarly, P content in both roots and shoots was unaffected by application of aluminium chloride and these observations were consistent in both non-mycorrhizal and mycorrhizal plants throughout the experiment (Table 5.2b).

Figs 5.15 and 5.16 show that there were no effects of aluminium chloride on total length of root infected or percentage infection at any harvest. Thus there was no evidence for aluminium toxicity in the plants. Toxicity would not have been expected at the pH of the soil used (pH 7.0).

5.4.2.2. The effect of dimethyl phosphonate on shoot dry weight, root dry weight and P nutrition determined from dried material, and on mycorrhizal infection

In mycorrhizal plants, shoot dry weight was unaffected by application of dimethyl phosphonate (Figs 5.9 - 5.11), but root dry weight was reduced at every harvest (Figs 5.12 - 5.14).

In non-mycorrhizal plants the effects of dimethyl phosphonate were more complex. In plants without P supplement (NMP0) there was no effect on shoot dry weight at the 3-week harvest, but at 6 and 9 weeks reductions were observed. There was no effect of dimethyl phosphonate on shoot growth of non-mycorrhizal, P-supplemented plants (NMP1). The root dry weight was markedly reduced, as with mycorrhizal plants (Figs 5.12 - 5.14). This observation was consistent throughout the experiment, in the presence and absence of P application.

Dimethyl phosphonate application increased P concentrations in shoots and roots (Table 5.2a). The increase was observed in both non-mycorrhizal and mycorrhizal plants at all three harvests. The increases in shoot P concentration were greater in non-mycorrhizal than in mycorrhizal plants.

P content in the shoots of both non-mycorrhizal and mycorrhizal plants was also increased following application of dimethyl phosphonate at all three harvests (Table 5.2b). Again, the increases occurred in both types of non-mycorrhizal plants. The effect of dimethyl phosphonate on P content of the root was not consistent. In general this treatment had no effect on root P content, with the exception that in NMP0 plants at the 6-week harvest a reduction was observed, while there was an increase in MP0 plants at the 9-week harvest.

The length of root infected was reduced (Fig. 5.15) whereas the percentage infection was unaffected (Fig. 5.16). These results were consistent in all harvests.

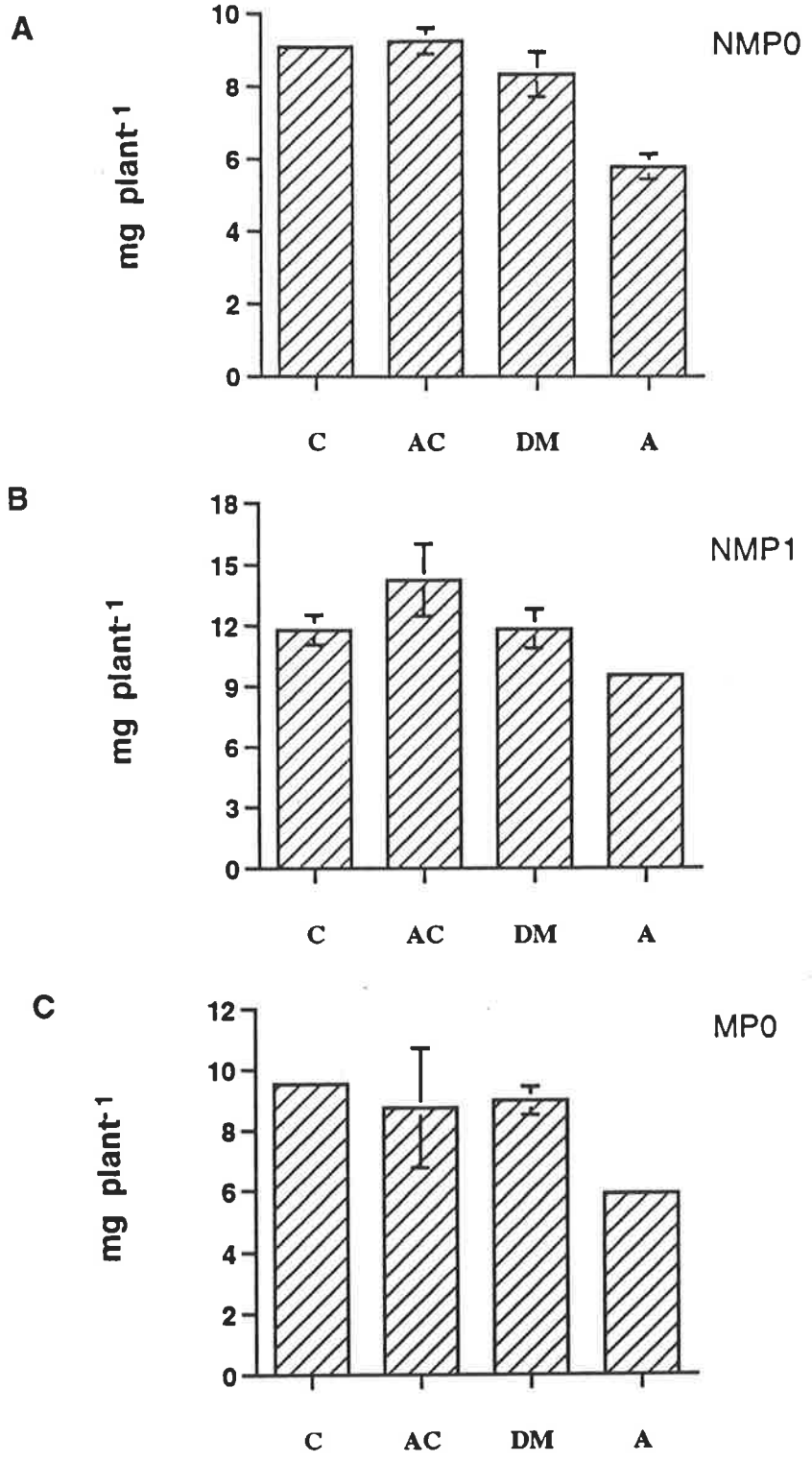


Figure 5.9. (Experiment 3). Effects of aluminium chloride (AC), dimethyl phosphonate (DM), Aliette (A) and a control (C) on shoot dry weight of *Allium cepa* L. at the 3-week harvest in non-mycorrhizal plants, no added P (NMP0)(A), non-mycorrhizal "matched" plants (NMP1)(B) and mycorrhizal plants (MP0)(C). Vertical bars represent standard errors of means, n=3; if no bar is shown, standard error is smaller than the line.

