



**The role of vesicular-arbuscular mycorrhizal fungi in
Linum usitatissimum L. production in Southern
Australian soils.**

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This thesis is dedicated to my wife Nicole, for all her love,
inspiration and encouragement.

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Abstract

This project consisted of two distinct sections. In the first, the role of indigenous VAM fungi in the growth and nutrition of linseed (*Linum usitatissimum* L.) grown in a field soil was examined. Screening of soils from two sites (Dunn and Merildin) near Clare, South Australia with different cropping histories and assessment of the VAM fungal infectivity of these soils revealed that linseed grown in Dunn soil was more heavily colonised by indigenous VAM fungi but grew poorly, whereas linseed grown in Merildin soil was lightly colonised but grew well. Linseed grown in Dunn soil also had significantly greater shoot P and Zn concentrations but lower shoot N concentrations than linseed grown in Merildin soil. Determination of the most probable number of propagules in these two soils revealed that there were many more infective VAM fungal propagules in Dunn compared to Merildin soil. This indicated that the difference in colonisation of linseed grown in these soils was at least partly due to a difference in VAM fungal propagule density.

The role of the indigenous VAM fungi in the growth and nutrition of linseed in Dunn soil was evaluated by eliminating the indigenous VAM fungi by autoclaving the soil. Results for this experiment showed that linseed grown in autoclaved Dunn soil reinoculated with untreated Dunn soil containing indigenous VAM fungal propagules responded positively to colonisation by indigenous VAM fungi. Increases in SDW and shoot P and Zn concentrations were observed indicating that the indigenous VAM fungi present in Dunn soil were potentially beneficial to linseed growth and nutrition. Because of this, the possibility of enhancing the potential benefit of the indigenous VAM fungi was assessed. Firstly, cropping Dunn soil with the known mycorrhizal host plants subterranean clover, linseed and vetch increased the VAM fungal inoculum potential of the soil. This in turn resulted in greater SDW and increased shoot P and Zn concentrations of linseed grown in the cropped compared to uncropped soil. Secondly, the effect of soil P concentration on colonisation by the indigenous VAM fungi present in Dunn soil was assessed. This experiment also assessed whether the different plant responses to VAM fungal colonisation observed in previous experiments were a result of the different P concentrations of the soils used in these experiments. However, plant growth was restricted and although the cause of this restriction is unclear, it is most likely nutritional.

Overall, these experiments established that the indigenous VAM fungi present in Dunn soil were beneficial to linseed growth and nutrition in soils with moderate soil P concentrations and that cropping Dunn soil with mycorrhizal plants enhanced this benefit. Furthermore, results from these experiments suggest that the poor growth of linseed in Dunn soil in the initial screening of the Clare field soils was not due to a VAM fungal induced growth depression, decline in VAM fungi or Zn deficiency as originally suspected from field observations but not confirmed. Instead, the poor growth of linseed in Dunn soil in the initial screening of the Clare field soils was possibly due to N deficiency.

In the second section of this project, the aim was to examine the role of VAM fungi in the Zn nutrition of *L. usitatissimum*. This required a soil with P and Zn concentrations that would support VAM fungal colonisation and at the same time induce Zn deficiency of *L. usitatissimum*. Hamilton soil from Pinnaroo, South Australia, was selected as it had apparently suitable P and Zn concentrations. Pot cultures of VAM fungi were raised in Hamilton soil using *L. usitatissimum* cv. 'LinolaTM' as a host plant and inoculum from these cultures was tested in inoculation trials. However, unexpected results were obtained. Colonisation of the pot cultures was delayed and in inoculation trials VAM fungal colonisation was low. Accordingly, the proposed studies of Zn uptake were not possible. However, the results obtained did have important agricultural implications and ecological significance.

Declaration

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

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

Benjamin Mark Thomas

May 2001

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Chapter 1 – General introduction and literature review

A. General introduction

This project had its origin in observations of poor growth and apparent zinc (Zn) deficiency in linseed (*Linum usitatissimum* L.) grown near Clare, South Australia. It was thought that these observations were a result of a decline in vesicular-arbuscular mycorrhizal (VAM) fungi in the soil due to a preceding canola (*Brassica napus* L.) crop, which does not support VAM fungi (Gavito and Miller, 1998). VAM fungi can play an important role in the uptake of nutrients by plants, particularly those nutrients that are relatively immobile in soil such as phosphorus (P) and Zn (Bowen, 1983; Russell, 1988). Compared with P, the role of VAM fungi in Zn nutrition has been poorly documented. Radioactive tracers have been used to study the uptake of Zn by VAM fungi (Bürkert and Robson, 1994), but in most cases only nutrient analyses of plant tissue have been used to illustrate the potential role of VAM fungi in the Zn nutrition of plants.

The general aims of this project, therefore, were: (1) to examine the role of indigenous VAM fungi in the growth of linseed in the field soil from Clare, and (2) to examine the effect of VAM fungi on the Zn nutrition of *L. usitatissimum* in an agricultural soil with particular emphasis placed on the mechanisms of uptake and transfer of Zn from the fungus to the plant

To address the first aim, experiments were conducted to: (a) screen the Clare field soils for VAM fungi and to examine the growth and nutrition of linseed in these soils, (b) assess the number of infective propagules in the soils and infectivity of the soils, (c) determine the role of the

indigenous VAM fungi in the growth and nutrition of linseed in Clare field soil by eliminating the VAM fungi, (d) examine the possibility of enhancing the indigenous VAM fungi through the use of crop rotations and, (e) determine the response of the indigenous VAM fungi to soil P concentration.

To address the second aim, it was necessary to screen for a soil that could be manipulated to favour VAM fungal colonisation whilst inducing Zn deficiency of *L. usitatissimum*. Hamilton soil from Pinnaroo, South Australia, was selected as it had apparently suitable P and Zn concentrations. However, unexpected results from preliminary trials showed that VAM fungal colonisation was delayed and/or low, so that the proposed analyses of the role of VAM fungi in Zn nutrition of *L. usitatissimum* were not possible.

The following review of the literature focuses on the role of VAM fungi in agricultural systems. This includes: a general review of the VAM association including colonisation processes, host plant nutrition and development, and fungal growth and development; a review of the factors affecting the VAM association in agricultural systems; and a review of the role of indigenous and introduced VAM fungi in agricultural systems.

B. Literature review

1.1 Introduction

Vesicular-arbuscular mycorrhizal (VAM) symbioses are associations between a VAM fungus and its host plant. They are widely distributed, occurring in various plant groups including almost all the major agricultural crops (Mosse, 1973) and with the exception of a few plant families belonging mainly to the Cyperaceae, Chenopodiaceae, Cruciferae, Juncaceae and Proteaceae (Gianinazzi-Pearson, 1986; Gerdemann, 1968). In addition, other plant types establish different types of mycorrhizas, for example ectomycorrhizas, ericoid and orchid mycorrhizas (Gianinazzi-Pearson, 1986; Smith and Read, 1997). In most cases, the symbioses are mutualistic with both the fungus and host plant benefiting from the association. The fungus supplies the host plant with essential nutrients such as phosphorus (P) and zinc (Zn) and receives plant carbohydrate needed for its own growth and development.

1.1.1 The colonisation process

Colonisation of a host plant by a VAM fungus is initiated by hyphae growing from soil-borne propagules such as spores, hyphae within dead roots, possibly excised hyphae or by external hyphae growing from neighbouring living colonised roots (Brundrett *et al.*, 1984; Garriock *et al.*, 1989; Sanders and Sheikh, 1983; Smith and Gianinazzi-Pearson, 1988). Colonisation depends on the density (number per unit volume) and infectivity of these propagules (Abbott and Robson, 1981; McGee *et al.*, 1999; Smith and Walker, 1981; Wilson and Trinick, 1983). Colonisation of the host commences when the root and hyphae growing from the propagules come into contact,

and in some cases an appressorium is formed on the root surface (Figure 1.1) (Garriock *et al.*, 1989; Giovannetti *et al.*, 1993; Harley and Smith, 1983).

Penetration of the root cells is initiated by hyphae directly or by hyphae growing from the appressorium. These hyphae are often called hyphal pegs or infective hyphae (Harley and Smith, 1983). The hyphae either penetrate between the epidermal cells of the root or through these cells. The penetrating hyphae then branch and grow longitudinally within and between the cells of the host root (see Figure 1.1) (Carling and Brown, 1982).

Development of the fungus continues within the root cells with the formation of intracellular arbuscules, intercellular or intracellular vesicles and coils (Figure 1.1) depending on whether the mycorrhiza is an *Arum* or *Paris* type. If it is an *Arum*-type mycorrhiza, arbuscules are formed whereas, if it is a *Paris*-type mycorrhiza, hyphal coils dominate (Gallaud, 1905; Smith and Smith, 1996; Smith and Smith, 1997). As only *Arum*-type mycorrhizas were studied in this project, only the processes regarding these mycorrhizas were reviewed. The large amount of fungal material that constitutes a mature arbuscule and the large increase in plant-fungal interface as the plant cell membrane invaginates to accommodate the growing fungus, makes the arbuscule the most likely site of nutrient transfer between the symbionts or, at least P transfer to the plant (Carling and Brown, 1982; Smith and Read, 1997; Toth and Miller, 1984).

Vesicles are lipid storage bodies, which contain many nuclei (see Figure 1.1) and may have similar function to the fungal spores, i.e. to supply the fungus with substrates in order for the fungus to locate and colonise a host plant (Biermann and Lindermann, 1983; Brundrett *et al.*, 1984). Alternatively, vesicles may provide substrates for the formation of spores. As vesicles

contain lipids (Cooper and Lösel, 1978; Cox *et al.*, 1975), they may be seen as an indication of the carbon allocation from the plant to the fungus.

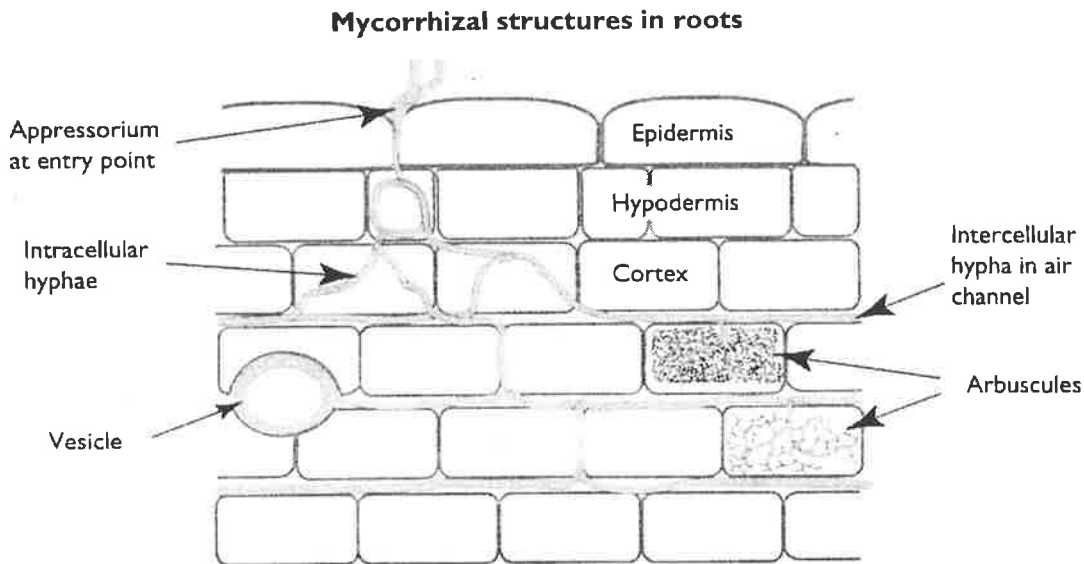


Figure 1.1. Intraradical anatomy of a well-formed *Arum*-type vesicular-arbuscular mycorrhiza. Appressoria, arbuscules, vesicles, intercellular hyphae and intracellular hyphae can be seen (Adapted from Brundrett *et al.*, 1996)

1.1.2 Host plant nutrition and growth

The effect of vesicular-arbuscular mycorrhizal fungi on plant growth and nutrition is well documented. In soils low in readily-extractable P, mycorrhizal plants often have more growth and dry matter production than non-mycorrhizal plants (Bethlenfalvay *et al.*, 1982a; Cox and Tinker, 1976; Hayman, 1982a; Sanders and Sheikh, 1983; Smith and Gianinazzi-Pearson, 1988; Smith and Read, 1997; Smith *et al.*, 1986). This increase in plant growth is normally a direct result of increased uptake of P and other nutrients such as Zn. Smith *et al.* (1986) investigated the

effect of mycorrhizal infection on glasshouse-grown *Allium cepa* L. and reported that the growth response associated with mycorrhizal colonisation was a result of increased inflows of P. This observation is well supported by other researchers (Sanders *et al.*, 1977; Sanders and Tinker, 1973; Smith *et al.*, 1994a) although the actual rates of inflow of P into mycorrhizal roots differ between reports because of the different fungi, host plant and methods used. Nutrient uptake by mycorrhizal plants is often greater than non-mycorrhizal plants because they possess a network of external hyphae that gives a larger and better absorbing surface (Sanders and Sheikh, 1983). In soils low in P, plant uptake of P is limited by the slow rates of movement of P ions in soil rather than the ability of the root to take them up. In mycorrhizal plants, the external hyphae are able to grow and exploit the nutrients beyond the nutrient depletion zone that surrounds the plant root (Gianinazzi-Pearson and Gianinazzi, 1983; Jakobsen, 1999; Jakobsen *et al.*, 1992; Pearson and Tinker, 1975; Smith and Gianinazzi-Pearson, 1988). This exploitation of P is also facilitated by the ability of the thin hyphae to utilise P from soil micropores inaccessible to roots or root hairs (Jakobsen, 1999). The fungus extracts these nutrients, and translocates the nutrients along the hyphae to the plant root where they are transferred to the host plant (Smith and Gianinazzi-Pearson, 1988; Pfeffer *et al.*, 1999).

1.1.3 Carbohydrate and fungal metabolism in vesicular-arbuscular mycorrhiza

A number of researchers have demonstrated that the carbohydrate demand of the vesicular-arbuscular mycorrhizal fungus is met by its host plant (Bevege *et al.*, 1975; Cox *et al.*, 1975; Gianinazzi-Pearson and Gianinazzi, 1983; Smith and Gianinazzi-Pearson, 1988). After the carbohydrate has been transferred from the host plant to the fungus, the fungus rapidly converts it into compounds not readily utilised by the plant, such as lipids and glycogen (Cox *et al.*, 1975; Gianinazzi-Pearson and Gianinazzi, 1983; Harris and Paul, 1987; Pfeffer *et al.*, 1999; Smith *et*

al., 1994b). Published values of transfer of C from the plant to the fungus range from 4 to 20% of photoassimilated carbon (see review by Jakobsen, 1999). The use of carbon by the fungus is often compensated for by an increase in photosynthesis by the plant. Without this compensation, the carbon drain imposed by the fungus on the plant would lead to serious growth depressions (Jakobsen, 1999). Growth depressions are reviewed further in Chapter 1.2.1.3.

1.1.4 VAM fungi and the zinc nutrition of plants

VAM fungi have been shown to improve the Zn nutrition of many plants (Barea, 1991). This includes subterranean clover (Bürkert and Robson, 1994), maize (Azaizeh *et al.*, 1995; Khalil *et al.*, 1994; Kothari *et al.*, 1991; Liu *et al.*, 2000), lucerne (Nielsen and Jensen, 1983), and linseed (Thompson, 1996). However, compared to P, research on the role of VAM fungi in the Zn nutrition of plants is poorly documented.

Like P, Zn is relatively immobile in soil (Bowen, 1983) and so under Zn limiting conditions, nutrient depletion zones may form around the plant root in a similar manner to P. The ability of VAM fungi to improve Zn uptake is therefore thought to involve similar mechanisms as their role in P uptake (Chapter 1.1.2). That is, in mycorrhizal plants, the external hyphae of the fungi are able to grow and exploit the nutrient reserves beyond the zone of Zn depletion surrounding the plant root.

1.2 Vesicular-arbuscular mycorrhizal fungi in agricultural systems

1.2.1 General environmental factors affecting VAM fungi

1.2.1.1 Soil pH

VAM fungi have been shown to vary greatly in their tolerance to soil pH. Similarly, soil pH has been shown to greatly influence the occurrence of different VAM fungi in different soil types. Hayman and Tavares (1985) showed that at pH 4, *Glomus clarum* greatly enhanced growth of alpine strawberry whereas a number of other *Glomus* species had no effect. They also showed that each fungus tested, which included various *Glomus*, *Acaulospora*, and *Gigaspora* sp., differed in their efficiencies at various soil pH.

Soil pH is thus an important factor to consider when attempting to manipulate indigenous VAM fungal populations or introduce more effective VAM fungal isolates or species. One of the requirements when attempting to inoculate soils with VAM fungi, is that the introduced fungi must be able to establish and persist in the target soil (Porter *et al.*, 1987). Porter *et al.* (1987) showed that inoculation of a field soil with introduced fungal species resulted in lower levels of colonisation of plant roots by the introduced fungi, fewer spores produced and a decrease in the germination of spores of the predominant VAM fungus. They discovered that soil pH was the major factor limiting the establishment of VAM fungi in 'foreign' soil. Therefore, the pH of the target soil must be taken into account as it may affect the success of inoculation with VAM fungi.

1.2.1.2 Phosphorus

In agricultural terms, the factor most important to mycorrhizal functioning is soil P. In most controlled soil experiments, the soil used has a low concentration of readily-extractable P so as to favour the mycorrhizal symbiosis. However, in agricultural soils, the concentration of readily-extractable P can be much higher, especially following fertilisation. This concentration of P is often higher than is optimal for mycorrhizal functioning. Hence, the role of P in the mycorrhizal symbiosis is critical when investigating mycorrhizal functioning in agricultural soils or systems.

High concentrations of P, often following applications of fertiliser, can have a detrimental effect on VAM fungi, neutralising the benefit of the fungus to the mycorrhizal symbiosis. As postulated by Hayman (1987), there are three factors involved in this effect of elevated P on the symbiosis (a) Physical effects, (b) Colonisation effects, and (c) Parasitic effects.

(a) *Physical effect* – One of the major functions of the fungal partner of the mycorrhizal symbiosis is a physical one. As discussed in Chapter 1.1.2, in soils low in P, plant uptake of P is limited by the diffusion of P to the root surface. In mycorrhizal plants, external hyphae of the fungus are able to grow and exploit nutrients beyond the nutrient depletion zone surrounding a plant root. However, when P concentrations are higher, sufficient P is maintained near the root surface by diffusion and this beneficial effect of the VAM fungus may be redundant (Hayman, 1987).

(b) *Colonisation effect* – High concentrations of P in the soil or plant, such as those following fertilisation, normally result in a reduction in VAM fungal colonisation (Amijee *et al.*, 1989; Bolan *et al.*, 1984; Braunberger *et al.*, 1991; Bruce *et al.*, 1994; Jasper *et al.*, 1979; Menge

et al., 1978; Same *et al.*, 1983; Schwab *et al.*, 1983; Thomson *et al.*, 1986). However, the addition of some soil P can aid spore germination (De Miranda and Harris, 1994; De Miranda *et al.*, 1989) and colonisation in severely deficient soils (Bolan *et al.*, 1984, De Miranda *et al.*, 1989).

It is generally accepted that the inhibitory effect of P on VAM fungal colonisation is a result of either a direct effect of soil P on the growth of the VAM fungus or an indirect effect associated with the P status of the plant (De Miranda *et al.*, 1989; Nagahashi *et al.*, 1996). De Miranda and Harris (1994) showed that additions of P above $12.5 \mu\text{g P g}^{-1}$ soil reduced spore germination whilst hyphal growth decreased significantly with additions above $37.5 \mu\text{g P g}^{-1}$ soil. Reductions in spore germination were also observed by Wilson *et al.* (1989) when soil P concentration exceeded $15 \mu\text{g P g}^{-1}$ soil. Reductions in spore germination and growth of external hyphae in soil would be expected to result in reduced VAM fungal colonisation by restricting root fungal contact. It is critical to remember that direct effects of soil P on VAM fungal development depend greatly on soil factors such as soil type, fixation capacity and soil biota. Furthermore, allowances must be made for any possible effects of the accompanying ion added with the P (eg. Hepper, 1983).

A number of explanations have been suggested for the effect of high plant P status on VAM fungal colonisation. Probably the most widely accepted of these is that an increase in the P status of the plant is associated with a decrease in the availability of fungal substrates from the root (Graham *et al.*, 1982; Thomson *et al.*, 1991). An increase in the concentration of P in a plant has been shown to result in a decrease in the concentration of soluble carbohydrates in roots, which was closely correlated with VAM fungal colonisation (Same *et al.*, 1983; Schwab *et al.*, 1983; Jasper *et al.*, 1979). Graham *et al.* (1981) postulated that when P deficient, plant membranes

become more permeable resulting in increased exudation and therefore an increase in colonisation. When P concentration increases, membrane permeability and hence root exudation decreases thus limiting mycorrhizal colonisation (Graham *et al.*, 1981; Menge *et al.*, 1978; Ratnayake *et al.*, 1978). Similarly, Schwab *et al.* (1983) proposed that the reduction in growth of external hyphae from primary infections and hence a reduction in the formation of secondary infections was due to a reduction in the leakage of compounds from host cells caused by improved P nutrition. Despite this, there are no reports of exudation actually favouring colonisation. Furthermore, it can be argued that the growth of external hyphae originating from the primary infection may have been restricted by high soil P concentration. This is supported by Thomson *et al.* (1986), who showed that there appeared to be little effect of P supply on the permeability of plant membranes to soluble carbohydrates and amino acids. High P status of the plant has also been reported to have an adverse effect on the growth of infection units (Amijee *et al.*, 1989; Bruce *et al.*, 1994).

The effect of increased P supply on VAM fungal colonisation is also complicated by the fact that root growth is stimulated by the high P supply. In many cases, high P supply stimulated root growth but had little effect on the root length colonised by VAM fungi resulting in a decrease in percentage colonisation (Bruce *et al.*, 1994; De Miranda *et al.*, 1989; Smith, 1982; Thomson *et al.*, 1990).

In summary, although some P is required for spore germination and colonisation in severely deficient soils, greater additions often result in a decrease in colonisation of the plant roots by VAM fungi, most likely mediated by a combination of soil and plant factors.

It is interesting to note that despite the negative effects of P on colonisation, some fungi, especially those found in agricultural soils where fertilisation has occurred for some time, have adapted to high concentrations of P. These fungi can still form mycorrhizas despite further additions of P (Hall, 1984; Hayman, 1982b; Jasper *et al.*, 1979; Porter *et al.*, 1978). In some cases, VAM fungi can still aid plant nutrition (e.g. P concentration) even under high P conditions (Hayman, 1987) although the response will depend on the sensitivity of the plant to VAM fungal colonisation and high P conditions.

The benefits of a VAM fungus able to exist and function in a high P soil and still remain beneficial to plant nutrition and growth are obvious, especially if other soil nutrients, the uptake of which is enhanced by VAM fungi, are limiting. Unfortunately, however, even if a fungus is able to exist in a high P soil, it may cause plant growth depressions (see (c) below and Chapter 1.2.1.3) rather than enhance growth. It is important to remember that the main role of VAM fungi in agriculture should be more efficient usage of applied P fertilisers not replacement of fertilisers (Gianinazzi-Pearson, 1986; Menge, 1983) ideally resulting in reduced fertiliser use without a subsequent drop in yield. VAM fungi that are able to survive applications of P fertiliser or high soil P concentrations and still promote plant nutrition and growth would have great agricultural value.

(c) *Parasitic effects* – If soil P is high enough to negate the beneficial effect of VAM fungi but not high enough to curtail fungal colonisation, then the fungus may cause a carbon drain on the plant resulting in reduced plant growth (Hayman, 1987; Smith, 1980a). In agricultural terms, the possibility of VAM fungi being detrimental to plant growth is a very important issue. This issue, therefore, will be further discussed in section 1.2.1.3.

1.2.1.3 Mutualism and parasitism in the VAM symbiosis

The VAM symbiosis is generally considered to be mutualistic (Francis and Read, 1995; Smith and Read, 1997), but under certain conditions the symbiosis can change from mutualism to parasitism. The difference between mutualism and parasitism is determined by the benefits and costs of the symbiosis for the two symbionts. As VAM fungi are considered obligate mycotrophs (Smith and Read, 1997; Tinker *et al.*, 1994; Rouhier and Read, 1998; Koide and Elliott, 1989), meaning that they can not survive without the plant, then the fungi always benefits from the association. However, the plant can benefit (mutualism), not be affected (commensalism), or suffer (parasitism) from the association.

In an association such as the VAM symbiosis, the relationship between the partners is governed by costs and benefits. In plant terms, mutualism occurs when the benefits of the symbiosis (normally improved nutrition) exceed the costs (carbohydrate supplied to the fungal partner). Parasitism occurs when the costs exceed the benefits (Bethlenfalvay *et al.*, 1982a; Johnson *et al.*, 1997; Koide and Elliott, 1989; Tinker *et al.*, 1994). It must be remembered that loss of carbon by the plant to the fungus is only a cost if that carbon was to be used for plant growth and/or development. Similarly, improved nutrition is only a benefit to the plant if the nutrient in question is limiting (Johnson *et al.*, 1997). Parasitism is usually observed as reduced plant growth of mycorrhizal plants when compared to non-mycorrhizal controls. Such depressions in growth have been reported in various plant species including soybean (*Glycine max* L.) (Bethlenfalvay *et al.*, 1982a; Bethlenfalvay *et al.*, 1982b), *Citrus* spp. (Antunes and Cardoso, 1991; Graham and Eissenstat, 1998; Graham and Eissenstat, 1994; Peng *et al.*, 1993), subterranean clover (*Trifolium subterraneum* L.) (Tester *et al.*, 1985), ryegrass (*Lolium perenne* L.) (Buwalda and Goh, 1982), *Trifolium hybridum* and *Medicago sativa* (Crush, 1976) and maize

(*Zea mays* L.) (Daft and El-Giahmi, 1978). There are three factors that govern VAM fungal parasitism of a host plant (a) Development, (b) Genotype, and (c) Environment.

(a) *Development* – Fungal parasitism of a host plant can occur at particular stages of plant development. For instance, plant growth depressions induced by VAM fungi have often been observed at early stages of plant growth (Johnson *et al.*, 1997; Bethlenfalvay *et al.*, 1982a; Bethlenfalvay *et al.*, 1982b). This occurs because the benefit of the symbiosis to the plant is low as the seedling is still receiving sufficient nutrients from its seed reserves. However, the costs of the fungus are high especially as the photosynthetic rate of the plant is low. These growth depressions are often temporary and as the photosynthetic area and photosynthesis of the plant increase and the nutrient supplied by the fungus is required following exhaustion of seed reserves, the association will often become mutualistic (Bethlenfalvay *et al.*, 1982a; Harris and Paul, 1987; Snellgrove *et al.*, 1986).

(b) *Genotype* – The potential for parasitism in the mycorrhizal symbiosis depends on the plant and VAM fungal species involved. This is related to the dependency of the plant to VAM fungal colonisation. Good examples of dependent plant species include cassava and oil palm, which grow poorly unless colonised by VAM fungi, whereas many cereals show very little response even when extensively colonised and are considered less dependent (Smith and Read, 1997).

It is possible to hypothesise that the greater the dependency of a plant on VAM fungi, the less chance of a mycorrhizally induced plant growth depression due to a greater cost:benefit differential (Johnson *et al.*, 1997). Alternatively, a more dependent plant may expend more carbon (photosynthate) to support high rates of VAM fungal colonisation (Graham *et al.*, 1997).

Under certain conditions such as high P, when the relative benefit of the symbiosis is lower, the failure of more dependent plants to restrict unnecessary or uncontrolled VAM fungal colonisation of plant roots may result in parasitism (Johnson *et al.*, 1997; Graham and Eissenstat, 1994). *L. usitatissimum* is generally considered to be a highly dependent on mycorrhizal fungi (Thompson, 1994; Thompson, 1996; Thingstrup *et al.*, 1998; Thingstrup *et al.*, 2000) and as such may be susceptible to growth depressions induced by VAM fungi.

VAM fungal species can also differ in their effectiveness. A number of fungal species or isolates grown on a single host species can increase, decrease or have no effect on plant growth (see references in Johnson *et al.*, 1997). Therefore, the potential for a plant growth depression may depend on the fungal species of isolate involved.

(c) *Environment* – Environmental factors are critical to determine the effects of VAM fungi on plant growth and the potential for parasitism. Of most importance in agriculture soils is the fertility of the soil but other environmental factors affecting the functioning of the mycorrhizal symbiosis include photon irradiance, defoliation, and competition. Fertilisation of a soil can cause a VAM fungal induced plant growth depression by decreasing the benefits of the symbiosis without subsequent reduction in costs. The role of soil fertility in parasitism in a VAM symbiosis has already been discussed (Chapter 1.3.1.2 (c)).

Low photon irradiance or light intensity can result in a plant growth depression induced by VAM fungi by making the relative costs of the symbiosis to the plant increase whilst benefits remain unchanged (Johnson *et al.*, 1997). Low light intensity causes a decrease in photosynthesis, resulting in less production of photosynthate. If the fungal costs remain unchanged, then there is less photosynthate for plant growth and development and the significance of the carbohydrate

drain by the VAM fungus increases, possibly resulting in depressed plant growth (Son and Smith, 1988; Tester *et al.*, 1985). Low light intensity has been shown to reduce plant growth (or induce plant growth depressions) in both growth room and glasshouse conditions (Daft and El-Giahmi, 1978; Son and Smith, 1988; Tester *et al.*, 1985). The significance of low light under field conditions is unclear, as it is very difficult to control.

It is interesting to note that CO₂ enrichment can increase mycorrhizal colonisation of plant roots. This suggests increase sequestration of carbon by the fungus. It is possible that under elevated CO₂ conditions, the photosynthesis of the plant will increase, supplying more carbon to the fungus reducing the chance of fungal induced growth depressions and in fact, may enable the fungus to scavenge for nutrients more extensively (Rouhier and Read, 1998).

Defoliation by either grazing or harvesting may cause a growth depression by limiting photosynthesis and depleting plant carbon reserves to the extent that the cost of the symbiosis on the plant outweighs its benefits (Daft and El-Giahmi, 1978).

Competition between plants is often experienced in agricultural crop systems. Where large numbers of plants are growing together, nutrient depletion zones will overlap and roots and mycorrhizal hyphae will compete for nutrients. The result is often reduced growth of all the individual plants compared with those plants well spaced (Evans and Miller, 1990; Baath and Hayman, 1984; Hayman, 1982b; Johnson *et al.*, 1997).

1.2.2 Specific agricultural factors affecting the VAM symbiosis

1.2.2.1 Soil disturbance

Soil disturbance or tillage can have a detrimental effect on VAM fungi, reducing colonisation and possibly nutrition of mycorrhizally responsive plants (Evans and Miller, 1988; Fairchild and Miller, 1988; O'Halloran *et al.*, 1986). This is supported by evidence that zero-till plots seem to favour mycorrhizas compared with conventional-till plots with greater colonisation of plant roots by VAM fungi (Ryan *et al.*, 1994). The adverse effects of tillage on mycorrhizas can be attributed to a destruction of the external hyphal network of the VAM fungi associated with a previous crop (Jasper *et al.*, 1989; Evans and Miller, 1990). This network enables rapid colonisation and effective P acquisition of plants sown directly into the soil (McGonigle and Miller, 2000). As shown by Fairchild and Miller (1988), maize plants that had access to an existing hyphal network in undisturbed soil, derived a large nutritional benefit from mycorrhizal colonisation as early as 14 days after sowing. Tillage destroys this network of hyphae often leading to a reduction in the inoculum capacity of the soil (Evans and Miller, 1988) and hence colonisation of plants sown into the soil is slower and less extensive (Evans and Miller, 1990). Furthermore, tillage may adversely affect nutrient absorption even though VAM fungal colonisation may not be reduced by destroying the continuity of the hyphal network (Evans and Miller, 1990; Fairchild and Miller, 1988). If colonisation is not reduced by tillage but the nutrient absorption is reduced, then the costs of the fungus to plant may exceed benefits especially early in plant growth, resulting in a growth depression.

The effect of management on VAM fungi is also seen in long-fallow disorder of field crops. Long-fallow disorder is characterised by the poor growth of cereal, oilseed or pulse crops after a

long period of weed-free fallow as a result of poor P and Zn nutrition (Duncan, 1967; Thompson, 1987; Thompson, 1994). Weed-free long fallow is a practice that is often adopted in many cropping areas of Australia as it enhances water and nutrient storage and also controls weeds and some diseases (Leeper and Uren, 1993). Long-fallow disorder can be corrected by the application of Zn and P fertilisers (Thompson, 1987). However, Thompson (1987) showed that the colonisation of the roots of crops such as wheat, chickpea and maize suffering from long-fallow disorder and the number of intact VAM fungal propagules were much lower than plants growing in an adjacent field where the fallow period had been much shorter. Subsequent studies showed that reinoculation of the long fallow soil with either recently cropped soil (Thompson, 1987), spores and root pieces recovered from recently cropped soil by wet sieving (Thompson, 1994), or inoculation of long fallow disordered soil with cultured inoculum of the VAM fungal isolates *Glomus mosseae* or *G. etunicatum* (Thompson, 1996) greatly enhanced the colonisation of linseed and also the uptake of P and Zn. It was therefore concluded by Thompson (1987, 1994 and 1996) that long fallow disorder of crops, especially linseed, is caused by a decline in the density of VAM fungal propagules in the soil leading to a decrease in VAM fungal colonisation of plant roots and hence VAM fungal contribution to plant nutrition.

1.2.2.2 Crop rotations

Crop rotations can have a marked effect on VAM fungal populations and hence the VAM fungal contribution to plant nutrition. Plants differ in terms of mycorrhizal responsiveness and mycorrhizal status. Thus, the use of certain crop species or agricultural practices in rotation can influence the VAM status of a soil either positively or negatively depending on the plant species or practice involved. This effect may be important in relation to other crop species involved in

the rotation. Non-mycorrhizal crop plants have been shown to have a negative effect on VAM fungi and on the growth and yield of subsequent mycorrhizally responsive crop species (Arihara and Karasawa, 2000; Black and Tinker, 1979; Fontenla *et al.*, 1999). This includes species such as canola (*Brassica napus* L.) (Gavito and Miller, 1998), mustard (*Brassica juncea* L.) (Harinikumar and Bagyaraj, 1988) and kale (*Brassica oleracea* L.) (Black and Tinker, 1979). Conversely, VAM fungal inoculum potential may be improved by including mycorrhizally responsive plants in the rotation. For example, Black and Tinker (1979) showed that VAM spore numbers and subsequent infections of barley roots were largest following barley (a mycorrhizal plant species) and were lowest following a fallow period or kale (a non-mycorrhizal plant species) crop. Similarly, winter wheat cover-cropping in the Northern Hemisphere instead of traditional fallow, has been shown to increase VAM fungal inoculum potential of a field soil, as assessed by a maize bioassay during the following growing season. This higher colonisation was correlated with higher maize growth and yield (Boswell *et al.*, 1998). The mycorrhizal cover crop serves as a VAM fungal host, thus maintaining or improving the VAM fungal potential during the winter (or non-cropping) season (Dodd and Jeffries, 1986). Pre-cropping a soil with a known VAM fungal host has also been shown to improve subsequent VAM responsive crops. Pre-cropping a field soil with the known VAM fungal hosts cassava (*Manihot esculenta* L.), kudzu (*Pueraria phaseoloides* L.) or sorghum (*Sorghum bicolor* L.), increased VAM fungal spore densities (Dodd *et al.*, 1990b) and the colonisation of cowpea (*Vigna unguiculata* L.) or *Stylosanthes capitata* (Dodd *et al.*, 1990a). This increased colonisation was associated with increased yields of these two plant species (Dodd *et al.*, 1990a). Similar positive effects on VAM fungal colonisation and subsequent plant growth have been found when cowpea was cropped following a previous cowpea crop (Harinikumar and Bagyaraj, 1988), for sunflower (*Helianthus annuus* L.) following cotton (*Gossypium hirsutum* L.), sorghum or sunflower (Thompson, 1987) and for maize following several mycorrhizal crops (Arihara and Karasawa, 2000).

This research highlights the possibility of planning crop rotation strategies to influence the VAM fungal population present in a field soil. Cropping a soil with a known VAM fungal host plant species may enhance VAM fungal propagule production. Depending on the VAM fungal species involved, subsequent VAM responsive crops could then be more extensively colonised and gain greater benefit from enhanced VAM fungal contribution to plant growth and nutrition. This strategy would be of particular interest if the VAM fungal species involved were found to enhance growth and nutrition of crops grown in this soil. Conversely, cropping a soil with known non-mycorrhizal plant species such as canola may adversely affect VAM fungal propagule production. In this case, subsequent VAM responsive crops may be less colonised. This would be preferential if the VAM fungal species involved are inefficient and/or causing little or negative effects on the growth of VAM responsive crops.

1.3 Indigenous and inoculant VAM fungi

The effect of VAM fungi (either indigenous or introduced) on plant production in agricultural systems depends on their ability to colonise plant roots rapidly and extensively, enhance nutrient uptake and plant growth and persist in soil (Abbott and Robson, 1982a). Rapid and extensive colonisation results in the VAM fungus (if effective) being able to supply the plant with nutrients during early vegetative stages of growth when they are most required. The ability of VAM fungi to enhance plant nutrient uptake and growth is termed its effectiveness. A more effective fungus may produce large amounts of external hyphae thus providing the plant with the ability to explore more soil. Effectiveness also depends on the ability of the fungus to absorb soil nutrients, transport them to the plant root and transfer them to the plant. Persistence refers to the ability of

the fungus to maintain a high inoculum potential in a soil even during periods where no host plant is available.

In order to investigate the role of indigenous VAM fungi in field soils, it is necessary to produce non-mycorrhizal controls for comparison. Unfortunately, evaluation of the results from such studies is often complicated because the methods used to produce non-mycorrhizal controls, eg. sterilisation, can also cause changes to non-mycorrhizal soil factors such as soil nutrient concentrations and other soil biota. Despite these complications, indigenous VAM fungi have been shown to have varying effects on plant growth (Frey and Ellis, 1997; Harinikumar and Bagyaraj, 1988). Positive effects have been reported for field pea (*Pisum sativum* L.) (Jakobsen, 1986), linseed (Thingstrup *et al.*, 1998; Thompson, 1987; Thompson, 1994) and barley (*Hordeum vulgare* L.) (Baon *et al.*, 1992). Hayman (1987) also stated that "there is considerable circumstantial evidence that indigenous endophytes can increase yields of some temperate crops by 50% and some tropical crops by several hundred percent." Conversely, indigenous VAM fungi have been shown to cause growth depressions of legumes (*Trifolium repens* L. and *T. pratense* L.) (Crush, 1976) and citrus (Graham and Eissenstat, 1998). Growth depressions are heavily dependent on a number of factors already reviewed in Chapter 1.2.1.3.

A number of strategies have been used to enhance or augment the indigenous VAM fungi population in an attempt to increase VAM fungal colonisation and contribution to plant nutrition and hence improve plant production. This can take the form of manipulation of the current indigenous VAM fungal population or introduction of more effective fungal species.

The indigenous VAM fungal population may be manipulated either positively or negatively by using agricultural practices such as crop rotations, fertilisers and tillage practices. These practices

have been discussed in Chapters 1.2.2. Briefly, if the indigenous VAM fungi are found to be beneficial to plant growth, agricultural practices such as reduced tillage, less fertilisation and crop rotations involving known VAM fungal hosts may be adopted to favour the VAM fungi (Boddington and Dodd, 2000; Daft, 1992).

Attempts have often been made to introduce more effective isolates of VAM fungi to field soils in order to improve plant production. Hall (1984) showed that inoculation of field soils in New Zealand with various fungal species resulted in dry matter increases of white clover ranging from *ca.* 5% to *ca.* 30%, depending on the fertility of the soil. Increased plant growth following inoculation with VAM fungi has also been shown for barley (Clarke and Mosse, 1981), subterranean clover (Abbott and Robson, 1978; Abbott *et al.*, 1983), and onion (Mosse and Hayman, 1971). Inoculation success depends heavily on the fungal species to be introduced, with different species having various effects on plant growth following inoculation (Abbott and Robson, 1978). In most cases, inoculation of field soils with VAM fungi would be expected to increase plant growth in soils which have either a low abundance of indigenous VAM fungi or which contain species which are less effective than inoculant fungi (Abbott and Robson, 1982a). However, inoculation of field soil with supposedly more effective isolates can be unsuccessful, resulting in a lack of response to inoculation. Possible reasons for a lack of response to inoculation are: (1) the isolate introduced to a field soil is no more effective than the indigenous fungi (Hall, 1988); (2) the introduced isolate is unable to become established due to environmental and soil factors; or (3) the introduced fungal endophyte is unable to compete with the indigenous fungal species (Abbott and Robson, 1982a; Abbott *et al.*, 1983).

Chapter 2 – Materials and Methods

Several experiments were conducted in this project using field soils collected from Clare, South Australia (Merildin and Dunn) or Pinnaroo, South Australia (Hamilton). This chapter contains general materials and methods used in these experiments. Specific materials and methods are referred to in the appropriate chapter.

2.1 Materials

2.1.1 Host plants and vesicular-arbuscular mycorrhizal fungi

2.1.1.1 Host plants

Host plants used in the experiments were linseed (*Linum usitatissimum* L. cv. "Glenelg"), a variety supplied by M. Faulkner, Clare, South Australia; Linola™ (*L. usitatissimum* L. cv. "Eyre") supplied by CSIRO Division of Plant Industry, Canberra, A.C.T. (Linola™ is a trademark of CSIRO, Australia); subterranean clover (*Trifolium subterraneum* L. cv. "Mt. Barker") supplied by Mt. Barker Agricultural Seeds, South Australia; vetch (*Vicia sativa* L.) collected from field site near Clare, South Australia; and canola (*Brassica napus* L. cv. "Drum") supplied by Seed Services, Adelaide, South Australia.

2.1.1.2 Vesicular-arbuscular mycorrhizal fungi

In experiments with Clare field soils (Chapter 3), soil containing indigenous isolates of VAM fungi was used in all experiments and in no case were any foreign fungal isolates added.

In experiments with Hamilton soil (Chapter 4), 3 VAM fungal species were utilised. They were: *Scutellospora calospora* (Nicol. & Gerd.) Walker and Sanders WUM 12 from University of Western Australia, Crawley, Western Australia; *Glomus intraradices* (Schenk and Smith) DAOM 181602 from A. Fortin, Montreal, Canada; and *Glomus coronatum* WUM 2 also from University of Western Australia, Crawley, Western Australia. Dried inoculum of these fungi was supplied by D. Miller, Department of Soil and Water, University of Adelaide, Adelaide, South Australia.

2.1.1.3 *Rhizobium*

Rhizobium leguminosarum bv. *trifolii* was used to inoculate subterranean clover plants, whereas *R. leguminosarum* bv. *viciae* was used to inoculate vetch plants. Both are commercially marketed as “Nodulaid” by Biocare Technology Pty. Ltd., Somersby, New South Wales, Australia. In all cases, slurry of the appropriate *Rhizobium* inoculant was prepared as per manufacturer's instructions, applied to the soil surface after the seed was sown and watered in.

2.1.2 Soil

In experiments with Clare field soils, details of the soil used in each experiment are described in the appropriate chapter (Chapter 3). In all cases, the soil was collected from the top 10 cm, air-dried, crushed but not sieved prior to use.

Hamilton soil was collected (0-10 cm depth) from a farm property near Pinnaroo, South Australia. It was a light brown loamy sand with approximately 8.4 mg readily extractable P kg⁻¹ soil (Colwell) (Chapter 2.2.1), 0.3 mg Zn kg⁻¹ soil (DTPA extractable, Chapter 2.2.3), and pH 6.0 (0.01 M CaCl₂, Chapter 2.2.2). This soil was passed through a 2 mm sieve, autoclaved twice at 121°C for 60 minutes to eliminate indigenous VAM fungi and dried at 105°C prior to use.

Mallala soil was collected (0-10 cm depth) from scrubland near Mallala, South Australia. This soil, a red brown loam, was passed through a 2 mm sieve, autoclaved twice at 121°C for 60 minutes to eliminate indigenous VAM fungi and dried at 105°C prior to use. In all cases, Mallala soil was mixed 1:9 with autoclaved milled sand to produce Mallala soil:sand mix. This mix had 0.83 mg readily extractable P kg⁻¹ (Colwell, 1963; Chapter 2.2.1) and pH 7.4 (0.01M CaCl₂, Chapter 2.2.2). Milled sand was supplied by Plant Growth Services, SARDI, Waite Campus, Adelaide, South Australia. It was autoclaved twice at 121°C for 60 minutes and dried at 105°C prior to use.

2.2 General Methods

2.2.1 Determination of readily-extractable soil phosphorus

Readily extractable P in soils was determined by the method of Colwell (1963). In some cases, analysis of the soil extract was performed using an autoanalyser with a Bemas sampler, Technicon pump, Chemlab spectrophotometer and Omniscribe chart recorder, following the method described by Rayment and Higginson (1992) as this allows greater numbers of samples to be processed and measured at one time.

2.2.2 Soil pH

Soil pH was determined using 0.01M CaCl₂ at a ratio of 1:5 soil:CaCl₂ following the method described by Rayment and Higginson (1992).

2.2.3 DTPA-extractable Zn, Mn, Fe and Cu in soils.

Concentrations of DTPA extractable Zn, Mn, Fe and Cu were measured using a method based on that described by Lindsay and Norvell (1978) and Rayment and Higginson (1992). Soil was extracted with a mixture of diethylenetriamine penta-acetic acid (DTPA), triethanolamine (TEA) and CaCl₂ and the concentrations of Zn, Mn, Fe and Cu in this extract determined using Intra Coupled Plasma Atomic Emission Spectroscopy (ICP-AES). ICP-AES determination of soil extracts was performed by the Analytical Chemistry Unit, CSIRO Division of Land and Water, Adelaide.

2.2.4 Surface sterilisation, germination and inoculation of seeds

All seeds were surface sterilised by placing them in a 3% NaOCl solution for 10 minutes. The seeds were then rinsed at least 5 times with reverse osmosis (R.O.) water. The seeds were then imbibed in R.O. water for 20 minutes. Surface sterilised, imbibed seeds were placed on to filter papers moistened with R.O. water and incubated at 25°C for 48 hours to allow germination.

Subterranean clover and vetch seeds were inoculated with *Rhizobium* to establish nodulation and nitrogen fixation (Chapter 2.1.1.3).

2.2.5 Growth conditions, watering and nutrients

In all experiments, plants were grown in a growth room with a 14/10-hour light/dark cycle at temperatures of 20 and 15°C for the light and dark periods respectively. Lights supplied 300-450 $\mu\text{mol m}^{-2}\text{s}^{-1}$.

Pots were watered three times weekly with R.O. water. The amount of water added to each pot is given in the appropriate chapter. Where indicated in chapters, plants received modified Long Ashton nutrient solution (Cavagnaro *et al.*, 2001) once weekly. Volumes and composition of modified Long Ashton solution depended on host plant, pot size and experimental aim. In each case, the composition is designated in the appropriate chapter. For example, Long Ashton nutrient solution (+N, -P, +Zn) represents the solution as described by Cavagnaro *et al.* (2001), whereas modified Long Ashton nutrient solution (+N, -P, -Zn) represents the same solution without $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$.

2.2.6 Harvesting

At each harvest, plants were washed free of soil and rinsed thoroughly with R.O. water. The plants were then separated into roots and shoots. Fresh weights of roots and shoots were measured and the roots subsampled by weight. Shoots and one subsample of roots were dried at 80°C, dry weights recorded and the dried material used for ICP-AES analysis of plant nutrient concentrations and for total nitrogen determination where required. Total dry weights of roots were calculated using FW/DW ratio of the subsamples. The other root subsample was cleared and stained to allow examination of VAM fungal colonisation (Chapter 2.2.7).

2.2.7 Clearing and staining of roots

Roots were cleared and stained with aniline blue using a method based on that described by Grace and Stribley (1991). Harvested root subsamples were cut into pieces approximately 1-3cm long, placed into 10% KOH and left to clear for several days at room temperature. Cleared roots were rinsed well with R.O. water, acidified in 1M HCl until they turned white and stained in 0.05% aniline blue in 70% glycerol for 2-3 hours depending on age and type of roots. After they were stained, the roots were rinsed with R.O. water, placed into lactic acid : glycerol (1:1) to destain and stored at room temperature.

2.2.8 Colonisation assessment and determination of root length

Two methods were used to assess VAM fungal colonisation of the plant roots – the gridline-intersect technique and magnified intersects technique. The gridline-intersect technique was first used by Tennant (1975) to determine root length and was adapted to also give percent VAM colonisation as well as colonised root length (Giovannetti and Mosse, 1980). In the majority of experiments, VAM fungal colonisation of plant roots was assessed using the gridline-intersect technique described by Giovannetti and Mosse (1980). Any fungal structure (arbuscule, vesicle or hypha) intersecting a gridline was scored positive for mycorrhizal colonisation allowing percentage colonisation to be calculated. Percentage colonisation was used as the measure of VAM fungal colonisation for all experiments.

The magnified intersect technique developed by McGonigle *et al.* (1990) allows more precise description of the colonisation as it enables enumeration of the different fungal structures (i.e.

arbuscules, vesicles, intraradical hyphae) present in the root. It also allows estimation of the root length of the plant, however, it takes time and is laborious and so was not used for this purpose.

2.2.9 Nutrient analysis of plant material other than nitrogen

Dried plant material was digested in HNO_3 , and the resultant digest analysed for elements other than N by ICP-AES based on the method of Zarcinas *et al.* (1987). ICP-AES analyses were conducted by Waite Analytical Services, Waite Campus, University of Adelaide, Adelaide, South Australia.

2.2.10 Total nitrogen in soil or plant tissue

Total nitrogen concentrations in soils or plant tissues were determined using the Kjeldahl method (Bremner, 1996). Soil or plant material was digested in concentrated H_2SO_4 to which one Kjeldahl catalyst tablet (Ajax Cat. No. 2206-1000) and 30% w/v hydrogen peroxide had been added. Analyses of digests for $\text{NH}_4\text{-N}$ using an autoanalyser were performed by C. Rivers, Department of Soil and Water, University of Adelaide, Adelaide, South Australia based on the method described by Rayment and Higginson (1992).

2.2.11 Data Analysis

In all experiments, data were analysed using Genstat 5 (Genstat 5 Committee, 1987) and significant differences between treatments determined using analysis of variance (ANOVA). At all times, significance was determined at the 5% level. When a significant *F*-value was obtained ($P < 0.05$), Tukey's honestly significant difference statistic (Zar, 1999) was used to determine

significant differences between treatment means. In all experiments, data on percentage mycorrhizal colonisation were subjected to arcsin transformation prior to analysis (Zar, 1999).

Chapter 3.1 – Screening Clare field soils for indigenous VAM fungi

3.1.1 Introduction

Indigenous VAM fungi have been shown to be both beneficial and detrimental to plant growth, with the response depending on the fungus, host plant and environmental conditions (Harinikumar and Bagyaraj, 1988; Frey and Ellis, 1997). As discussed in Chapter 1.3, there are two approaches to manipulating VAM fungi in agricultural systems. If the indigenous VAM fungi are sufficiently beneficial to plant growth then it may be possible to enhance the positive effects of these fungi through the use of agricultural practices that favour VAM fungal colonisation, persistence and functioning (Abbott *et al.*, 1992; Boddington and Dodd, 2000; Daft, 1992). Such practices include minimum tillage, cover-cropping and controlled fertiliser applications. If the indigenous VAM fungal population is low or ineffective in improving plant nutrition and growth, the introduction of more effective VAM fungal species or isolates may be considered (Abbott and Robson, 1982a; Scheltema *et al.*, 1987). In either situation, the role of the indigenous VAM fungi needs to be assessed before making any recommendations regarding the management of VAM fungi in a given field soil.

Two field sites (Merildin and Dunn) near Clare, South Australia were observed to have very different cropping histories. Dunn had been cropped, after a long pasture phase, with canola, linseed and most recently, wheat. In comparison, Merildin had been cropped for many more years (i.e. no pasture phase) and had most recently been cropped with canola.

Linseed grown at the Dunn site following canola (*Brassica napus* L.) grew poorly and appeared to be suffering a nutrient deficiency that resembled zinc deficiency, although this was not confirmed. Given the negative effect of non-mycorrhizal host crops such as canola on VAM fungal colonisation and functioning (Arihara and Karasawa, 2000; Black and Tinker, 1979; Gavito and Miller, 1998; Harinikumar and Bagyaraj, 1988; Hayman *et al.*, 1975), it is possible that cropping with canola had led to decreased VAM fungal inoculum potential and/or infectivity in Dunn soil. This would lead to poor VAM fungal colonisation of the following linseed crop, possibly resulting in poor nutrition and growth of linseed. Furthermore, it was possible that a similar effect would be seen at the Merildin site given that this site had recently been cropped with canola.

This experiment investigated the indigenous VAM fungal colonisation and subsequent nutrition of linseed grown in Merildin and Dunn soils to formulate possible reasons for the observed poor growth of linseed in Dunn soil in the field. This experiment will be referred to here and elsewhere as the Screening Experiment.

3.1.2 Materials and Methods

Soil from the top 10 cm was collected from the two sites in June 1996. The soil was mixed thoroughly but not sieved or dried. Selected properties of these soils are shown in Table 3.1.1. Moist field soil, equivalent to 500 g oven-dry (O.D.) soil, was placed into black tubing pots 25 cm long and 5 cm in diameter. Fifteen surface-sterilised (Chapter 2.2.4) linseed seeds were sown into each pot to a depth of 1 cm, and there were 5 replicates of each treatment for each harvest time (21, 42 and 63 days after sowing).

	SOIL	
SOIL PROPERTY	MERILDIN	DUNN
Crop History ¹	Canola, <u>Oaten Hay</u>	Pasture, Canola, Linseed, Wheat, <u>Vetch</u>
Soil Texture	Medium clay	Medium clay
Soil pH ²	6.3	7.4
Soil P (mg/kg soil) ³	207	105
Soil Zn (mg/kg soil) ⁴	1.4	0.7
Soil Mn (mg/kg soil) ⁵	99	20

Table 3.1.1 – Selected properties of Merildin and Dunn soils. Soil samples (10 cm depth) collected in 1996, air-dried and sieved (<2 mm) prior to analysis. ¹1996 crop underlined. Soil pH determined using 0.01M CaCl₂ (Chapter 2.2.2) ³Bicarbonate-extractable soil P (Colwell, 1963) (Chapter 2.2.1). ⁴DTPA-extractable soil Zn (Lindsay and Norvell, 1978) (Chapter 2.2.3). ⁵DTPA-extractable soil Mn (Lindsay and Norvell, 1978) (Chapter 2.2.3).

Plants were grown in a growth room, given no additional nutrients, and were watered to weight (0.25 g H₂O/g O.D. soil) thrice weekly with R.O. water (Chapter 2.2.5). The plants were harvested after 21, 42 and 63 days (Chapter 2.2.6). Roots were cleared with 10% KOH and stained with aniline blue (Chapter 2.2.7) and colonisation of the root system by VAM fungi was determined using the gridline-intersect technique (Chapter 2.2.8). Dried plant shoots and roots were weighed and the plant nutrient status determined by digestion of this plant material

followed by ICP-AES (Chapter 2.2.9) or by Kjeldahl test for total N (42 day old shoot material only) (Chapter 2.2.10).

3.1.3 Results

Linseed grown in Merildin or Dunn soil had similar shoot dry weight (SDW) after 21 days. However, after 42 and 63 d, linseed grown in Merildin soil had significantly greater SDW than linseed grown in Dunn soil (Figure 3.1.1). VAM fungal colonisation of roots was significantly greater in linseed grown in Dunn soil at all harvest times (Figure 3.1.2). After 21 days, the concentration of P in the shoots of linseed grown in Dunn soil was lower than linseed grown in Merildin soil whilst the concentration of Zn and Mn was similar. However, after 42 and 63 days, linseed grown in Dunn soil had significantly greater concentrations of P and Zn in shoots but significantly lower concentration of Mn in shoots than linseed grown in Merildin soil (Figures 3.1.3, 3.1.4 and 3.1.5). Concentration of N in shoots after 42 days was significantly higher in linseed grown in Merildin soil ($28.7 \pm 0.3 \text{ mg N kg}^{-1}$) than Dunn soil ($15.1 \pm 1.0 \text{ mg N kg}^{-1}$).