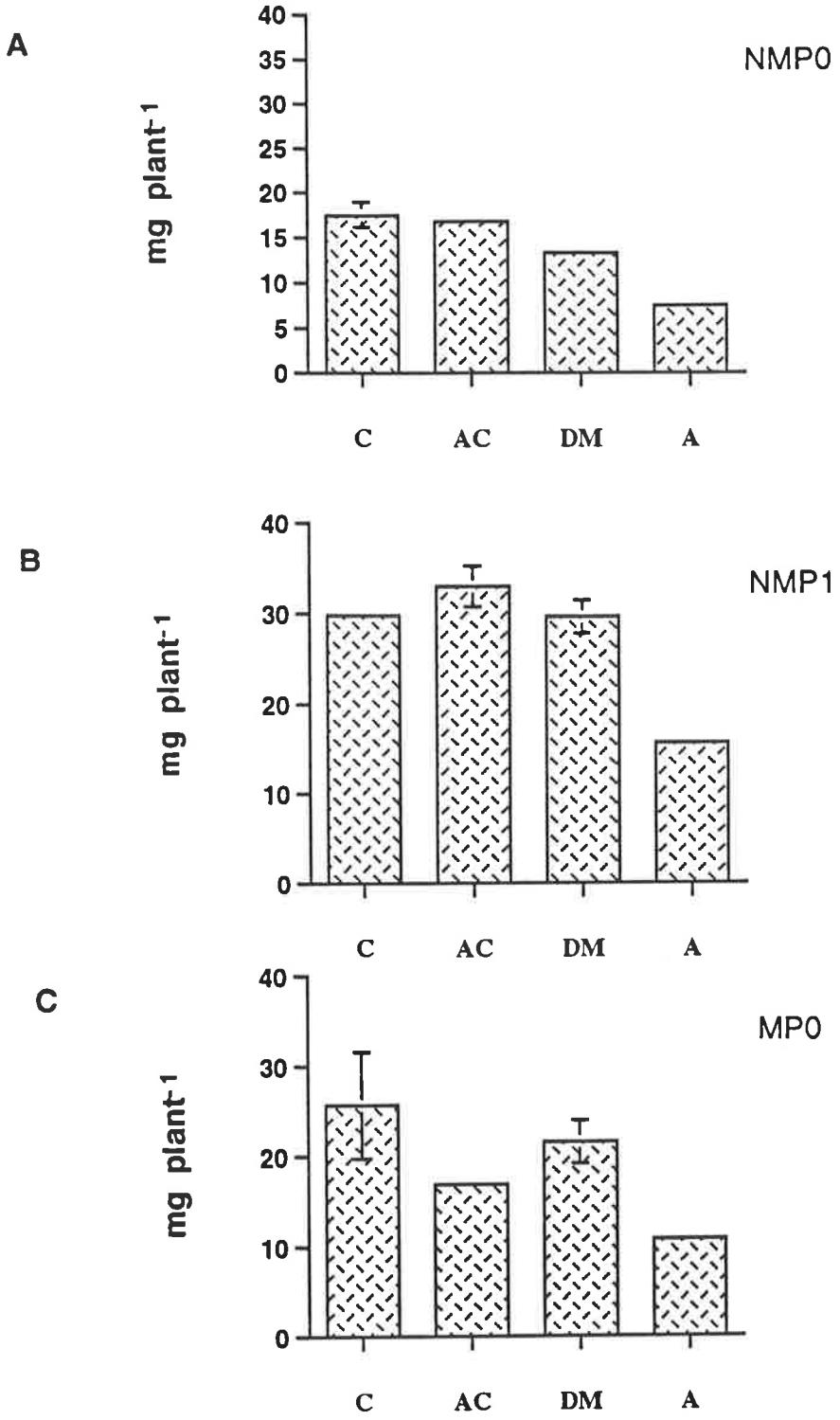


Figure 5.10. (Experiment 3). Effects of aluminium chloride (**AC**), dimethyl phosphonate (**DM**), Aliette (**A**) and a control (**C**) on shoot dry weight of *Allium cepa* L. at the 6-week harvest in non-mycorrhizal plants, no added P (NMP0)(A), non-mycorrhizal "matched" plants (NMP1)(B) and mycorrhizal plants (MP0)(C). Vertical bars represent standard errors of means, n=3; if no bar is shown, standard error is smaller than the line.

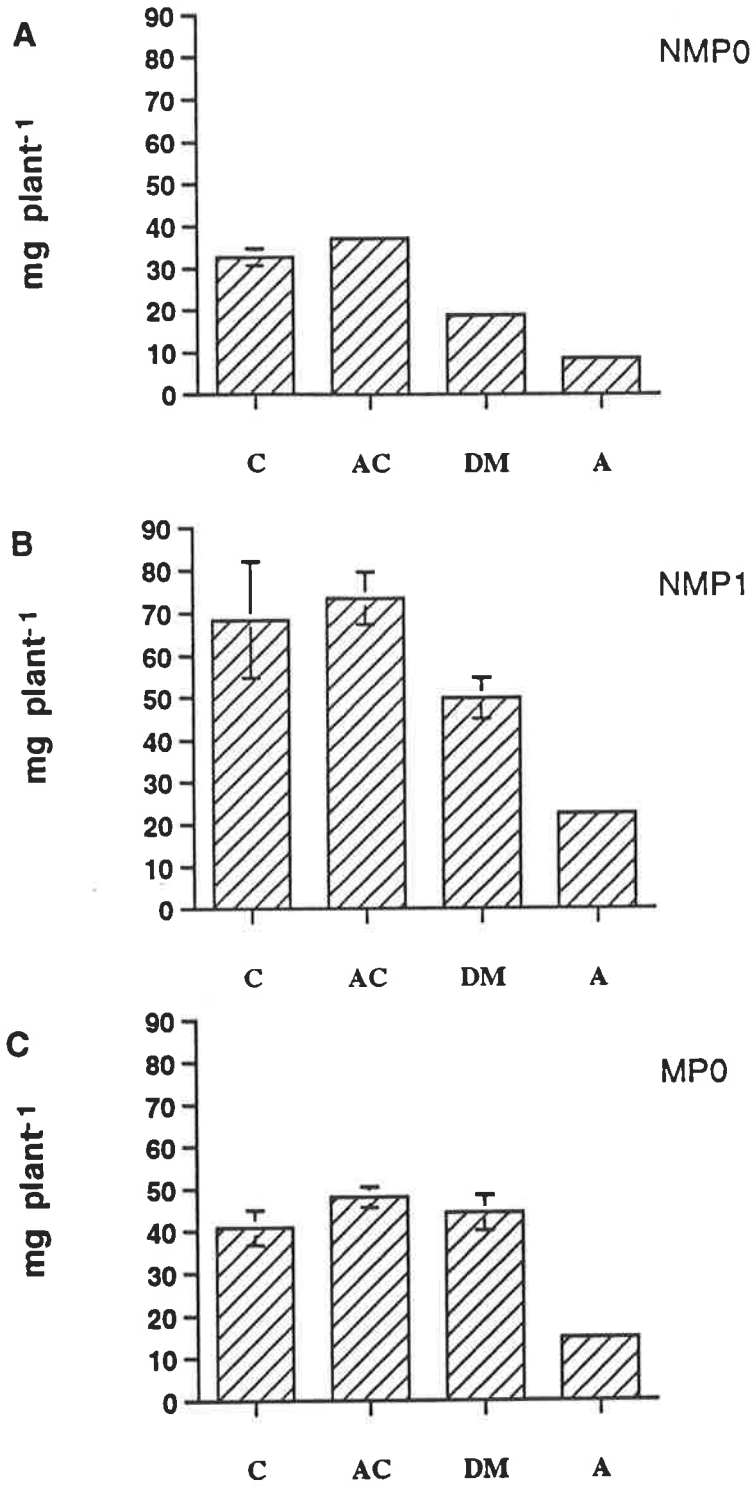


Figure 5.11. (Experiment 3). Effects of aluminium chloride (**AC**), dimethyl phosphonate (**DM**), Aliette (**A**) and a control (**C**) on shoot dry weight of *Allium cepa* L. at the 9-week harvest in non-mycorrhizal plants, no added P (NMP0)(A), non-mycorrhizal "matched" plants (NMP1)(B) and mycorrhizal plants (MP0)(C). Vertical bars represent standard errors of means, n=4; if no bar is shown, standard error is smaller than the line.