3.1.4 Discussion

Linseed grew larger (greater SDW) in Merildin soil than in Dunn soil (Figure 3.1.1). The poor growth of linseed in Dunn soil supports the observations made in the field. However, VAM fungal colonisation of linseed was much greater when grown in Dunn soil than Merildin soil (Figure 3.1.2). It did not therefore appear that VAM fungi were playing a positive role in the nutrition and growth of linseed, which was surprising given the high mycorrhizal responsiveness or dependence previously reported for linseed (Thompson, 1994; Thompson, 1996; Thingstrup *et al.*, 1998; Thingstrup *et al.*, 2000). It should be noted, however, that mycorrhizal responsiveness

Figure 3.1.1 – Shoot dry weight of linseed grown in Merildin or Dunn soil after 21, 42 and 63 days. Bars represent standard error of the mean of 5 replicates. Columns with different letters are statistically different at the 5% level (Chapter 2.2.11). Data for each harvest time were statistically analysed separately.

Figure 3.1.2 – Percentage of the root length of linseed colonised by indigenous VAM fungi when grown in Merildin and Dunn soils. For explanation of error bars and column labelling refer to Figure 3.1.1. Data for each harvest time were statistically analysed separately and statistical analysis followed arcsin transformation of the colonisation data (Chapter 2.2.11).

Figure 3.1.1.

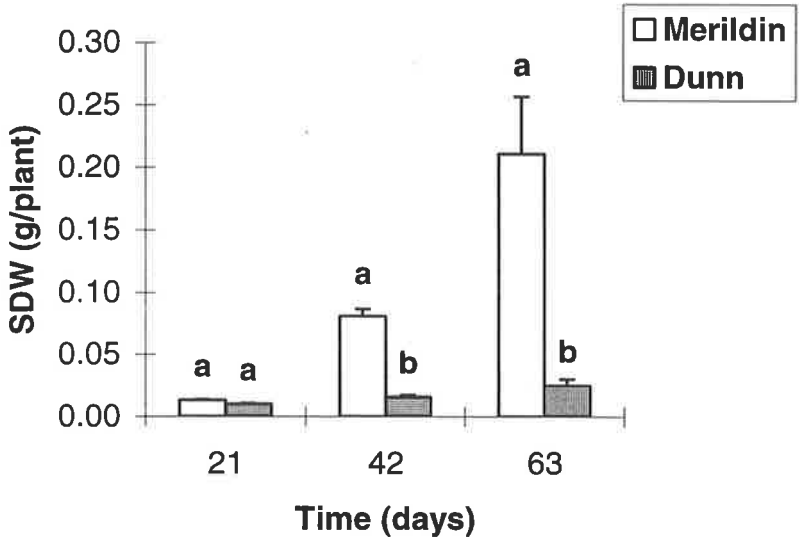


Figure 3.1.2

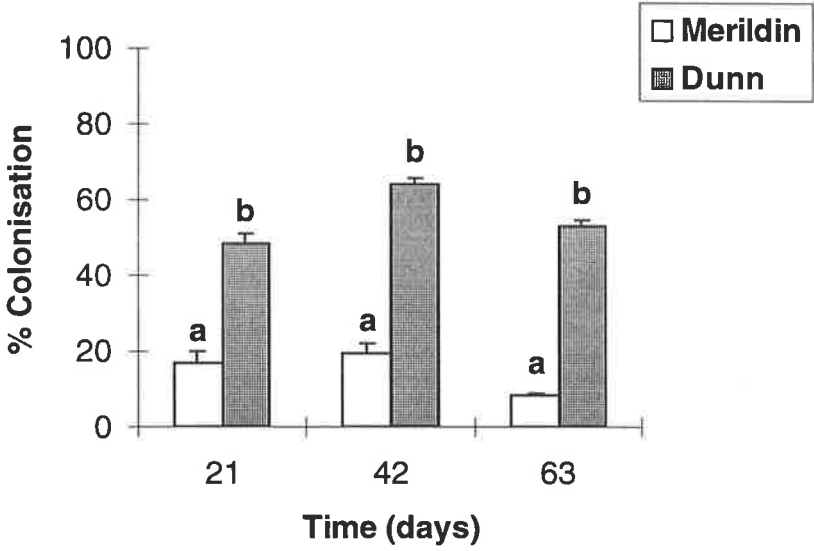


Figure 3.1.3 – Concentration of P in shoots of linseed grown in Merildin and Dunn soil. For explanation of error bars and column labelling refer to Figure 3.1.1. Data for each harvest time were statistically analysed separately

Figure 3.1.4 – Concentration of Zn in shoots of linseed grown in Merildin or Dunn soil. For explanation of error bars and column labelling refer to Figure 3.1.1. Data for each harvest time were statistically analysed separately.

Figure 3.1.3

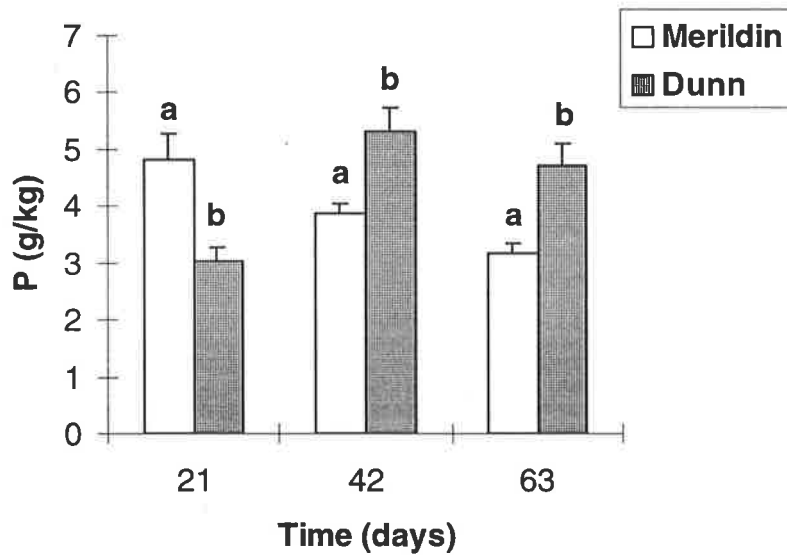


Figure 3.1.4

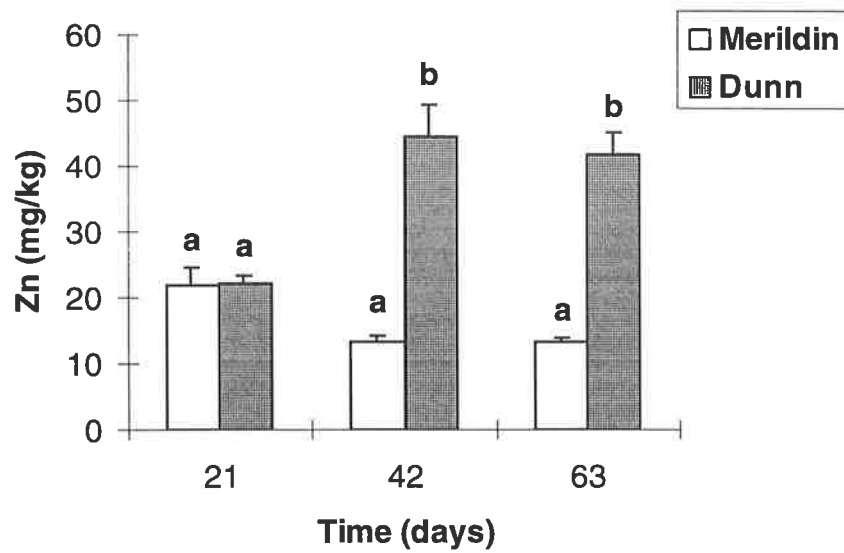
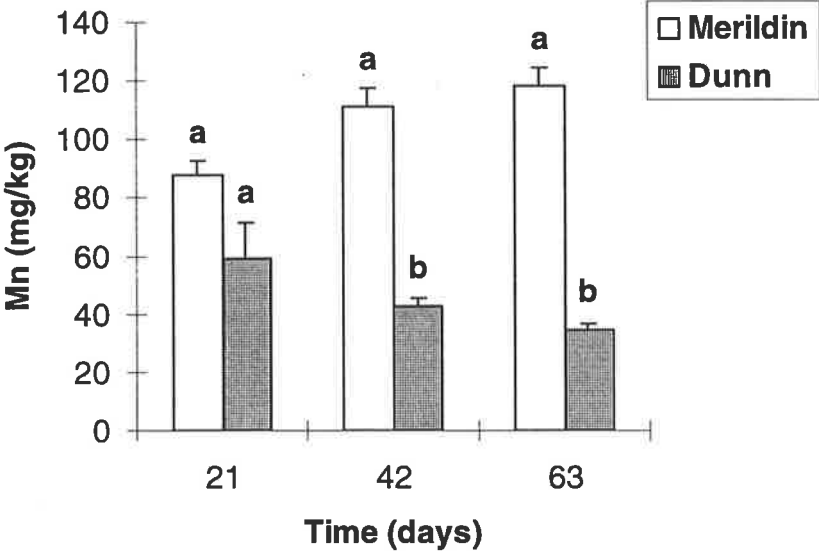


Figure 3.1.5 – Concentration of Mn in shoots of linseed grown in Merildin and Dunn soil. For explanation of error bars and column labelling refer to Figure 3.1.1. Data for each harvest time were statistically analysed separately.

Figure 3.1.5



or dependence of plants depend on the propagule density and chemistry of the soil used (especially soil P concentration), which may explain the different mycorrhizal responsiveness reported for linseed.

The difference in VAM fungal colonisation of linseed when grown in Merildin or Dunn soil may have been caused by a difference in the density of VAM fungal propagules between the soils. Differences in propagule density have been shown to result in different rates of colonisation (McGee *et al.*, 1999; Smith and Walker, 1981). Furthermore, any difference in propagule density may be, at least in part, a response to the different cropping histories of the two soils. As shown in Table 3.1.1, Dunn soil has a cropping history of pasture, canola, linseed, wheat and vetch whereas Merildin has a history of canola and oaten hay. In fact, Merildin soil had a longer history of cropping, whereas Dunn soil had a long period of pasture prior to being cropped. As pasture results in an increase in VAM fungal propagule density and subsequent colonisation (Hayman and Stovold, 1979; Smith, 1980b), then Dunn soil might be expected to have a higher number of VAM fungal propagules than Merildin soil, hence the greater VAM fungal colonisation of linseed grown in Dunn compared with Merildin soil. Moreover, Merildin soil was cropped with canola the year before collection whereas Dunn soil has been cropped with linseed and wheat (both known to be colonised by VAM fungi) since being cropped with canola. Therefore, the adverse effect of the canola rotation on VAM fungi was more likely to be observed with Merildin soil than with Dunn soil. The possibility of a difference in VAM fungal propagule density for Merildin and Dunn soils leading to differences in colonisation of linseed grown in these soils was investigated and is described at a later stage (Chapter 3.2 and 3.3).

Another possible explanation of the difference in VAM fungal colonisation of linseed grown in Merildin and Dunn soils relates to the P status of the two soils. With many hosts, high soil P

concentrations, especially following fertilisation, result in a reduction in VAM fungal colonisation (see Chapter 1.2.1.2). As Merildin soil has a higher concentration of P than Dunn soil, this could be a reason for the difference in colonisation of linseed. Interestingly, colonisation of roots of linseed grown in Dunn soil reached 60-70% (Figure 3.1.2) despite the high concentration of P in Dunn soil ($105 \mu\text{g P g}^{-1}$ soil (Colwell)). Thingstrup *et al.* (1998) also observed high VAM fungal colonisation (39%) of *Linum* sp. in soil containing $80 \mu\text{g P g}^{-1}$ soil (Olsen). It should be noted that Thingstrup *et al.* (1998) used the Olsen method of determining the concentration of bicarbonate-extractable P in soil, whereas the Colwell method was used in this project. Therefore, comparison of results obtained by Thingstrup *et al.* (1998) with results obtained in this project should be performed with care. One possible reason for this extensive colonisation is that the indigenous VAM fungi present in Dunn soil have become tolerant to the high soil P concentration at least with respect to colonisation. Such adaptation has previously been reported (see Chapter 1.2.1.2). If the VAM fungi present in Dunn soil improve plant nutrition and growth as well as being tolerant to high soil P concentrations, they would have great agricultural value, being able to improve plant nutrition even following P fertilisation. Just how tolerant these indigenous VAM fungi are to high soil P concentrations was investigated (Chapter 3.6).

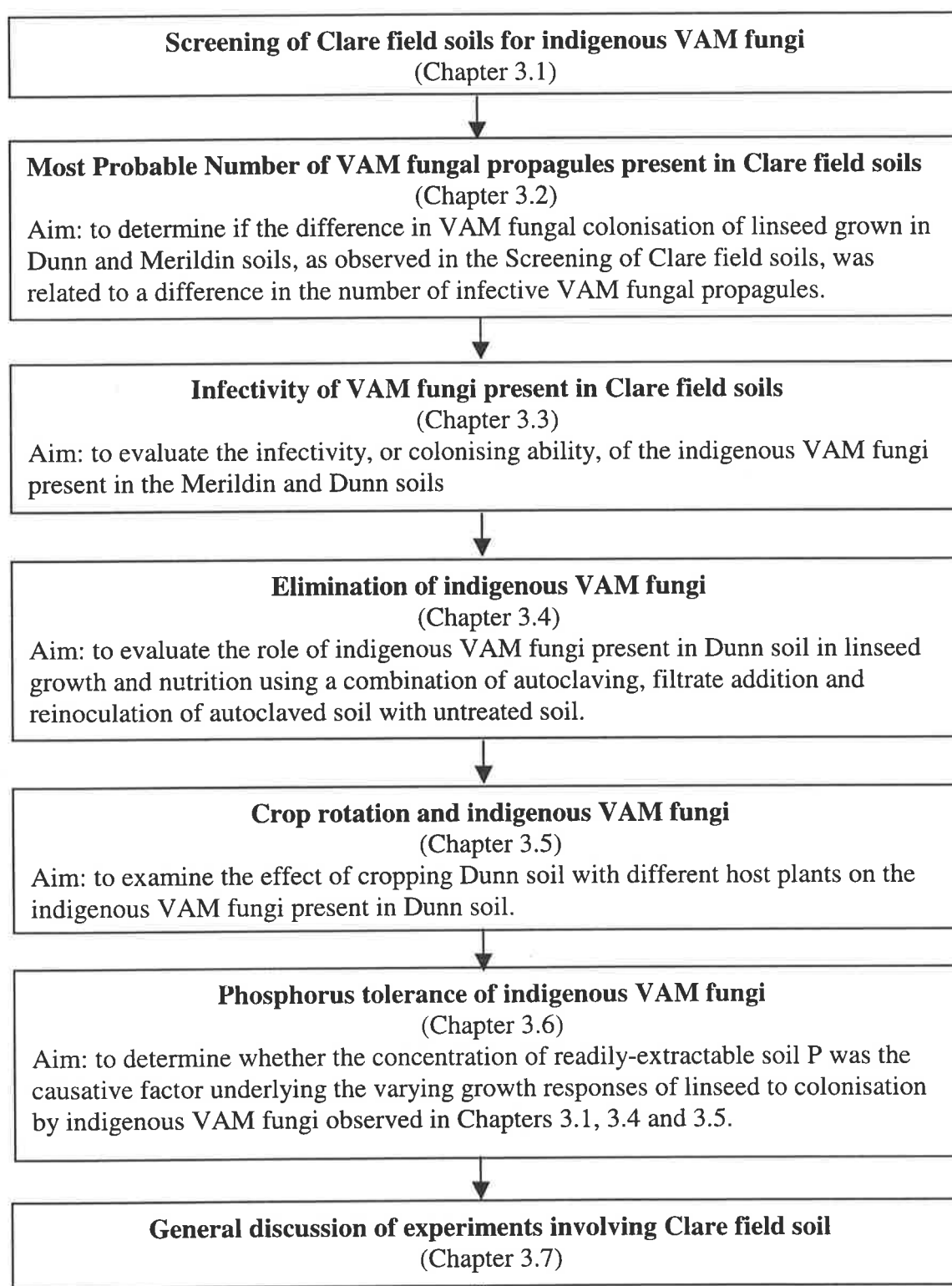
The nutritional analysis of the linseed shoots revealed that, after 42 and 63 days, linseed grown in Dunn soil had greater concentrations of P and Zn and lower concentrations of Mn than linseed grown in Merildin soil. These findings are consistent with previous research involving VAM fungi, which has shown that VAM fungal colonised plants often have higher concentrations of P and Zn (Azaizeh *et al.*, 1995; Barea, 1991; Kothari *et al.*, 1991; Liu *et al.*, 2000) but lower concentrations of Mn (Azaizeh *et al.*, 1995; Arines *et al.*, 1989; Kothari *et al.*, 1991; Liu *et al.*, 2000). It is also possible that the higher concentration of P in the shoots of linseed grown in

Dunn soil may have been a result of an accumulation effect due to the poor growth of these plants. The higher concentration of Mn in shoots of linseed grown in Merildin soil may also have been a reflection of the higher concentration of soil Mn in that soil (Table 3.1). Despite higher colonisation by VAM fungi and higher concentrations of P and Zn, linseed grown in Dunn soil had lower SDW. This suggests that some other factor was restricting the growth of linseed in Dunn soil and that it was not Zn deficiency as originally suspected.

There are several possible reasons for the poor growth of linseed in Dunn soil. One suggested by the results is nitrogen (N) deficiency. The concentration of N in shoots of linseed grown in Dunn soil ($15.1 \text{ mg N kg}^{-1}$) was much lower than linseed grown in Merildin soil ($28.7 \text{ mg N kg}^{-1}$). Unfortunately, data regarding critical concentrations of N in shoots of *Linum* sp. are variable and limited (for example, see Hocking *et al.*, 1987 or Reuter and Robinson, 1997). Furthermore, N deficiency may provide an explanation for the higher concentration of shoot P and Zn in linseed grown in Dunn soil. High concentrations of nutrients can indicate that another factor or nutrient is limiting (Lambert *et al.*, 1979). In the present situation, the uptake of P and Zn may have been similar for plants grown in Merildin and Dunn soil. However, because the growth of linseed in Dunn soil was limited by N deficiency, P and Zn become concentrated in the smaller shoots of linseed grown in Dunn soil.

Another possible explanation for the poor growth of linseed in Dunn soil is that the very extensive colonisation by indigenous VAM fungi induced a plant growth depression (see Chapter 1.2.1.3). Unfortunately, although poor growth of linseed in Dunn soil might have been caused by the indigenous VAM fungi, this could not be confirmed, as there were no non-mycorrhizal controls in this experiment.

In summary, this initial experiment showed that linseed grown in Merildin and Dunn soils differed greatly in VAM fungal colonisation, shoot dry weight and nutrition. To determine the cause of these differences, further experiments were required. These experiments are shown in the following flow diagram:



Chapter 3.2 - Most Probable Number of VAM fungal propagules present in Clare field soils

3.2.1 Introduction

The significantly greater colonisation of linseed grown in Dunn soil compared with Merildin soil observed whilst screening the Clare soils (Chapter 3.1) could have been due to a number of factors. These include differences in VAM fungal propagule density or infectivity and nutrient status of the soil and/or plant.

VAM fungal propagules include spores, hyphae within dead root pieces, possibly excised external hyphae and hyphae growing from nearby living roots (Smith and Read, 1997; Brundrett and Abbott, 1994; Brundrett *et al.*, 1996). Colonisation depends on the density and the infectivity or 'quality' of these propagules (Abbott and Robson, 1981; McGee *et al.*, 1999; Smith and Walker, 1981; Wilson and Trinick, 1983) and is controlled by factors governing spore germination or hyphal regrowth such as soil P concentration, temperature and water.

Often VAM fungal propagule density is estimated by spore counts following wet sieving of soil. However, these counts do not take into account the other types of propagules (Abbott and Robson, 1991b; An *et al.*, 1990) and fail to include those VAM fungal species that form no spores or spores too small to be sieved and/or counted (Abbott and Robson, 1982b; Abbott and Robson, 1982a; An *et al.* (1990); Brundrett and Abbott, 1994; Porter 1979). Spore counts may also include spores that are dead or dormant, thus overestimating propagule density (Baon *et al.*, 1992). Furthermore, there is no guarantee that wet sieving removes from the soil all the spores

large enough to be sieved and counted (Porter, 1979) and spore counts are not always related to VAM fungal root colonisation in the same soil (Hayman, 1984; Hayman and Stovold, 1979).

One alternative method that has often been used to estimate the density of infective VAM fungal propagules in soil is the Most Probable Number (MPN) method. Originally, the MPN method, which permits estimation of a population density without an actual count of cells or colonies (Alexander, 1982; Cochran, 1950), was developed for use with bacteria. The method was adapted for VAM fungal research allowing estimation of propagule density without actual spore or propagule counts (Porter, 1979). Porter (1979) concluded that the MPN method appeared to give a more realistic estimate of the number of infective VAM fungal propagules than spore counts. It is important to keep in mind that there are several assumptions associated with MPN estimations of VAM fungal propagules. These assumptions, which are not always met by VAM fungi, include (a) the VAM fungal propagules are randomly distributed throughout the soil, (b) the propagules are single and not aggregated, (c) dilution of the original soil sample is proportional to the number of propagules, and (d) if one individual propagule is present, it will be detected by the assay (Tommerup, 1992). Furthermore, for VAM fungi, detection depends on the use of a trap plant so that MPN estimation of VAM fungal propagules is dependent on factors such as temperature, time of harvest, specificity of the assay host plant, and nutrient status of the soil or plant, particularly soil P concentration, all of which influence the rate of colonisation and hence sensitivity of detection (Wilson and Trinick, 1982).

Despite the above complications and assumptions, the MPN method was chosen for use in this experiment to determine if the difference in VAM fungal colonisation of linseed grown in the Merildin and Dunn soils, as observed in the Screening of the Clare field soils (Chapter 3.1), was caused by a difference in the number of infective VAM fungal propagules.

3.2.2 Materials and Methods

A baiting technique was used with linseed or Linola™ as the host plant. Dilution series (10-fold) of the Dunn and Merildin soils with autoclaved sand were performed by mixing 100 g of soil with 900 g of autoclaved sand. This was repeated five times to give final concentrations of each soil of 10^0 through to 10^{-5} . Six replicate samples of 50 g of each dilution were placed into compartments of a seedling tray.

Seeds of linseed and Linola™ were surface sterilised and germinated (Chapter 2.2.4). One seedling was planted in each compartment to a depth of 1 cm, with planting started in the lowest dilution to prevent cross-contamination between dilutions. Plants were grown under growth room conditions, watered with R.O. water thrice weekly and each compartment was given 0.5 ml modified Long Ashton nutrient solution (+N, +Zn, -P) weekly (Chapter 2.2.5).

After 21 days, i.e. the same growth period as for the first harvest in the Screening Experiment (Chapter 3.1), the plants were removed from the compartments and the entire root system cleared in 10% KOH and stained with aniline blue (Chapter 2.2.7). Colonisation (absence or presence of fungi) of each root system was assessed and the MPN of VAM fungal propagules per gram of soil determined using the MPN-program in GW Basic devised by Cuthbertson (1986).

3.2.3 Results

The most probable numbers of propagules in the Dunn and Merildin soils are shown in Table 3.2.1. The number of infective VAM fungal propagules was much higher in the Dunn than the

Merildin soil, and there was no difference in MPN of infective VAM fungal propagules if linseed or Linola™ was used as the host plant.

Soil	Host plant	MPN of VAM fungal propagules per g soil	Standard error
Merildin	Linseed	6.1	3.3
Merildin	Linola™	6.1	3.3
Dunn	Linseed	58.4	36.3
Dunn	Linola™	58.4	36.3

Table 3.2.1 – Most probable number of VAM fungal propagules in Dunn and Merildin soils using linseed or Linola™ as the host plant.

3.2.4 Discussion

The results show that the MPN of infective VAM fungal propagules was higher in Dunn soil than Merildin soil irrespective of plant host. This indicates that the greater colonisation of linseed grown in Dunn soil observed in the Screening Experiment (Chapter 3.1) was at least partly due to a greater density of infective VAM fungal propagules. Similar results for agricultural soils were observed by Baon *et al.* (1992). They showed that two soils, Peake and Avon, differed greatly in MPN value (28 for Peake compared to 0.9 for Avon). Furthermore, cereal plants grown in Peake soil had a higher percentage of root length infected by VAM fungi, reflecting the greater MPN value for Peake soils.

However, as highlighted in the introduction, there are numerous assumptions and problems associated with MPN estimations of VAM fungal propagules. In the current experiment, a similar host plant, experimental conditions and harvest time were used in an attempt to minimise any influence of these factors on the MPN value obtained. However, the two soils tested did have different P concentrations. Therefore, despite using only a small amount of inoculum in comparison to the total soil volume in an attempt to minimise any differences in soil nutrient concentration (Porter, 1979; Wilson and Trinick, 1982), it is possible that the different P concentrations of Merildin and Dunn soils may have had a significant effect on the MPN value obtained. As shown in Table 3.1.1, Merildin soil had a higher concentration of readily-extractable soil P than Dunn soil. High soil P concentrations can have a negative effect on VAM fungal colonisation (see Chapter 1.2.1.2). Therefore, the lower VAM fungal colonisation of linseed or Linola reflected in a lower MPN value for these plants grown in Merildin soil compared to Dunn soil may be partly a result of the higher soil P concentration in Merildin soil.

The MPN method of estimating the number of infective VAM fungal propagules also gives no indication of the extent or activity of colonisation that would arise from these propagules (Abbott and Robson, 1982b; An *et al.*, 1990; Hayman and Stovold, 1979; Scheltema *et al.*, 1987) and there is a large standard error associated with the method. Increasing the number of replicate pots per dilution and/or decreasing the dilution at each step can reduce this error. However, both greatly increase the number of plants that need to be grown and scored making the method unfeasible. This, along with the reasons discussed earlier, and the fact that the MPN method utilises a number of assumptions not necessarily met by VAM fungi, led to the decision to utilise an alternative method of assessing the mycorrhizal status of the Clare field soils with respect to plant growth and nutrient uptake. The method chosen was an infectivity assessment or bioassay, which is described in Chapter 3.3.

Chapter 3.3 –Infectivity of indigenous VAM fungi present in Clare field soil

3.3.1 Introduction

In Chapter 3.2, the MPN of infective VAM fungal propagules present in Clare field soil was determined. Although this provides valuable information on the number of infective propagules in these soils, an MPN scores the presence or absence of colonisation but does not indicate the extent of colonisation that would arise from these propagules (Abbott and Robson, 1991a). This information can be obtained from an infectivity assessment.

Infectivity of a soil refers to the amount of VAM fungal colonisation that will arise when a mycorrhizal host plant is grown in that soil. An infectivity assessment has the advantage that many of the assumptions used with MPN estimations do not apply such as that there is a random distribution of propagules. Infectivity assessments also permit the study of plant growth and nutrition in response to VAM fungal colonisation. Furthermore, as with the MPN assessment, if the environmental conditions, growing times and same host plant are used, an infectivity assessment allows comparative studies of the infectivity of field soils collected at different sites or times.

This experiment aimed to assess the VAM fungal infectivity of Clare field soils collected in 1996. Infectivity of Dunn soil collected in 1998 was also assessed and compared to results obtained for 1996 soil, which will indicate whether 1998 soil could be used in subsequent experiments given that there was insufficient 1996 soil. This experiment will be referred to here and elsewhere as the Infectivity Assessments.

3.3.2 Materials and Methods

Field soils from both Merildin and Dunn were collected in 1996. In 1998, soil was collected only from the Dunn site as the Merildin site had been treated with a pre-emergent herbicide. This site had been cropped with vetch (1996) and oaten hay (1997) and was sampled prior to the 1998 crop. Infectivity of each soil was assessed immediately following sampling in the following manner.

Samples of air-dried soil (125 g) were placed into black tubing pots 24 cm long and 2 cm in diameter. Three replicates for each soil were included. Linseed (*L. usitatissimum* L. cv 'Glenelg') seeds were surface sterilised and germinated (Chapter 2.2.4). Germinated linseed seeds (3) were planted in each pot to a depth of 1 cm, and the plants thinned to one per pot after emergence. Plants were grown in a growth room, watered to weight (75% field capacity w/w) thrice weekly with R.O. water and given no additional nutrients (Chapter 2.2.5).

After 21 days, the plants were harvested (Chapter 2.2.6). Shoots were removed and dried for dry weight measurements whilst the roots were rinsed in R.O. water, cleared in 10% KOH and stained with aniline blue (Chapter 2.2.7). VAM fungal colonisation of the plant root was assessed using the grid-line intersect method (Chapter 2.2.8). Dried plant shoots were analysed for nutrient status (other than N) by nitric acid digestion of the shoot material followed by ICP-AES (2.2.9). Unfortunately, total N analysis of the plant shoots was not possible due to a lack of plant material.

3.3.3 Results

VAM fungal colonisation of linseed was significantly greater in Dunn compared to Merildin soil (Table 3.3.1). However, linseed grown in Merildin soil had significantly greater SDW than that grown in Dunn soil. Linseed grown in Merildin soil also had significantly greater shoot P, Zn and Mn concentrations than linseed grown in Dunn soil but the difference in Zn concentration was significant at the 10% level only whereas the difference in P and Mn concentration was significant at the 5% level. Linseed grown in Dunn soil collected in 1996 and 1998 had similar VAM colonisation, SDW, and shoot P, Zn and Mn concentrations.

3.3.4 Discussion

Linseed grown in 1996 Merildin soil had significantly greater SDW than plants grown in 1996 Dunn soil but had significantly lower VAM fungal colonisation (Table 3.3.1). Nutrient analyses showed that linseed grown in Merildin soil had greater P, Zn and Mn concentrations than linseed grown in Dunn soil. These results reflect those obtained after 21 days in the Screening Experiment (Chapter 3.1). This indicates that this infectivity assessment reflects the colonisation and growth that would arise if larger pots were used and it can therefore be used for rapid assessment of the infectivity of Clare field soils.

One of the principal aims of these infectivity assessments was to determine whether growth and colonisation of linseed was similar when grown in Dunn soil collected in 1996 and 1998. The results indicate that there was little difference in the growth, colonisation and P and Zn nutrition of linseed in Dunn soil collected in 1996 and 1998. This is important in relation to the experiments described in the following chapters, namely the Elimination Experiment (Chapter

Soil	Year of collection	Percentage of root length colonised	Shoot dry weight (g/plant)	Shoot P concentration (g/kg)	Shoot Zn concentration (mg/kg)	Shoot Mn concentration (mg/kg)
Merildin	1996	3.8 (0.7)a	0.09 (0.011)b	4.6 (0.2)b	12.8 (1.6)b	96.7 (12.5)b
Dunn	1996	36.0 (2.1)b	0.04 (0.004)a	3.3 (0.1)a	8.0 (0.4)a	47.3 (7.6)a
Dunn	1998	31.4 (1.9)b	0.03 (0.003)a	3.0 (0.5)a	8.5 (0.9)a	38.4 (1.9)a

Table 3.3.1 – VAM fungal colonisation, growth and nutrition of linseed grown for 21 days in an infectivity assessment of Merildin and Dunn soils. Infectivity of the soils was tested immediately following collection of soil. Merildin soil was not tested in 1998. Values are the means of 3 replicates with the standard errors of the means in parentheses. Values with the same letters are not significantly different at the 5% level (Chapter 2.2.12) except for shoot Zn concentration where values with the same letters are not significantly different at the 10% level.

3.4), Crop Rotation Experiment (Chapter 3.5) and Phosphorus Tolerance Experiment (Chapter 3.6) of indigenous VAM fungi (Chapter 3.3). As there is little difference in the infectivity of soil collected from the Dunn site in 1996 and 1998, then similar colonisation, growth and nutrition of linseed observed in the Screening Experiment (Chapter 3.1) would be likely if linseed plants were grown in Dunn 1998 soil. Therefore, Dunn soil collected in 1998 could be used in the Elimination Experiment, Crop Rotation Experiment and Phosphorus Tolerance Experiment.

Chapter 3.4 -Elimination of indigenous VAM fungi

3.4.1 Introduction

The Screening Experiment (Chapter 3.1) and Infectivity Assessments (Chapter 3.3) showed that linseed grew poorly in Dunn soil despite being extensively colonised by indigenous VAM fungi. It is possible that the indigenous VAM fungi might have contributed to the poor growth of linseed in Dunn soil. However, as there were no non-mycorrhizal controls in either of these experiments, the contribution of the indigenous VAM fungi to the growth and nutrition of linseed in Dunn soil remained unclear.

To examine the role of VAM fungi in nutrition and growth of their host plants in field soil, an appropriate control treatment is required to eradicate or reduce the VAM fungal contribution and allow comparisons between mycorrhizal and non-mycorrhizal plants to be made. Autoclaving, fungicides, soil solarisation, fumigation and γ -irradiation have all been shown to have adverse effects on VAM fungi. Of these methods, fungicides and autoclaving are the most widely used.

Fungicides have been used extensively in VAM research to reduce the colonisation and hence the contribution of VAM fungi to the symbiosis. A review of the various fungicides and their effect on VAM fungi by Menge (1982) gives excellent general information on many of the fungicides previously used in VAM research. Of the available fungicides, benomyl has been used extensively and has been shown to reduce VAM colonisation of several host plants including onion (*Allium cepa* L.) (Kough *et al.*, 1987; Manjunath and Bagyaraj, 1984; Sukarno *et al.*, 1993), pea (*Pisum sativum* L.) (Schreiner and Bethlenfalvay, 1996), and bluebell (*Hyacinthoides*

non-scripta L.) (Merryweather and Fitter, 1996). However, the application of fungicides such as benomyl has given variable results. In pot experiments, Sukarno *et al.* (1993) showed a massive decrease in colonisation of onion plants by VAM fungi following application of benomyl. However, Pedersen and Sylvia (1997) observed minimal reduction in colonisation of maize by VAM fungi in the field even after several applications of benomyl. Schreiner and Bethlenfalvay (1997) concluded their study of fungicides and VAM fungi by stating that "Interactions of AM fungi and fungicides are highly variable and biological responses depended on the fungus-fungicide combinations and environmental conditions." Of particular interest is the effect of soil type on the success of fungicide application. As with fertilisers, it is possible that in soils with high clay or organic matter content, fungicide applied to the soil surface will bind to soil or organic matter particles (Pedersen and Sylvia, 1997). The fungicide may then be unavailable or may not penetrate deeply into the soil thus having less effect on VAM fungal populations especially at deeper soil layers (P. O'Connor pers. comm.). To incorporate the fungicide under these conditions, the fungicide would need to be mixed into the soil. In heavy textured soils, this would not be possible without destroying the soil structure. Fungicides may also have phytotoxic (chlorosis or biomass reduction of susceptible plant species) or phytobeneficial (improved plant nutrition or removal of plant pathogens) effects (Kahiluoto *et al.*, 2000; Kahiluoto and Vestberg, 2000).

The possibility of poor control of VAM fungi in the medium heavy clay soil such as Merildin and Dunn by fungicides due to poor soil penetration, as well as possible phytotoxic and phytobeneficial effects, led to the decision not to use them to eliminate or control the VAM fungi in this experiment.

Autoclaving is often used to control VAM fungi present in soil. It is an effective treatment for pot experiments, eliminating all VAM fungi from the target soil. Unfortunately, autoclaving may alter the structure and physico-chemical properties of the soil as well as eliminating other soil organisms including plant pathogens (Smith and Smith, 1981a). Autoclaving may also result in the release of nutrients (e.g. N, P and Mn), which may be beneficial or toxic to plants depending on the amount released (Jakobsen and Andersen, 1982; Skipper and Westerman, 1973; Smith and Smith, 1981a). These effects ultimately have an effect on plant growth in that soil. Furthermore, due to these effects, comparisons between untreated and autoclaved soil are difficult. One approach to overcome this is to autoclave all the soil and then reinoculate one batch with VAM fungal propagules in the form of untreated soil (Smith and Smith, 1981a; Smith and Smith, 1981b). The advantage of this approach is that the basic soil properties will be similar for all treatments allowing comparisons to be made. The disadvantage is that neither the autoclaved or reinoculated soil relates to the untreated soil (Smith and Smith, 1981a). Nevertheless, with correct planning and effective control measures, autoclaving is a very effective treatment for the elimination of VAM fungi from soil and was the method chosen for this experiment.

To combat the effects of autoclaving on microorganisms and soil fertility, filtrate of non-sterilised soil may be added to the autoclaved soil (without adding VAM fungal propagules) in an attempt to reintroduce soil microorganisms (Smith and Smith, 1981a). Koide and Li (1989) observed a growth depression when filtrate was added to autoclaved soil growing sunflower or mustard. They suggested that this might have been due to the reintroduction of pathogens or a sufficient number of saprophytic microorganisms that competed with the plant for nutrients and hence reduced plant growth. Reduced plant growth due to competition for nutrients between the plant and microorganisms were also observed by Daniels-Hetrick *et al.* (1988) and McLaughlin

and Alston (1986). Conversely, reinoculation of soil with soil microorganisms has been reported to improve plant growth by removing phytotoxins that may have formed during autoclaving (Rovira and Bowen, 1966). On balance, it appears that the advantages of addition of filtrate to sterilised soil outweigh the disadvantages. This led to the decision to include addition of filtrate as a treatment in this experiment.

Therefore, this experiment used a combination of treatments including autoclaving, addition of soil filtrate and reinoculation of autoclaved soil with untreated soil to evaluate the role of indigenous VAM fungi in the growth and nutrition of linseed in Dunn soil. It will be referred to here and elsewhere as the Elimination Experiment.

3.4.2 Materials and Methods

Soil collected in June 1998 from Dunn was used in this experiment. It will be referred to as Dunn-1998 soil. This soil was collected adjacent to the site of the 1996 collection. The soil was air-dried and crushed prior to use and contained $74 \mu\text{g}$ readily-extractable P g^{-1} soil (Colwell, 1963; Chapter 2.2.1). Several treatments were developed to take account of potential effects of autoclaving on other soil properties (e.g. soil nutrient concentrations) and allow comparisons to be made between these treatments. In all treatments, autoclaved soil refers to soil that was autoclaved twice at 121°C for 60 minutes. The 5 soil treatments and the rationale behind each treatment were as follows.

(a). Untreated soil (U) – 100% Dunn-1998 soil not autoclaved. Representative of untreated field soil.

(b). Autoclaved soil (A) – 100% autoclaved Dunn-1998 soil. All microorganisms including VAM fungi eliminated.

(c) Autoclaved soil reinoculated with untreated soil only (AR) – 90% autoclaved Dunn-1998 soil reinoculated with 10% (w/w) untreated Dunn-1998 soil. Reinoculation of autoclaved soil with untreated soil containing VAM fungal propagules will allow mycorrhizas to be re-established in the autoclaved soil. This treatment is the mycorrhizal equivalent of soil A (treatment b).

(d) Autoclaved soil reinoculated with soil filtrate only (AF) – 100% autoclaved Dunn-1998 soil to which $<8\ \mu\text{m}$ filtrate from 4 g untreated soil (equivalent to 1%) is added to the soil surface and watered in. This treatment examines the effect of addition of soil filtrate (no VAM fungal propagules) to autoclaved soil on plant nutrition and growth.

(e) Autoclaved soil, reinoculated with untreated soil (10% w/w) and soil filtrate (ARF) – As for treatment (c) AR except $<8\ \mu\text{m}$ filtrate from 4 g untreated soil (equivalent to 1%) is also added to the soil surface and watered in. This treatment examines the combined effect of addition of soil filtrate and reinoculation with VAM fungal propagules in the form of untreated soil on the growth and nutrition of plants in autoclaved soil. It is the mycorrhizal equivalent of soil AF (treatment d).

Soil filtrate

In treatments (d) and (e), filtrate from untreated soil was added in an attempt to reintroduce some of the soil micro-organisms other than VAM fungal propagules to the soil. It was unknown what spores or fungal species present in Dunn-1998 soil would be re-introduced in this way and so a trial filtration was conducted. Dunn-1998 soil (75 g) was stirred in 150 ml of R.O. water for 60 minutes. This suspension was then filtered through a Whatman No. 541 filter paper (20-25 μm aperture). Observation of this filtrate under a dissecting microscope revealed no spores but a large number of hyphal fragments. Living hyphal fragments can act as VAM fungal propagules

(Arihara and Karasawa, 2000), although the infectivity of hyphal fragments declines rapidly in moist soil (Daft *et al.*, 1987). Therefore, it was unclear if the hyphal fragments in the 20-25 μm fraction would be infective and so the 20-25 μm filtrate was passed through a Millipore filter (8 μm aperture) to remove the hyphal fragments. Observation of the 8 μm filtrate revealed no hyphae. A gram stain showed that the 8 μm filtrate contained bacteria and a dilution plate series of the 8 μm filtrate was then conducted to determine the number of colony forming units (CFU) present. 10-fold serial dilutions of the 8 μm filtrate with sterile R.O. water were produced to give dilutions from 10^0 through to 10^{-8} . For fungi, 0.1 ml of each dilution was spread on 1% (w/v) half-strength potato dextrose agar with 0.05% (w/v) streptomycin and 0.05% (w/v) penicillin. For bacteria, 0.1 ml of each dilution was spread on to 1.5% (w/v) nutrient agar with 0.075% (w/v) cycloheximide. For both fungi and bacteria, there were 5 replicates per dilution. All plates were incubated at 25°C for 72 hours and CFU counted. This resulted in a total count of approximately 3600 CFU (bacteria) and 60 CFU (non-mycorrhizal fungi) in 8 μm filtrate from 1 g of Dunn soil. Therefore, 8 μm filtrate from 4 g of the untreated soil contains 14400 CFU (bacteria) and 240 CFU (fungi) which was added to the surface of each pot in soil treatment (d) and (e) and watered in.

Plant growth and conditions

In all treatments, the total weight of soil in each pot was 400 g OD equivalent. Seeds of linseed (*Linum usitatissimum* L. cv. "Glenelg") and canola (*Brassica napus* L. cv. "Drum") were surface sterilised and germinated (Chapter 2.2.4). Canola was included in this experiment because it is a non-host for VAM fungi and so any differences in growth of canola in the various soil treatments will be independent of VAM fungi. The use of non-mycorrhizal host plants in this manner has

also been reported by Koide and Li, (1989) and Smith and Smith (1981a). Seven surface sterilised germinated seeds (either linseed or canola) were sown in each pot and the plants were thinned to 3 per pot following seedling emergence. There were 5 replicate pots per treatment.

Each pot was watered to weight (75% field capacity) with R.O. water three times a week and the plants were given **no** additional nutrients. The plants were grown under growth room conditions (Chapter 2.2.5) and were harvested after 21 and 42 days (Chapter 2.2.6). Plant roots were cleared with 10% KOH and stained with aniline blue (Chapter 2.2.7) and colonisation of the root system by VAM fungi was carried out using the gridline-intersect technique (Chapter 2.2.8). Dried plant shoots and roots were analysed for nutrient status (other than N) by nitric acid digestion of this plant material followed by ICP-AES (Chapter 2.2.9) or by Kjeldahl test for total N (Chapter 2.2.10).

3.4.3 Results

Plant growth

After 21 and 42 days, there was significantly greater shoot dry weight (SDW) of canola grown in all treatments involving A soil compared with U soil, suggesting an effect of autoclaving on soil nutrient release (Figure 3.4.1A). However, there was little or no difference in the growth of canola grown in the 4 soil treatments utilising A soil.

After 21 days, there was no significant difference in the SDW of linseed grown in any of the variously treated soils (Figure 3.4.1B). After 42 days there was significantly more SDW of

Figure 3.4.1 – Shoot dry weight of (A) canola and (B) linseed grown in variously treated Dunn-1998 soil. Soil treatments are U = Untreated soil, A = Autoclaved soil, AF = Autoclaved soil reinoculated with filtrate only, AR = Autoclaved soil reinoculated with untreated soil only, and ARF = Autoclaved soil reinoculated with untreated soil and filtrate. Bars represent the standard error of 5 replicates. Columns with different letters are significantly different at 5% level (Chapter 2.2.11). Data for each harvest time were statistically analysed separately.

Figure 3.4.1 A - Canola

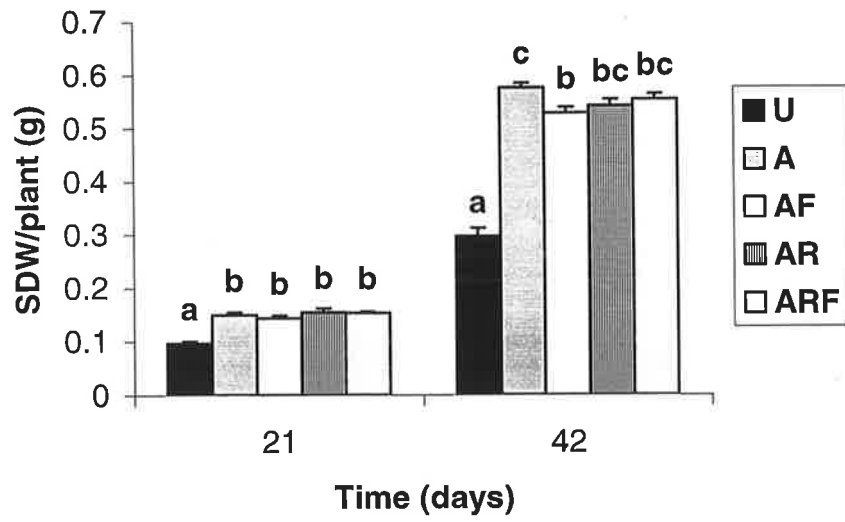
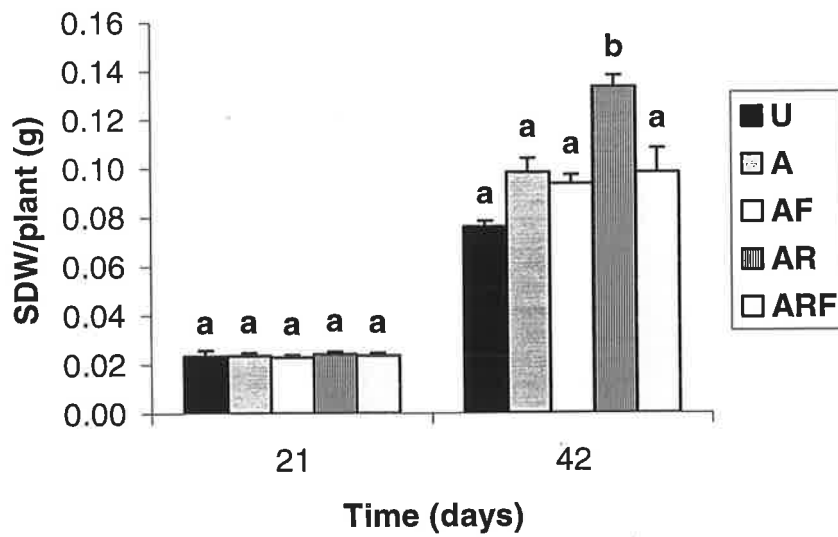


Figure 3.4.1 B - Linseed



linseed grown in AR soil compared with the other soil treatments. There was no significant difference in SDW of linseed grown in U soil compared with A, AF or ARF soil.

VAM fungal colonisation

After 21 and 42 days, there was no colonisation of canola roots by VAM fungi.

No colonisation was seen in linseed plants grown in A and AF soil after 21 or 42 days (Figure 3.4.2). After 21 days, linseed grown in U soil had significantly greater percent root length colonised by VAM fungi than the linseed plants grown in AR or ARF soil. After 42 days, there was no significant difference in percent VAM fungal colonisation of linseed grown in U and AR soil or AR and ARF soil. However, percent root length colonised by VAM fungi was significantly greater for linseed grown in U soil compared to ARF soil.

Plant nutrition

After 21 days, there were quite small but statistically significant differences in the concentration of P in the shoots of canola grown in the various soil treatments (Figure 3.4.3 A). Plants grown in AR or ARF soil had significantly lower shoot P concentrations than plants grown in A or AF soil whilst the shoot P concentration of plants grown in U soil was not significantly different from plants grown in any of the other soils. After 42 days, there were no significant differences in the concentration of P in shoots of canola grown in any of the soils.

After 21 days, there was significantly less P in shoots of linseed grown in U soil than in the other soil treatments except for ARF soil (Figure 3.4.3 B). Of the other soil treatments there was

Figure 3.4.2 – VAM fungal colonisation of linseed grown in variously treated Dunn-1998 soil. For explanation of soil treatments, error bars and column labelling refer to Figure 3.4.1. Data for each harvest time were statistically analysed separately and statistical analysis was performed following arcsin transformation of data (Chapter 2.2.11).

Figure 3.4.2

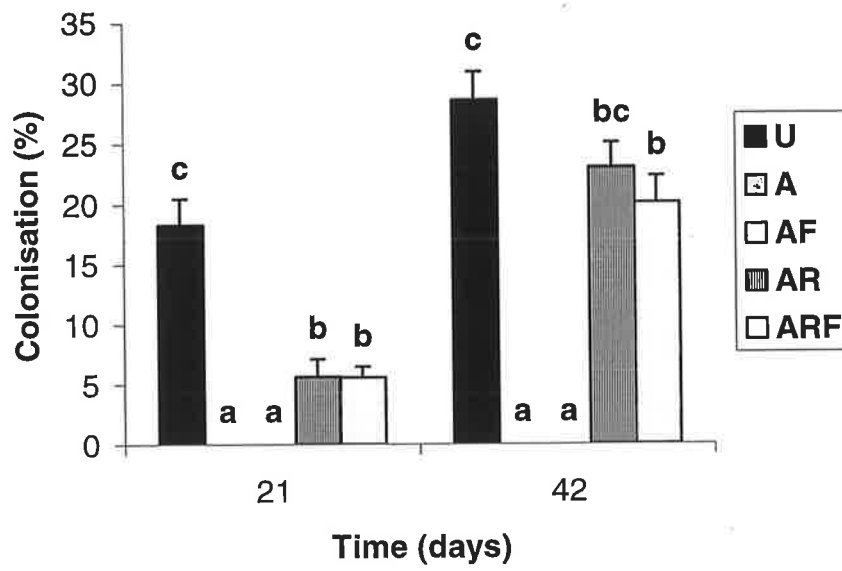


Figure 3.4.3 – Concentration of P in shoots of (A) canola and (B) linseed grown in variously treated Dunn-1998 soil. For explanation of soil treatments, error bars and column labelling refer to Figure 3.4.1. Data for each harvest time were statistically analysed separately.

Figure 3.4.3 A - Canola

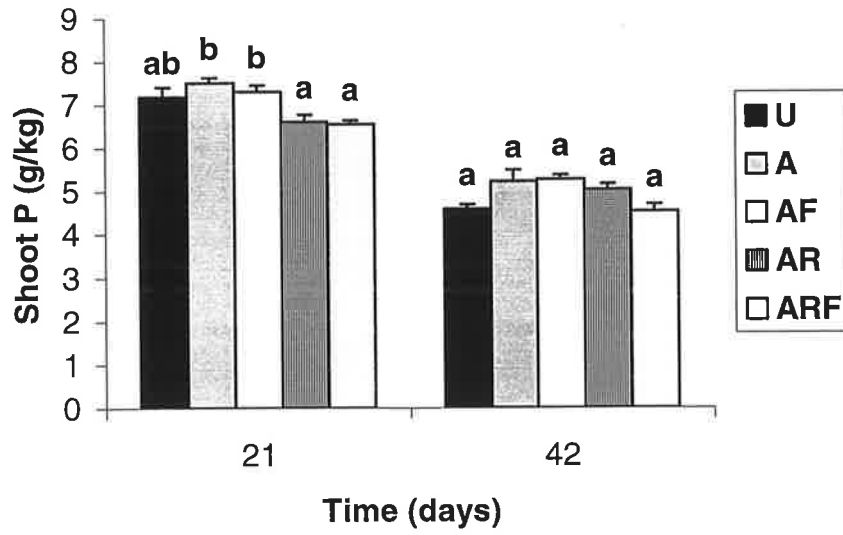
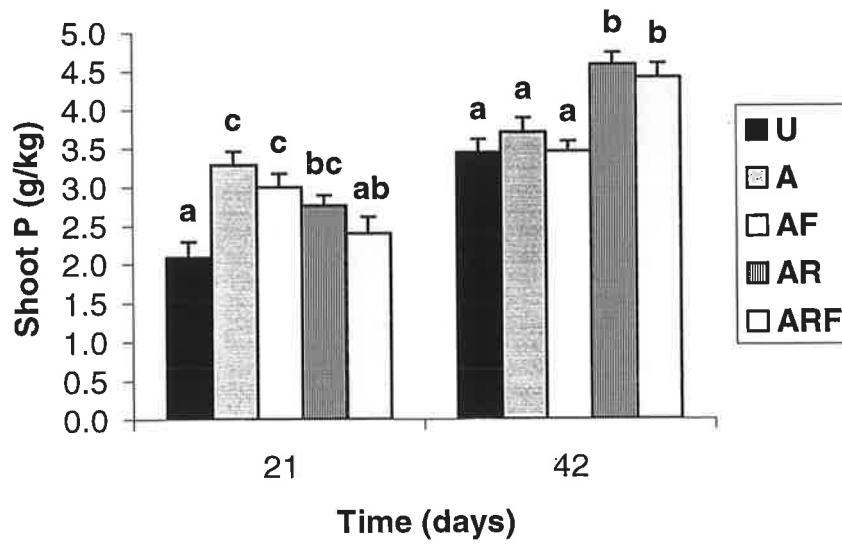


Figure 3.4.3 B - Linseed



significantly more P in shoots of linseed grown in A or AF than in ARF soil. After 42 days, there were no significant differences in concentration of P in shoots of linseed grown in U, A or AF soil. However, linseed grown in AR or ARF soil had significantly higher shoot P concentrations than linseed grown in U, A or AF soil.

After 21 days, there was significantly more Zn in shoots of canola grown in A or AF soil than canola grown in U, AR or ARF soil (Figure 3.4.4 A). This difference, although significant, was not great. After 42 days, there was no significant difference in concentration of Zn in shoots of canola grown in any of the soil treatments.

Linseed grown in A, AF or AR soil had significantly more Zn in shoots than linseed grown in U soil after 21 days (Figure 3.4.4 B). Furthermore, linseed grown in A soil also had significantly greater shoot Zn concentration than linseed grown in ARF soil after 21 days. As with canola, although these differences were significant, they were not great. After 42 days, there was no significant difference in the concentration of Zn in shoots of linseed grown in any of the soils.

After 21 days, there was significantly less N in shoots of canola grown in U soil than A, AF, AR or ARF soil (Figure 3.4.5 A). A similar pattern was observed for canola after 42 days except that, in general, the concentrations were much lower.

As with canola, there was significantly less N in shoots of linseed grown in U soil than in any of the other soil treatments after 21 days (Figure 3.4.5 B). After 42 days, however, there were no significant differences in the concentration of N in shoots of linseed grown in any of the soils.

Figure 3.4.4 – Concentration of Zn in shoots of (A) canola and (B) linseed grown in variously treated Dunn-1998 soil. For explanation of soil treatments, error bars and column labelling refer to Figure 3.4.1. Data for each harvest time were statistically analysed separately.

Figure 3.4.4 A - Canola

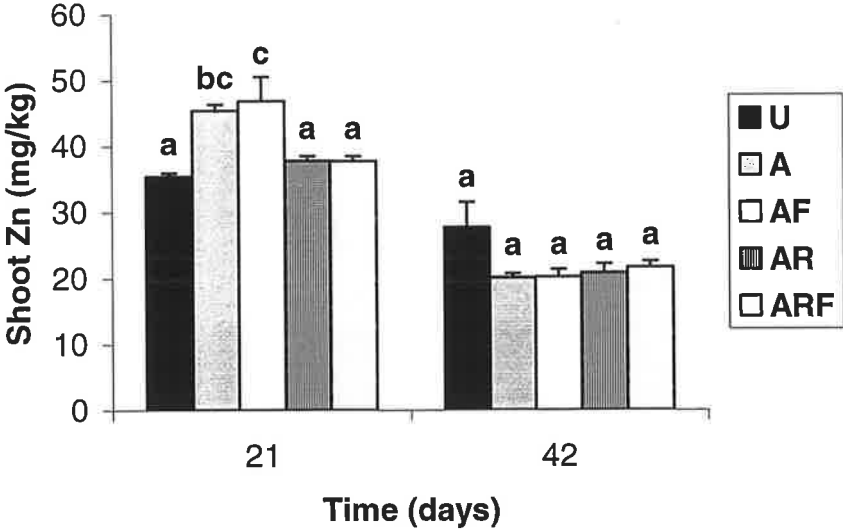


Figure 3.4.4 B - Linseed

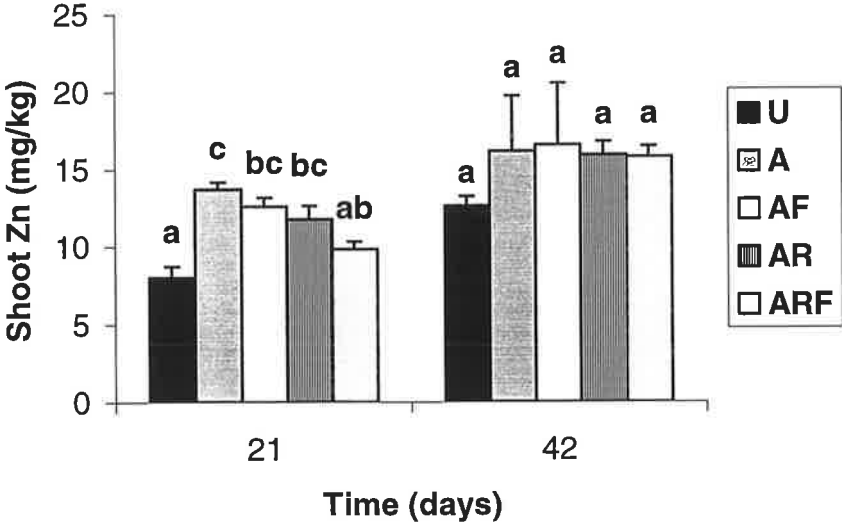


Figure 3.4.5 – Concentration of N (total) in shoots of (A) canola and (B) linseed grown in variously treated Dunn-1998 soil. For explanation of soil treatments, error bars and column labelling refer to Figure 3.4.1. Data for each harvest time were statistically analysed separately.