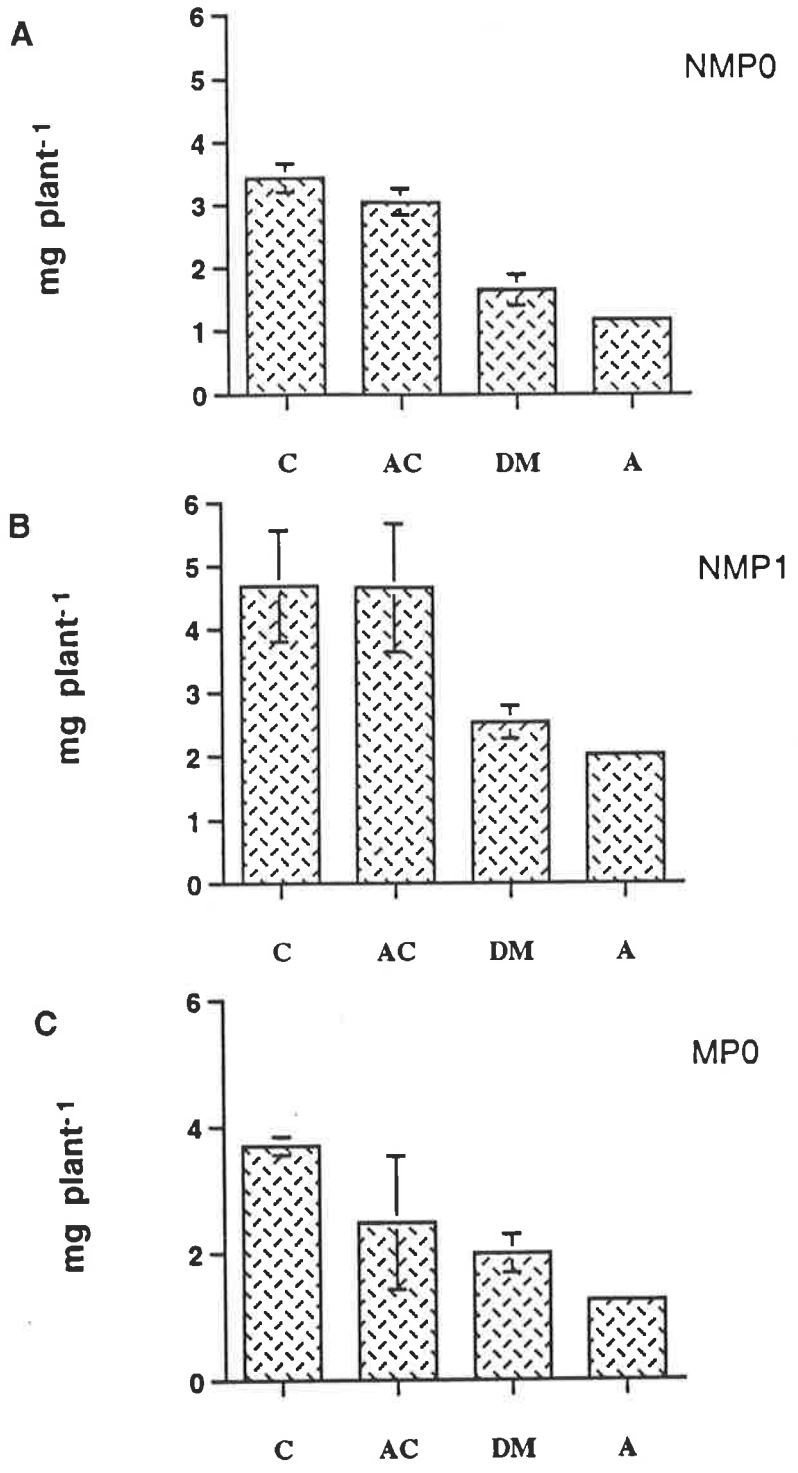


Figure 5.12. (Experiment 3). Effects of aluminium chloride (**AC**), dimethyl phosphonate (**DM**), Aliette (**A**) and a control (**C**) on root dry weight of *Allium cepa* L. at the 3-week harvest in non-mycorrhizal plants, no added P (NMP0)(A), non-mycorrhizal "matched" plants (NMP1)(B) and mycorrhizal plants (MP0)(C). Vertical bars represent standard errors of means, n=4; if no bar is shown, standard error is smaller than the line.

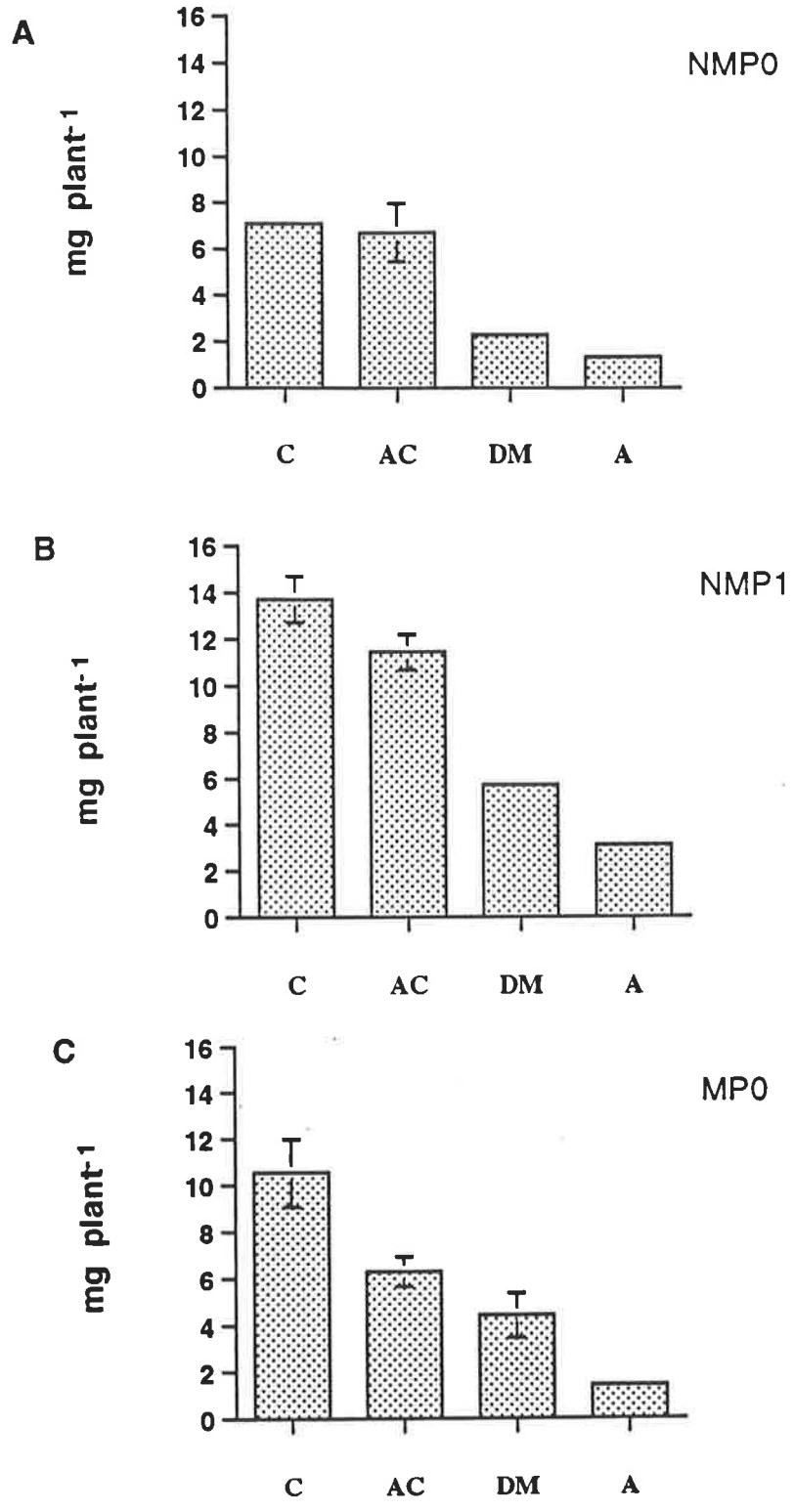


Figure 5.13. (Experiment 3). Effects of aluminium chloride (**AC**), dimethyl phosphonate (**DM**), Aliette (**A**) and a control (**C**) on root dry weight of *Allium cepa* L. at the 6-week harvest in non-mycorrhizal plants, no added P (NMP0)(A), non-mycorrhizal "matched" plants (NMP1)(B) and mycorrhizal plants (MP0)(C). Vertical bars represent standard errors of means, n=4; if no bar is shown, standard error is smaller than the line.

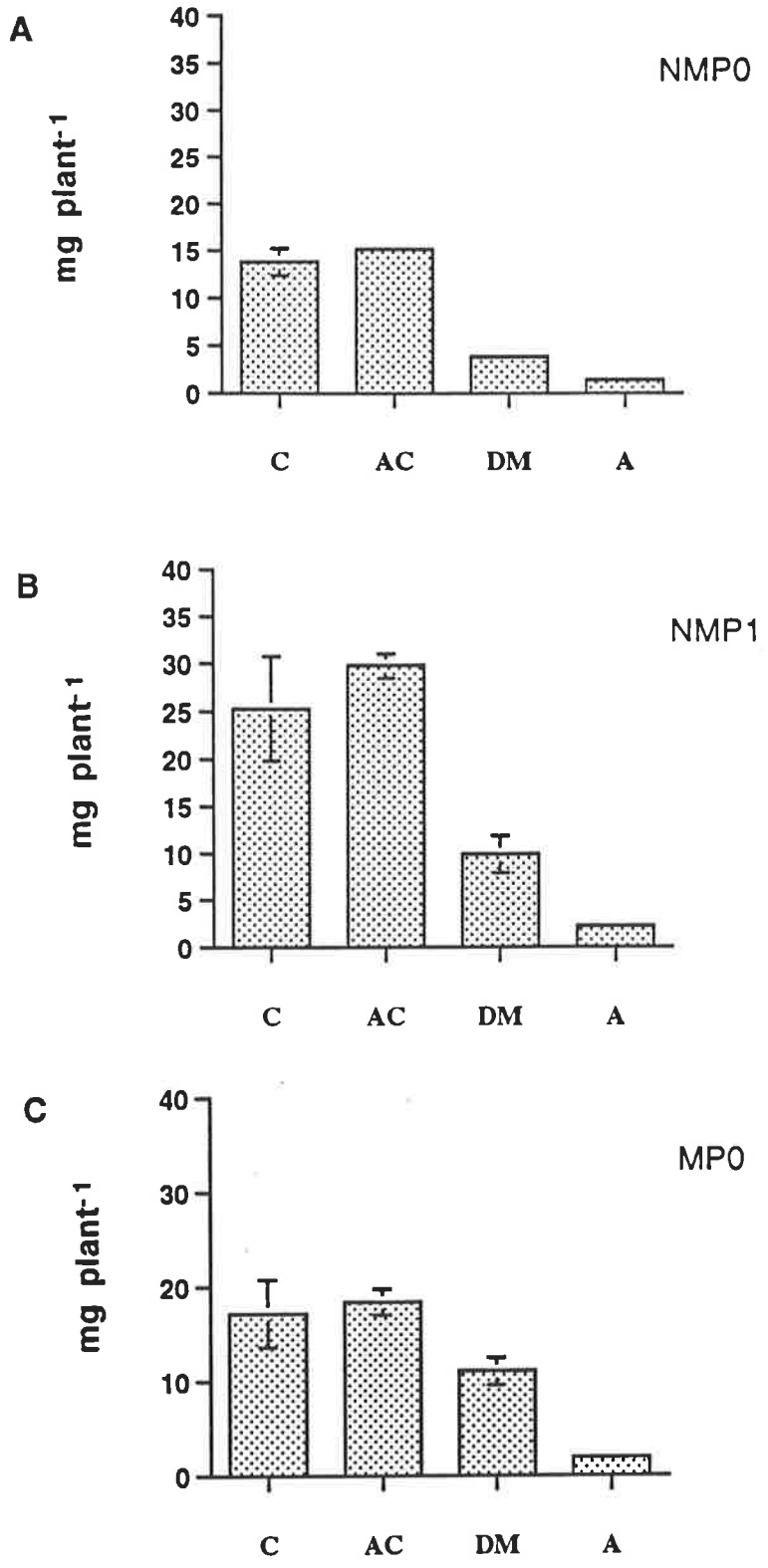


Figure 5.14. (Experiment 3). Effects of aluminium chloride (**AC**), dimethyl phosphonate (**DM**), Aliette (**A**) and a control (**C**) on root dry weight of *Allium cepa* L. at the 9-week harvest in non-mycorrhizal plants, no added P (NMP0)(A), non-mycorrhizal "matched" plants (NMP1)(B) and mycorrhizal plants (MP0)(C). Vertical bars represent standard errors of means, n=4; if no bar is shown, standard error is smaller than the line.

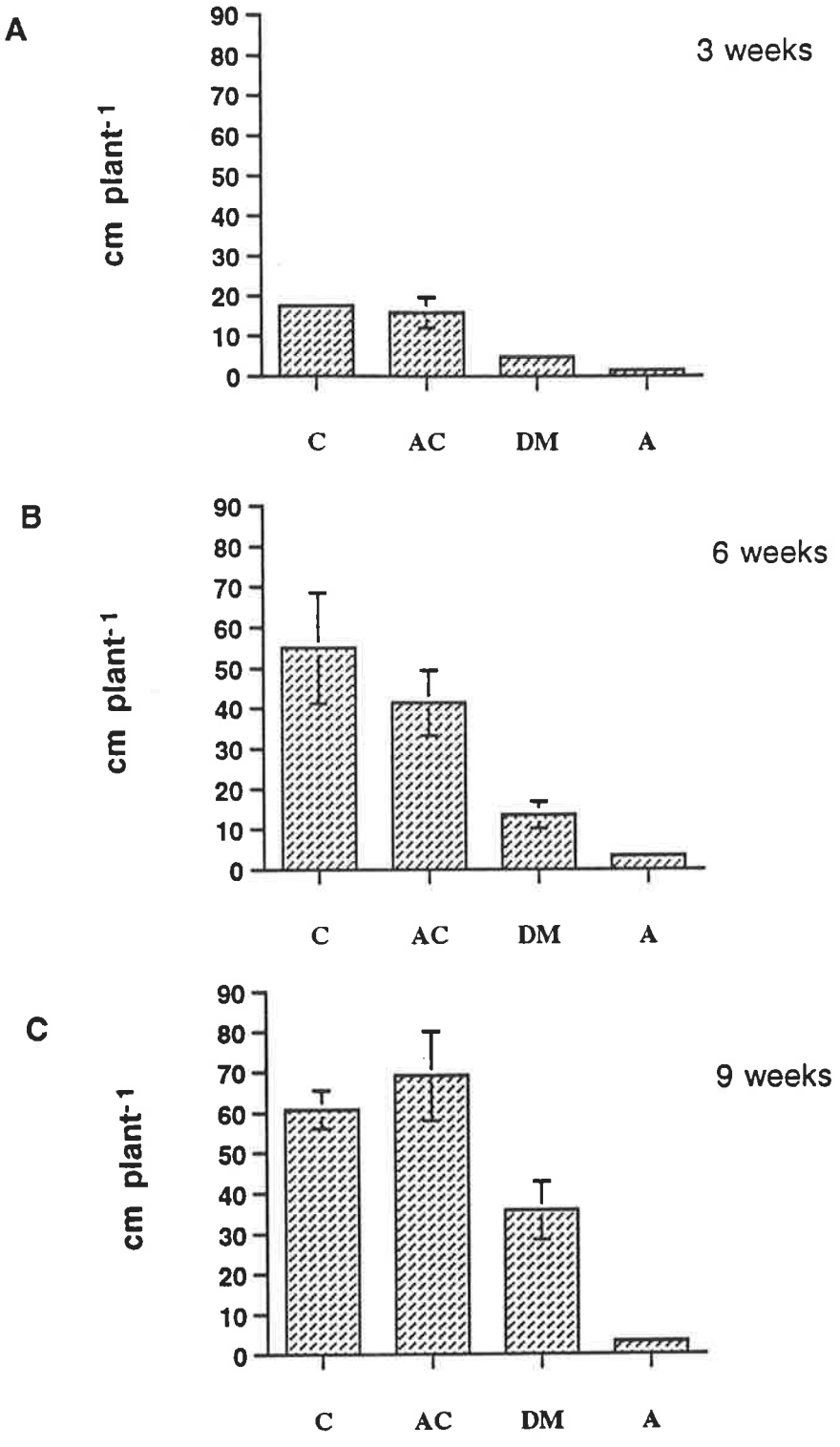


Figure 5.15. (Experiment 3). Effects of aluminium chloride (**AC**), dimethyl phosphonate (**DM**), Aliette (**A**) and a control (**C**) on infected root length of *Allium cepa* L. associated with *Glomus* sp. "City Beach" (WUM 16) at the 3- (A), 6- (B) and 9-week (C) harvests. Vertical bars represent standard errors of means, n=3; if no bar is shown, standard error is smaller than the line.

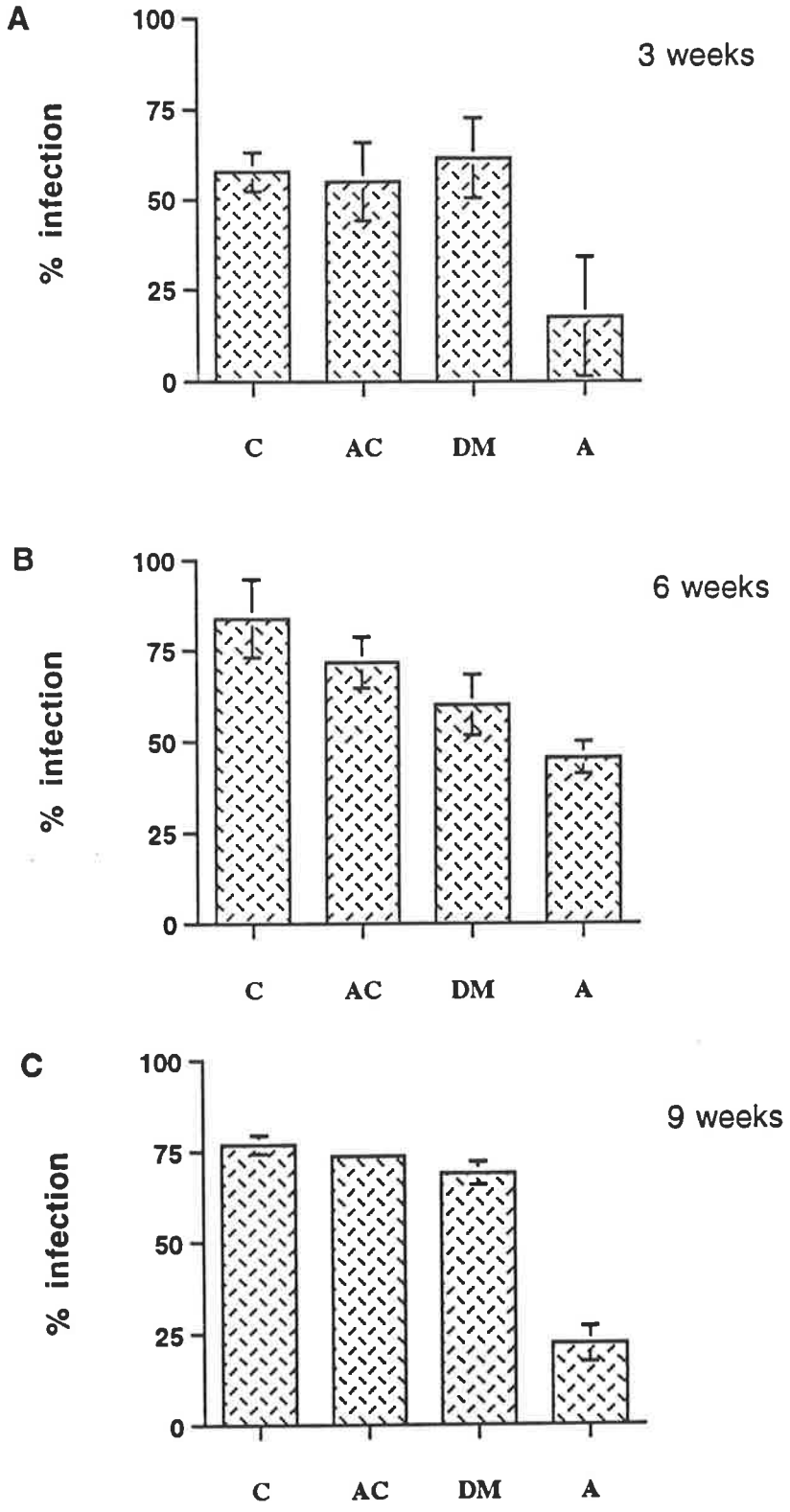


Figure 5.16. (Experiment 3). Effects of aluminium chloride (AC), dimethyl phosphonate (DM), Aliette (A) and a control (C) on percentage infection (%) of *Allium cepa* L. associated with *Glomus* sp. "City Beach" (WUM 16) at the 3- (A), 6- (B) and 9-week (C) harvests. Vertical bars represent standard errors of means, n=3; if no bar is shown, standard error is smaller than the line.