Figure 3.4.5 A - Canola

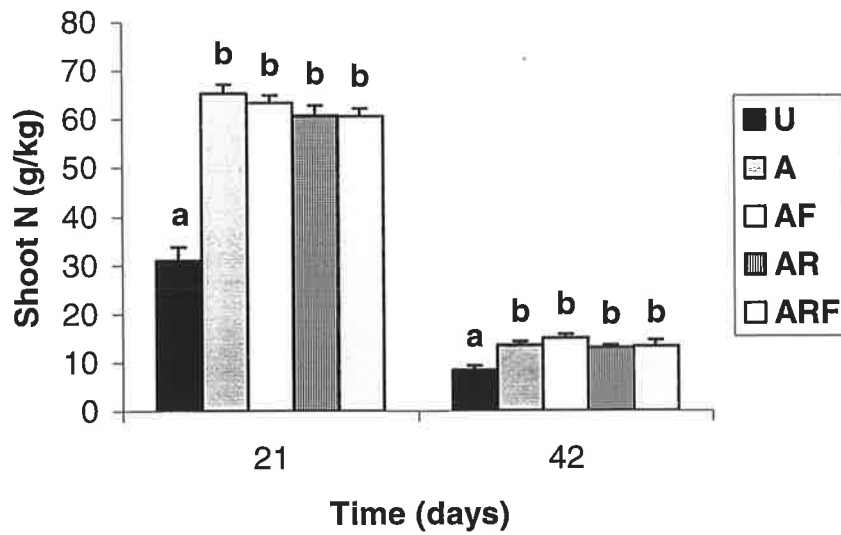


Figure 3.4.5 B - Linseed

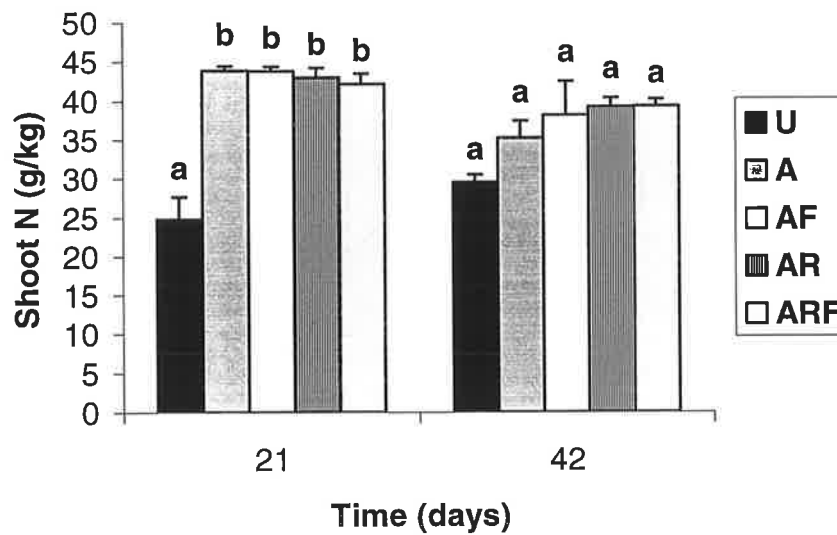


Figure 3.4.6 – Concentration of Mn in shoots of (A) canola and (B) linseed grown in variously treated Dunn-1998 soil. For explanation of soil treatments, error bars and column labelling refer to Figure 3.4.1. Data for each harvest time were statistically analysed separately.

Figure 3.4.6 A - Canola

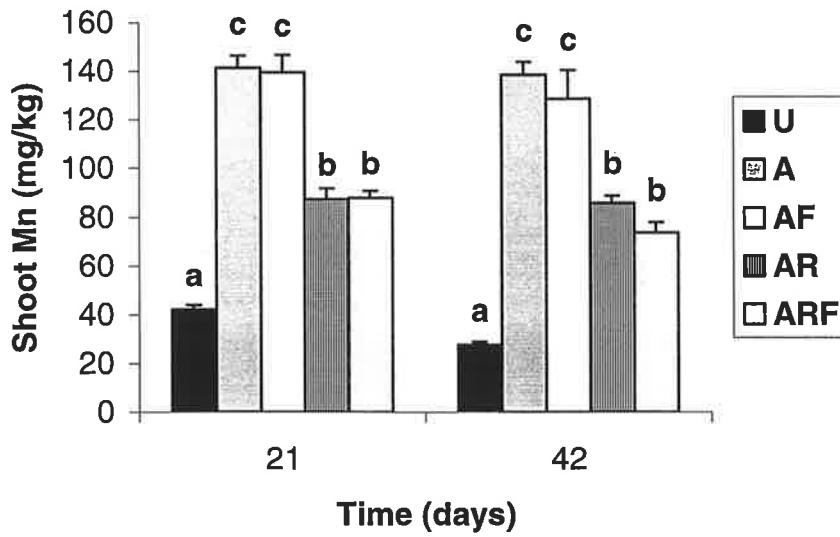
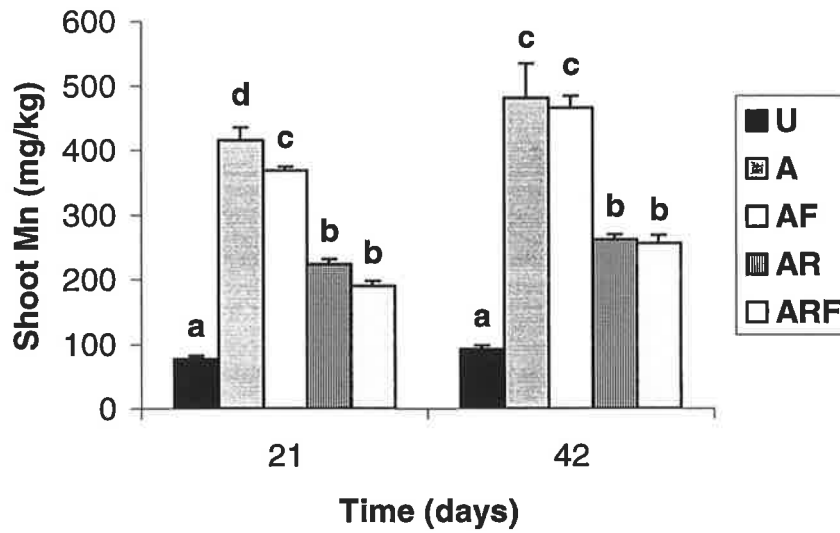


Figure 3.4.6 B - Linseed



The concentration of Mn in shoots of canola or linseed grown in the various soil treatments followed a similar pattern (Figure 3.4.6 A and B). After either 21 or 42 days, plants grown in U soil had significantly lower shoot Mn concentration than plants grown in AR or ARF soil. These in turn had significantly lower shoot Mn concentrations than plants grown in A or AF soil.

3.4.4 Discussion

The difference in growth of canola plants grown in U soil compared with the other soils shows that the U soil differed in factors other than VAM colonisation. The most likely factor is soil fertility, as autoclaving has been shown to increase the fertility of soil particularly with respect to N, P and some micronutrients (Jakobsen and Andersen, 1982; Skipper and Westerman, 1973; Smith and Smith, 1981a). This is confirmed by the nutrient analysis of the canola shoots which showed that canola grown in autoclaved soil treatments had higher shoot N and Mn concentrations than canola grown in U soil (Figure 3.4.5 A and 3.4.6 A). This result is important when comparing the growth of linseed in the variously treated soils. Differences in SDW of linseed grown in the U soil compared with linseed grown in the other soils must therefore be considered with care, as the effect may be at least partly due to nutritional differences.

It is interesting that the concentrations of Mn and to a lesser extent P and Zn, in shoots of canola were lower when the plants were grown in reinoculated AR and ARF soil compared with plants grown in A and AF soil (Figure 3.4.3 A, 3.4.4 A and 3.4.6 A). One treatment included in this experiment (AF) involved the addition of soil filtrate in an attempt to reintroduce some soil biota to autoclaved soil. This treatment appeared to have little effect on canola growth, with few differences observed between plants grown in A and AF soil or AR and ARF soil. However, reinoculation of the autoclaved soil with 10% untreated soil had a large effect on Mn nutrition of

canola, with canola grown in AR or ARF soil having significantly lower shoot Mn concentrations compared to plants grown in A or AF soil. The addition of filtrate was designed to reintroduce some soil biota that can help to destroy phytotoxins and reduce the concentration of potentially toxic nutrients such as Mn that arise following autoclaving (Rovira and Bowen, 1966). However, it is possible that reinoculating soil with untreated soil reintroduced soil microorganisms not retained in the $<8\mu\text{m}$ filtrate. These microorganisms may have reduced the concentration of plant-available Mn in the soil and would explain why the concentration of Mn in shoots of canola was lower for plants grown in AR and ARF soil compared to plants grown in A and AF soil. The same reasoning can be applied to explain the differences in shoot Mn concentration of linseed grown in AR or ARF soil compared to linseed grown in A or AF soil (Figure 3.4.6 B).

Despite the effect of reinoculation with untreated Dunn-1998 soil on Mn in shoots, canola grown in A, AF, AR or ARF soil had quite similar growth and concentrations of other nutrients including P, Zn and N (Figures 3.4.3 A, 3.4.4 A and 3.4.5 A). This indicates that these soils probably did not differ in these nutrients. Therefore, it is feasible to compare linseed grown in A, AF, AR or ARF soil as any differences are unlikely to be due to non-mycorrhizal factors and could be attributed to VAM fungal colonisation. Even so, it is important to keep in mind that linseed and canola have different nutritional requirements, and so caution regarding conclusions is required.

After 21 days, there was significantly greater colonisation (percent root length colonised) of linseed grown in U soil compared with the other potentially mycorrhizal soil treatments (i.e. AR and ARF) (Figure 3.4.2). After 42 days, however, this difference was only apparent between plants grown in U and ARF soils. The difference at 21 days is most likely a result of differences in VAM fungal propagule density between the treatments. U soil consisted entirely of Dunn-1998

soil, which has already been shown to be highly infective (Chapter 3.3). The AR and ARF treatments only contained 10% Dunn-1998 soil, and it is reasonable to assume that the pots contained approximately 10% of the infective propagules present in U soil. Initial colonisation of linseed in AR and ARF soil would therefore be expected to be slower and less extensive. However, after 42 days colonisation of linseed grown in the U, AR and ARF soils is similar as the initial difference in propagule density and accompanying lag phase in root colonisation is overcome. A similar effect was reported by McGee *et al.* (1999), who showed that initial colonisation by 10 propagules was slower and lower than colonisation from 100 propagules. However, after 36 days, the colonisation percentage was similar from 10 or 100 propagules.

After 21 days, there were no differences in SDW of linseed grown in any of the soils (Figure 3.4.1 B). However, there was significantly more growth of linseed grown in AR soil than the other treatments after 42 days. This is most likely due to greater VAM fungal colonisation of linseed grown in AR soil compared to the other autoclaved soil treatments (Figure 3.4.2) and hence a greater contribution of VAM fungi to plant nutrition in this soil. Nutritional analyses support this explanation with shoot P concentration of linseed grown in AR and ARF soils being significantly greater than that of linseed grown in A or AF soil after 42 days (Figure 3.4.3 B). As the canola plants grown in these soils had similar shoot P concentration then the difference in shoot P concentration of linseed grown in these soils was a mycorrhizal effect and not a non-mycorrhizal effect.

It is noteworthy that there was no difference in VAM fungal colonisation and shoot P concentration of linseed grown in AR and ARF soils. However, linseed grown in AR soil had significantly greater SDW and therefore must have absorbed more P. The only difference between the AR and ARF soils was the addition of soil filtrate. It is possible that soil micro-

organisms added with the soil filtrate have utilised some of the soil nutrients and so less was available for plant growth hence less SDW of linseed grown in ARF soil. This effect of soil micro-organisms on soil nutrient status and subsequent plant growth has been shown by Koide and Li (1989). However, as already discussed for canola, the addition of filtrate appeared to have little influence on the nutrition of either canola or linseed with the nutrition of plants grown in A or AR soil not differing from those grown in AF or ARF soil respectively (Figures 3.4.4, 3.4.5 and 3.4.6). Therefore, reasons for this difference in SDW of linseed grown in AR and ARF soils remain unknown.

SDW of linseed grown in U soil was not significantly different to that of linseed grown in A or AF soil after 42 days (Figure 3.4.1 B). This was in contrast to the growth pattern of canola, which showed that SDW of canola grown in the autoclaved soil treatments was significantly greater than that of canola grown in U soil. As well as SDW, shoot P concentration of linseed grown in U soil was not significantly different to linseed grown in A or AF soil. It is possible that the VAM fungi have improved the growth and nutrition of linseed in U soil leading to similar growth as linseed grown in A or AF soil despite the differences between the soils that did not relate to mycorrhizal status. In other words, it appears that VAM fungal colonisation and consequent improved nutrition and growth of linseed grown in U soil are promoting linseed growth to a level similar to that of linseed grown in A or AF soil.

SDW of linseed in U (Dunn-1998) soil was also much greater than that of linseed grown in Dunn-1996 soil (Chapter 3.1). Comparisons of results from these two experiments indicates that linseed grown in U soil had a much higher shoot N concentration than linseed grown in Dunn-1996 soil (29.5 compared to 15.1 g N kg⁻¹ SDW). This is the likely cause of the difference in

growth of linseed and also supports the theory that poor growth of linseed in Dunn-1996 soil (Chapter 3.1) was due to poor N nutrition.

The results obtained in this experiment for linseed suggest that the indigenous VAM fungi present in Dunn-1998 soil are beneficial for linseed growth. This is in direct contrast to results obtained for the Screening Experiment (Chapter 3.1) that suggested that the indigenous VAM fungi might have been detrimental to linseed growth. It is important to remember that autoclaving the soil in this Elimination Experiment has obviously changed soil fertility as shown by the growth and nutrition of canola in the various soil treatments. Therefore, changing the fertility of the Dunn-1998 soil may have corrected a nutrient deficiency or imbalance, allowing the effect of the indigenous VAM fungi to be observed.

In conclusion, this experiment showed that: (i) addition of soil filtrate had little effect on linseed growth or VAM fungi, (ii) reinoculation of autoclaved soil with untreated Dunn-1998 soil (AR) resulted in VAM fungal colonisation similar to U soil after 42 days, and (iii) improved growth of linseed in AR soil can be attributed to VAM fungal contribution to plant P nutrition. This final conclusion suggests that the indigenous VAM fungi present in Dunn-1998 soil are potentially beneficial to linseed nutrition and growth. However, autoclaving may have corrected a nutrient deficiency or imbalance enabling the positive effect of VAM fungi on plant nutrition and growth to be seen.

Given that this experiment showed the indigenous VAM fungi present in Dunn soil to be potentially beneficial to linseed growth, it would be favourable to promote these fungi. This was addressed in Chapter 3.5.

Chapter 3.5 – Crop rotation and indigenous VAM fungi

3.5.1 Introduction

The Elimination Experiment (Chapter 3.4) revealed that the indigenous VAM fungi present in Dunn soil are more likely to be beneficial to linseed growth than detrimental, as first thought (see Chapter 3.1). Therefore, it may be advantageous to promote these indigenous VAM fungi, enhancing colonisation and possibly improving the VAM fungal contribution to plant nutrition and growth. As discussed in Chapter 1.2.2, different management strategies can be beneficial or detrimental to VAM fungi present in the field soil consequently affecting other mycorrhizally responsive plants grown in the rotation. One strategy involves cropping the soil with either non-mycorrhizal or mycorrhizal crops (Chapter 1.2.2.2). The choice of crop depends on whether the effect desired is to promote (use of mycorrhizal crop plant) or reduce (use of non-mycorrhizal crop plant or fallow) the indigenous VAM fungi.

This experiment aimed to assess the possibility of promoting the indigenous VAM fungi present in Dunn soil by cropping the soil with three mycorrhizal host plants - subterranean clover, linseed and vetch. The VAM fungal colonisation, growth and nutrition of linseed grown in these cropped soils were also examined. This experiment is referred to here and elsewhere as the Crop Rotation Experiment.

3.5.2 Materials and Methods

Cropping of Dunn 1996 and Dunn 1998 soils with subterranean clover, vetch or linseed

Soil collected in 1996 from Dunn was air dried and crushed prior to use. It was then mixed 1:1 with autoclaved sand as there was insufficient soil to permit the use of 100% soil¹. Samples of this soil/sand mix equivalent to 300 g oven dry (O.D.) soil were placed into 500 ml white pots. Seeds of subterranean clover, vetch or linseed were surface sterilised and germinated (Chapter 2.2.4). For subterranean clover, 3 germinated seeds were sown in each pot to a depth of 1 cm and inoculated with *Rhizobium leguminosarum* bv. *trifolii* (Chapter 2.1.1.3). For vetch, 2 germinated seeds were sown in each pot to a depth of 1 cm and inoculated with *Rhizobium leguminosarum* bv. *viciae* (Chapter 2.1.1.3). For linseed, 4 germinated seeds were sown in each pot to a depth of 1 cm. In all cases the plants were thinned to one plant per pot after seedling emergence, and three replicate pots were included for each host plant.

Plants were grown in a growth room, watered to weight (80% field capacity or 0.135 g/g) three times a week with R.O. water and given no additional nutrients (Chapter 2.2.5). After 12 weeks, the cultures were cored using a stainless steel corer 1.5 cm in diameter and 25 cm long, and the plants roots washed from the soil core. The roots were then cleared in 10% KOH and stained with aniline blue (Chapter 2.2.7). VAM fungal colonisation of the plant roots was assessed using the grid-line intersect technique (Chapter 2.2.8). After 16 weeks, plants were placed in transparent sunbags (Sigma Cat. No. B-7026) to protect the cultures from a blackfly outbreak,

Note: there was insufficient Dunn-1996 soil to conduct the Elimination Experiment, however, there was sufficient Dunn-1996 soil to permit this experiment.

watering ceased and the plants left in sunbags to dry slowly. Once the plants and cropped soils were dry, shoots were removed and the cropped soil stored under cool dry conditions.

Cropping of Dunn-1998 soil with subterranean clover, vetch, or linseed was conducted in a similar manner, but with the following differences. Samples of 1:1 soil/sand mix equivalent to 500 g O.D. soil were placed into each pot and 5 replicates were included for each host plant. Five germinated subterranean clover, 4 germinated vetch or 5 germinated linseed seeds were sown into separate pots to a depth of 1 cm, but as with Dunn-1996 soil, plants were thinned to one per pot following seedling emergence. As with Dunn-1996 soil, the plants were watered to weight (80% field capacity or 0.135 g/g) three times a week and given no additional nutrients. However, plants grown in Dunn-1998 soil were not placed into sunbags as there was no blackfly problem. Instead, after 16 weeks growth, the plants and soil were slowly dried off over 2 weeks by gradually reducing watering from 80% to 45% field capacity (0.135 g/g to 0.076 g/g), after which the plant tops were removed and the cropped soil stored under dry cool conditions.

Infectivity of cropped soils

Infectivity of each cropped soil was evaluated using the technique given in Chapter 3.3. One replicate pot of each soil cropped with linseed, subterranean clover or vetch with similar levels of colonisation was selected for this experiment. Root pieces in each cropped soil were cut into 1-2 cm pieces and the soil mixed thoroughly to distribute potential inoculum pieces evenly. The concentration of P in each of the cropped soils, determined using the autoanalyser method (Chapter 2.2.1), ranged from 30-70 $\mu\text{g P g}^{-1}$ soil.

For each cropped Dunn-1996 soil, a weight of soil equivalent to 310 g O.D. cropped soil was mixed thoroughly with 155 g O.D. autoclaved Dunn-1998 soil and 155 g O.D. autoclaved sand. Autoclaved Dunn-1998 soil and sand mix was included to provide sufficient nutrients and was preferred over fertiliser solutions, as it more closely resembled the original soil and natural balances, apart from the dilutions caused by adding autoclaved sand, would be more closely maintained. Samples (150 g) of the resultant mix were placed into black tubing pots 20 cm long and 2 cm in diameter. Four replicate pots were included for each cropped soil. For comparison, original Dunn-1996 soil was diluted with autoclaved sand (1:1) and tested in the same manner as the cropped soils. Hereafter, this soil will be referred to as orig-96. It must be stressed that orig-96 soil differs greatly from that used in the Screening Experiment (Chapter 3.1) and Infectivity Assessments (Chapter 3.3) due to the addition of autoclaved Dunn-1998 soil and sand. As such comparisons of the results from these experiments are invalid.

Seeds of linseed were surface-sterilised and germinated (Chapter 2.2.4). Three germinated linseed seeds were sown in each pot, and the plants were thinned to one per pot after emergence. Plants were grown in a growth room (Chapter 2.2.5) watered to weight (0.19 g H₂O/g O.D. soil) three times weekly with R.O. water and given no additional nutrients. After 21 days, plants were harvested (Chapter 2.2.6), roots cleared and stained with aniline blue (Chapter 2.2.7) and VAM fungal colonisation of the roots assessed using the grid-line intersect technique (Chapter 2.2.8). Dried shoots were analysed for nutrients using nitric acid digestion of the shoot material followed by ICP-AES (Chapter 2.2.9). There was insufficient plant material to permit the analysis of total N in shoots.

Infectivity of the Dunn 1998 soil cropped with linseed, subterranean clover and vetch was assessed as for Dunn-1996 soil with the following differences. For each cropped Dunn-1998 soil

a weight of soil equivalent to 390 g O.D. soil was mixed thoroughly with 195 g O.D. of autoclaved Dunn-1998 soil and 195 g O.D. autoclaved sand. Samples (150 g) of the resultant mix were placed into black tubing pots 20 cm long and 2 cm in diameter. Five replicates were included for each crop plant. As with Dunn-1996 soil, original Dunn-1998 soil was diluted with autoclaved sand (1:1) and tested in the same manner as the cropped Dunn-1998 soils for comparison. Hereafter, this soil will be referred to as orig-98. As with Dunn-1996 soil, it must be stressed that orig-98 soil differs greatly from the Dunn-1998 soil tested in the Infectivity Assessments (Chapter 3.3) and Elimination Experiment (Chapter 3.4) due to the addition of autoclaved Dunn-1998 soil and sand. As such comparisons of the results for Dunn-1998 soil from these experiments are invalid.

3.5.3 Results

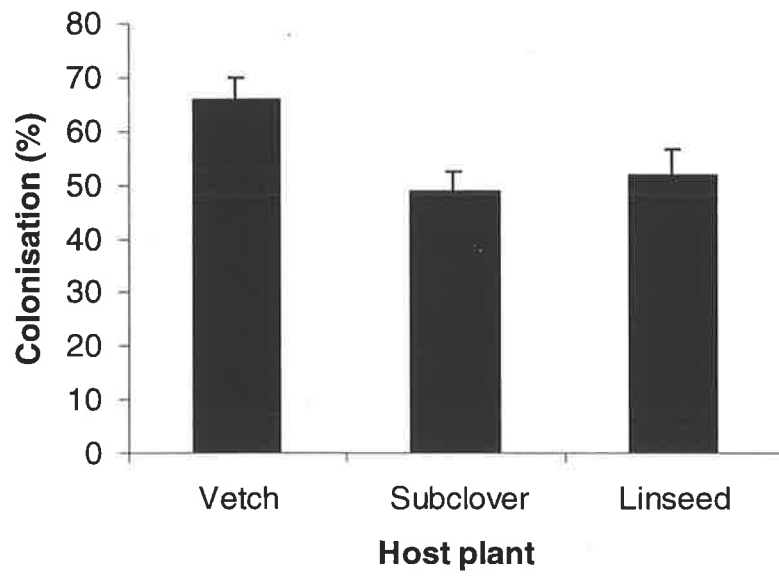
Cropping of Dunn 1996 and Dunn 1998 soil with subterranean clover, vetch or linseed

VAM fungal colonisation was extensive (>40% root length colonised) for all host plants irrespective of soil (Figure 3.5.1). Colonisation of vetch was greater than colonisation of either subterranean clover or linseed when grown in Dunn-1996 or Dunn-1998 soils. Furthermore, there were only small differences in colonisation between the two soils, with values for Dunn-1998 soil slightly higher than Dunn-1996 soil (compare Figure 3.5.1 A and B).

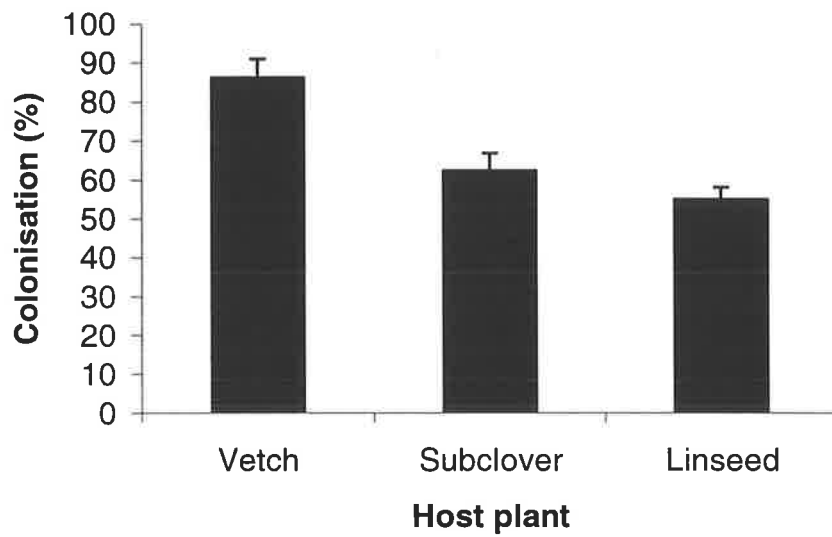
Figure 3.5.1 – VAM fungal colonisation of linseed, subterranean clover and vetch trap plants grown in (A) Dunn-1996 and (B) Dunn-1998 soil after 84 days. Bars represent standard error of the mean of 3 replicates for Dunn-1996 soils and standard error of 5 replicates for Dunn-1998 soils.

Figure 3.5.1

A – Dunn-1996 soil



B – Dunn-1998 soil



Infectivity of cropped soils

Linseed grown in Dunn-1996 or Dunn-1998 soil, which had been cropped with linseed, subterranean clover or vetch, had significantly greater VAM fungal colonisation than linseed grown in orig-96 or orig-98 soils respectively (Figure 3.5.2).

Shoot dry weight (SDW) of linseed was significantly greater when grown in Dunn-1996 soil cropped with either linseed or subterranean clover when compared with growth in orig-96 soil (Figure 3.5.3). There was no significant difference in SDW of linseed grown in Dunn-1996 soil cropped with vetch or orig-96 soil. SDW of linseed was also significantly greater when linseed was grown in Dunn-1998 soil cropped with vetch or subterranean clover when compared to growth in orig-98 soil. However, SDW of linseed grown in Dunn-1998 soil cropped with linseed was not significantly different to that of linseed grown in orig-98 soil.

Concentration of P was significantly greater in shoots of linseed grown in Dunn-1996 soil cropped with vetch, linseed or subterranean clover compared with linseed grown in orig-96 soil (Figure 3.5.4). Concentration of P was also significantly greater in shoots of linseed grown in Dunn-1998 soil cropped with vetch or subterranean clover compared with linseed grown in orig-98 soil. Although there was no significant difference in concentration of P in shoots of linseed grown in Dunn-1998 soil cropped with linseed compared with orig-98 soil, there was a trend toward greater concentration of P in shoots of linseed grown in Dunn-1998 soil cropped with linseed.

Figure 3.5.2 – VAM fungal colonisation of linseed grown in Dunn-1996 or Dunn-1998 soil that had been cropped with vetch, linseed or subterranean clover after 21 days. Bars represent the standard error of the mean of 5 replicates. Columns with different letters are statistically different at the 5% level (Chapter 2.2.11). Dunn-1996 and Dunn-1998 were statistically analysed separately and statistical analysis followed arcsin transformation of the colonisation data (Chapter 2.2.11).