Table 5.2a. (Experiment 3). Effects of Aliette, dimethyl phosphonate and aluminium on P concentration of dried material of mycorrhizal and non-mycorrhizal plants using Bartlett's method. Plants were harvested at 3, 6 and 9 weeks.

	3 weeks	6 weeks	9 weeks
Treatment	µg P mg^{-1} dry weight	µg P mg^{-1} dry weight	µg P mg^{-1} dry weight
Shoot			
MP0 control	2.13	1.72 ± 0.12	1.40 ± 0.13
MP0 Aliette	6.83	6.84 ± 0.10	6.45 ± 0.10
MP0 dimethyl phosphonate	3.77	3.59 ± 0.03	3.67 ± 0.08
MP0 aluminium chloride	2.46	1.92 ± 0.17	1.43 ± 0.05
NMP0 control	1.87	1.80 ± 0.18	1.60 ± 0.04
NMP0 Aliette	8.74	8.50 ± 0.64	8.49 ± 0.52
NMP0 dimethyl phosphonate	4.70	2.81 ± 0.16	3.08 ± 0.10
NMP0 aluminium chloride	2.01	1.87 ± 0.06	1.37 ± 0.03
NMP1 control	2.78	1.73 ± 0.18	1.95 ± 0.17
NMP1 Aliette	8.50	8.61 ± 0.15	9.02 ± 0.10
NMP1 dimethyl phosphonate	4.97	4.20 ± 0.10	3.81 ± 0.13
NMP1 aluminium chloride	2.54	1.91 ± 0.17	2.27 ± 0.18
Root			
MP0 control	3.23	2.88 ± 0.22	2.36 ± 0.14
MP0 Aliette	8.41	7.10 ± 0.32	6.55 ± 0.32
MP0 dimethyl phosphonate	5.61	6.27 ± 0.20	5.53 ± 0.34
MP0 aluminium chloride	3.62	3.47 ± 0.03	2.19 ± 0.15
NMP0 control	2.26	2.95 ± 0.21	2.02 ± 0.12
NMP0 Aliette	6.69	5.11 ± 0.27	4.83 ± 0.34
NMP0 dimethyl phosphonate	4.23	4.59 ± 0.08	4.32 ± 0.04
NMP0 aluminium chloride	2.28	3.10 ± 0.17	1.98 ± 0.06
NMP1 control	3.27	2.35 ± 0.31	1.91 ± 0.17
NMP1 Aliette	9.08	8.82 ± 0.58	6.02 ± 0.11
NMP1 dimethyl phosphonate	7.64	5.20 ± 0.27	4.31 ± 0.12
NMP1 aluminium chloride	3.81	2.89 ± 0.36	2.21 ± 0.24

Values are means and standard errors from three replicate pots, except for 3-week harvest as the replicates were pooled; NM, non-mycorrhizal; M, mycorrhizal; P0, no added P; P1, 15 µg P kg^{-1} soil.

Table 5.2b. (Experiment 3). Effects of Aliette, dimethyl phosphonate and aluminium on P content of dried material of mycorrhizal and non-mycorrhizal plants using Bartlett's method. Plants were harvested at 3, 6 and 9 weeks.

	3 weeks	6 weeks	9 weeks
Treatments	µg P shoot^{-1} or root^{-1}	µg P shoot^{-1} or root^{-1}	µg P shoot^{-1} or root^{-1}
Shoot			
MP0 control	20.28	44.34 ± 11.11	55.37 ± 6.94
MP0 Aliette	40.37	73.72 ± 0.82	95.46 ± 1.91
MP0 dimethyl phosphonate	33.82	77.71 ± 8.93	162.71 ± 17.37
MP0 aluminium chloride	22.04	32.67 ± 4.04	69.01 ± 5.67
NMP0 control	16.93	31.69 ± 5.00	52.24 ± 2.59
NMP0 Aliette	50.34	63.17 ± 2.02	72.25 ± 1.46
NMP0 dimethyl phosphonate	38.78	37.15 ± 2.45	57.57 ± 4.60
NMP0 aluminium chloride	18.75	31.25 ± 1.96	50.73 ± 2.26
NMP1 control	32.67	51.63 ± 5.37	137.61 ± 38.00
NMP1 Aliette	81.26	134.65 ± 5.23	203.37 ± 11.50
NMP1 dimethyl phosphonate	58.75	124.06 ± 5.52	190.13 ± 18.00
NMP1 aluminium chloride	35.31	63.85 ± 9.46	166.15 ± 12.63
Root			
MP0 control	11.95	29.99 ± 3.68	39.74 ± 7.12
MP0 Aliette	10.51	10.07 ± 0.60	12.78 ± 2.11
MP0 dimethyl phosphonate	11.00	27.75 ± 3.93	61.45 ± 9.86
MP0 aluminium chloride	9.41	21.79 ± 2.14	39.96 ± 1.50
NMP0 control	7.75	20.97 ± 1.88	27.46 ± 1.09
NMP0 Aliette	7.89	6.71 ± 0.66	6.84 ± 0.84
NMP0 dimethyl phosphonate	6.85	10.40 ± 0.42	16.49 ± 1.98
NMP0 aluminium chloride	7.14	20.30 ± 2.94	29.79 ± 1.29
NMP1 control	15.27	31.74 ± 2.84	49.78 ± 13.93
NMP1 Aliette	18.33	27.54 ± 4.24	13.85 ± 2.81
NMP1 dimethyl phosphonate	19.33	29.66 ± 0.68	42.29 ± 7.75
NMP1 aluminium chloride	17.72	32.58 ± 2.05	65.37 ± 4.84

Values are means and standard errors from three replicate pots, except for 3-week harvest as the replicates were pooled; NM, non-mycorrhizal; M, mycorrhizal; P0, no added P; P1, 15 µg P kg^{-1} soil.

5.4.2.3. *The effects of Aliette on shoot dry weight, root dry weight and P nutrition determined from dried material, and on mycorrhizal infection*

Aliette markedly reduced shoot (Figs 5.9 - 5.11) and root dry weights (Figs 5.12 - 5.14) at all harvests of both non-mycorrhizal and mycorrhizal plants, and the effects of Aliette were more severe than for an equivalent concentration of dimethyl phosphonate.

Aliette application had effects similar to phosphonate application on P concentration. Aliette increased P concentrations of shoots and roots of both non-mycorrhizal and mycorrhizal plants (Table 5.2a) In comparison with phosphonate, the increase in P concentration following Aliette application was relatively high. This was observed in shoots and roots in both non-mycorrhizal and mycorrhizal plants. In addition, the increase in the total P (phosphonate plus phosphate) concentration of shoots in non-mycorrhizal plants was higher than that in mycorrhizal plants.

Aliette markedly increased shoot P content of both non-mycorrhizal and mycorrhizal plants at all harvests (Table 5.2b) The effects on root P content, on the other hand, were not consistent, there being either no effect or a reduction.

Fig. 5.15 shows that Aliette reduced the length of root infected at all harvests. Similarly, percentage infection was reduced markedly by application of Aliette (Fig. 5.16).

Despite stunted growth of roots and shoots, plants treated with Aliette had higher P concentrations in both. Furthermore, the high concentration of P was not consistent with reduction of fungal growth inside the root. These

observations were in agreement with the results reported in Chapter 3. The reduction in percentage infection did not agree with the results in Experiment 2, but reduction in the length of root infected was consistent in all experiments.

5.4.2.4. The effect of aluminium chloride, dimethyl phosphonate and Aliette on concentration of phosphonate and phosphate in the plants, measured using NMR spectroscopy

This information was derived from data obtained by NMR spectroscopy. As the material from three replicate pots was pooled before preparing the extracts for NMR spectroscopy it was not possible to apply the same statistical analysis as carried out for the measurement of dry weight or total P. Again, as the plant growth, especially of roots, was stunted following phosphonate and Aliette application, the amount of material available for analysis in some cases was so small that neither phosphate nor phosphonate could be detected in the extracts. The data available for roots confirm the observation on shoots (see Table 5.3).