Figure 3.5.3 – Shoot dry weight of linseed grown in Dunn-1996 or Dunn-1998 soil that had been cropped with vetch, linseed or subclover after 21 days. For explanation of error bars and column labelling refer to Figure 3.5.2. Dunn-1996 and Dunn-1998 soil were statistically analysed separately.

Figure 3.5.2

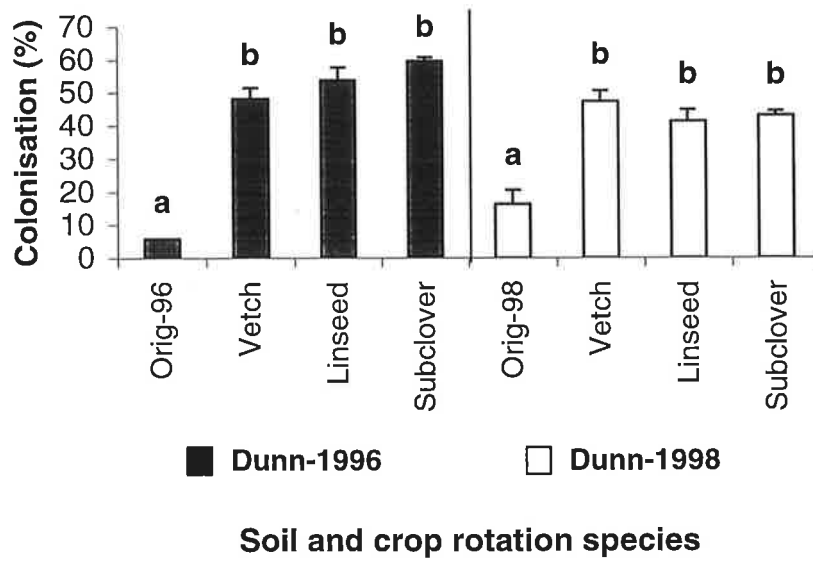


Figure 3.5.3

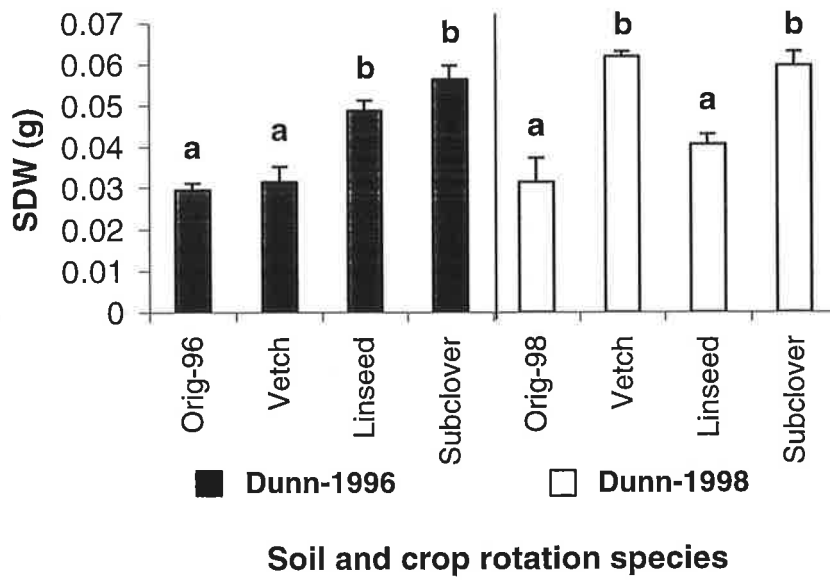


Figure 3.5.4 – Concentration of P in shoots of linseed grown in Dunn-1996 or Dunn-1998 soil that had been cropped with vetch, linseed or subclover after 21 days. For explanation of error bars and column labelling refer to Figure 3.5.2. Dunn-1996 and Dunn-1998 soil were statistically analysed separately.

Figure 3.5.5 – Concentration of Zn in shoots of linseed grown in Dunn-1996 or Dunn-1998 soil that had been cropped with vetch, linseed or subclover after 21 days. For explanation of error bars and column labelling refer to Figure 3.5.2. Dunn-1996 and Dunn-1998 soil were statistically analysed separately.

Figure 3.5.4

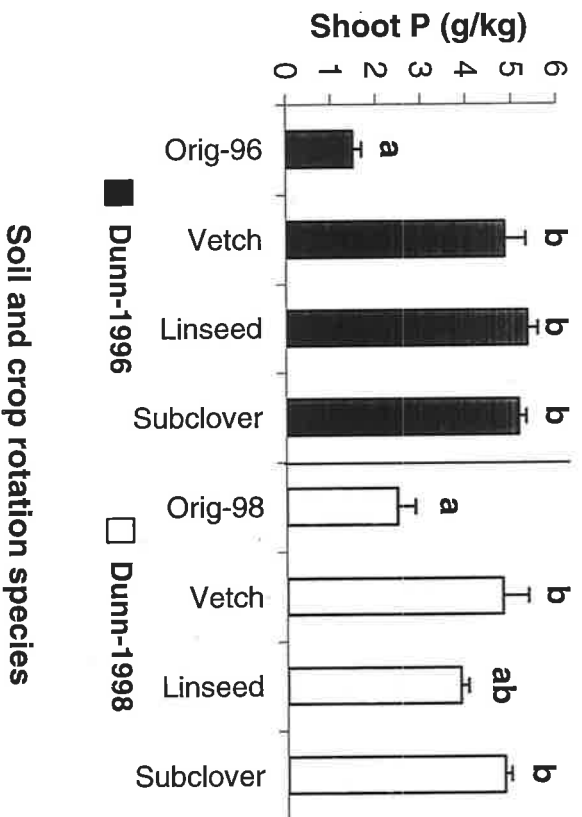


Figure 3.5.5

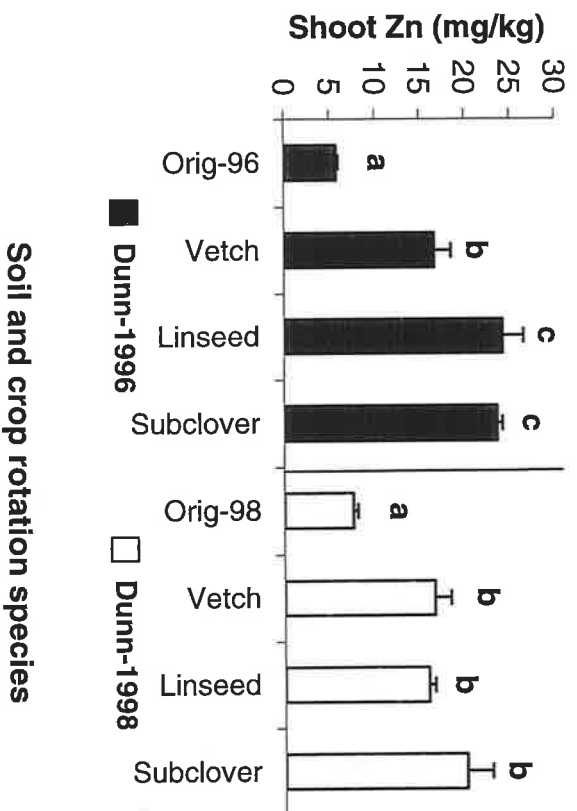


Figure 3.5.6 – Total P content of shoots of linseed grown in Dunn-1996 or Dunn-1998 soil that had been cropped with vetch, linseed or subclover after 21 days. For explanation of error bars and column labelling refer to Figure 3.5.2. Dunn-1996 and Dunn-1998 soil were statistically analysed separately.

Figure 3.5.7 – Total Zn content of shoots of linseed grown in Dunn-1996 or Dunn-1998 soil that had been cropped with vetch, linseed or subclover after 21 days. For explanation of error bars and column labelling refer to Figure 3.5.2. Dunn-1996 and Dunn-1998 soil were statistically analysed separately.

field soil, and would be likely to have different nutritional balances and ratios as well as different soil physical and biological characteristics that may have affected plant growth and nutrition independent from any VAM fungal effect. For example, in the original Dunn-1996 soil, N may have been limiting (Chapter 3.1). Addition of autoclaved soil and sand to cropped Dunn-1996 soil may have alleviated this nutrient deficiency possibly resulting in the better growth of linseed in these soils. Moreover, it is possible that by alleviating a nutrient deficiency with the addition of autoclaved soil and sand, the positive effects of the indigenous VAM fungi on linseed growth and nutrition became more apparent.

Another possible reason for the positive effects of VAM fungi on linseed growth and nutrition observed in this experiment but not the Screening Experiment (Chapter 3.1) involves the relationship between VAM fungi, plant growth and nutrition, and soil P status. As discussed in 1.2.1.3, plant growth depressions induced by VAM fungi may occur under high P conditions. This was thought to be a possible reason for the poor growth of linseed in the Screening Experiment (Chapter 3.1). After cropping the Dunn soil with linseed, subterranean clover or vetch, and then mixing these soils with autoclaved Dunn soil and sand, the concentration of phosphorus in the resultant soil mix ($30\text{--}75 \mu\text{g P g}^{-1}$ soil) was much lower than the original Dunn-1996 soil used in the Screening Experiment ($105 \mu\text{g P g}^{-1}$ soil). Despite adding some nutrients in the form of autoclaved Dunn soil and sand mix, the cropped soil used in the infectivity assessment was less fertile than the original Dunn-1996 soil. Therefore, the concentration of P in the cropped soil, which is known to influence VAM fungal colonisation as well as the possibility of VAM fungal induced plant growth depressions, may be in the range that results in benefit to the plant from the VAM association. This may explain the growth responses and better P and Zn nutrition of linseed in the infectivity assessment of the cropped soils compared to linseed grown in original Dunn-1996 soil (Screening Experiment).

To conclude, this host plant experiment has shown that the indigenous VAM fungi present in Dunn soil are potentially beneficial to plant growth and nutrition. Furthermore, growing subterranean clover, linseed or vetch in Dunn soil increases the VAM fungal infectivity of Dunn soil as shown by higher colonisation of linseed in the infectivity assessment. This greater colonisation of linseed by VAM fungi was related to greater SDW of the linseed plants and higher shoot P and Zn concentrations of these plants. However, it is important to note that the fertility of the cropped soil differed from that of original Dunn-1996 or Dunn-1998 soils, and this is likely to have a large influence on the growth and nutrition of the plants, and the contributions made to plant growth and nutrition by VAM fungi. Of particular interest is the effect of soil P on the indigenous VAM fungi and growth and nutrition of linseed in Dunn soil. This was examined further in Chapter 3.6.

Chapter 3.6 – Phosphorus tolerance of indigenous VAM fungi

3.6.1 Introduction

It was initially thought that the poor growth of linseed in Dunn soil observed in the Screening Experiment (Chapter 3.1) might have been a manifestation of a growth depression induced by the indigenous VAM fungi present in Dunn soil, especially given the high concentration of P in this soil. However, results from the Elimination Experiment (Chapter 3.4) and Crop Rotation Experiment (Chapter 3.5), indicate that the indigenous VAM fungi present in Dunn soil can be beneficial to linseed growth. The soil used in the Elimination and Crop Rotation Experiments differed from the original Dunn soil either because the soil used was from a different collection or had been cropped. As a result, these soils had different readily-extractable soil P concentrations from the soil used in the Screening Experiment, i.e. $75 \mu\text{g P g}^{-1}$ soil (Elimination Experiment) and $30\text{-}70 \mu\text{g P g}^{-1}$ soil (Crop Rotation Experiment) compared with $105 \mu\text{g P g}^{-1}$ soil (Screening Experiment). These different soil P concentrations may have been a major causative factor in the different growth responses of linseed to VAM fungal colonisation observed in these experiments. In the Crop Rotation Experiment (low to moderately high readily-extractable soil P concentration) VAM fungal colonisation was beneficial for linseed growth whereas in the Elimination Experiment (moderately high readily-extractable soil P concentration) growth of linseed appeared to benefit only slightly from VAM fungal colonisation. Finally, in the Screening Experiment (high readily-extractable soil P concentration) growth of linseed may have been inhibited by a growth depression caused by high VAM fungal colonisation although, as previously stated, it was not possible to confirm this, as there were no non-mycorrhizal controls in this experiment. Positive responses of *Linum* sp. to soil P and VAM fungal colonisation have

been reported by Thingstrup *et al.* (1998). They showed that indigenous VAM fungi are responsible for the maintenance of yield of flax when grown at intermediate soil P concentrations (ca. 40 $\mu\text{g P g}^{-1}$) but not moderately high soil P concentrations (82 $\mu\text{g P g}^{-1}$). This was despite extensive mycorrhiza formation at the higher P concentration. As previously discussed, care should be taken when discussing results obtained by Thingstrup *et al.* (1998) as they determined soil P concentrations using the Olsen technique whereas in this project the Colwell method was adopted. This experiment was therefore conducted to determine whether the concentration of readily-extractable soil P was the causative factor underlying the varying growth responses of linseed to colonisation by indigenous VAM fungi observed in the Screening, Elimination and Crop Rotation Experiments.

3.6.2 Materials and Methods

Soil collected from the Dunn site in 1998 (Dunn 1998) was used in this experiment. It was air dried, crushed but not sieved prior to use. The concentration of readily-extractable P in this soil was 74 $\mu\text{g P g}^{-1}$ soil. To obtain soil with a lower concentration of readily-extractable P, this soil was diluted with autoclaved sand in a ratio of 1 part soil to 4 parts sand. The concentration of readily-extractable P in the resultant mix was 19 $\mu\text{g P g}^{-1}$ soil. It will be referred to as Dunn-20. It must be stressed that this soil differs greatly to the Dunn-1998 soil used in the Infectivity Assessments (Chapter 3.3), Elimination Experiment (Chapter 3.4) and Crop Rotation Experiment (Chapter 3.5) due to the addition of autoclaved sand. As such, comparisons of the results from these experiments with results for this experiment are invalid.

A P addition curve for Dunn-20 soil (no plants) was constructed to determine how much P to add to the Dunn-20 soil to obtain target soil P concentrations. Different volumes of a $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ solution containing 2 mg P/ml was added to 200 g of Dunn-20 soil and the soil mixed thoroughly. These soils were allowed to undergo several wetting and drying cycles by watering the pots to weight (0.157 g/g or 75% field capacity) three times weekly. After several cycles, a core from each pot was taken, the soil dried and readily-extractable soil P concentration determined using the autoanalyser method (Chapter 2.2.1). A P addition curve for Dunn-20 soil (see Figure 3.6.1) was constructed to give the following equation

$$\begin{aligned} \text{Actual soil P concentration} &= 1.0091(\text{Amount of P added}) + 20.849 \\ (\mu\text{g P/g soil}) & \quad (\mu\text{g P/g soil}) \end{aligned}$$

This equation was used to determine how much $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ to add to 500 g of Dunn-20 to give a target soil P concentration.

For example, for a target actual soil P concentration of 50 $\mu\text{g P/g soil}$

$$50 = 1.0091(\text{Amount of P added}) + 20.849$$

($\mu\text{g P/g soil}$)

$$\text{Amount of P to add} = 28.9 \mu\text{g P/g soil}$$

Dunn-20 soil was adjusted in this manner to give five soil P concentrations from very low to greater than the high values encountered in the field. These were:

P0 (low) – Dunn-20 soil with no addition of P

P50 (medium) – Dunn-20 soil adjusted to 50 $\mu\text{g P/g soil}$.

P100 (high) – Dunn-20 soil adjusted to 100 $\mu\text{g P/g soil}$.

P150 (v. high) – Dunn-20 soil adjusted to 150 $\mu\text{g P/g soil}$.

P250 (extreme) – Dunn-20 soil adjusted to 250 $\mu\text{g P/g soil}$.

Samples (500 g) of Dunn-20 soil were placed into pots 24 cm long with a diameter of 4.5 cm. The required amount of P was added and the soil mixed thoroughly. Each pot was then watered to weight (0.157 g/g or 75% field capacity) and left for several days to allow the P concentration of the soil to equilibrate. Five replicate pots at each soil P concentration were included.

Linseed seeds were surface sterilised and germinated (Chapter 2.2.4). Germinated seeds (5) were sown in each pot to a depth of 1 cm and thinned to 3 plants per pot following emergence. The plants were grown in a growth room, watered to weight (0.157 g H₂O/g O.D. soil) with R.O. water thrice weekly and given 5 ml of modified Long Ashton nutrient solution (-P, +N, +Zn) once weekly (Chapter 2.2.5). Plants were harvested after 21 and 42 days (Chapter 2.2.6). Shoots were removed and dried for dry weight measurements and nutrient determination. Plant roots were rinsed with R.O. water, cleared in 10% KOH and stained with aniline blue (Chapter 2.2.7). Colonisation of the root system by VAM fungi was determined using the grid-intersect technique (Chapter 2.2.8). Dried plant shoots were analysed for nutrient status (other than N) by nitric acid digestion of this plant material followed by ICP-AES (Chapter 2.2.9) or by Kjeldahl test for total N (Chapter 2.2.10).

3.6.3 Results

After 21 and 42 days, there was little or no difference in the SDW of linseed grown in soil with various P concentrations (Figure 3.6.2). After 21 days, linseed grown in P0, P50 or P100 soil had significantly greater root colonisation by VAM fungi than linseed grown in P150 or P250 soil (Figure 3.6.3). This result was also seen after 42 days, except that linseed grown in P0 and P50 soil had significantly greater VAM fungal colonisation than linseed grown in P100 soil and these

Figure 3.6.1 – P addition curve for Dunn-20 soil. Bars represent the standard error of the mean of 5 replicates.

Figure 3.6.2 – Shoot dry weight of linseed grown in Dunn-20 soil with different P concentrations after 21 and 42 days. Bars represent standard error of the mean of 5 replicates. Columns with different letters are statistically different at the 5% level (Chapter 2.2.11). Data for each harvest time were statistically analysed separately.

Figure 3.6.1

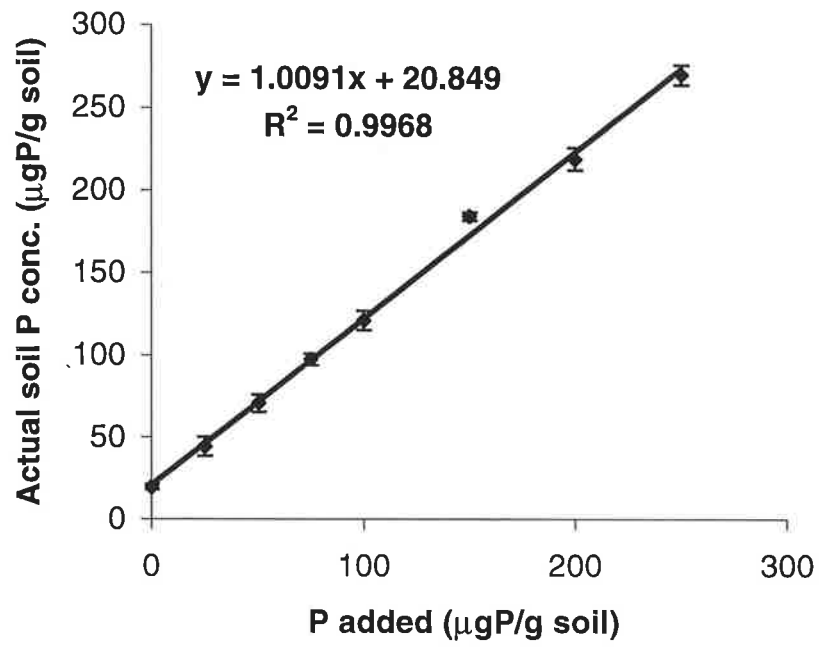
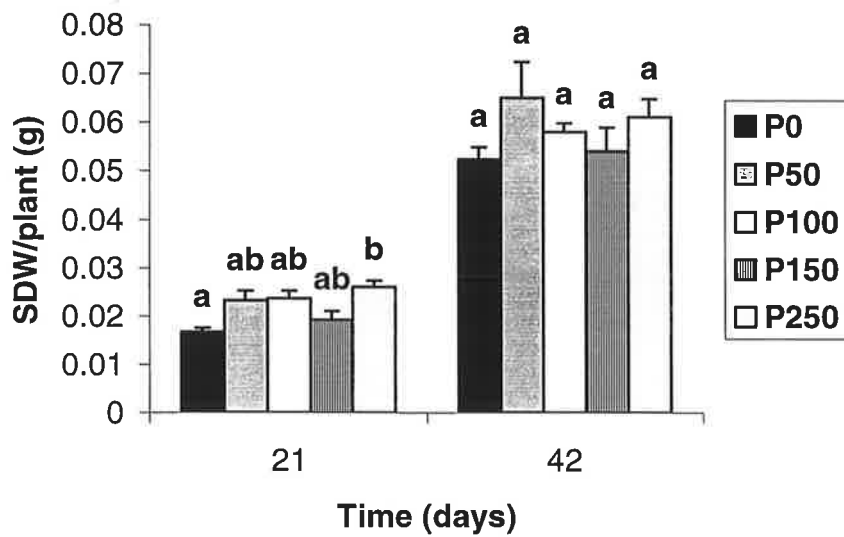


Figure 3.6.2



plants in turn had significantly greater VAM fungal colonisation than linseed grown in P150 and P250 soil.

The concentration of P in shoots of linseed grown in the various soils increased as soil P concentration increased (Figure 3.6.4) after 21 and 42 days. After 21 days, only linseed grown in P0 soil had shoot P concentrations less than the approximate critical deficiency limit. After 42 days, linseed grown in all P treatments had shoot P concentrations greater than the approximate critical deficiency limit. There were no signs of P toxicity in the linseed plants despite the high shoot P concentrations.

A similar pattern to shoot P concentration was observed for the concentration of Na in shoots of linseed, which increased as the concentration of soil P increased (Figure 3.6.5) after 21 and 42 days.

After 21 days, there were no significant differences in the concentrations of Zn in shoots of linseed grown in the various soils (Figure 3.6.6). After 42 days there was significantly less Zn in shoots of linseed grown in P250 soil than the other soils. Furthermore, after 21 days, concentration of Zn in shoots of linseed grown in any of the soils did not exceed the approximate critical deficiency limit (10 mg/kg). Unfortunately, there is no data on the approximate critical deficiency limit after 42 days growth.

After 21 days, the concentration of N in shoot of linseed grown in the various soil P treatments increased with increasing soil P concentration although this effect was not significant at the 5% level (Figure 3.6.7). After 42 days there was no difference in the concentration of N in shoots of linseed grown in the various soil P treatments. Furthermore, the concentration of N in shoots of

Figure 3.6.3 – VAM fungal colonisation of linseed grown in Dunn-20 soil with different P concentrations after 21 and 42 days. For explanation of error bars and column labelling refer to Figure 3.6.2. Data for each harvest time were statistically analysed separately.

Figure 3.6.4 – Concentration of P in shoots of linseed grown in Dunn-20 soil with different P concentrations after 21 and 42 days. For explanation of error bars and column labelling refer to Figure 3.6.2. Horizontal line represents approximate critical deficiency limit (Reuter and Robinson, 1997). Data for each harvest time were statistically analysed separately.

Figure 3.6.3

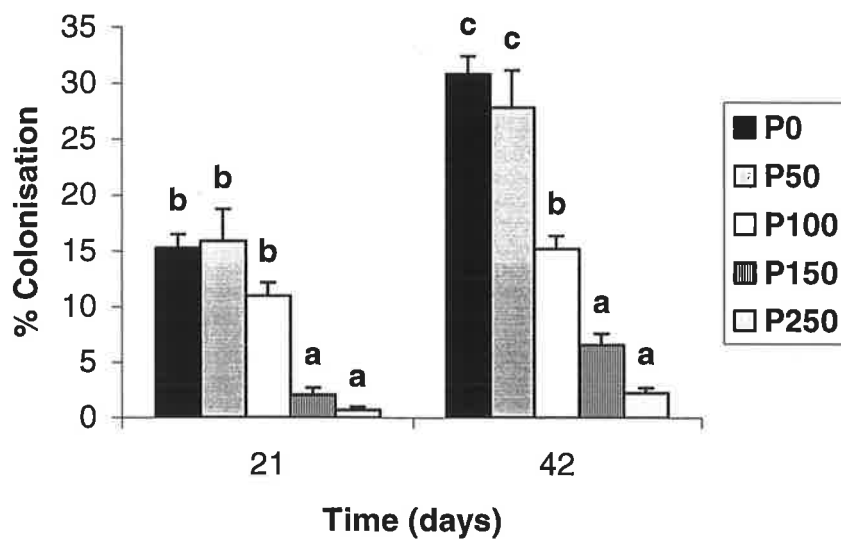


Figure 3.6.4

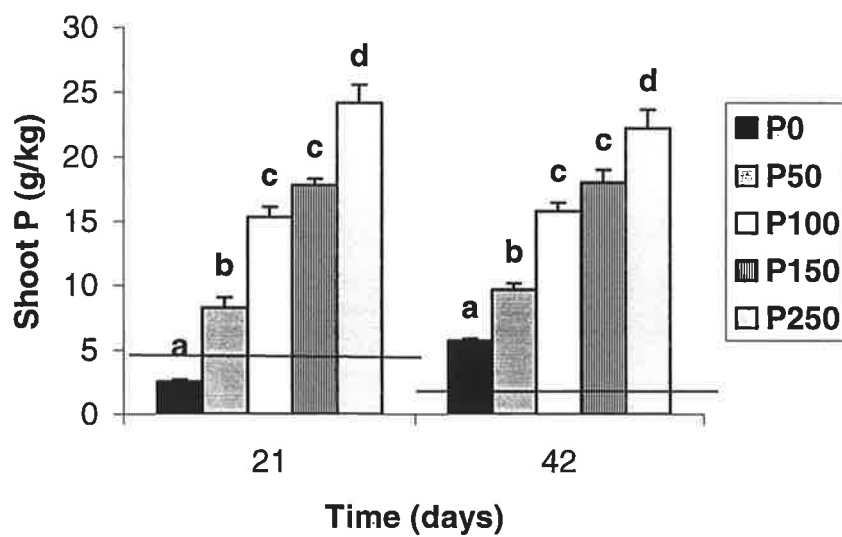


Figure 3.6.5 – Concentration of Na in shoots of linseed grown in Dunn-20 soil with different P concentrations after 21 and 42 days. For explanation of error bars and column labelling refer to Figure 3.6.2. Data for each harvest time were statistically analysed separately.

Figure 3.6.6 – Concentration of Zn in shoots of linseed grown in Dunn-20 soil with different P concentrations after 21 and 42 days. For explanation of error bars and column labelling refer to Figure 3.6.2. Horizontal line represents approximate critical deficiency limit (Reuter and Robinson, 1997). Data for each harvest time were statistically analysed separately.

Figure 3.6.5

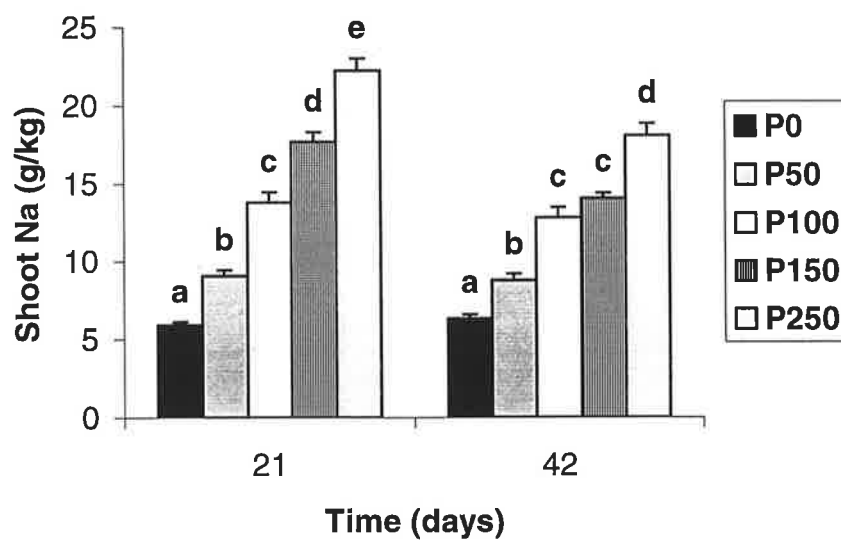


Figure 3.6.6

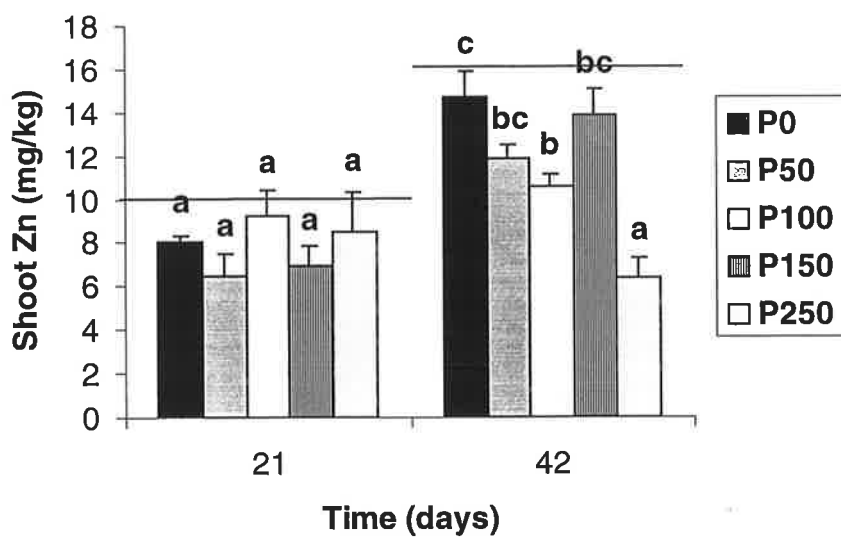


Figure 3.6.7 - Concentration of N in shoots of linseed grown in Dunn-20 soil with different P concentrations after 21 and 42 days. For explanation of error bars and column labelling refer to Figure 3.6.2. Horizontal line represents approximate critical deficiency limit (Reuter and Robinson, 1997). Data for each harvest time were statistically analysed separately.

Figure 3.6.8 – N:P ratio in shoots of linseed grown in Dunn-20 soil with different P concentrations after 21 and 42 days. For explanation of error bars and column labelling refer to Figure 3.6.2. Data for each harvest time were statistically analysed separately.

Figure 3.6.7

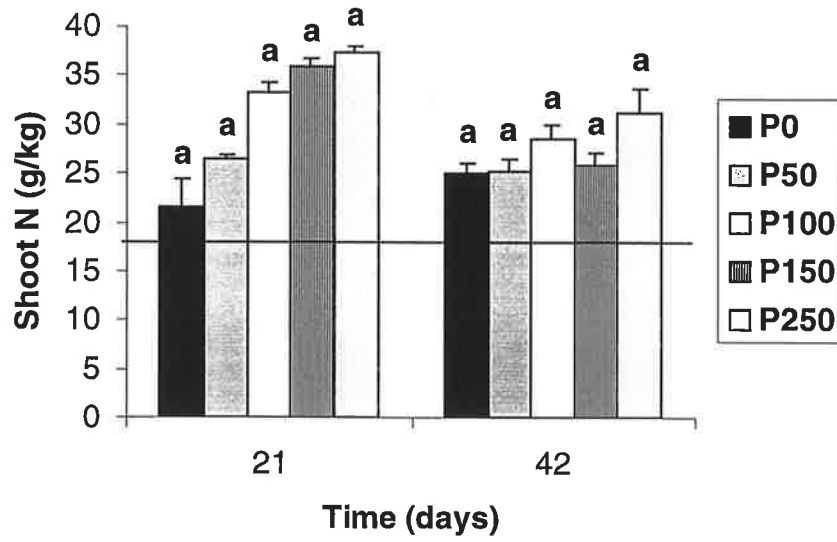
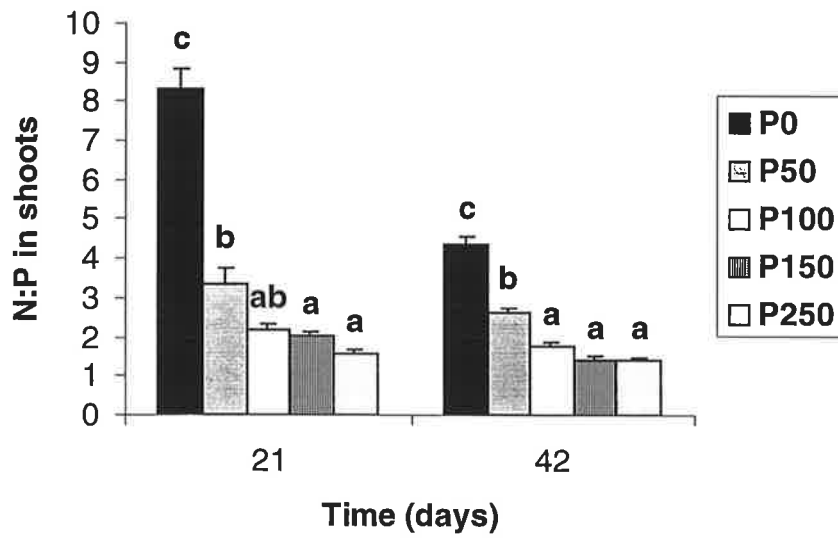


Figure 3.6.8



linseed grown in the various soil P treatments at either the 21 or 42-day harvest always exceeded the approximate critical deficiency limit.

After 21 days, the N:P ratio in the shoots of linseed was significantly higher in plants grown in P0 soil (Figure 3.6.8). The ratio then decreased with increasing soil P concentration. A similar trend was observed for 42 days.

3.6.4 Discussion

Increasing the concentration of readily-extractable P in Dunn-20 soil resulted in a decrease in VAM fungal colonisation after 21 days when the concentration exceeded $100 \mu\text{g P g}^{-1}$ soil., whilst after 42 days, $50 \mu\text{g P g}^{-1}$ soil significantly reduced colonisation (Figure 3.6.3). This adverse effect of increasing readily-extractable P concentration in soil on VAM fungal colonisation has been previously reported by many researchers with other species (see Chapter 1.2.1.2). However, there were only small differences in growth of linseed in the various soils after 21 days, whilst no significant differences in SDW were observed after 42 days (Figure 3.6.2). The lack of a difference in SDW with the addition of P might be attributed to the positive effect of VAM fungi on plant nutrition and growth in the P0, P50 and to a lesser extent the P100 soils. This positive effect may result in plant growth and nutrition similar to that of non-mycorrhizal plants grown in soil with a higher concentration of P (Smith *et al.*, 1986). In this case, however, there appeared to have been no effects of VAM fungi on the growth and shoot P concentration of linseed grown with the P0, P50 and P100 soil treatments (Figure 3.6.4). This indicates that growth of linseed in the various soil treatments was restricted by factor(s) other than P and VAM fungal colonisation.

Irrespective of VAM fungal colonisation, the difference in shoot P concentration of linseed grown with the various soil P treatments was not reflected in a difference in SDW. One theory regarding the lack of a growth response to P fertilisation involves the availability of N to the plants. N:P ratios in plant shoots may indicate whether either N or P is limiting plant growth. Under conditions of high P supply and low N supply, a low N:P ratio would be expected (Koerselman and Mueleman, 1996). N:P ratios for linseed plants grown for 42 days in the Elimination Experiment (Chapter 3.4), which did not appear to be suffering any nutrient deficiency, ranged from 8-10. Similarly, the N:P ratio of linseed shoots calculated using data from Hocking *et al.* (1987) for N and P concentrations adequate for linseed growth after 63 days was approximately 9. This suggests, therefore, that for unlimited growth of linseed, the N:P ratio in the shoots should be 8-10. If the N:P ratio is less than 8, N may be limiting growth, whereas if the N:P ratio is higher than 10, P may be limiting. As shown in Figure 3.6.8, linseed grown for 42 days in P0 soil had a N:P ratio of 4.4. The ratio then decreased as P concentration increased. In all treatments, the N:P ratio was lower than 8, which suggests that the growth of linseed in all treatments was limited by N availability. This is despite the weekly addition of 0.84 mg N per pot in the form of modified Long Ashton nutrient solution, although it is possible that this was insufficient for plant requirements. However, N:P ratios can be misleading under conditions where neither N nor P are limiting. For example, if the availability of N is sufficient for plant growth but P is high, then there may be luxury uptake of P particularly if another nutrient is limiting (Menge 1983). This would result in a low N:P ratio in the plant even though N was sufficient. This may have occurred in this experiment, given that the concentration of N in shoots of linseed exceeded the approximate critical deficiency limit. N deficiency, therefore, may not be the main cause of the poor growth of linseed.

Another possible reason for the poor growth of linseed, which arises from the data, is Zn deficiency. As shown in Figure 3.6.6, concentration of Zn in shoots of linseed is low, which may indicate that Zn availability is limiting growth even though 25 ng Zn was added to each pot on a weekly basis in the form of modified Long Ashton nutrient solution. However, it is unlikely that Zn deficiency is the main cause of the poor growth. If it were, SDW would be expected to fall as the uptake and concentration decreased with additions of P as a result of a P-induced Zn deficiency (Marschner, 1995). That SDW was not affected by additions of P, and there appeared to be no correlation between SDW and shoot Zn concentration, suggests that Zn availability was not limiting growth.

The increase in concentration of Na in shoots of linseed as soil P concentration increased is most likely to have been due to the use of $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ to increase the concentration of P in the soil. As the concentration of P in the soil increased so would the concentration of Na. Assuming that this would result in greater uptake of Na by the linseed plants, this would explain the increase in Na concentration in linseed shoots as soil P increased.

In conclusion, this P response experiment has shown that indigenous VAM fungi are able to colonise linseed grown in Dunn-20 soil with a P concentration up to 50 mg P kg^{-1} soil. Above this concentration, colonisation was heavily reduced. However, any differences in colonisation were not reflected in SDW nor shoot P or Zn concentrations. Finally, factors responsible for the poor growth of linseed in all the soils are unclear, although it is likely to be related to nutrition. To determine which factors were responsible, separate elements could be added to the soil to identify which element was limiting growth. In the meantime, the aims of this experiment could be addressed in another experiment examining the response of indigenous VAM fungi present in

Dunn soil to varying soil P concentrations ensuring all elements other than P were not limiting by adding a basal nutrient dressing containing sufficient quantities of all elements.

Chapter 3.7 – General discussion of experiments involving Clare field soils

One of the difficulties encountered when interpreting results obtained from experiments involving Clare field soils was the need to use soils collected at different times. Although allowances were made for this, the differences between the soils complicated experimental design and interpretation of the results. In retrospect, a single large soil collection at the start of the experiments would have overcome these problems. Nevertheless, it is possible to come to some clear conclusions about the roles of the indigenous VAM fungi in linseed growth and nutrition, especially in Dunn soil.

Observations at the Dunn site showed that linseed grown after canola grew poorly and appeared to be suffering from Zn deficiency, although this was not confirmed in field crops. Canola is a non-mycorrhizal crop and would therefore be expected to have a negative impact on VAM fungal propagule density and subsequent colonisation of mycorrhizal crop plants (Black and Tinker, 1979; Fontenla *et al.*, 1999). This posed the following question; could poor growth and apparent Zn deficiency of linseed at the Dunn site be related to poor VAM fungal colonisation following canola? For comparison, a second site (Merildin) that had been recently cropped with canola was also selected. Screening of the Clare field soils (Chapter 3.1) and infectivity assessments (Chapter 3.3) showed that colonisation of linseed by indigenous VAM fungi when grown in Dunn soil was high (36.0% and 31.4% for Dunn soil collected in 1996 and 1998 respectively) whereas colonisation of linseed grown in Merildin soil was low. Furthermore, in pot experiments the linseed plants grown in Dunn or Merildin soil did not suffer from Zn deficiency. Determination of the Most Probable Number of VAM fungal propagules in the two soils (Chapter 3.2) indicated that Dunn soil contained 58.4 ± 36.3 VAM fungal propagules whereas

Merildin soil contained 6.1 ± 3.3 propagules. Therefore, the difference in colonisation of linseed grown in Dunn and Merildin soils was at least partly due to a difference in the number of VAM fungal propagules between these soils.

Despite the high VAM fungal colonisation of linseed grown in Dunn soil, growth of these plants was poor. Therefore, if the poor growth of linseed in Dunn soil was not related to low VAM fungal colonisation or Zn deficiency, what was it related to? The first possibility was that it was a result of a growth depression induced by very high colonisation of the plant by indigenous VAM fungi. In order to test this, it was necessary to eliminate the indigenous VAM fungi from Dunn soil so that their role in the growth and nutrition of linseed grown in this soil could be evaluated (Chapter 3.4). This was conducted by autoclaving the soil, which in itself posed problems especially in regard to the design of appropriate control treatments given the effect of autoclaving on soil properties such as nutrient concentrations and soil organisms (Smith and Smith, 1981a). Results for this experiment revealed that linseed responded positively to high colonisation by indigenous VAM fungi when grown in autoclaved Dunn soil that was reinoculated with untreated Dunn soil reflected in greater SDW and shoot P and Zn concentrations. This suggests that the indigenous VAM fungi present in Dunn soil were potentially beneficial to linseed growth and nutrition. However, colonisation in the reinoculated treatments after 21 days was 5.7% and 5.6% for AR and ARF soil respectively, compared with 18.3% in untreated soil. Therefore, it remains possible that the high propagule density and rapid colonisation of linseed in untreated Dunn soil may induce a plant growth depression. The experimental methods used could not address this possibility. It is unclear what methods could be used to address this possibility, without causing changes to factors other than the VAM fungal component making unequivocal interpretation of the results extremely difficult.

Another possibility for the poor growth of linseed in Dunn soil that was not considered in the design of the project, but which appeared during the project was that the poor growth was due to limiting soil N. To confirm this, a further experiment should have been conducted where N was added to Dunn-1996 soil. Unfortunately, there was insufficient Dunn-1996 soil remaining to permit such an experiment.

As it appears that the indigenous VAM fungi present in Dunn soil are potentially beneficial for plant growth, the next question was: can management strategies be used to increase this potential benefit? Firstly, the use of crop rotations was assessed (Chapter 3.5). Cropping soil with mycorrhizal plants has been reported to increase the density of VAM fungal propagules in a soil which in turn results in greater VAM fungal colonisation and improved growth and nutrition of subsequent mycorrhizal crop plants (Arihara and Karasawa, 2000; Boswell *et al.*, 1998; Dodd *et al.*, 1990b; Harinikumar and Bagyaraj, 1988; Thompson, 1987). Cropping of Dunn-1998 soil with the mycorrhizal host plants subterranean clover, vetch or linseed, increased VAM fungal inoculum potential of the soil as shown by higher colonisation of linseed compared to uncropped soil. This higher colonisation resulted in greater SDW and higher shoot P and Zn concentrations of these plants. Secondly, the effect of soil P concentration on indigenous VAM fungal colonisation, growth and nutrition of linseed was assessed. VAM fungal functioning is strongly influenced by soil P concentration (Hayman, 1987). Given that the soils used in the screening (Chapter 3.1), elimination (Chapter 3.4), and cropping (Chapter 3.5) experiments had different P concentrations, it is possible that the different growth responses of linseed to indigenous VAM fungal colonisation in these experiments were related to these different concentrations of soil P. Furthermore, it was unclear what concentration of soil P was best for maximum benefit from the indigenous VAM fungi present in Dunn soil. Therefore, an experiment examining the effect of various soil P concentrations on the indigenous VAM fungal colonisation, growth and nutrition

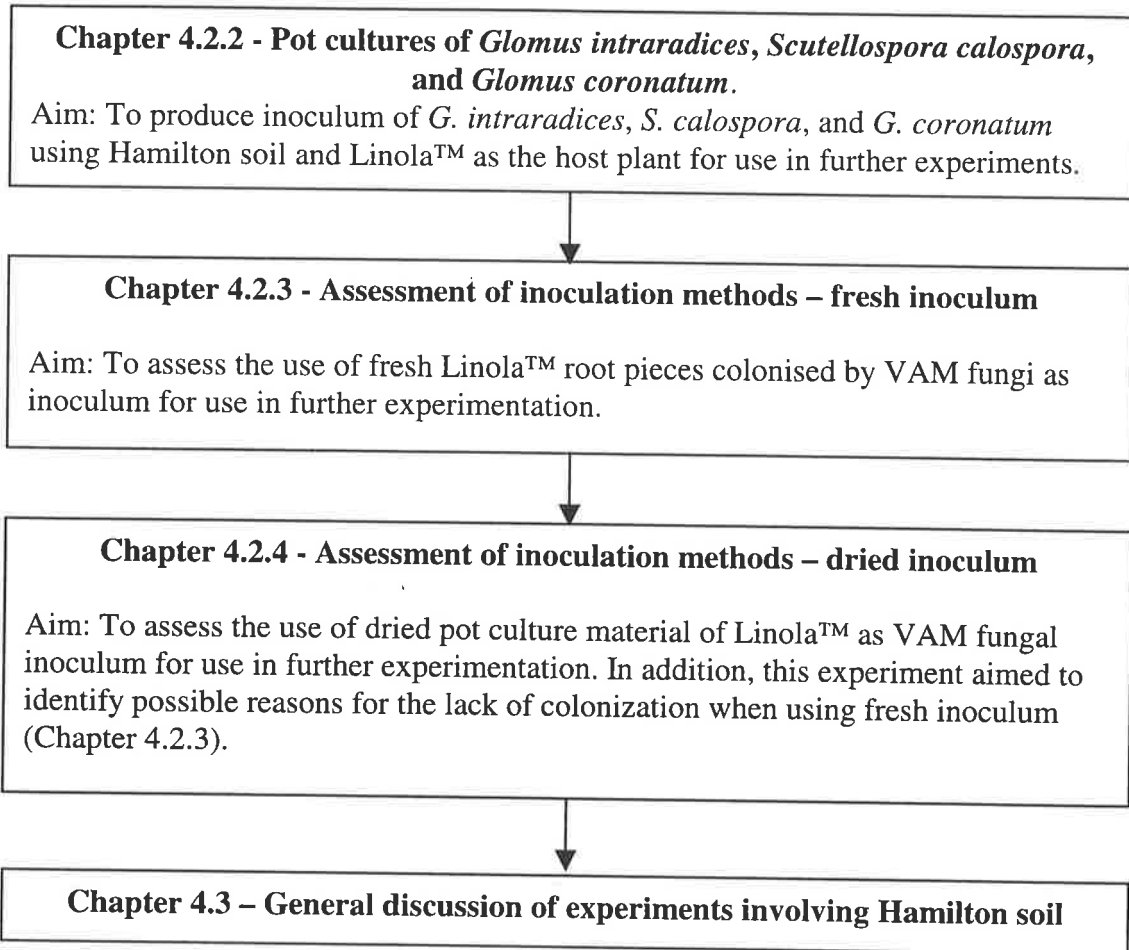
of linseed was conducted (Chapter 3.6). However, plant growth was limited by an as yet unidentified factor probably of nutritional nature and no responses to VAM fungal colonisation were observed. The issue would be resolved by performing another P response experiment ensuring all nutrients other than P were not limiting. This would permit evaluation of the growth responses of linseed to indigenous VAM fungal colonisation at varying soil P concentrations.

In summary, it is evident from these experiments that the poor growth of linseed in Dunn soil observed in the field and Screening Experiment was not due to a growth depression induced by indigenous VAM fungi, a decline in indigenous VAM fungal populations, or Zn deficiency. Instead, it is probable that the poor growth of linseed was caused by N deficiency, however there was insufficient Dunn 1996 soil to confirm this. Further experimentation revealed that the indigenous VAM fungi present in Dunn soil were potentially beneficial to linseed growth and nutrition at moderate soil P concentrations (30-70 $\mu\text{g P g}^{-1}$ soil) and cropping Dunn soil with mycorrhizal host plants enhanced the VAM fungal infectivity of the soil, which in turn resulted in better growth and nutrition of linseed. These results therefore suggest that to gain the potential beneficial effects from the indigenous VAM fungi present in Dunn soil, management strategies that promote these fungi should be implemented at the site. These might include the use of mycorrhizal crops in rotations to maintain VAM propagule density at a sufficient level and controlled fertiliser inputs to maintain a soil P concentration at which the indigenous VAM fungi present in Dunn soil have shown the most potential benefit, i.e. 30-75 $\mu\text{g P g}^{-1}$ soil. However, prior to implementing such strategies, it would be necessary to conduct trials in the field to optimise crop rotations and fertiliser inputs. To realise the potential benefits of the indigenous VAM fungi present in Dunn soil, it is also important to ensure that non-mycorrhizal factors (e.g. nutrition) are not limiting plant growth.

Chapter 4.1 – General introduction to experiments involving Hamilton soil

When this project commenced, its main objective was to examine the role of VAM fungi in the Zn nutrition of *Linum usitatissimum*. Unlike the role of VAM fungi in plant P nutrition, which has received extensive attention, the influence of VAM fungi on plant Zn nutrition has been little researched. To examine the role of VAM fungi in Zn nutrition, a soil with low concentrations of P and Zn was required that could be manipulated to enable identification of target concentrations of P and Zn that would support VAM fungal colonisation and at the same time promote Zn deficiency of *L. usitatissimum*. P and Zn interact in soil so that high P concentrations can cause Zn deficiency in plants (often referred to in the literature as “P-induced Zn deficiency”) (Marschner, 1995). Therefore, to induce Zn deficiency in *L. usitatissimum*, the concentration of P in the soil needs to be high. However, as discussed in Chapter 1.2.1.2, high soil P concentrations can lead to a reduction in VAM fungal colonisation. Therefore, various soils were screened to select one that could be manipulated to favour both VAM fungal colonisation and Zn deficiency of *L. usitatissimum*.

Hamilton soil from Pinnaroo, South Australia, was selected as it had apparently suitable P and Zn concentrations (see Chapter 4.2.2.1). Linola™ (*L. usitatissimum* cv. “Eyre”) a modified form of linseed with improved oil properties was chosen as it has more agricultural potential than linseed. Pot cultures (Chapter 4.2.2) and experiments using various VAM fungal species, soils and host plants (Chapter 4.2.3 and 4.2.4) were conducted in this soil. The following flow diagram is included to make these experiments easier to follow.



In each of these experiments, VAM fungal colonisation of the host plant was delayed and/or poor. Because of this unexpected result, it was not possible to investigate the role of VAM fungi in the Zn nutrition of Linola™.

Chapter 4.2 – Inoculum production and assessment of inoculation methods

4.2.1 Introduction

Prior to commencing experiments examining the role of VAM fungi in the Zn nutrition of linseed, sufficient inoculum of target VAM fungal species needed to be produced using pot culture methods. Pilot experiments were also required to investigate possible strategies for inoculation of soils with VAM fungi. This chapter addresses these aims and should be considered in the context of the original main aim, which was to examine the role of VAM fungi in the Zn nutrition of *Linum usitatissimum*.

Zn is a trace element required in low concentrations by plants compared with macronutrients such as N, P and K. Zn deficiency of plants may only be subclinical and any introduction of Zn by way of contaminants could be enough to overcome such a deficiency. Therefore, it was necessary to keep the introduction of Zn to the experimental system to a minimum. One potential source of Zn is the VAM fungal inoculum particularly when using dried inoculum consisting of soil, spores, colonised plant roots and VAM fungal hyphae, which is normally produced in pot cultures using fertilisers (containing Zn). To combat this potential source of Zn (and also P), pot cultures of VAM fungi were prepared using Hamilton soil, which had low P and Zn concentrations.

Following the production of pot cultures of VAM fungi, the method of inoculation of soil with VAM fungi was assessed. Inoculation with a band of fresh inoculum was tested in the first of these experiments (Chapter 4.2.3). Fresh inoculum consists of fresh roots colonised by VAM

fungi, so that very little soil is introduced into the experimental pots with inoculation, reducing the introduction of Zn and P. In the case that banding of fresh inoculum was unsuccessful, dried soil inoculum containing soil, roots, VAM colonised roots, VAM fungal hyphae and possibly spores (not assessed) mixed throughout the soil was also tested (Chapter 4.2.4). Results of both trials were assessed in terms of VAM fungal colonisation of plant roots. The trial of dried soil inoculum was also assessed in terms of plant growth.

4.2.2 Pot cultures of *Glomus intraradices*, *Scutellospora calospora*, and *Glomus coronatum*

4.2.2.1 Materials and Methods

Fungal inoculum was produced using standard pot culture procedures (see Brundrett *et al.*, 1996), which utilises dried soil inoculum from previous cultures to facilitate the rapid production of inoculum.

Hamilton soil that has a low concentration of readily extractable P and Zn (8.4 and 0.3 mg kg⁻¹ soil respectively) was collected from a depth of 0-10 cm near Pinnaroo, South Australia, passed through a 2 mm sieve, autoclaved and dried (Chapter 2.1.2.1). Dried inoculum of the VAM fungal species, *Glomus intraradices*, *G. coronatum*, and *Scutellospora calospora* produced using Mallala soil mixed with sand was used to inoculate the new pot cultures. This inoculum was produced using single spore cultures of the various VAM fungal species and was supplied by D. Miller, Department of Soil and Water, University of Adelaide. The inoculum was mixed and the roots chopped into pieces 1-2 cm long, taking care to avoid cross contamination of the different fungal species. These fungal species were chosen as they have been successfully used in many studies in our research group.

Dried inoculum was mixed with Hamilton soil at a ratio of 1 part inoculum to 9 parts soil. Inoculated Hamilton soil (500 g) was placed into acid-washed white plastic pots 25 cm long and 4.5 cm in diameter. There were 5 replicate pots for each fungal species. Control (NM) treatments were not inoculated with any fungal species.

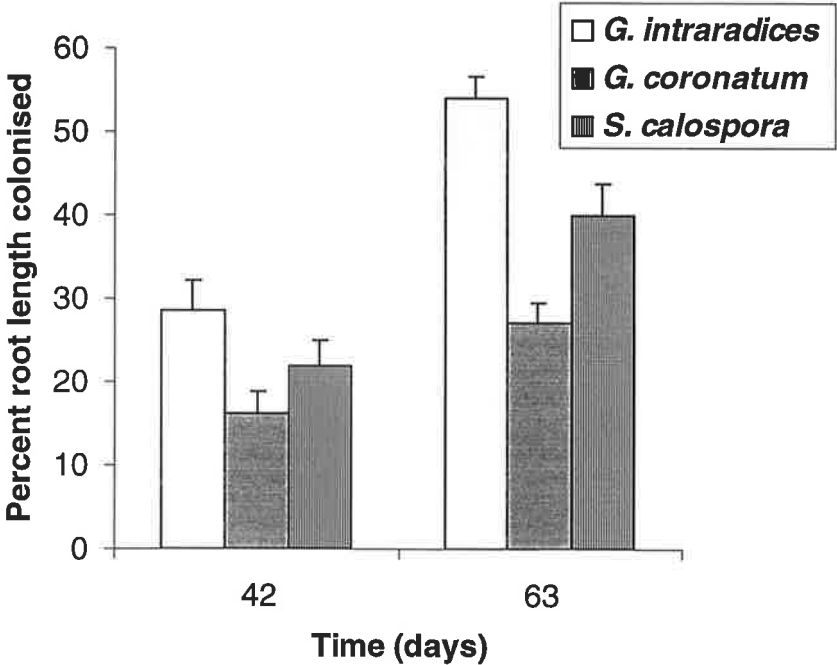
Linola™ seeds were surface sterilised and germinated (Chapter 2.2.4). Five germinated seeds were sown in each pot and plants were thinned to three per pot, following emergence. Plants were grown in a growth room, given 5 ml of modified Long Ashton nutrient solution (+N, -P, -Zn) and watered thrice weekly to weight (0.125 g H₂O/g O.D. soil) with R.O. water (Chapter 2.2.5). Cores of each culture were taken after 42 and 63 days using a stainless steel corer 25 cm long and 1.5 cm in diameter. Roots were washed from each core, cleared in 10% KOH and stained with aniline blue (Chapter 2.2.7). Colonisation of roots by VAM fungi was assessed using the gridline intersect method (Chapter 2.2.8). Fractional vesicular colonisation of the plants by the VAM fungi was also determined using the magnified intersects method (Chapter 2.2.8). After 84 days, one pot of each fungus was used as fresh inoculum whilst the remaining cultures were left to dry slowly to produce dried soil inoculum.

4.2.2.2 Results

Colonisation of Linola™ by the three VAM fungal isolates at 42 and 63 days is shown in Figure 4.2.1. Colonisation was greatest for Linola™ inoculated with *G. intraradices* followed by *S. calospora* and *G. coronatum*. Colonisation of Linola™ by any of the fungal species was initially delayed. However, by 63 days, colonisation of Linola™ was considered sufficient for effective inoculum production, especially for *G. intraradices*. After 63 days no vesicles had been formed

Figure 4.2.1 – Percentage colonisation of Linola™ by various VAM fungal species grown in pot cultures using Hamilton soil after 42 and 63 days. Bars represent standard error of the mean of 5 replicates.

Figure 4.2.1



by any of the fungal treatments and no intraradical spores were observed in roots colonised by *G. intraradices*.