Table 5.3 shows that both non-mycorrhizal and mycorrhizal plants took up phosphonate as well as phosphate following Aliette or dimethyl phosphonate application. In untreated control plants and plants treated with aluminium chloride, phosphate only was detected. These observations were consistent throughout the experiment except for the aluminium chloride treatment MP0 at the 6-week harvest already mentioned. The results for the Aliette treatments were in agreement with those of Experiment 2 reported in this chapter.

Aliette and phosphonate treatments in the absence of NaH_2PO_4 application increased the concentration of P in both roots and shoots compared to the untreated controls (Table 5.3). These effects were observed in both non-mycorrhizal and mycorrhizal plants and similar differences between the treatments were observed at all three harvests, except for MP0 at 9 weeks where Aliette and dimethyl phosphonate reduced root P concentration. The high concentration of phosphate in these treatments suggests that phosphonate had been converted to phosphate in the plants.

Comparisons between mycorrhizal and non-mycorrhizal plants in the absence of P application (Table 5.3) indicated that mycorrhizal plants had lower phosphonate concentrations than non-mycorrhizal plants at all harvests.

There was no clear trend in the concentrations of phosphate and phosphonate in the non-mycorrhizal plants to which both phosphate (NMP1) and Aliette had been applied. This may due to competition between NaH_2PO_4 and dimethyl phosphonate or Aliette for uptake by roots.

Table 5.3. (Experiment 3). Effects of Aliette, dimethyl phosphonate and aluminium chloride on concentration (µg mg^{-1}dry weight) of phosphate and phosphonate of mycorrhizal and non-mycorrhizal plants determined by ^{31}P NMR analysis. Plants were harvested at 3, 6 and 9 weeks.

	3 weeks		6 weeks		9 weeks	
Treatments	**Phosphate**	**Phosphonate**	**Phosphate**	**Phosphonate**	**Phosphate**	**Phosphonate**
Shoot						
MP0 control	0.29	ND	0.32	ND	0.71	ND
MP0 aliette	0.39	1.16	1.15	0.58	0.29	0.18
MP0 phosphonate	0.64	0.41	0.78	0.09	0.34	0.03
MP0 aluminium	0.46	ND	0.38	0.34[c]	1.18	ND
NMP0 control	0.21	ND	0.06	ND	0.34	ND
NMP0 aliette	0.65	2.96	1.78	3.04	0.72	1.85
NMP0 phosphonate	1.24	0.67	1.10	0.16	0.61	0.07
NMP0 aluminium	0.35	ND	0.38	ND	ND	ND
NMP1 control	0.71	ND	0.61	ND	0.57	ND
NMP1 aliette	0.72	0.67	0.35	0.39	0.87	0.79
NMP1 phosphonate	0.57	0.32	0.33	0.09	0.24	0.17
NMP1 aluminium	0.63	ND	0.58	ND	0.10	ND
Root						
MP0 control	0.53	ND	0.41	ND	2.59	ND
MP0 aliette	1.83	4.36	ND	1.43	1.91	ND
MP0 phosphonate	ND	ND	0.82	0.64	1.32	0.56
MP0 aluminium	ND	ND	0.70	ND	1.61	ND
NMP0 control	0.59	ND	0.64	ND	0.35	ND
NMP0 aliette	6.17	ND	1.77	3.04	2.26	ND
NMP0 phosphonate	0.63	0.69	4.64	1.35	1.74	0.66
NMP0 aluminium	0.37	ND	0.51	ND	1.15	ND
NMP1 control	0.56	ND	0.52	ND	0.87	ND
NMP1 aliette	1.48	6.48	0.52	2.25	0.87	1.52
NMP1 phosphonate	1.55	3.44	1.04	0.96	0.61	0.19
NMP1 aluminium	2.40	ND	0.34	ND	0.77	ND

NM, non-mycorrhizal; M, mycorrhizal; P0, no added P; P1, 15 µg P kg^{-1} soil; ND, not detected; [c] Contaminated by Aliette or dimethyl phosphonate.

5.4.2.5. P analysis from extracts of plants

Comparison of data for P concentration determined from dried materials and P concentration derived from extract materials of untreated control plants (either mycorrhizal or non-mycorrhizal) indicated that about 50 to 60% of the P in the dried plant tissues (compare Tables 5.2a and 5.4) was extracted by the solvent system consisting of methanol:chloroform:water.

It is noted that there are differences in the absolute amounts of P measured by Bartlett's method and by NMR spectroscopy with the latter being consistently lower than the former. It is not known at this time why the NMR method gives lower values but could indicate that the line broading effects of paramagnetic species have not been completely eliminated from the NMR measurement. Furthermore, this discrepancy could be due to the extraction of a number of water soluble P-containing compounds from the plant tissue which could not be detected as they did not give a sufficient signal to noise response in the NMR experiments.

Table 5.4. (Experiment 3). Effects of Aliette, dimethyl phosphonate and aluminium chloride on total P concentration (phosphate and phosphonate) from extract material of mycorrhizal and non-mycorrhizal plants determined by Bartlett's method. Plants were harvested at 3, 6 and 9 weeks.

	3 weeks	6 weeks	9 weeks
Treatments			
Shoot			
MP0 control	1.16	2.33	0.84
MP0 Aliette	4.93	4.78	3.39
MP0 dimethyl phosphonate	1.95	2.73	1.36
MP0 aluminium chloride	1.19	1.06	0.90
NMP0 control	1.12	0.62	0.71
NMP0 Aliette	6.47	9.68	7.87
NMP0 dimethyl phosphonate	2.76	2.21	7.63
NMP0 aluminium chloride	0.93	1.06	0.81
NMP1 control	1.45	1.18	1.00
NMP1 Aliette	6.70	6.07	5.38
NMP1 dimethyl phosphonate	3.13	8.31	1.51
NMP1 aluminium cloride	1.34	1.34	1.07
Root			
MP0 control	1.57	1.65	1.52
MP0 Aliette	5.48	30.89	3.21
MP0 dimethyl phosphonate	3.23	3.25	2.63
MP0 aluminium chloride	0.74	1.89	1.55
NMP0 control	1.58	2.17	0.66
NMP0 Aliette	4.27	9.68	3.59
NMP0 dimethyl phosphonate	3.98	3.69	2.99
NMP0 aluminium chloride	1.68	2.04	1.25
NMP1 control	2.22	1.31	1.51
NMP1 Aliette	11.38	3.52	4.36
NMP1 dimethyl phosphonate	6.65	3.37	3.27
NMP1 aluminium chloride	1.67	1.49	1.52

M, mycorrhizal; P0, no added P; P1, 15 μg P kg^{-1} soil; ND, not detected.

5.5. General Discussion

The information gained from all three experiments (Experiments 1 - 3) is included here.

This study not only confirmed that plants took up phosphonate following application of Aliette or dimethyl phosphonate as reported previously (Coffey & Ouimette, 1989; Despatie et al., 1989; Fenn & Coffey, 1985; Seymour et al.,1994), but also demonstrated that plants were able to convert phosphonate to phosphate. This finding contrasts with previous observations of Robertson & Boyer (1956), Despatie et al. (1989) and Guest & Grant (1991) who reported that plants were not able to convert phosphonate to phosphate. Furthermore, the high P concentration in onion plants following Aliette application observed in Experiment 3 was confirmed to be due to phosphonate accumulation and the consequent reduction in growth of plants was also confirmed.

Phosphonate concentrations detected in onion shoots following Aliette application ranged between 1.85 - 3.04 and 0.18 - 1.16 μg mg^{-1} dry weight for non-mycorrhizal and mycorrhizal plants, respectively. Coffey & Ouimette (1989) measured phosphonate concentration in tops (shoots and leaves) of avocado seedlings following application of Aliette as a soil drench at a concentration 10 times higher than the concentration applied in this experiment. They reported that the phosphonate concentration was 0.25 - 1.19 μg phosphonate mg^{-1} fresh weight between 1 and 8 weeks. Using 10% as a conversion factor from fresh weight to dry weight, it is possible to estimate the

concentration based on dry weight. The estimated phosphonate concentration would be 2.5 - 11.9 µg phosphonate mg^{-1} dry weight, which is of the same order of magnitude as in the experiment reported here. This is unexpected because of the higher Aliette concentration applied in their experiment. A possible explanation for this may be that there were differences in the clay content of the soils used and hence differences in capacity to adsorb fungicide. The soil used in this experiment had a lower clay content (soil:sand = 10:90) than that used in their experiment.

It was confirmed that aluminium alone (applied as aluminium chloride) had no effects on the growth of non-mycorrhizal and mycorrhizal plants, as would be expected at a soil pH of 7.0 and above. Aluminium toxicity associated with poor root growth, inhibition of root cell mitosis and root elongation (Fiskesjo, 1990; Rengel, 1992), is normally observed at a low pH.

In contrast to aluminium chloride, application of either dimethyl phosphonate or Aliette caused the growth to be stunted. This stunted growth was related to the concentration of phosphonate that was accumulated by the plants. Therefore, with the dimethyl phosphonate treatment it can be concluded that stunted growth was due solely to the phosphonate present in the plant. Furthermore, the relative concentration of phosphonate to phosphate in non-mycorrhizal plants treated with Aliette was positively correlated with the concentration of Aliette applied. This finding is in agreement with that reported by Seymour et al. (1994) who showed that application of Aliette and phosphonic acid resulted in a reduction in dry weight in the tops of maize. The data does not support the observations of Despatie at al. (1989) and Guillemin

& Gianinazzi (1991), who reported that single foliar applications of Aliette to non-mycorrhizal plants increased root biomass of onion and pineapple, but had no effect on shoot biomass. In mycorrhizal plants, root and shoot biomass were unaffected by either single or multiple applications.

This study shows that it is unlikely that the increased P concentration in mycorrhizal plants following application of Aliette was correlated with enhancement of fungal growth in the root, expressed as percentage infection, as had been reported previously (Jabaji-Hare & Kendrick, 1987).

Comparisons between Aliette and dimethyl phosphonate treatments indicated higher phosphonate concentrations in the Aliette-treated plants, resulting from possible synergistic effects of phosphonate and aluminium, which led to the plant taking up more phosphonate. This contrasts with effects of Aliette and phosphorous acid observed in several *Phytophthora* spp. (Coffey & Joseph, 1985; Fenn & Coffey, 1984, 1985; Cohen & Coffey, 1986). Aliette generally has low activity against *Phytophthora* spp. *in vitro.* whereas phosphorous acid showed a high inhibitory effect. Because of this, it was suggested that Aliette may act indirectly against the pathogens by triggering a defence mechanism in the host (Bompeix et al., 1980). The mechanisms of action of Aliette and phosphonate (or phosphorous acid) in the plant and the possible complicating effects of aluminium are not fully understood (Grant, 1990; Coffey & Ouimette, 1989; Cohen & Coffey, 1986).

Both non-mycorrhizal and mycorrhizal plants took up phosphonate as well as phosphate, following either phosphonate or Aliette application, and plants treated with phosphonate or Aliette had higher concentrations of phosphate in

comparison with the corresponding untreated control. This was observed in both non-mycorrhizal and mycorrhizal plants, suggesting that in both treatments phosphonate may be converted to phosphate either by the plants or the fungus or both. This has not been reported in the literature previously.

There has been one attempt to quantify phosphonate in roots of non-mycorrhizal and mycorrhizal onion plants following Aliette application (Despatie et al., 1989). However, neither the concentration of phosphate nor the phosphonate/phosphate ratio was determined. Consequently, the interpretation (Despatie et al., 1989) that neither the plant nor the fungus could convert phosphonate to phosphate must be viewed with caution.

With dimethyl phosphonate treatments, mycorrhizal plants had lower concentrations of phosphonate compared to non-mycorrhizal plants, which was correlated with greater growth. This suggests that the mycorrhizal fungus converted phosphonate to phosphate more efficiently than non-mycorrhizal plants. The immediate question is where does the conversion occur? In mycorrhizal plants, the conversion of phosphonate to phosphate may occur either in the fungus, the root and/or the shoots, whereas in non-mycorrhizal plants it could occur in the roots and/or the shoots. Further work will be required to identify which tissues are involved. In addition, it will be important to study possible mechanisms of competition between phosphonate and phosphate for uptake by the roots or mycorrhizal fungus. In pathogenic fungi, it has been proposed by Grant (1990) that competition occurs between phosphonate and phosphate for common membrane transport proteins. He also postulated that a high affinity phosphonate/phosphate accumulation

system may sometimes operate in plant tissues. Therefore, it is likely that phosphonate/phosphate transport depends on the relative concentrations of the two compounds in the medium and/or the quantity of phosphonate/phosphate transporter proteins present in cell membranes. This needs further investigation because the values for the concentrations of phosphonate and phosphate in the tissues are the result both of uptake (which may differ with different phosphonate:phosphate ratios in soil) and of possible conversion.

CHAPTER 6. GENERAL DISCUSSION

The findings of this investigation demonstrated that the systemic fungicides Aliette, Benlate and Ridomil had complex and differing effects on the VA mycorrhizal symbiosis. Benlate appeared to act directly on the fungus whereas Aliette and Ridomil inhibited both plant growth and fungal colonisation of the roots. This investigation represents the first attempt to determine effects of the fungicides on growth of P-sufficient plants in the absence of infection, as a pre-requisite to understanding the effects of fungicides on the VA mycorrhizal symbiosis. The three fungicides clearly had different effects on the growth of non-mycorrhizal *Allium cepa*. Benlate had no effect on plant growth, whereas Aliette and Ridomil reduced it markedly.

This study also demonstrated greater inhibition of fungal colonisation by the fungicides than had been indicated previously. Benlate not only reduced mycorrhizal colonization and suppressed metabolic activity of mycorrhizal fungus in the root and P inflow via the fungus, as previously reported (Fitter & Nichols, 1988; Jalali & Domsch, 1975; Kough et al., 1987; Hale & Sanders, 1982), but also reduced development of the living external mycelium, area of living interface between plant and fungus and the P flux to the plant across the living plant-fungal interface. All of these factors led to a relatively small contribution of the VA mycorrhizal fungus to P acquisition by the plant and a considerably reduced growth response when P supply in the soil was limiting. Inhibition of arbuscule formation and development of hyphae in soil is in line with effects of methyl benzimidazole-2yl-carbamates (to which group Benlate

belongs) on fungal development. These compounds reduced the number of cytoplasmic microtubules and apparently inhibited intracellular transport of vesicles of *Fusarium acuminatum* and caused abnormal growth of hyphal tips and hence branching patterns of *Aspergillus nidulans* (Howard & Aist, 1980; Jochova et al., 1993). Such changes may also occur in tip cells of VA mycorrhizal fungi. If this is so, arbuscule formation would be detrimentally affected, as shown in this investigation, with respect to number of arbuscules per section of root, particularly at harvests immediately following Benlate application. It would be possible to make a detailed study of the effects of Benlate on arbuscular branching using transmission or scanning electron microscopy. Furthermore, the recent use of confocal microscopy to study an important system of pleiomorphic tubules in the ectomycorrhizal fungus *Pisolithus tinctorius* (Shepherd et al., 1993) opens up a new avenue to study inhibition of this system, including effects of Benlate.

The lack of phytotoxic effects and marked reduction in mycorrhizal contribution to P uptake and growth mean that Benlate can continue to be used to generate non-mycorrhizal control plants in field experiments (Carey et al., 1992; Fitter, 1986; West et al., 1993a,b). Problems in interpretation of the results have arisen with application of Benlate in the field due to complexities of the interaction between pathogens and mycorrhizas (West et al., 1993b). For example, if Benlate kills both mycorrhizal and pathogenic fungi it is possible that application of the fungicide may stimulate growth of non-mycorrhizal control plants due to elimination of the pathogens. Furthermore, negative effects on saprophytic fungi in soil, as shown by reduction in length of active hyphae in uninoculated pots (Chapter 3) must not be ignored and may indicate

possible side-effects of Benlate application on organic matter turnover and nutrient cycling. Suppression of *Penicillium* sp., a common soil fungus, has also been reported by West et al. (1993a) following Benlate application in the field.

From a commercial standpoint, the lack of phytotoxicity of Benlate is clearly also important. However, this fungicide should not be applied in situations where plant productivity is dependent on a mycorrhizal contribution in highly mycorrhiza-dependent species such as onion, leek, carrot, faba bean, sweet corn, linseed, citrus, oil palm and cassava (Plenchette et al., 1983; Thompson, 1991; Graham et al., 1991; Blal et al., 1990; Sieverding, 1991). In this study, the growth of mycorrhizal onion plants with respect to root length and shoot dry weight was reduced significantly following Benlate application. The work reported in this thesis has confirmed and amplified previous results for Benlate and also describes a more detailed analysis on the effects of this fungicide on the VA mycorrhizal symbiosis.