4.2.3 Assessment of inoculation methods – fresh inoculum

4.2.3.1 Materials and Methods

After 84 days growth, one replicate pot culture of each VAM fungal species was used as fresh inoculum. These cultures were cored using a stainless steel corer (Chapter 4.2.2.1) and the plant roots rinsed from the soil, cleared with 10% KOH and stained with aniline blue (Chapter 2.2.7). Colonisation of these Linola™ roots was then assessed using the gridline-intersect method (Chapter 2.2.8). The aim was to minimise any differences in inoculum density between the fungal species and so the same colonised root length of each fungus was added to pots. Given the amount of root material available for the fungal treatments, 315 cm of colonised root was added to each pot. The weight of roots equivalent to 315 cm colonised root for each fungal treatment was 0.25 g, 0.55 g, and 0.45 g for *G. intraradices*, *S. calospora*, and *G. coronatum* respectively. As a control measure, non-mycorrhizal (NM) treatments were included and these were inoculated with 0.45 g of NM roots (average weight of fresh roots of the three fungal treatments).

Sieved (2 mm), autoclaved and dried Hamilton soil (500 g) was placed into acid-washed white pots 25 cm long with a diameter of 4.5 cm. The fresh inoculum was added as a band placed 2.5 cm below the soil surface. Five replicate pots of each VAM fungal species were prepared.

Linola™ seeds were surface sterilised and germinated (Chapter 2.2.4). Germinated seeds (5) were sown in each pot and the plants thinned to three per pot following emergence. Plants were grown in a growth room, given 5 ml modified Long Ashton nutrient solution (+N, -P, -Zn) weekly and watered to weight (0.125 g H₂O/g O.D. soil) with R.O. water thrice weekly (Chapter 2.2.5).

Cores of soil and roots were taken from each pot after 21 and 42 days using a stainless steel corer (Chapter 4.2.2.1). Roots were rinsed from the soil, cleared in 10% KOH and stained with aniline blue (Chapter 2.2.7). Colonisation of the roots was assessed using the gridline intersect technique (Chapter 2.2.8). Old inoculum roots were distinguishable from new roots as they were darker and less firm.

4.2.3.2 Results

There was no colonisation of Linola™ by any of the VAM fungal species after 21 or 42 days. Not surprisingly, there were no visible differences in shoot growth of Linola™ inoculated with any of the fungi compared with NM controls (visual growth observations only).

4.2.4 Assessment of inoculation methods – dried soil inoculum

As no VAM fungal colonisation of Linola™ was observed in Hamilton soil using fresh root inoculum, this next experiment compared colonisation and growth of 3 host plants grown in both Hamilton and Mallala soil inoculated with dried soil inoculum of *G. intraradices*. The rationale of using three host plants was to indicate if host plant type affected colonisation. The host plants chosen were Linola™, linseed and subterranean clover. Linseed (Chapter 3; Thingstrup *et al.*,

1998; Thompson, 1987) and subterranean clover (Abbott and Robson, 1979) are readily colonised by VAM fungi. Mallala soil was included as it has been shown to support extensive VAM fungal colonisation of host plants by *G. intraradices* (Rosewarne *et al.*, 1997) and other fungi including *S. calospora* (Dickson *et al.*, 1999) and would indicate if there were factors specific to Hamilton soil that affect VAM fungal colonisation. Only the infectivity of the *G. intraradices* inoculum was assessed as it had the greatest VAM fungal colonisation of plant roots (Figure 4.2.1) and was therefore considered to have the greatest VAM fungal inoculum potential.

4.2.4.1 Materials and Methods

Mallala and Hamilton soils were prepared as shown in Chapter 2.1.2. Dried inocula of the two fungal treatments (*G. intraradices* and NM) from the pot cultures raised using Hamilton soil and Linola™ were prepared by mixing the inocula thoroughly, cutting any dried roots into 1-2 cm pieces. Hamilton or Mallala soil was inoculated with either *G. intraradices* or NM by adding 1 part dried inoculum to 9 parts soil and mixing thoroughly, taking care not to cross contaminate the two fungal treatments. Inoculated soil (500 g) was placed into white pots 25 cm long and diameter 4.5 cm. There were three replicate pots per treatment.

Seeds of Linola™, linseed and subterranean clover were surface sterilised and germinated (Chapter 2.2.4). Germinated seeds (6) of each plant type were sown into each pot (one plant species per pot) to a depth of 1 cm and the plants thinned to 3 per pot following seedling emergence. Subterranean clover seeds were inoculated with a slurry of *Rhizobium* (Chapter 2.1.1.3). Plants were grown in a growth room, given 5 ml modified Long Ashton nutrient solution (+N, -P, +Zn for Linola™ and linseed; -N, -P, +Zn for subterranean clover) weekly and watered to weight (0.125 g H₂O/g O.D. soil) thrice weekly with R.O. water (Chapter 2.2.5). It

should be noted that whereas Zn was not added in the pot cultures (Chapter 4.2.2) and assessment of fresh inoculum (Chapter 4.2.3) in order to restrict the introduction of Zn into the system, Zn was added in the fertiliser in this experiment as it was more important to ascertain possible mechanisms for the poor colonisation observed when fresh inoculum was used rather than restrict the introduction of Zn.

Plants were harvested after 21 and 42 days (Chapter 2.2.6). Shoot fresh weight was measured and the shoots dried for SDW measurements. Root fresh weight was measured and the roots separated into 2 sub-samples. One sub-sample was dried to give RDW measurements and the other sub-sample was cleared in KOH and stained with aniline blue (Chapter 2.2.7). Colonisation of the root by VAM fungi was determined using the gridline intersect technique (Chapter 2.2.8).

4.2.5 Results

There was no VAM fungal colonisation of Linola™, linseed or subterranean clover after 21 days growth. After 42 days growth, VAM fungal colonisation of Linola™ and subterranean clover in Hamilton and Mallala soils was very low (Figure 4.2.2). Colonisation of linseed in Mallala soil was slightly higher. Subterranean clover had the least VAM fungal colonisation of the three host plants.

Growth (SDW/plant) of the three host plants after 21 and 42 days was significantly greater in Hamilton soil than in Mallala soil (Figure 4.2.3 - only 42 day data shown as the trend was identical after 21 and 42 days). There were no significant differences in the SDW/plant of plants inoculated with *G. intraradices* compared with the non-mycorrhizal control (NM) irrespective of the host plant.

Figure 4.2.2 – VAM fungal colonisation of Linola™, linseed or subterranean clover by *G. intraradices* grown in Hamilton or Mallala soil after 42 days. Bars represent standard error of the mean of 5 replicates. Columns with different letters are statistically different at the 5% level (Chapter 2.2.11). Data for each host plant type was statistically analysed separately.

Figure 4.2.3 – Shoot dry weight of Linola™, linseed or subterranean clover inoculated with either NM or *G. intraradices* after 42 days. Bars represent standard error of the mean of 5 replicates. Columns with different letters are statistically different at the 5% level (Chapter 2.2.11). Data for each host plant type was statistically analysed separately.

Figure 4.2.2

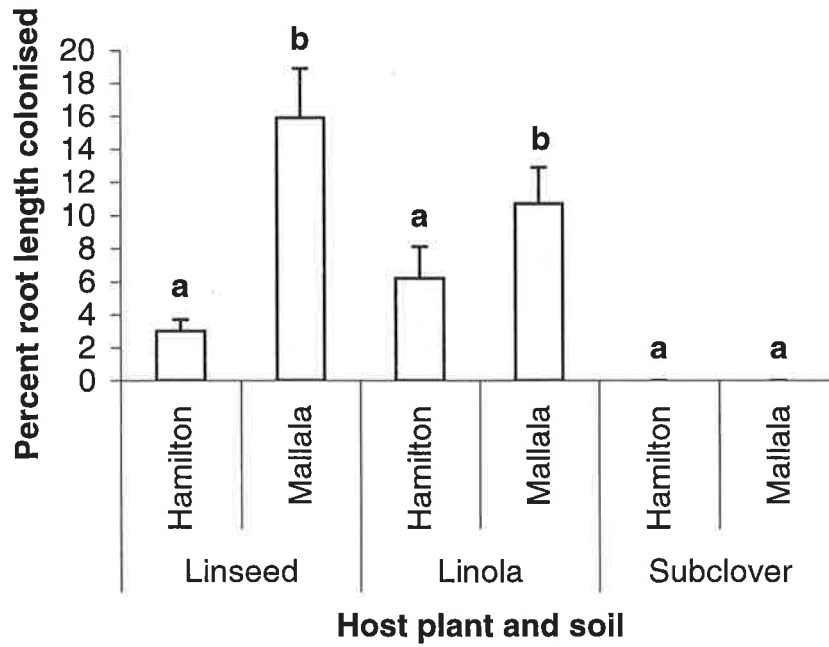
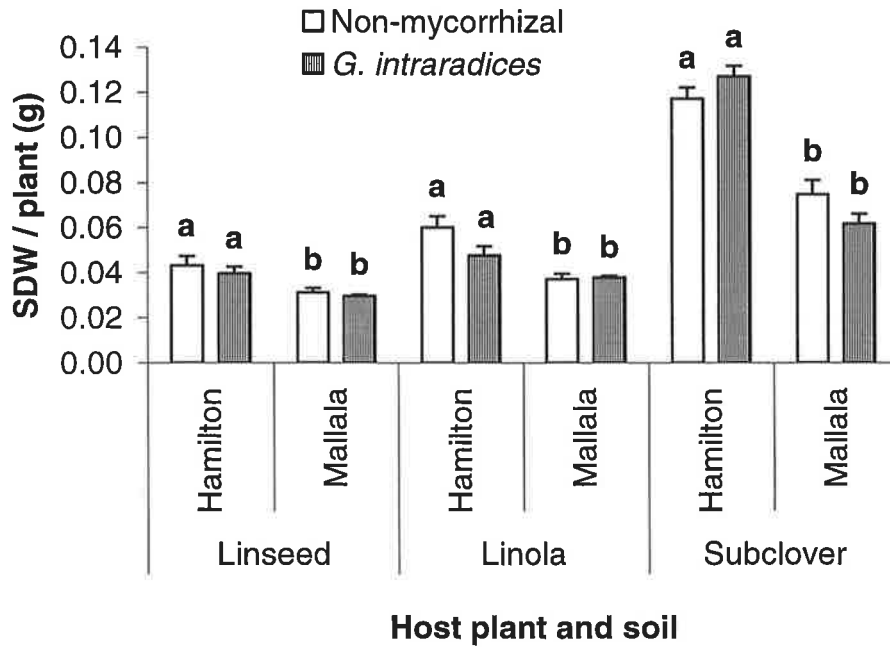


Figure 4.2.3



4.2.5 Discussion

Colonisation of the pot cultures (Figure 4.2.1) was initially low. After 63 days, however, colonisation had reached 54%, 27% and 40% for *G. intraradices*, *G. coronatum* and *S. calospora* respectively indicating that colonisation of the pot cultures was delayed rather than low. Colonisation of plants in Hamilton soil inoculated with fresh root inoculum from the pot cultures was absent after 42 days whereas plants grown in Hamilton or Mallala soil inoculated with dried soil inoculum of *G. intraradices* from the pot cultures was low (<20%).

The pattern of colonisation of a plant root system is sigmoidal and consists of three phases; an initial lag phase, a phase of rapid development and a maintenance or plateau phase which signifies the maximum length of root that is mycorrhizal (Abbott and Robson, 1984; Bowen, 1987; Smith and Gianinazzi-Pearson, 1988). The initial lag phase is related to primary colonisation or the recognition and entry of the VAM fungus into the plant root (Bowen, 1987). The rapid development and plateau phases are related to the spread of these primary colonisations either within or outside the root (Bowen, 1987; Smith and Walker, 1981).

The delay in colonisation of the pot cultures, or long initial lag phase, indicates that **primary** colonisation of the plant roots by the VAM fungi was slow. Possible causes of this include low plant root density or low propagule density of the VAM fungus, as both would reduce the probability of root/fungal contact, which in turn would result in fewer primary infections and consequently a longer initial lag phase in colonisation (Nehl *et al.*, 1998). Low root density is unlikely, given the large number of roots produced by Linola™, whilst low VAM fungal propagule density (not assessed) is possible. McGee *et al.* (1999) showed that colonisation from 10 VAM fungal propagules was less and initiation of colonisation delayed compared to 100

VAM fungal propagules. In order to confirm this hypothesis, the propagule density of the inoculum used to raise these pot cultures could be determined using a combination of spore counts and bioassays.

It is possible that Hamilton soil is not favourable to VAM fungal propagule germination and/or hyphal growth resulting in slow initial colonisation of the pot cultures. Reasons for this may be due to either physical or chemical properties of this soil (Abbott and Robson, 1984). A review of factors affecting propagule germination and hyphal growth in soil is given by Bowen (1987). The VAM fungal propagule density of the inoculum used to raise the pot cultures may have been sufficient for rapid extensive colonisation but if Hamilton soil does not support VAM fungal propagule germination and/or hyphal growth then a delay in initial colonisation would be expected. Once again, measurement of the propagule density of the inoculum used to raise these pot cultures would clarify this issue.

The results for the two inoculation trials showed that both fresh and dried inoculum prepared from pot cultures raised on Linola™ in Hamilton soil established only low levels of colonisation of host plants. Localised placement of inoculum, such as that employed with fresh root inoculum (Chapter 4.2.3), has been shown to result in a delay in colonisation (or long initial lag phase) compared to inoculum spread throughout the soil (McGee *et al.*, 1999; Smith and Smith, 1981b). Even so, in the trial of banded fresh root inoculum, no colonisation was observed even at the site of inoculum placement suggesting that the inoculum had poor infectivity. Furthermore, dried soil inoculum failed to colonise subterranean clover in Mallala soil. This is surprising given that this combination has been used successfully in pot cultures for many years in the Department of Soil and Water, University of Adelaide (D. Miller pers. comm.) and indicates that the lack of colonisation was a result of poor infectivity of the inoculum. The absence of vesicles in the roots

of the Linola™ plants in the pot cultures supports this explanation. The presence of intraradical vesicles increases the inoculum potential of mycorrhizas (Biermann and Linderman, 1983). Therefore, the absence of vesicles would indicate that the inoculum has poor infectivity. This is supported by the lack of intraradical spores in cultures of *G. intraradices*.

The poor infectivity of the inoculum produced in the pot cultures raised using Linola™ in Hamilton soil may be a result of host and/or soil effects. However, it was not possible to isolate the cause. It is not possible to declare that Hamilton soil does not support VAM fungi as Linola™ was colonised in the pot culture and it is unknown if unsterilised Hamilton soil contains VAM fungi which would indicate whether or not it supports VAM fungal reproduction. To determine if Hamilton soil was a cause of the poor infectivity of the inoculum, further pot cultures need to be produced using Hamilton soil and plants other than Linola™. If the resultant inoculum had poor infectivity then Hamilton soil could be seen as not supporting VAM fungal reproduction.

Linola™ is a mutant genotype of linseed developed to produce oil with a low linolenic acid content (Green and Marshall, 1984). As the only difference between Linola™ and linseed is the fatty acid composition of the oil, the agronomic characteristics and fertiliser requirements of Linola™ should be the same as linseed (Hocking, 1995a; Hocking, 1995b). It was also expected that, like linseed, Linola™ would establish VAM associations, which was confirmed by the colonisation of the pot cultures. However, the ability of Linola™ to favour VAM fungal reproduction is unknown. Further pot cultures using Linola™ and other soils and VAM fungal sp. could be used to assess this.

In addition to soil and host effects, the use of only 10% inoculum in the assessment of dried soil inoculum (Chapter 4.2.4) may have restricted the number of infective propagules in the test soil such that only low levels of colonisation were obtained. A further experiment conducted at the completion of these trials (Appendix) however, showed that after 21 days there was no colonisation of subterranean clover or linseed grown in 100% *G. intraradices* inoculum raised from the pot cultures using Hamilton soil and Linola™. This suggests that low colonisation levels were not due to the use of 10% inoculum but that the infectivity of the inoculum was very poor.

Chapter 4.3 – General discussion of experiments involving Hamilton soil

The experiments involving Hamilton soil were designed to examine the effect of VAM fungi on the Zn nutrition of *Linum usitatissimum*. However, it was not possible to address this aim because of poor VAM fungal colonisation in all the experiments. This poor colonisation was attributed to poor infectivity of the inoculum produced using Hamilton soil and Linola™ (*L. usitatissimum* L. cv. 'Eyre'). It is unclear if this poor infectivity of the inoculum was due to Hamilton soil, Linola™ or a combination of both.

To address the original aim of these experiments would require the screening and selection of another soil low in P and Zn that also supports VAM fungi, colonisation and growth of *L. usitatissimum*. Alternatively, more VAM fungi could be screened to identify species or isolates able to persist in Hamilton soil and colonise *L. usitatissimum*. Another approach, using Hamilton soil, is the use of "nurse" pots. Nurse pots are constructed by growing a known VAM fungal host plant such as leek (*Allium porrum* L.) in a soil that results in the formation of a hyphal network in that soil. Target plants are transplanted directly into this established mycorrhizal system, resulting in extensive colonisation of the target plants by hyphae originating from the nurse plants that is more rapid than traditional procedures (Rosewarne *et al.*, 1997). In the case of Hamilton soil, VAM fungal colonisation of Linola™ by *Glomus intraradices*, *Scutellospora calospora*, and *G. coronatum* in pot cultures did eventually occur (Chapter 4.2.2). Therefore, nurse pots could be constructed using leek, the "usual" nurse plant host, and Hamilton soil and any of these fungal species. Once sufficient colonisation of the leek plants has occurred, *L. usitatissimum* could be transplanted into these pots permitting studies on the role of VAM fungi in the Zn nutrition of *L. usitatissimum* to be conducted.

Poor infectivity of the inoculum raised in pot cultures using Linola™ and Hamilton soil may have been caused by either Hamilton soil, Linola™ or a combination of both. This issue could be resolved by raising more pot cultures of VAM fungi using Hamilton soil and host plants other than Linola™, or Linola™ and soils other than Hamilton soil and assessing the infectivity of the resultant inoculum. It is also unknown if unsterilised Hamilton soil supports VAM fungi as only sterilised Hamilton soil was used in the experiments. This could be resolved by screening unsterilised Hamilton soil for VAM fungi using trap plants which may indicate whether Hamilton soil or Linola™ was the cause of the poor infectivity of the inoculum produced using this host plant and soil combination.

There is a possibility that Hamilton soil does not contain or support VAM fungi but this seems unlikely. It is generally agreed that VAM fungi occur in most soils (Abbott and Robson, 1991; Menge, 1983) and agricultural or cultivated soils have been shown to contain more VAM fungal spores than native or grassland soils (Hayman and Stovold, 1979; Mosse and Bowen, 1968). However, there are few, if any, reports of an agricultural soil, such as Hamilton soil, not supporting VAM fungi. Furthermore, the only published surveys of areas similar to the Mallee region were conducted near Murray Bridge, South Australia (McGee, 1986), and other Australian locations, of which none were of the Mallee region (Mosse and Bowen, 1968). Examination of roots collected from field grown plants growing in Hamilton soil would resolve this issue.

Chapter 5 - Final conclusions and suggestions for future work

This project investigated the role of VAM fungi in the growth and nutrition of *Linum usitatissimum* L. in agricultural soils in southern Australia. It had two general aims: (1) to examine the role of indigenous VAM fungi in the growth and nutrition of linseed (*L. usitatissimum* L. cv. 'Glenelg') in field soil collected near Clare, South Australia; and (2) to examine the effect of VAM fungi on the Zn nutrition of Linola™ (*L. usitatissimum* L. cv 'Eyre').

To address the first of these aims, experiments were conducted using two field soils (Merildin and Dunn) collected near Clare, South Australia. Results from an initial experiment using soils collected in 1996 showed that linseed grown in Dunn soil was heavily colonised by indigenous VAM fungi yet grew poorly compared with linseed grown in Merildin soil, which was only slightly colonised. This was surprising given that linseed has been shown to be highly dependent on VAM fungi (Thompson, 1994; Thompson, 1996; Thingstrup *et al.*, 1998; Thingstrup *et al.*, 2000). Results from further experiments showed that this poor growth was not due to a decline in indigenous VAM fungal populations, a growth depression caused by the indigenous VAM fungi, or Zn deficiency as suggested by but not confirmed from initial field observations. Instead, comparison of the SDW and total N content of shoots of linseed grown in 100% Merildin or Dunn soil collected in 1996 and 100% Dunn soil collected in 1998 (Table 5.1) suggests that the poor growth of linseed in Dunn soil collected in 1996 (Dunn 1996) soil was possibly due to N deficiency. An experiment to determine the effect of adding N to Dunn 1996 soil would have confirmed this. Unfortunately, this was not possible as there were inadequate supplies of this soil. Results from further experimentation revealed that the indigenous VAM fungi present in

Dunn soil were potentially beneficial to growth and nutrition of linseed and this potential could be enhanced by the use of crop rotations that included mycorrhizal host plants.

Soil	Year of collection	SDW/plant of linseed after 42 days (g)	Total N concentration in shoots after 42 days (g/kg)
¹ Merildin	1996	0.081 (0.006)	28.7 (0.3)
¹ Dunn	1996	0.016 (0.002)	15.1 (1.0)
² Dunn	1998	0.076 (0.002)	29.5 (1.0)

Table 5.1 - Shoot dry weight (SDW) and total N content of shoots of linseed grown in Merildin or Dunn soil collected in 1996 or Dunn soil collected in 1998. ¹Results from Screening Experiment (Chapter 3.1). ²Results from Elimination Experiment, soil treatment U (Chapter 3.4). Values are the means of 5 replicates with the standard errors of the means in parentheses.

Suggestions for future work involving the indigenous VAM fungi present in Dunn soil include:

- Testing potential limitation of linseed growth by N in new collections of Dunn soil, by pot experiments with a range of additions of N. These experiments must take into account the basal N concentration of the soil, which will vary between collections.
- Measuring the effectiveness of the indigenous VAM fungi in P or Zn uptake and supply to linseed by using mesh containers buried in the root zone containing either ³²P or ⁶⁵Zn labelled soil with the aim being to provide direct measurements of uptake of nutrients by VAM fungi (Schweiger and Jakobsen, 1999; Schweiger *et al.*, 1999). The mesh size permits VAM fungal hyphae but not linseed roots to enter the interior of the container. ³²P or ⁶⁵Zn labelled soil is placed into the centre of these containers and surrounded by a buffer zone of unlabelled soil to

prevent any root uptake. These containers are then buried next to the plant. As only VAM external hyphae can enter the container and access the labelled soil, any ^{32}P or ^{65}Zn entering the plant must have been taken up by the VAM fungus and transferred to the plant. This method has been used in the field (Schweiger and Jakobsen, 1999) and in glasshouse trials (Schweiger *et al.*, 1999). Recently, Jakobsen *et al.* (2001) successfully applied the technique to intact soil cores.

A similar method using mesh containers that does not require the use of radioactive isotopes was described by Schweiger and Jakobsen (2000). Plant root growth is restricted to a small volume of soil separated by mesh from a large root free volume of soil (see Jakobsen *et al.*, 1992). Only VAM external hyphae have access to this root free soil and so the VAM fungal contribution to plant nutrition can be measured as the difference in nutrient uptake by plants with and without VAM fungal access to this root free soil. This method can also be applied in the field, by growing plants in mesh bags ensuring that the bags are small enough that plant growth will eventually be limited by the nutrient in question. Both methods using mesh containers have the main advantage that no fungicides or soil sterilisation are required. This avoids affects of these latter techniques on non-mycorrhizal factors (see Chapter 3.4).

- Examine the role of indigenous VAM fungi in Dunn soil in plant growth and nutrition in the field. So far, all experiments using Dunn soil have been conducted under controlled conditions. Therefore, the effect of the indigenous VAM fungi present in Dunn soil on plant growth and nutrition under field conditions is unclear. Field trials could assess the contribution of the indigenous VAM fungi to plant nutrition by using mesh containers (see above). Field trials could also be conducted that assess the effect of crop rotation on VAM fungal populations and any subsequent effect on the VAM contribution to plant growth and nutrition.

- Study the effects of indigenous VAM fungi present in Dunn soil on other mycorrhizal crop plants, which may differ in mycorrhizal dependency, e.g. vetch (*Vicia sativa* L.), wheat

(*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.). These experiments could be conducted under controlled conditions using mesh containers or soil sterilisation and reinoculation, or under field conditions using mesh containers.

- Examine the possibility of re-establishing indigenous VAM fungal populations in Merildin soil. This may involve the use of crop rotations that include mycorrhizal plants and reducing P fertilisation to a level that maintained soil P concentrations in a range that maximised the VAM fungal contribution and plant yield. These experiments would initially be conducted under controlled conditions and then in the field.

To address the second aim of this project, an agricultural soil (Hamilton) with P and Zn concentrations that could be manipulated to promote VAM fungal colonisation and induce Zn deficiency was collected from Pinnaroo, South Australia. However, it was not possible to examine the effect of VAM fungi on the uptake of Zn by Linola™ in this soil in the time available because of poor VAM fungal colonisation.

Lack of VAM fungal colonisation in experiments using Hamilton soil was attributed to poor infectivity of the inoculum used. This inoculum had been raised using Linola™ in Hamilton soil. It is unclear if the poor infectivity of this inoculum was due to the use of Hamilton soil, Linola™ or a combination of both. This situation could be clarified by raising pot cultures of VAM fungi using Hamilton soil and host plants other than Linola™ or using Linola™ and soil other than Hamilton. Assessing the infectivity of the resultant inoculum would indicate if the poor infectivity of the inoculum produced using Linola™ and Hamilton soil was caused by Linola™, Hamilton soil or both.

Suggestions for future work examining the role of VAM fungi in the Zn nutrition of their host plant include:

- Using trap plants to screen unsterilised Hamilton soil for the presence of indigenous VAM fungi. To this point, all experiments examining the role of VAM fungi in the Zn nutrition of *Linola*TM in Hamilton soil have been conducted using sterilised soil. Screening unsterilised Hamilton soil for VAM fungi would indicate whether the soil supports any indigenous VAM fungi. This would answer the question raised in Chapter 4.3, regarding the possibility of Hamilton soil not supporting VAM fungi.
- Examining the role of VAM fungi in Zn nutrition of *Linola*TM in Hamilton soil using “nurse” pots (see Chapter 4.3), which should enable rapid and extensive colonisation of the *Linola*TM plants. Mesh containers, described previously, could also be used to provide direct measurement of VAM fungal uptake of Zn.

In conclusion, the experiments in this thesis illustrated the well established point that work with VAM in field soils is not easy. The two main difficulties encountered were that (i) each field soil, including different collections from the same site, has different chemical, physical and biological characteristics, which may differently affect the VAM symbiosis, and (ii) it is difficult, if not impossible to isolate and evaluate the fungal component of a VAM symbiosis without causing concurrent changes to non-mycorrhizal factors, which in turn may affect plant growth.

APPENDIX

Testing the infectivity of *G. intraradices* inoculum raised in pot cultures using Hamilton soil and Linola™.

Introduction

Poor colonisation of in the assessment of dried inoculum of *G. intraradices* raised in pot cultures using Hamilton soil and Linola™ (Chapter 4.2.4) was suspected to be a result of poor infectivity of this inoculum. However, it was possible that poor colonisation may have been exaggerated by the use of only 10% inoculum (w/w) in this assessment. Therefore, this preliminary experiment aimed to assess the colonisation of linseed and subterranean clover in 100% *G. intraradices* inoculum raised in pot cultures using Hamilton soil and Linola™.

Materials and Methods

Dried inoculum of *G. intraradices* used in Chapter 4.2.4 was assessed in this experiment. Samples (50 g) of 100% inoculum were placed into compartments of a seedling tray and there were 3 replicates per host treatment.

Seeds of linseed and subterranean clover were surface sterilised and germinated (Chapter 2.2.4). Germinated seeds (3) of each plant type were sown into each compartment (one plant species per compartment) to a depth of 0.5 cm and the plants thinned to 1 per pot following emergence. Subterranean clover seeds were inoculated with a slurry of *Rhizobium* (Chapter 2.1.1.3). Plants

were grown in a growth room, given 0.5 ml modified Long Ashton nutrient solution (+N, -P, +Zn for linseed; -N, -P, +Zn for subterranean clover) weekly and watered thrice weekly with R.O. water (free draining compartments) (Chapter 2.2.5).

Plants were grown for 21 days, at which time they were harvested (Chapter 2.2.6). Roots were cleared in 10% KOH, stained in aniline blue (Chapter 2.2.7) and colonisation of the root system by VAM fungi assessed using the gridline intersect method (Chapter 2.2.8). No plant growth measurements were taken.

Results

No VAM fungal colonisation was observed in roots of linseed or subterranean clover after 21 days.

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