Both Aliette and Ridomil reduced total root length infected and percentage infection, with the effects of Aliette being more marked than those of Ridomil. There was considerable inhibition of plant growth by both of these fungicides, which could have resulted from the combined effects of phytotoxicity and reduced contribution of the mycorrhizal fungus to plant nutrition. There was no indication that Aliette increased percentage infection, as reported previously by Jabaji-Hare & Kendrick (1987) for *Allium porrum*, regardless of the concentration used (see Chapters 3 and 5). Poor root growth following application of Aliette (as observed here) could have brought about this result if fungal growth had not also been affected. Jabaji-Hare & Kendrick (1987) applied Aliette to 55-day old plants in which the infection had already become

well established. Differences in the method of fungicide application, the timing of application relative both to plant age and stage of mycorrhizal colonization, the duration of the experiment and the species of host plant may explain these discrepancies. Use of Aliette as a soil drench to mycorrhiza-dependent plants cannot be recommended. However, application of the fungicide as a foliar spray is likely to be less damaging to mycorrhizal colonization than soil drenches. The amount of fungicide reaching the roots following foliar application depends on the ability of the plant to absorb and translocate the fungicide through the phloem, consequently the amount reaching the roots may be lower and also delayed compared with a situation where the fungicide can be absorbed directly by the roots. Fungicides applied as foliar sprays act only within the root, influencing colonization of cortex but not directly on fungal growth in soil. The stage of fungal and plant development at the time of application may also be important. For example, application of Aliette to plants where the infection is already established may have a different effect from that when applied prior to planting. It could be envisaged that an increase in percentage infection following Aliette application after the infection had become established (as observed by Jabaji-hare & Kendick, 1987) was due to the reduction of root growth being proportionally greater than the reduction in fungal growth inside the root. The effect of Aliette on fungal growth inside the root could be determined in a multiple harvest experiment of the type used by Tester et al. (1986) and Bruce et al. (1994) to model the rates of different processes contributing to infection. Comparison of both rates of infection from soil and growth within the root could be made following foliar spraying and soil drenching.

Time of harvesting may also be an important factor, as it was clearly demonstrated in this study that initial stunted growth of mycorrhizal plants treated with Aliette was followed by recovery. This emphasizes the importance of relatively long experiments with multiple harvests in elucidating the interactions. The absence of reports in the literature on the reduction of growth of mycorrhizal plants following Aliette application may be due to the plant being fully recovered from initial stunted growth at the time of harvest. Phytotoxic effects of Aliette on growth and the ability of plants to recover may vary between species. This requires further investigation.

It was demonstrated that both non-mycorrhizal and mycorrhizal plants took up phosphonate as well as phosphate following application of Aliette or dimethyl phosphonate to the soil. Accumulation of phosphonate therefore accounts for the high P concentrations in the tissues reported here and by Seymour et al.(1994). The relative concentration of phosphonate to phosphate was correlated with the phytotoxic effects of Aliette. Another important finding was that mycorrhizal plants had lower relative concentrations of phosphonate to phosphate following Aliette application than non-mycorrhizal plants and that the lower ratio was associated with better growth. This effect has not been reported before. It seems likely that the mycorrhizal fungus (or the mycorrhizal plant) is able to convert phosphonate to phosphate more effectively than uninfected plants. Alternatively, the mycorrhizal hyphae (although less extensive and active after treatment with Aliette) absorbed phosphate more rapidly than phosphonate. This might imply that the phosphate/phosphonate transporters in the plant and fungus had different affinities for the two ions (see Grant et al.,

1990 and Chapter 5). This point would need to be followed up in uptake studies using ^{32}P-labelled phosphate and phosphonate.

After treatment with Aliette the MP0 plants grew better than NMP1 plants, which was the reverse of the situation when no fungicide was applied. The most likely explanation is that the mycorrhizal fungi not only improved P nutrition, but also increased the uptake of other nutrients, compensating for the poor root growth and resulting in generally improved plant nutrition. The consequence of this would be increased tolerance of the phytotoxic effects of Aliette. The role of VA mycorrhizal fungi in taking up nutrients other than P, such as N, Zn and Cu has now been well documented (Raven et al., 1978; Jakobsen & Rosendahl, 1990; Cooper & Tinker, 1978; Thompson, 1989; Li et al., 1991). It is likely that hyphal networks in the soil may replace the function of roots stunted by other mechanisms including naturally poorly developed magnoloid roots (Baylis, 1975), aluminium toxicity (Rengel, 1992) and compacted soil. This area is of importance and requires more investigation with respect to management of the potential contribution of mycorrhizas to plant production.

In conclusion, widespread application of Aliette in the field should be preceded by investigations of its effects on both plant growth and the VA mycorrhizal symbiosis using a wide range of species of symbionts.

Another issue of practical importance in optimising the effects of Aliette on disease control is the competition between phosphate and phosphonate with respect to uptake. The reports of effects of phosphate on the efficacy of phosphonate (or phosphorous acid) in inhibiting growth of *Phytophthora* spp. are inconsistent. Bompeix et al.(1980) reported that addition of phosphate to

the medium reduced the inhibitory effect of phosphorous acid on *Phytophthora capsici* grown on tomato leaflets, whereas Dolan & Coffey (1988) found the converse to be true for *Phytophthora palmivora* on tomato seedlings. It has been suggested that different responses may be due to differences in the species of fungi and phosphate concentration in the medium (Griffith et al., 1989a,b). Another factor that may influence the interaction between phosphate and phosphorous acid in controlling pathogens is timing of phosphate application (Bompeix et al., 1980). Simultaneous application of phosphate and phosphorous acid resulted in lower efficacy of the fungitoxic compound, possibly via competition for uptake sites. On the other hand, application of phosphate either before or after phosphorous acid application had no effect (see Coffey & Ouimette, 1989).

If the relative concentration of phosphonate to phosphate (applied simultaneously) is crucial for controlling fungal pathogens, and this ratio is similar to that required to eliminate phytotoxicity, then the situation becomes very complicated, particularly with respect to interactions with mycorrhizal fungi. Plants associated with mycorrhizal fungi normally have higher tissue P concentrations than non-mycorrhizal plants of the same size (e.g. Stribley et al., 1980; Oliver et al., 1983) and it will be most important to determine whether this increase (coupled with increased phosphonate/phosphate conversion) is sufficient to alter the phosphate/phosphonate ratio to a level sufficient to reduce the fungicidal effects of Aliette on pathogens. Further studies are required to understand the different mechanisms of uptake and of conversion of phosphonate to phosphate in plants and fungi.

Ridomil had similar effects to Aliette, reducing the length of infected root, development of living intercellular hyphae, arbuscules and vesicles and hence area of living interface between plant and fungus. The length of living external hyphae per gram of soil was also reduced. Again, this fungicide had negative effects on growth of non-mycorrhizal plants. Thus, the reduction in P uptake and hence growth of mycorrhizal plants following Ridomil application is the result of multiple effects of this fungicide on the fungus-plant interaction. The reduction in mycorrhizal colonization and hence growth of mycorrhizal plants supported the finding of Jabaji-Hare & Kendrick (1987) but not that of Nemec (1980). In general, Ridomil had smaller effects on the development of intercellular hyphae, arbuscules, vesicles and also fungal development in the soil than Benlate. However, negative effects of this fungicide on plant growth complicated the analysis of its effects on the VA mycorrhizal symbiosis. Ridomil clearly affects both plant and fungus and its use should be carefully controlled. In this context it is interesting to hear reports that its use has already been banned in some countries (A.W. Gunawan, personal communication).

Both Ridomil and Aliette have been shown to have negative effects on the growth of onion plants, making it difficult to analyse the mycorrhizal interaction. This may help to explain the conflicting data in the literature. This investigation has made it quite clear that a simplistic approach to the analysis of mycorrhizal effects in terms of percentage infection is inadequate.

VA mycorrhizas play a very significant role in agriculture, particularly in low input agricultural systems, where they are important for absorption of nutrients from the soil and may play roles in drought tolerance, disease tolerance and in increasing soil structural stability (Auge & Stodola, 1990; Linderman, 1992;

Tisdall, 1991; Tisdall & Oades, 1980; Nelson, 1993). The potential ability of the networks of VA mycorrhizal hyphae in the soil to stabilise aggregates and hence reduce soil erosion is likely to be of increasing importance in agricultural systems where minimum or reduced tillage is practised. In tropical countries with high rainfall the importance of mycorrhizas is probably significant both in prevention of erosion and in rapid nutrient cycling to minimise leaching. It will be necessary to evaluate the perceived benefits of fungicide application to reduce crop losses from pathogens, against the possible disadvantages of reducing or eliminating mycorrhizal fungi.

The findings of this investigation emphasise the importance of considering plant growth in the absence of the fungus when investigating the effects of systemic fungicides (or any other external factor) on VA mycorrhizal symbiosis and also highlight the need for detailed investigations when analysing the VA mycorrhizal symbiosis.

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APPENDIX

(Reprints of papers)

1. Sukarno N, Smith SE, Scott ES. 1993. The effects of fungicides on vesicular-arbuscular mycorrhizal symbiosis. I. The effects on vesicular-arbuscular mycorrhizal fungi and plant growth. *New Phytologist* **125**: 139-147.

2. Sukarno N, Smith SE, Scott ES. 1994. The effects of fungicides on transport of phosphorus from fungus to plant in vesicular-arbuscular mycorrhizal symbiosis. In: Pankhurst, ed. *Soil biota: management in sustainable farming systems (poster papers).* Adelaide: CSIRO Australia, 111-113.

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